

SÃO PAULO STATE UNIVERSITY “JÚLIO DE MESQUITA FILHO”

School of Pharmaceutical Sciences – Araraquara Campus

Postgraduate Program in Biosciences and Biotechnology Applied to Pharmacy



NATHALIA VIEIRA DOS SANTOS

Development of sustainable platforms for the stabilization and purification of the recombinant Green Fluorescent Protein produced by *Escherichia coli*

Araraquara – São Paulo

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Faculdade de Ciências Farmacêuticas – Campus de Araraquara

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**Desenvolvimento de plataformas sustentáveis para estabilização e purificação da
Proteína Verde Fluorescente recombinante produzida por *Escherichia coli***

Tese apresentada ao Programa de Pós-Graduação em Biociências e Biotecnologia aplicadas à Farmácia, área de concentração: Biotecnologia Diagnóstica, Bioprodutos e Biofármacos, da Faculdade de Ciências Farmacêuticas, UNESP, como parte dos requisitos para obtenção do título de Doutora em Biociências e Biotecnologia Aplicadas à Farmácia.

Orientador: Prof. Dr. Jorge F. B. Pereira

Coorientador: Prof. Dr. Fernando L. Primo

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This thesis was presented to the Postgraduate Program in Biosciences and Biotechnology Applied to Pharmacy, area of Diagnostic Biotechnology, Bioproducts and Biopharmaceuticals, Faculty of Pharmaceutical Sciences, UNESP, as part of the requirements for the degree of Doctor in Biosciences and Biotechnology Applied to Pharmacy.

Supervisor: Prof. Dr. Jorge F. B. Pereira

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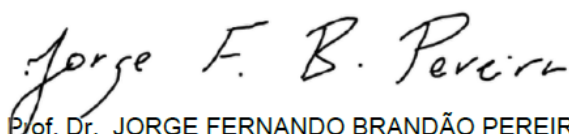
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*I dedicate this work to my mother, the strongest and most loving person in my life.
I am only able to be where I am because of your support and faith in me.
Mom, you are my inspiration. I love you.*

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“A journey of a thousand miles begins with a single step.”

Lao Tzu

RESUMO

A biotecnologia permitiu o desenvolvimento de novas biomoléculas para aplicações médicas e industriais. No entanto, o uso de muitos produtos biológicos notáveis ainda é limitado em grande escala devido às suas baixas estabilidades (que dificultam distribuição, armazenamento e manuseio) e preços muito elevados (principalmente devidos aos processos complexos e caros de produção e purificação). Um melhor entendimento das propriedades e estabilidade das biomoléculas, aliados ao desenvolvimento de plataformas mais simples, baratas e sustentáveis para estabilização e purificação de produtos biotecnológicos, pode ajudar a superar essas limitações e expandir seus usos comerciais. Assim, o objetivo do trabalho foi melhorar as aplicações em larga escala de biomoléculas usando a Proteína Verde Fluorescente Melhorada (EGFP, do inglês *Enhanced Green Fluorescent Protein*, importante biomarcador e biossensor) como uma prova de conceito. Nesse sentido, a pesquisadora projetou estudos aprofundados da estrutura de EGFP e suas propriedades de fluorescência, avaliou sua estabilidade na presença de diferentes solventes ou sob condições de estresse, e desenvolveu novos sistemas sustentáveis para a estabilização e purificação da EGFP usando Líquidos Iônicos (LIs), sais orgânicos e polímeros compatíveis com biomoléculas. Nos estudos de propriedades da EGFP, foi possível detectar um pico de fluorescência adicional e nunca relatado para esta proteína, resolvendo uma controvérsia científica sobre as conformações e atividade da EGFP. Além disso, nos estudos de estabilidade em pH, foi possível demonstrar a relação entre a fluorescência da EGFP e pH, assim como elucidar os impactos do pH ácido na estrutura da proteína. Essas informações podem ajudar no desenvolvimento de novos biossensores de pH neutro-a-ácido. Os estudos da atividade da EGFP na presença de LIs e sob condições de estresse mostraram que LIs podem ter diferentes impactos na estabilidade de proteínas, dependendo de sua natureza, concentração e comprimento da cadeia alquílica. Por exemplo, alguns LIs como cloreto de colina, octanoato de colina e decanoato de colina protegeram a fluorescência de EGFP, enquanto LIs baseados em imidazólios com maior comprimento de cadeia alquílica reduziram sua atividade. Além disso, foi possível encontrar LIs que triplicaram a preservação a longo prazo da atividade da EGFP em temperatura ambiente, que podem potencialmente ser aplicados como estabilizadores de proteínas em formulações biofarmacêuticas. Na etapa de purificação, uma plataforma sustentável foi desenvolvida associando sistemas bifásicos aquosos (compostos por polímeros e sais) e ultrafiltração, obtendo EGFP > 97 % pura e também recuperando mais de 60% dos solventes utilizados no processo. Em conclusão, os estudos da atividade e estabilidade da EGFP, aliados ao desenvolvimento de técnicas alternativas sustentáveis de purificação de baixo custo, podem ampliar seu uso comercial e tem aplicação potencial para outras biomoléculas.

Palavras-chave: Proteína Verde Fluorescente; Purificação de Proteínas; Estabilidade de Proteínas; Líquidos Iônicos; Sistemas Aquosos Bifásicos; Biossensores.

ABSTRACT

Biotechnology allowed the development of novel biomolecules for medical and industrial applications. However, there are still limitations to the use of many outstanding biological products at large-scale due to their low stability (which hinders their distribution, storage, and handling) and very high prices (mostly caused by their intricate and costly production and purification processes). A better understanding of the properties and stability of biomolecules, allied with the development of simpler, cheaper, and more sustainable stabilization and purification platforms for biotechnological products, can help to overcome these limitations and expand their commercial use. Hence, the goal of this work was to improve the large-scale applications of biomolecules using the Enhanced Green Fluorescent Protein (EGFP, an important biomarker and biosensor) as a proof of concept. In this sense, the researcher designed in-depth studies of EGFP structure and fluorescence properties, evaluated its stability in the presence of different solvents or under stress conditions, and developed novel sustainable stabilization and purification systems for EGFP using Ionic Liquids (ILs), organic salts, and polymers compatible with biomolecules. In the studies of EGFP properties and structure, it was possible to detect an additional and never reported fluorescence peak for this protein and solve a scientific controversy regarding EGFP conformations and activity. Additionally, on the pH stability studies, it was possible to demonstrate the pH-dependence of the fluorescence of EGFP and elucidate the impacts of acidic pH on the structure of the protein, which can aid in the development of novel neutral-to-acidic pH-biosensors. The studies of EGFP activity in the presence of ILs and under stress conditions showed ILs have different impacts on the stability of proteins, depending on their nature, concentration, and alkyl chain length. For example, some ILs like cholinium chloride, cholinium octanoate, and cholinium decanoate protected EGFP fluorescence, while imidazolium-based ILs with longer alkyl chain length reduced the activity of the protein. Furthermore, it was possible to find ILs that tripled the long-term preservation of EGFP activity at room temperature, which could potentially act as protein stabilizers in biopharmaceutical formulations. For the purification stage, it was possible to develop a sustainable purification platform associating aqueous biphasic systems (composed of polymers and salts) and ultrafiltration, obtaining > 97 % pure EGFP while recovering 60 % of the solvents used in the process. In conclusion, the study of EGFP activity and stability, allied with the development of sustainable alternative low-cost purification techniques, can broaden its commercial use and have potential applications for other biomolecules.

Keywords: Green Fluorescent Protein; Protein Purification; Protein Stability; Ionic Liquids; Aqueous Biphasic Systems; Biosensors.

LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
ABNT	<i>Associação Brasileira de Normas Técnicas</i> (Brazilian Association of Technical Standards)
ABS	aqueous biphasic systems
ACS	American Chemical Society
ANSTO	Australia's Nuclear Science and Technology Organization
ANSTO-AS	ANSTO - The Australian Synchrotron
AMTPS	aqueous micellar two-phase systems
Apo A	apolipoprotein A
ATN	Australian Technology Network of Universities
ATPS	aqueous two-phase systems
ATR	attenuated total reflectance
BM	biomolecule
BSA	bovine serum albumin
CAGR	compound annual growth rate
CAPES	<i>Coordenação de Aperfeiçoamento de Pessoal de Nível Superior</i> (Coordination of Superior Level Staff Improvement)
CAPES-PROEX	<i>CAPES-Pró-Reitoria de Extensão da UNESP</i> (CAPES-Extension board from UNESP)
UNESP	
CD	circular dichroism
CHO	Chinese hamster ovary cells
CNPq	<i>Conselho Nacional de Desenvolvimento Científico e Tecnológico</i> (National Council for Scientific and Technological Development)
COSMO-RS	COnductor like Screening MOdel for Real Solvents
CP	contaminant proteins
DES	deep eutectic solvents
DIT	digital integration time
DOE	design of experiments
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EC	extinction coefficient
<i>e.g.</i>	<i>exempli gratia</i> (for example)
EGFP	enhanced green fluorescent protein
EOPO	copolymer of ethylene oxide and propylene oxide
<i>et al.</i>	<i>et alii</i> (and others)
EU	European Union
Fab'	antigen-binding fragment
FAPESP	<i>Fundação de Amparo à Pesquisa do Estado de São Paulo</i> (São Paulo Research Foundation)
FCPC	fast centrifugal partition chromatography
FCT	<i>Fundação para a Ciência e Tecnologia de Portugal</i> (Science and Technology Foundation of Portugal)
FDA	U.S. Food and Drug Administration

FI	fluorescence intensity
FP	fluorescent proteins
FTIR	Fourier transform infrared spectroscopy
FTIR-ATR	Fourier transform infrared spectroscopy with an attenuated total reflectance
GRAVY	grand average of hydropathicity
GFP	green fluorescent protein
hGH	human growth hormone
hGHA	human growth hormone antagonist
HEWL	hen egg white lysozyme
HIC	hydrophobic interaction chromatography
HPV	<i>Human papillomavirus</i>
HT	high tension
IFP	intrinsic fluorescence of proteins
IGF-I	human insulin-like growth factor-I
IgG	immunoglobulin G
IL	ionic liquids
IL-based ABS	ionic liquids-based aqueous biphasic systems
IMAC	immobilized metal affinity chromatography
IPTG	inductor β -D-1-thiogalactopyranoside
IUPAC	International Union of Pure and Applied Chemistry
Ka	ionization constant
LB	Luria Bertani broth
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LPS	lipopolysaccharides
m-EGFP	monomeric enhanced green fluorescent protein
mAb	monoclonal antibodies
MD	molecular dynamics
MRW	mean residue weight
MW	molecular weight
NaPA	sodium polyacrylate
NH	nano hydrogel
NH/EGFP	nano hydrogel with incorporated enhanced green fluorescent protein
OFR	oscillatory flow reactor
OspA	outer surface protein A fragment
<i>P. pastoris</i>	<i>Pichia pastoris</i>
PB	potassium phosphate buffer
PBS	phosphate-saline buffer
PC-SAFT	perturbed chain-statistical associating fluid theory
DNA	deoxyribonucleic acid
pDNA	plasmid of deoxyribonucleic acid
PEG	polyethylene glycol
PEG-OMe	polyethylene glycol methyl ether

PF	purification factor
pH	potential of hydrogen
Phe	phenylalanine
pKa	negative log of the ionization constant
PPG	polyethylene glycol
P _{uv}	amino acids with absorbance in ultraviolet light
QSAR	Quantitative Structure Activity Relationship
QY	quantum yield
R&D	research and development
Reppal	hydroxypropyl starch
RFP	red fluorescent protein
rhIFN α -1	recombinant human interferon α -1
rHSA	recombinant human serum albumin
RNA	ribonucleic acid
RSC	Royal Society of Chemistry
RSD	relative standard deviation
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
S1	first-order Rayleigh light scattering
S2	second-order Rayleigh light scattering
SAXS	small angle x-ray scattering
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC-SAXS	size-exclusion chromatography small angle x-ray scattering
sfGFP	superfolder green fluorescent protein
TNF	tumor necrosis factor
TNF mAb Fab'	tumor necrosis factor monoclonal antibody antigen-binding fragment
TP	total protein
Trp	tryptophan
Tyr	tyrosine
UA	units of absorbance
UF	unities of fluorescence
UNESP	<i>Universidade Estadual Paulista "Júlio de Mesquita Filho"</i> (São Paulo State University)
USA	United States of America
USD	American dollars
UV	ultraviolet light
viz.	<i>videlicet</i> (in other words)
WHO	World Health Organization
Wiley	John Wiley & Sons, Inc.
wtGFP	wild-type green fluorescent protein
YFP	Fezziwig yellow fluorescent protein

LIST OF ACRONYMS AND SYMBOLS

$\times g$	times gravity
$(\text{NH}_4)_2\text{SO}_4$	ammonium sulfate
$[\text{C}_1\text{mim}]\text{Cl}$	1,3-dimethylimidazolium chloride
$[\text{C}_2\text{mim}]\text{Cl}$	1-ethyl-3-methylimidazolium chloride
$[\text{C}_4\text{mim}][\text{N}(\text{CN})_2]$	1-butyl-3-methylimidazolium dicyanamide
$[\text{C}_4\text{mim}]\text{Cl}$	1-butyl-3-methylimidazolium chloride
$[\text{C}_4\text{mpyr}]\text{Cl}$	1-butyl-3-methylpyridinium chloride
$[\text{C}_6\text{mim}]\text{Cl}$	1-hexyl-3-methylimidazolium chloride
$[\text{C}_8\text{mim}]\text{Cl}$	1-octyl-3-methylimidazolium chloride
$[\text{C}_{10}\text{mim}]\text{Cl}$	1-decyl-3-methylimidazolium chloride
$[\text{C}_{12}\text{mim}]\text{Cl}$	1-dodecyl-3-methylimidazolium chloride
$[\text{Ch}][\text{Ac}]$	cholinium acetate
$[\text{Ch}][\text{Bit}]$	cholinium bitartrate
$[\text{Ch}][\text{But}]$	cholinium butanoate
$[\text{Ch}][\text{Dec}]$	cholinium decanoate
$[\text{Ch}][\text{Gly}]$	cholinium glycolate
$[\text{Ch}][\text{Hex}]$	cholinium hexanoate
$[\text{Ch}][\text{Lac}]$	cholinium lactate
$[\text{Ch}][\text{Oct}]$	cholinium octanoate
$[\text{Ch}][\text{Pen}]$	cholinium pentanoate
$[\text{Ch}][\text{Pro}]$	cholinium propanoate
$[\text{Ch}][\text{Bic}]$	cholinium bicarbonate
$[\text{Ch}]\text{Cl}$	cholinium chloride
$[\text{Ch}][\text{OH}]$	cholinium hydroxide
$[\text{Ch}]\text{X}$	cholinium-based ILs
$[\text{C}_n\text{mim}]\text{Cl}$	<i>n</i> -3-methyl-imidazolium-based ILs
$[\text{EGFP}]_0$	initial concentration of EGFP
$[\text{EGFP}]_B$	concentration of EGFP in the bottom phase
$[\text{EGFP}]_{\text{EGFP-rich phase}}$	concentration of EGFP in the EGFP-rich phase
$[\text{EGFP}]_{\text{rel}}$	relative concentration of EGFP by total protein
$[\text{EGFP}]_T$	concentration of EGFP in the top phase
$[\text{N}_{2,2,2,2}]\text{Br}$	tetraethylammonium bromide
$[\text{N}_{4,4,4,4}][\text{BES}]$	tetrabutylammonium 2-(bis(2-hydroxyethyl)amino)ethanesulfonate
$[\text{N}_{4,4,4,4}]\text{Br}$	tetrabutylammonium bromide
$[\text{TP}]_{\text{EGFP-rich phase}}$	concentration of total proteins in the EGFP-rich phase
$[\theta]$	mean residue ellipticity
$^{\circ}\text{C}$	Celsius degrees
$^1\text{H NMR}$	proton nuclear magnetic resonance
<i>A</i>	first constant obtained by the regression of the experimental binodal data

A_a	amplitude of agitation
B	second constant obtained by the regression of the experimental binodal data
C	third constant obtained by the regression of the experimental binodal data
C	carbon
$C_6H_7NaO_7$	monosodium citrate
cm	centimeter
C_{PT}	concentration of the protein in $mg.mL^{-1}$
Da	Dalton
deg	<i>degrees</i> of ellipticity
EAN	ethylammonium nitrate
EE_{EGFP}	EGFP extraction efficiency
EGFP/TP	concentration of EGFP over concentration of TP
F1	EGFP point of highest intensity of fluorescence 1
F1S	EGFP point of highest intensity of fluorescence 1 for the standard
F2	EGFP point of highest intensity of fluorescence 2
F2S	EGFP point of highest intensity of fluorescence 2 for the standard
f_a	frequencies of agitation
FI EGFP/FI TP	fluorescence intensity of EGFP over the fluorescence intensity of TP
FP/TP	relation between the fluorescence intensity of the FP over the fluorescence intensity of TP
$FP_{FP-poor\ phase}$	fluorescence intensity of the FP in the FP-poor phase
$FP_{FP-rich\ phase}$	fluorescence intensity of the FP in the FP-rich phase
g	gram
GuHCl	guanidine hydrochloride
h	hour
H_2O_2	hydrogen peroxide
H_3PO_4	phosphoric acid
HCl	hydrochloric acid
H_i	hydrophobicity value of the amino acid at certain position
i	position of an amino acid in a protein
I	scattering intensity
k	kilo
K	partition coefficient
K	Kelvin
K_2HPO_4	potassium hydrogen phosphate
K_3PO_4	potassium phosphate tribasic
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic
KOH	potassium hydroxide
L	liter
m	milli for units and meter for distances

m/v	mass/volume
MB _{EGFP}	EGFP mass balance
min	minute
<i>n</i>	total number of samples/variables
Na ₂ CO ₃	sodium carbonate
Na ₂ HPO ₄	sodium phosphate dibasic
Na ₂ SO ₄	sodium sulfate
NaCl	sodium chloride
NaH ₂ PO ₄	sodium dihydrogen phosphate
NaOH	sodium hydroxide
NaPA	sodium polyacrylate
OD _{600nm}	optical density at 600 nm
P	path length of the CD cell in mm
p(r)	pair distance distribution function
Pa	Pascal
pI	isoelectric point
q	scattering vector, also called momentum transfer
r	radius
R ²	coefficient of determination
RE _{FP}	relative extraction for the FP
res	amino acid residues
RFP/TP	relation between the fluorescence intensity of the RFP over the fluorescence intensity of TP
Rg	radius of gyration
RSD _n	RSD for each point of the curve
s	second
S	Siemens
SWOT	strengths, weaknesses, opportunities and threats
TP _{FP-rich phase}	fluorescence intensity of the TP in the FP-rich phase
U	enzyme unit
V ₀	initial volume
V _B	bottom volume
V _{EGFP-rich phase}	EGFP-rich phase volume
V _{inj}	injection velocity
V _T	initial volume
w1	weight fraction of PEG-2000
w2	weight fraction of K ₂ HPO ₄ /KH ₂ PO ₄
w3	weight fraction of H ₂ O
wt%	weight percentage
x	inorganic salt weight percentage
y	PEG-2000 weight percentage

y_{FI}	fluorescence intensity at LOD or LOQ
YFP/TP	relation between the fluorescence intensity of the YFP over the fluorescence intensity of TP
θ	ellipticity
κ	confidence level
λ	wavelength
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
μ	micro
μ_b	average of the blank signal
μ_x	average of the points of the curve
σ_b	standard deviation of the blank
σ_n	standard deviation for each point of the curve

LIST OF TABLES

Table 1 – Examples of biomolecules extracted from cell lysate* or fermented broth** using ABS.	71
Table 2 – Examples of bioproducts extracted and purified from different fermented broths using different IL-based ABS.	78
Table 3 – Examples of biopharmaceuticals approved by the FDA, obtained from bacteria as the heterologous expression system (Biopharma database).	89
Table 4 – Examples of biopharmaceuticals approved by FDA obtained from yeast as heterologous expression system (Biopharma database).	93
Table 5 – Weight fraction (wt%) composition of the components of each ABS. The references where the phase diagrams are described are also defined.	110
Table 6 – Overview of the main effects of IPTG concentration ([IPTG], agitation rate, and induction time over the bacterial growth (represented by optical density at 600 nm, OD _{600nm}) and EGFP production (indicated by EGFP concentration, [EGFP]) by <i>E. coli</i> BL21 (DE3) [pLysS; pET28(a)] at 30 °C.	119
Table 7 – Comparison of range and precision of EGFP calibration curves at fluorescence peaks F1 (λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (λ_{ex} 278 nm, λ_{em} 510 nm). Parameters presented are the upper limit of the curve, coefficient of determination (R^2), the limit of detection (LOD), the limit of quantification (LOQ), and relative standard deviation (RSD).	129
Table 8 – Detailed weight fraction compositions, physicochemical characteristics [pH, conductivity (mS.cm ⁻¹ at 25 °C), viscosity (mPa.S), density (g.cm ⁻³), water content (wt%)] of each ABS.	167
Table 9 – Comparison of different liquid-liquid extraction approaches (single step liquid-liquid extraction, or association with back-extraction or chromatography techniques) for the purification of GFP. GFP recovery (%), [GFP] _{rel} (%) (indicating GFP purity), purification factor (PF), and partition coefficient (<i>K</i>) values are presented when available.	181
Table 10 – Weight fraction (wt%) and RE _{FP} (%) for extraction of YFP and RFP with PPG-400/[Ch]Cl ABS. Results correspond to the average of three independent assays and respective standard deviations.	182

Table 11 – Weight fraction (wt%), EE_{EGFP} (%), MB_{EGFP} (%), and $[EGFP]_{rel}$ (%) for the PPG-400/[Ch]Cl ABS before and after solvent recycling using ultrafiltration. $[EGFP]_{rel}$ (%) of the polished EGFP, recovered after the ultrafiltration step is also presented. Results correspond to the average of three independent assays and respective standard deviations.....	185
Table 12 – Weight (g) of the recovered top and bottom phases after the ultrafiltration of the PPG-400/[Ch]Cl ABS phases; total mass of the PPG-400/[Ch]Cl ABS before and after the solvent recycling process, with the respective recovery percentage (wt%).	185
Table 13 – Composition and advantage of Aqueous Biphasic System s(ABS) from this study with the highest cost-benefit for applications that require or not high purity levels.	189
Table 14 – Parameters [amplitudes (A_a), frequencies of agitation (f_a), and injection velocity (V_{inj})] and results [$EGFP$ Extraction Efficiency (EE_{EGFP}), Fluorescence Intensity (FI) of $EGFP$ over the FI of total proteins (FI $EGFP$ /FI TP) and separation times] for the Oscillatory Flow Reactor (RFO) experiments for the scale-up of $EGFP$ purification (from 5 to 50 g) using PPG-400/[Ch]Cl ABS.	192

LIST OF FIGURES

Figure 1 – Schematic representation of the main issues and potential solutions for expanding the large-scale access to proteins of commercial interest.	37
Figure 2 – Schematic representation of the research structure and achievements of the Thesis project, grouped by experimental stages.	39
Figure 3 –A) Representation of the crystalline structure of the <i>Aequorea victoria</i> by <i>E. Coli</i> . B) Representation of the crystalline structure of EGFP expressed by <i>E. Coli</i> . The images present helixes in red, strands in green, and coils in blue. C) Overlay of wtGFP structure (color) and EGFP (gray).....	44
Figure 4 – General steps for the production, purification, and formulation of bioproducts employing microbial heterologous expression system.	62
Figure 5 – General steps for the development and application of aqueous biphasic systems (ABS) for the extraction and purification of biomolecules (BM) at an industrial scale.	66
Figure 6 – General SWOT analysis (strengths, weaknesses, opportunities, and threats) of IL-based ABS for the extraction and purification of biomolecules.....	82
Figure 7 – Chemical structure of studied ILs and salts.	101
Figure 8 – Chemical structure of studied denaturing agents.	101
Figure 9 – Chemical structure of studied polymers.....	102
Figure 10 – A) 3D fluorescence spectrum, B) emission spectrum (λ_{ex} 488 nm) and C) excitation spectrum (λ_{ex} 510 nm) of EGFP 5.4 $\mu\text{g.mL}^{-1}$ 25 °C in phosphate buffer (PB) pH 7.4. The emission spectrum in B) is equivalent to the red horizontal line in the 3D fluorescence spectrum in A) , and the excitation spectrum in C) comprises the purple line in A) . 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (unit of fluorescence, UF) according to the color scale from blue to red. F1 (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (around λ_{ex} 278 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity; S1 and S2 are first and second-order light scattering, respectively.	122
Figure 11 – 3D fluorescence spectra at 25 °C in phosphate buffer (PB) pH 7.4 of A) Blank and B) EGFP 5.4 $\mu\text{g.mL}^{-1}$, range λ_{ex} 240-550 nm and λ_{em} 470-570 nm, scale of fluorescence intensity 0-400 units of fluorescence (UF) and corresponding zoomed region of EGFP in a range	

of λ_{ex} 240-330 nm and λ_{em} 480-540 nm, scale of fluorescence intensity 0-120 UF. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (UF) according to the color scale from blue to red. F1 (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (around λ_{ex} 278 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity; S1 and S2 are first and second-order light scattering, respectively. **C)** EGFP ($5.4 \mu\text{g.mL}^{-1}$) fluorescence excitation spectra (λ_{ex} 240-550 nm) for emissions 509 (green), 510 (blue) and 511 nm (purple) and **D)** EGFP ($5.4 \mu\text{g.mL}^{-1}$) fluorescence emission spectra (λ_{em} 470-570 nm) for excitations 276 nm (yellow), 278 nm (pink), 280 nm (red), 486 nm (green), 488 nm (blue) and 490 nm (purple). **E)** EGFP (0.216 mg.mL^{-1}) absorbance spectrum (λ 250-800 nm) in units of absorbance (UA); F2 and protein intrinsic absorbance in UV (P_{uv}) have an overlapping peak at 276 nm and F1 a peak at 488 nm. The results were obtained in triplicate with a blank. A representative of the triplicate is presented for the 3D spectra, and the average of the results is shown for excitation, emission, and absorbance spectra. 123

Figure 12 – A) 3D fluorescence spectra at 25 °C of standard EGFP ($10 \mu\text{g.mL}^{-1}$) from quantification kit from Biovision® (in assay buffer pH 8.0), range λ_{ex} 240-530 nm and λ_{em} 470-570 nm, scale of fluorescence intensity 0-500 units of fluorescence (UF) and corresponding zoomed region of EGFP in a range of λ_{ex} 240-330 nm and λ_{em} 480-540 nm, scale of fluorescence intensity 0-60 UF. **B)** 3D fluorescence spectra of BSA in PB pH 7.4, range λ_{ex} 240-530 nm and λ_{em} 280-580 nm (EGFP concentration $10 \mu\text{g.mL}^{-1}$ and scale of fluorescence intensity 0-500 UF in **B.I** and 3 mg.mL^{-1} in 0-2500 UF in **B.II**. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (UF) according to the color scale from blue to red. F1S (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2S (around λ_{ex} 296 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity, and S1 and S2 are the first and second-order light scattering, respectively. 125

Figure 13 – A) 3D fluorescence spectra at PB pH 7.4 at 25 °C of different concentrations of EGFP (**A.I** – dil 10x - $21.6 \mu\text{g.mL}^{-1}$, **A.II** – dil 20x - $10.8 \mu\text{g.mL}^{-1}$, **A.III** – dil 30x - $7.2 \mu\text{g.mL}^{-1}$, **A.IV** – dil 40x - $5.4 \mu\text{g.mL}^{-1}$), range λ_{ex} 240-330 nm and λ_{em} 480-540 nm, scale of fluorescence intensity 0-500 UF. **B)** Calibration curve for EGFP F2 peak (λ_{ex} 278 nm, λ_{em} 510 nm), concentration of EGFP [EGFP] (mg.mL^{-1}) in the X-axis and fluorescence intensity (UF) in the Y-axis. **C)** 3D fluorescence spectra of EGFP ($5.4 \mu\text{g.mL}^{-1}$) in PB of different pH (**C.I** – pH 6.0 and **C.II** – pH 7.4). **D)** after denaturation by temperature in PB pH 7.4 (30 min at

90 °C), range λ_{ex} 240-550 nm and λ_{em} 470-570 nm, scale of fluorescence intensity (UF) 0-400. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (UF) according to the color scale from blue to red. F1 (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (around λ_{ex} 296 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity. The results correspond to triplicate measurements with corresponding blank assays for the pH, calibration curve, and high-temperature experiments. The 3D spectra are shown as a representative of the triplicate, and the calibration curve as the average of the experiments. 127

Figure 14 – Effect of pH in EGFP relative fluorescence intensity (FI) (%) over time (48 h). EGFP (5.4 $\mu\text{g}.\text{mL}^{-1}$) in PBS 0 h is the reference for 100%. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. 132

Figure 15 – Effect of pH in EGFP relative fluorescence intensity (FI) (%) after 30 min exposure to different pH (black bars), followed by return to pH 7.4 for 60 min (red bars). EGFP (5.4 $\mu\text{g}.\text{mL}^{-1}$) in PBS 0 h is the reference for 100%. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. 134

Figure 16 – Emission spectra at λ_{ex} 488 nm **A)** after 30 min of EGFP (5.4 $\mu\text{g}.\text{mL}^{-1}$) in different pH and **B)** after 60 min return to pH 7.4. Excitation spectra at λ_{ex} 510 nm **C)** after 30 min of EGFP (5.4 $\mu\text{g}.\text{mL}^{-1}$) in different pH and **D)** after 60 min return to pH 7.4. Data presented in fluorescence intensity (FI) in unities of fluorescence (UF). 135

Figure 17 – Mean residue ellipticity ($[\theta]$) from the CD analysis of EGFP (0.3 $\text{mg}.\text{mL}^{-1}$, 1 mm cell) in PB of different pH after **A)** 30 min exposure to different pH and **B)** 30 min exposure to different pH + 60 min return to pH 7.4. Percentage (%) of secondary structures of EGFP (CD analysis in Dichroweb CONTIN-LL, set SP175) after **C)** 30 min exposure to different pH and **D)** 30 min exposure to different pH + 60 min return to pH 7.4. Samples were prepared in triplicate with a blank and presented as the average of the results. 136

Figure 18 – Emission spectra of fluorescence of tyrosine and tryptophan of EGFP (5.4 $\mu\text{g}.\text{mL}^{-1}$ of EGFP) at the excitation wavelength of 280 nm after **A)** 30 min exposure to different pH and **B)** 30 min exposure to different pH + 60 min return to pH 7.4. Data presented as fluorescence intensity (FI) in unities of fluorescence (UF). 138

Figure 19 – Well-plate SAXS of EGFP (2.5 $\text{mg}.\text{mL}^{-1}$) after **A)** 30 min exposure to different pH and **B)** 30 min exposure to different pH + 60 min return to pH 7.4. Results are presented in the log of intensity (Log I) and log of q ($\text{\AA}^{-1}$). 139

Figure 20 – SEC-SAXS results of all EGFP (5 mg.mL⁻¹) fractions from HPLC in function of time after 30 min at different pH. **A)** SEC-SAXS average intensity. **B)** Radius of gyration (Rg). **C)** Molecular weight (MW). **D)** UV from HPLC for SEC-SAXS at wavelengths (λ) **D.1)** 214 nm, **D.2)** 260 nm, **D.3)** 280 nm and **D.4)** 450 nm; results are presented as Absorbance (Absorbance unities, AU). Time was adjusted so the SEC-SAXS highest intensity peaks for EGFP at different pH matched, and the offset was applied for the other results. 141

Figure 21 – Analysis of the monomeric fraction of EGFP (5 mg.mL⁻¹) from SEC-SAXS. **A)** SEC-SAXS and Guinier plot of EGFP after 30 min exposure to different pH. Results are presented in the log of intensity (Log I) and log of q ($\text{\AA}^{-1}$) for SAXS and the natural log of the intensity of q [$\ln I(q)$] and q^2 ($\text{\AA}^{-1}$) for the Guinier plot. **B)** csp(r) of EGFP after 30 min at different pH. **C)** Radius of gyration (Rg) and images of monomeric EGFP structure after 30 min at different pH. Structures generated in SREFLEX using SEC-SAXS scattering and fittings to the EGFP PDB ID 2Y0G file. The top images are the lateral view of the EGFP structure and the bottom images are the overview. EGFP PDB ID 2y0G is shown in Green and refined fit to data shown in Cyan. 143

Figure 22 – Average Fluorescence Intensity [FI, $\times 10^3$ unities of fluorescence (UF)] of EGFP (5.4 $\mu\text{g.mL}^{-1}$) over pH. FI was acquired at EGFP peak (F1, λ_{ex} 488 nm, λ_{em} 510 nm) in triplicate with a blank. Results were fitted to a growth/sigmoidal dose-response model to calculate pKa. 145

Figure 23 – Effect of ionic strength (NaCl concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol.L⁻¹ and PBS as control) on EGFP Relative FI (%) over time (48 h). EGFP in PBS at 0 h is the reference for 100 %. Results were evaluated in triplicate with a blank and presented as the average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 148

Figure 24 – Effect of imidazolium ILs (concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol.L⁻¹) on EGFP Relative FI (%) over time (48 h). EGFP in PBS at 0 h is the reference for 100 %. PBS is presented as a control. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 149

Figure 25 – Effect of cholinium ILs and salts (concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol.L⁻¹) on EGFP Relative FI (%) over time (48 h). EGFP in PBS at 0 h is the reference for 100 %. PBS is presented as a control. Results were evaluated in triplicate with a blank and

presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 151

Figure 26 – EGFP Relative FI (%) for 90 days (3 months), at 25 °C, and absence of light, in the presence of aqueous solutions of imidazolium-based ILs (0.100 mol.L⁻¹) and buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) controls. The results were evaluated in triplicate with a blank sample and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 152

Figure 27 – EGFP Relative FI (%) for 90 days (3 months), at 25 °C and absence of light, in the presence of aqueous solutions of cholinium-based ILs (0.100 mol.L⁻¹) and buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) controls. The results were evaluated in triplicate with a blank sample and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 153

Figure 28 – Effect of temperature (25 to 90 °C) on EGFP Relative FI (%) over time (48 h). EGFP in PBS 0 h is the reference for 100 %. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 155

Figure 29 – Effect of chemical denaturing agents on EGFP Relative FI (%) over time (48 h). **A)** GuHCl (acid), **B)** Urea (base), **C)** H₂O₂ (oxidizing agent) and **D)** SDS (surfactant). EGFP in PBS 0 h is the reference for 100 %. PBS is presented as a control. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. 156

Figure 30 – Effect of **A)** imidazolium-based ILs and **B)** cholinium-based ILs and salts (0.100 mol.L⁻¹) on EGFP fluorescence at 2 h and 25 °C in the presence of the surfactant SDS (0.175 mol.L⁻¹). Data is presented as Relative FI (%) of EGFP. A buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) in the presence of SDS (0.175 mol.L⁻¹) are control groups. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. 158

Figure 31 – Effect of **A)** imidazolium-based ILs and **B)** cholinium-based ILs and salts (0.100 mol.L⁻¹) on EGFP fluorescence at 2 h and 25 °C in the presence of the acid GuHCl (4.000 mol.L⁻¹). Data is presented as Relative FI (%) of EGFP. A buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) in the presence of GuHCl (4.000 mol.L⁻¹) are control groups. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. 160

Figure 32 – Effect of **A)** imidazolium-based ILs and **B)** cholinium-based ILs and salts (0.100 mol.L^{-1}) on EGFP fluorescence at 2 h and 25°C in the presence of the oxidizing agent H_2O_2 (1.500 mol.L^{-1}). Data is presented as Relative FI (%) of EGFP. A buffer (PBS) and conventional salt ($\text{NaCl } 0.100 \text{ mol.L}^{-1}$) in the presence of H_2O_2 (1.500 mol.L^{-1}) are control groups. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. 161

Figure 33 – EGFP extraction efficiency [EE_{EGFP} (%)] for the: **A)** PEG-rich phase using ABS composed of 15 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%); and 20 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%); **B)** PEG-rich phase using ABS composed of 15 wt% PEG-1500 + 15 wt% $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$; 15 wt% PEG-2000 + 15 wt% $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$; **C)** [Ch]Cl-rich phase using ABS composed of PEG-600 (38, 40 or 42 wt%) + [Ch]Cl (38, 40 or 42 wt%) and PPG-400 (40, 45 or 50 wt%) + [Ch]Cl (10, 12 or 14 wt%) (concentration: polymer wt%/[Ch]Cl wt%). The results represent the average of three independent experiments $\pm$ standard deviation errors. 170

Figure 34 – EGFP mass balance [MB_{EGFP} (%)] for the: **A)** PEG-rich phase using ABS composed of 15 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%); and 20 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%); **B)** PEG-rich phase using ABS composed of 15 wt% PEG-1500 + 15 wt% $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$; 15 wt% PEG-2000 + 15 wt% $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$; **C)** [Ch]Cl-rich phase using ABS composed of PEG-600 (38, 40 or 42 wt%) + [Ch]Cl (38, 40 or 42 wt%) and PPG-400 (40, 45 or 50 wt%) + [Ch]Cl (10, 12 or 14 wt%) (concentration: polymer wt%/[Ch]Cl wt%). The results represent the average of three independent experiments $\pm$ standard deviation errors. 171

Figure 35 – EGFP back-extraction diagram representation showing the integration of a 1st purification using PEG-600/NaPA-8000 + [Ch]Cl-based ABS, followed by a 2nd purification step using a PEG-600/[Ch]Cl-based ABS. The EE_{EGFP} (%) and MB_{EGFP} (%) of each system are also presented. MB_{EGFP} (%) considering the overall back-extraction process. CP acronym corresponds to contaminant proteins. 175

Figure 36 – 3D Fluorescence spectra (units of fluorescence, UF) of **A)** EGFP raw extract and the EGFP-rich phase of the following ABS: **B)** PEG-600/NaPA-8000 + [Ch]Cl, **C)** PEG-1500 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, **D)** PEG-2000 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, **E)** PEG-600/[Ch]Cl, **F)** back-extraction and **G)** PPG-400/[Ch]Cl. All 3D fluorescence spectra correspond to a diluted sample with an EGFP concentration of $12.7 \pm 0.2 \text{ mg.L}^{-1}$. The 3D Fluorescence Spectra shows the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and

the intensity of fluorescence is given by the color scale from blue to red. The EGFP/TP is the ratio between the EGFP and total protein fluorescence intensities (UF). 176

Figure 37 – Relative concentration of EGFP by TP ($[EGFP]_{rel}$ (%)) for the EGFP raw extract and the EGFP-rich phases for the ABS composed of: 15 % PEG-600, 15 % NaPA-8000 + [Ch]Cl (1, 3 or 5%); 20 % PEG-600, 15 % NaPA-8000 + [Ch]Cl (1, 3 or 5%); 15 wt% PEG-1500 + 15 wt% K_2HPO_4/KH_2PO_4 ; 15 wt% PEG-1200 + 15 wt% K_2HPO_4/KH_2PO_4 ; [Ch]Cl/PEG-600, back-extraction and [Ch]Cl/PPG-400. The results are the average of three independent experiments $\pm$ standard deviation. 177

Figure 38 – SDS-PAGE electrophoresis of the EGFP extract and the ABS containing the same mass of EGFP in each well (15 μ g). Ladder (lane 1), EGFP raw extract (lane 2) and extracts after purification using the ABS: PEG-600/NaPA-8000 + [Ch]Cl (lane 3); PEG-600/[Ch]Cl (lane 4); back-extraction (lane 5); PPG-400/[Ch]Cl (lane 6); PEG-1500 + K_2HPO_4/KH_2PO_4 (lane 7); PEG-2000 + K_2HPO_4/KH_2PO_4 (lane 8). EGFP has 27 kDa and is represented by the strongest bands between 25-35 kDa. 179

Figure 39 – 3D Fluorescence spectra (units of fluorescence, UF) of **A)** YFP raw extract and **C)** RFP raw extract and FP-rich phase of the PEG-600/[Ch]Cl ABS for the purification of **B)** YFP and **D)** RFP. All 3D fluorescence spectra correspond to a diluted sample to present the same intensity of fluorescence in the peak of each FP (600 ± 8 UF). The 3D Fluorescence Spectra shows the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the intensity of fluorescence is given by the color scale from blue to red. The FP/TP relation (FP being YFP or RFP) is the ratio between the FP and total protein fluorescence intensities (UF). 183

Figure 40 – Diagram of the integrative process developed for the purification of EGFP using PPG-400/[Ch]Cl ABS and the recycling of the phase-forming agents using ultrafiltration. *Composition (wt%) of the new ABS after solvent recycling. 186

Figure 41 – Overview of the stages of the economic analysis of EGFP production and purification with ABS. 188

Figure 42 – Process diagram of the EGFP purification process using ABS in FCPC. 191

SUMMARY

CHAPTER 1.....	32
1 INTRODUCTION.....	33
1.1 GENERAL CONTEXT	33
1.2 MOTIVATIONS AND STRUCTURE FOR THE THESIS.....	37
1.3 FLUORESCENT PROTEINS (FP)	42
1.3.1 Enhanced Green Fluorescent Protein (EGFP)	45
1.3.1.1 EGFP specific properties and indexes	47
1.3.2 GFP behavior at different pH and its potential for the development of pH-biosensors	49
1.4 STABILITY OF GFP	51
1.4.1 Stabilization of proteins with ionic liquids.....	52
1.5 PURIFICATION OF GFP	56
1.5.1 Purification of biomolecules using liquid-liquid extraction.....	59
1.5.1.1 Liquid-liquid extraction.....	63
1.5.1.2 ABS for the purification of biomolecules	65
1.5.1.3 Trends and prospects for ABS for purification of biomolecules	76
1.6 BIOPHARMACEUTICALS: DEFINITIONS, PRODUCTION AND FUTURE OPPORTUNITIES.....	85
1.6.1 Biopharmaceuticals overview	85
1.6.2 Upstream processing of biopharmaceuticals	87
1.6.1 Future opportunities for biopharmaceuticals.....	94
2 OBJECTIVES	97
2.1 GENERAL OBJECTIVES	97
2.2 SPECIFIC OBJECTIVES	97
3 MATERIALS AND METHODS	99
3.1 RIGHTS AND PERMISSIONS	99
3.2 MATERIALS.....	99
3.2.1 Buffers	99
3.2.2 ILs and salts	100
3.2.3 Denaturing agents.....	101
3.2.4 Polymers	102
3.2.5 Proteins and protein standards	102

3.2.6	Other materials	103
3.3	PROTOCOLS	103
3.3.1	Protocols for the “EGFP fluorescence properties and structure” study ...	103
3.3.1.1	<i>EGFP absorbance and fluorescence analysis</i>	103
3.3.1.2	<i>3D spectra of EGFP standard and BSA</i>	103
3.3.1.3	<i>Effect of concentration, pH, and high temperature in EGFP peaks</i>	103
3.3.1.4	<i>Calibration curves and analytical parameters for F1 and F2</i>	104
3.3.2	Protocols for the “EGFP activity and structure at different pH” study ...	105
3.3.2.1	<i>EGFP fluorescence activity in pH over time</i>	105
3.3.2.2	<i>EGFP fluorescence activity and structural analysis at different pH</i>	105
3.3.2.3	<i>EGFP fluorescence activity relationship with pH</i>	106
3.3.3	Protocols for the “Effect of ionic liquids on EGFP activity” study	106
3.3.3.1.	<i>Effect of different concentrations of aqueous solutions of salts and ILs on EGFP fluorescence</i>	106
3.3.3.2.	<i>Effect of physical and chemical stress on EGFP fluorescence</i>	106
3.3.3.3.	<i>Protective/destabilizing effect of ILs on EGFP fluorescence under denaturation by chemical agents</i>	107
3.3.4	Protocols for the “Purification of EGFP using aqueous biphasic systems” study	107
3.3.4.1	<i>EGFP expression</i>	107
3.3.4.2	<i>Quantification of EGFP and total protein</i>	108
3.3.4.3	<i>Extraction and purification of EGFP</i>	109
3.3.4.4	<i>Determination of relative hydrophobicity of proteins</i>	111
3.3.4.5	<i>Back-extraction of EGFP</i>	111
3.3.4.6	<i>Extraction and purification of other recombinant fluorescent proteins produced by E. coli using PPG-400/[Ch]Cl ABS</i>	111
3.3.4.7	<i>Solvent recycling and EGFP polishing</i>	112
3.3.4.8	<i>Physicochemical characterization of ABS coexisting phases</i>	113
3.3.4.9	<i>Evaluation of EGFP purification by SDS-PAGE</i>	113
3.4	INSTRUMENTAL METHODS	114
3.4.1	Fluorescence analysis	114
3.4.1.1	<i>3D fluorescence spectroscopy</i>	114
3.4.1.2	<i>Single point fluorescence</i>	114
3.4.1.3	<i>Intrinsic fluorescence of proteins (IFP)</i>	115

3.4.2	Physical-chemical analysis.....	115
3.4.2.1	<i>pH and conductivity calibration and determination</i>	<i>115</i>
3.4.2.2	<i>Density and viscosity determination</i>	<i>115</i>
3.4.2.3	<i>Water content measurement with Karl-Fischer titration</i>	<i>115</i>
3.4.2.4	<i>Water content measurement with a gravimetric method.....</i>	<i>116</i>
3.4.3	Circular Dichroism (CD) analysis.....	116
3.4.4	Small-Angle X-Ray Scattering (SAXS)	117
3.4.5	FTIR-ATR analysis	118
3.4.6	SDS-PAGE	118
4	RESULTS AND DISCUSSION.....	119
4.1	EGFP PRODUCTION	119
4.2	EGFP FLUORESCENCE PROPERTIES AND STRUCTURE	121
4.3	EGFP ACTIVITY AND STRUCTURE AT DIFFERENT PH.....	132
4.3.1	Fluorescence activity of EGFP in the function of pH and time.....	132
4.3.2	Study of the reversibility of fluorescence activity of EGFP in pH	133
4.3.3	Structural alterations of EGFP in the function of pH.....	136
4.3.4	Impacts of pH on the activity and structure of EGFP	144
4.3.5	EGFP as analytical neutral to acidic pH-biosensors	145
4.4	EFFECT OF IONIC LIQUIDS ON EGFP ACTIVITY	147
4.4.1	Short-term effect of aqueous solutions of ILs on EGFP fluorescence	147
4.4.2	Long-term effect of aqueous solutions of ILs on EGFP fluorescence.....	152
4.4.3	Effect of physical and chemical stress on EGFP fluorescence.....	154
4.4.4	Effect of ILs solutions on EGFP fluorescence under chemical stress conditions	157
4.5	PURIFICATION OF EGFP USING AQUEOUS BIPHASIC SYSTEMS	165
4.5.1	EGFP extraction	165
4.5.2	Back-extraction of EGFP	174
4.5.3	Purification of EGFP.....	175
4.5.4	Recycling of the ABS phase-former components and EGFP polishing.....	183
4.6	ECONOMIC ANALYSIS OF EGFP PRODUCTION AND PURIFICATION WITH ABS	188
4.7	SCALE-UP OF EGFP PURIFICATION WITH ABS	190
4.7.1	Fast Centrifugal Partition Chromatography (FCPC) for scale-up of EGFP purification.....	190

4.7.2	Oscillatory Flow Reactor (OFR) for scale-up of EGFP purification.....	192
5	CONCLUSIONS	194
6	REFERENCES.....	197
	CHAPTER 2.....	233
	LIST OF PUBLICATIONS	234
	APPENDIX	244
	APPENDIX A – EGFP INFORMATION	245
	APPENDIX B – CALIBRATION CURVES	247
	APPENDIX C – SAMPLES AND PARAMETERS DETAILS FOR EGFP AT DIFFERENT PH STUDIES.....	249
	APPENDIX D – 3D FLUORESCENCE SPECTRA OF BSA AND EGFP	253
	APPENDIX E – FTIR-ATR ANALYSIS OF ABS PHASES.....	254
	APPENDIX F – 3D FLUORESCENCE SPECTRA ANALYSIS OF EGFP STABILITY IN PH.....	258
	APPENDIX G – CIRCULAR DICHROISM ANALYSIS	260
	APPENDIX H – PHYSICAL-CHEMICAL PROPERTIES OF SOLUTIONS OF SALTS, ILS, AND DENATURING AGENTS.....	261
	ANNEX	263
	ANNEX I – PHASE DIAGRAM DETERMINATION	264

CHAPTER 1

1 INTRODUCTION

1.1 GENERAL CONTEXT

Advances in biotechnology have allowed the development of many novel biomolecules (particularly recombinant proteins) with medical and industrial applications. Nevertheless, despite their great potential, these biological products are still inaccessible to many disadvantaged communities and low-income countries (FERRARI, 2019; OLIVEIRA *et al.*, 2015) primarily due to their low stability (which hinders their distribution, storage, and handling) (PATEL *et al.*, 2011) and their very high prices (mainly caused by intricate and costly production and purification processes). (DÁVILA, 2011) This issue is especially crucial for vaccines and other biopharmaceuticals, considering their fundamental role in preventing and treating life-threatening diseases. (LAGE, 2007) For example, according to the World Health Organization (WHO), vaccines already save 2 to 3 million lives every year, but vaccination coverage expansion could save an extra 1.5 million. (WHO, 2020) Thus, a better understanding of the properties and behavior of biopharmaceuticals, allied with the development of sustainable platforms for their stabilization and purification, can contribute to democratize access to these products and improve the health of millions of people. (HUMPHREYS, 2011; ZAFFRAN *et al.*, 2013) In this context, Enhanced Green Fluorescent Protein (EGFP, a biomarker and biosensor) was selected as a case of study to increase the understanding of protein properties and stability, as well as for the development of novel and more sustainable platforms for protein stabilization and purification.

Proteins are remarkably sophisticated biological structures that, in addition to their complex organization, are flexible and can be easily rearranged according to the conditions of the surrounding environment. (EBELING *et al.*, 1987; MONGAN; CASE, 2005; SMELLER, 2002) Although there are thousands of studies on protein composition, arrangement, and behavior, scientists are still scratching the surface of this topic. There are more than 340 million proteins registered in the non-redundant database of UniProt (UniParc), (UNIPROT, 2020) and 20,000 different proteins in the human body alone. (PONOMARENKO *et al.*, 2016) Impressively, this estimate considers unique proteins since a single yeast cell can contain 42 million of them. (HO *et al.*, 2018)

In addition to its great diversity, every protein is also an intricate system by itself. The structure of a protein is composed of a chain of amino acids in a three-dimensional arrangement,

divided into four organizational levels. (SCHULZ; SCHIRMER, 2013) The primary structure is the amino acid chain, the secondary comprehends the interactions of polypeptide chains (*e.g.*, α -helix, β -sheets, coils), the tertiary corresponds to the overall three-dimensional folding of the protein structure, and the quaternary describes the packing of different subunits of proteins formed by multiple polypeptide chains. (SCHULZ; SCHIRMER, 2013) The arrangement of these structures is not static, and a plethora of conditions [*e.g.*, potential of hydrogen (pH), temperature, pressure, ionic strength, molecular interactions, and presence of chemical compounds] can alter them, reversibly or irreversibly. (MANNING *et al.*, 2010) Considering that the activity of proteins, like of many other biomolecules, is intrinsically dependent on the integrity of their structure, a deeper understanding of the properties, structure, behavior, and stability of proteins is essential for their effective and safe use. (MANNING *et al.*, 2010) Notably, the stability (and sometimes enhancement) studies are fundamental not only for developing biomolecules-based applications but also for envisaging their industrial manufacturing. Additionally, this will impact the viability of their transport and handling on a large scale. Hence, one of the goals of this research is to provide novel insights into the characteristics, behavior, and stability of proteins.

Considering the low stability of proteins, especially their weak resistance to different temperatures, it is usually necessary to establish a complex cold-chain to transport, store, and handle them. Cities and rural settlements distant from large urban centers in low-income countries (or with limited access to the electrical grid) do not have the minimal conditions to guarantee the refrigeration of protein-based food and pharmaceuticals during their transport and storage. (HUMPHREYS, 2011; ZAFFRAN *et al.*, 2013) For example, this issue limits the access of these disadvantaged communities to essential biopharmaceuticals, such as vaccines and insulin, generating critical Public Health concerns. In this sense, improvements in the stability of bioformulations can help to alleviate this matter by increasing production yields through reducing losses in their processing and by facilitating their transport and storage. (MANNING *et al.*, 1989, 2010) Besides, this could potentially allow the creation of new uses for protein-based products, considering that their instability under stress conditions (such as acidic and basic pH environments, high temperatures, and presence of denaturing substances) is a considerable barrier for disruptive applications. (MANNING *et al.*, 1989, 2010)

Among the substances with the potential to stabilize protein-based products and other biomolecules, compounds such as ionic liquids (ILs) are promising candidates, especially considering some of them have appropriate characteristics for clinical and food applications.

(KUNZ; HÄCKL, 2016) For example, it is possible to design ILs to present useful properties for pharmaceutical applications, viz., biocompatibility with cells and biodegradability, high thermal stability, the capacity to solvate a wide range of compounds, and water solubility. (FREIRE, 2016; KUNZ; HÄCKL, 2016) Previous studies have already demonstrated the capacity of different ILs to stabilize proteins, including recent works of our research group. (MAGRI *et al.*, 2019; NASCIMENTO *et al.*, 2019b, 2019a) Based on previous findings, one of the main aims of this Thesis project was to explore the application of IL aqueous solutions for the stabilization of EGFP as a proof-of-concept, envisaging the future use of ILs as protein stabilizers.

Regarding the cost of bioproducts for therapeutic and diagnostic purposes, there is a deep association between their steep prices and the high degree of purity needed for their commercialization, which usually requires a costly and long chain of operations. (GRONEMEYER *et al.*, 2014) Biomolecules for commercial applications, particularly for medical uses, demand high purification and multi-step processing after their production (*i.e.*, biosynthesis) to recover, concentrate, purify and formulate the final bioproducts. This sequence of operations, called downstream processing, generally includes the most complex and expensive stages in the manufacture of biological products. (GRONEMEYER *et al.*, 2014) During the downstream processing of recombinant proteins, besides removing microorganisms, cell debris, host cell proteins, DNA, endotoxins, and viruses, there are also product-related impurities, like misfolded or aggregated proteins, which are very similar to the target molecule and need to be carefully separated. (HANKE; OTTENS, 2014) Therefore, there is the challenge of removing protein aggregates and degraded compounds, which are particularly dangerous for biotherapeutics since they can generate improper immunogenic reactions. (GUIOCHON; BEAVER, 2011) Hence, it is usually necessary to completely purify the target biomolecules to minimize risks. That is why the purity requirements of bioproducts for biological and medical use can be above 99 %, (KALYANPUR, 2002) levels that dramatically increase the difficulties, stages, and costs of downstream processing. For example, specifically for biopharmaceuticals (which require the highest purity degrees), downstream processing can correspond to 80 % of the total manufacturing costs. (WALSH, 2010) Therefore, when dealing with the reduction of total production expenses, the choice and design of extraction and purification stages are critical.

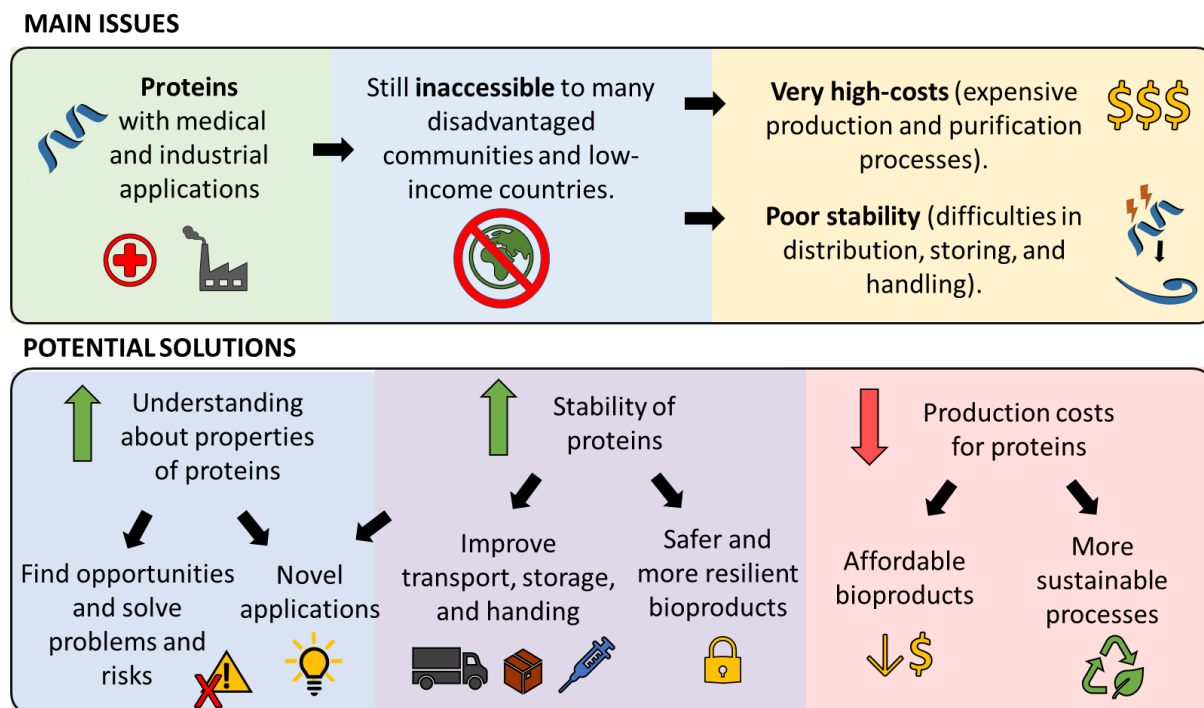
The design of simpler purification methods can help to decrease the costs and complexity of the downstream processing of biomolecules. In this sense, liquid-liquid

extraction systems (LLE) have great potential to improve the manufacturing of biomolecules because the traditional pharmaceutical industry already applies LLE very successfully. However, as the researchers designed the conventional LLE operations to extract small organic molecules (which are simpler and more resistant than macromolecules), unfortunately, most of the traditional units are not compatible with the usually unstable protein-based products. In this regard, the development of milder LLE, like Aqueous Biphasic Systems (ABS), is essential, as these offer a separation environment rich in water and very biocompatible with biologically active substances and cells (*i.e.*, protein-friendly environment). (BANIK *et al.*, 2003; DOS SANTOS *et al.*, 2018a)

ABS are mixtures of at least two different compounds (*e.g.*, polymers, salts, surfactants, amino acids) in water, which separate into two water-rich phases above specific concentrations and under certain conditions (*e.g.*, temperature and pH). (FREIRE *et al.*, 2012; PEREIRA *et al.*, 2013a; ROSA *et al.*, 2010) Depending on their phase-forming components, ABS can allow the selective partition of solutes between each of the coexisting phases, which leads to the separation and the purification of water-soluble solutes. (SANTOS *et al.*, 2011; WOLSKI *et al.*, 2011) Additionally, these systems have low costs, reduced energetic and operational demands, are also easier to be scaled-up and integrated with other downstream units, in comparison with the other conventional techniques (*e.g.*, chromatography and filtration). (FREIRE, 2016) Considering that ABS are adequate alternatives to concentrate and purify biomolecules, as well as the possibility of designing ABS-based platforms to be cheaper and sustainable, another main goal of this Thesis project was to evaluate the potential of ABS composed of ILs, salts, and polymers as environmentally-friendly, and low-cost platforms for the purification of EGFP.

To elucidate the main issues and potential solutions for expanding the large-scale access to proteins of commercial interest, **Figure 1** visually schematized the fundamental topics introduced above.

Figure 1 – Schematic representation of the main issues and potential solutions for expanding the large-scale access to proteins of commercial interest.



Source: Produced by the author.

As presented in **Figure 1**, a broader understanding of protein properties, the increase of their stability, and the reduction of their production costs are critical to create innovative applications for protein-based products, improve their transport, storage, and handling, and generate safer, more affordable, and resilient bioproducts through sustainable processes.

1.2 MOTIVATIONS AND STRUCTURE FOR THE THESIS

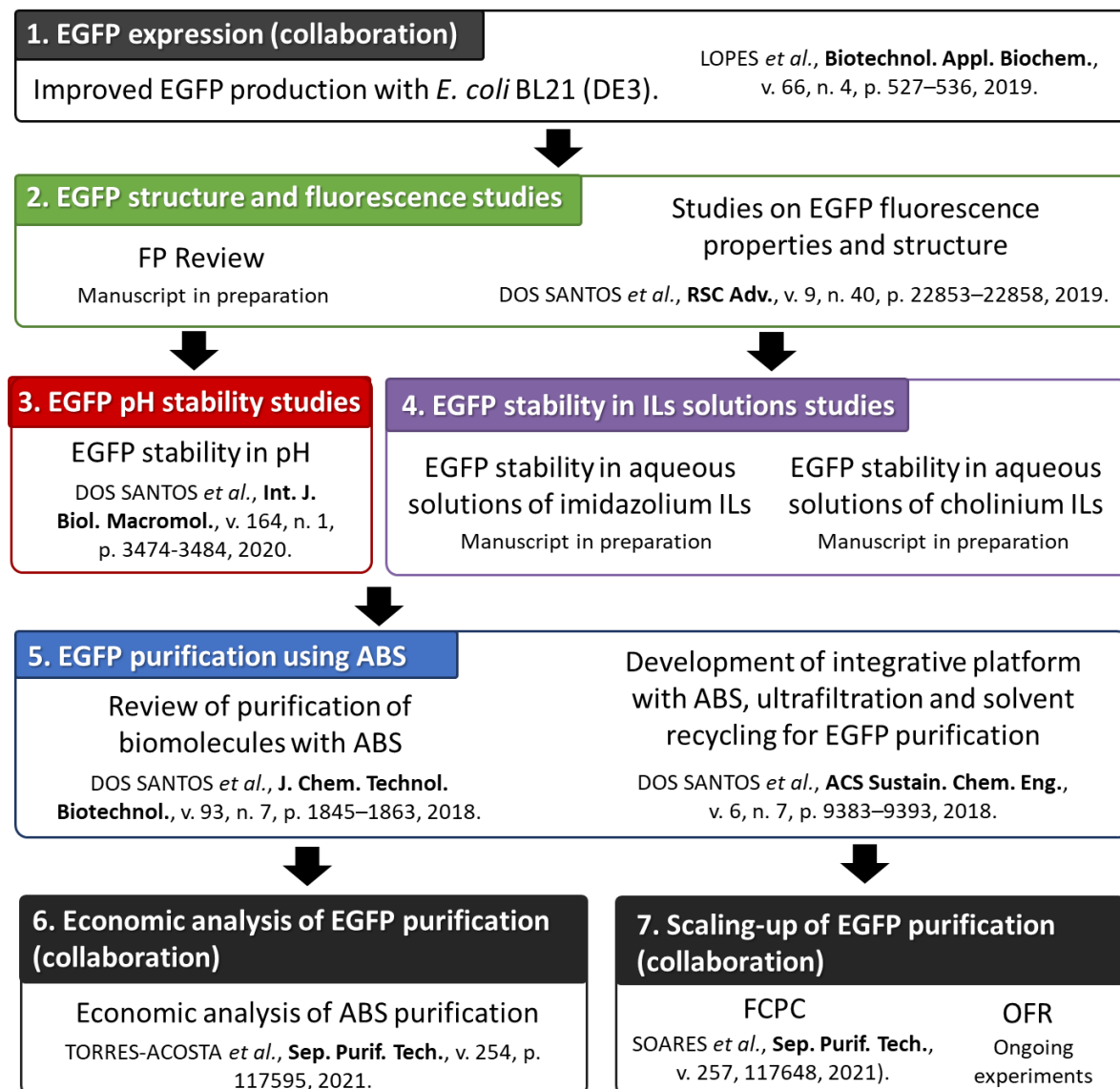
In light of the importance and applications of stabilizing bioformulations and lowering the cost of purification platforms for proteins, this work studied the Enhanced Green Fluorescent Protein (EGFP) as a proof-of-concept for developing novel platforms to stabilize and purify proteins. EGFP is a protein with an intense and natural fluorescence, which facilitates its monitoring and quantification. (ZIMMER, 2002) Although there are already applications of EGFP as a biomarker and biosensor, with uses in the medical and biological fields, further studies regarding its structure, properties, stabilization, and purification will contribute to expanding the range of commercial applications.

Considering the previous assumptions, the main motivations of this work were: I) to characterize and study in-depth the EGFP structure, properties, and stability at different environments and stress conditions; and II) to develop novel sustainable stabilization and

purification platforms for EGFP using aqueous solutions and ABS composed of ILs, organic salts, and polymers, components which are environmentally-friendly and biocompatible with the target-macromolecule (*i.e.*, protein-friendly).

To contextualize the scope of this project, it is noteworthy to mention that this research was part of two broader international joint-projects, namely: 1) “Optimization and scale-up of novel ionic-liquid-based purification processes for recombinant Green Fluorescent Protein produced by *Escherichia coli* ‘GFPurIL’” co-funded by “*Fundação de Amparo à Pesquisa do Estado de São Paulo*” (FAPESP, Brazil) and “*Fundação para a Ciência e Tecnologia de Portugal*” (FCT), FAPESP 2014/19793-3, in partnership with the University of Aveiro; and 2) “Understanding the molecular interactions between ionic liquids and biopharmaceuticals: the key for a proper design of downstream processing” co-funded by FAPESP and Australian Technology Network of Universities (ATN), FAPESP 2018/50009-8, in collaboration with the Royal Melbourne Institute of Technology (RMIT University) and ANSTO (Australia's Nuclear Science and Technology Organization) - The Australian Synchrotron (ANSTO-AS, Proposal M14460). Therefore, to elucidate the structure of this Thesis and the association with the joint-projects, **Figure 2** depicts a schematic representation of the research phases, objectives, and results. This scheme presents the work according to the different experimental research stages, its organization, and corresponding main outputs (*i.e.*, published articles or manuscripts in preparation). This Thesis document will extensively cover the results from stages 2, 3, 4, and 5, in which the student was fully involved [*i.e.*, N. V. dos Santos obtained all results and published (or will publish) the findings in international journals as the first author]. On the other hand, the results obtained from the collaborative works, specifically from stages 1, 6 and 7, will only be briefly summarized through this Thesis document, because although the scientific findings are directly related to this research, they also include experimental results that were not obtained exclusively by Nathalia, and in which she participates as co-author of the manuscripts.

Figure 2 – Schematic representation of the research structure and achievements of the Thesis project, grouped by experimental stages.



Source: Produced by the author.

1. EGFP expression (collaboration): In the first stage of the project, the group improved the EGFP expression in recombinant *E. coli* BL21 (DE3) by increasing the EGFP production yields while reducing the concentration of the costly inducer used in the process. The research group published these results in **Biotechnology and Applied Biochemistry** (DOI: [10.1002/bab.1749](https://doi.org/10.1002/bab.1749)) (LOPES *et al.*, 2019). This work was the initial stage of the Thesis project, considering the protocol to produce EGFP from the article was subsequently applied (with slight modifications) during all the experimental research.

2. EGFP structure and fluorescence studies: The second stage of the work started with a state-of-art on the characterization and understanding of fluorescent proteins (FP) structure and

properties. A critical review regarding FP origins, properties, and stabilities is under preparation, which also includes the use of bioinformatics tools to evaluate and compare the physical-chemical characteristics and stabilities of the 60 FP variants most applied in research. (DOS SANTOS *et al.*, 2020a) Afterward, an experimental study on EGFP fluorescent properties revealed a novel fluorescent peak (from another protonated state) for EGFP and solved an academic controversy for this protein (published in **RSC Advances**, DOI: [10.1039/C9RA02567G](https://doi.org/10.1039/C9RA02567G)). (DOS SANTOS *et al.*, 2019) The student obtained part of the results from stages 2, 3, and 4 during the one-year Ph.D. internship at RMIT University (Melbourne, Australia).

3. EGFP pH stability studies: The third stage focused on studies of EGFP fluorescence activity and structure at different pH, to understand the reversibility of the pH-based quenching and dequenching of EGFP fluorescence in acidic and alkaline environments. Despite previous studies on EGFP behavior at different pH, this topic required a scientific update considering the elucidation of the crystalline structure of EGFP in 2011, which demonstrated that are two protonated states for this protein instead of one, as was previously reported. This study also provided new insights into the development of EGFP pH-biosensors. The group recently published the results from stage 3 in the **International Journal of Biological Macromolecules** (DOI: [10.1016/j.ijbiomac.2020.08.224](https://doi.org/10.1016/j.ijbiomac.2020.08.224)) (DOS SANTOS *et al.*, 2020d).

4. EGFP stability in ILs solutions studies: Stage 4 of the project focused on stability studies of EGFP using ILs solutions under different stress conditions, aiming to find chemical stabilizers with the ability to protect biopharmaceuticals under stress environments, as well as to increase protein stability at room temperature. This stage also intended to provide further insights about which ILs have more potential to act as additives for the development of more stable biopharmaceutical formulations. The student is preparing two manuscripts to compile all novel findings for the use of imidazolium- and cholinium-based ILs as EGFP stabilizers, respectively. (DOS SANTOS *et al.*, 2020b, 2020c)

5. EGFP purification using ABS: In the fifth stage, as a result of an in-depth analysis of the state-of-art, a critical review on the purification of biopharmaceuticals using LLE technologies, including ABS, was written and published in the **Journal of Chemical Technology & Biotechnology** (DOI: [10.1002/jctb.5476](https://doi.org/10.1002/jctb.5476)). (DOS SANTOS *et al.*, 2018a) This review, together with the stability studies of EGFP in ILs solutions (from stage 4), were crucial for the selection of the most promising ABS compositions for the purification of EGFP. In an original study in collaboration with the University of Aveiro research team, a novel and sustainable purification

platform for EGFP using ABS and ultrafiltration was developed, with the respective results published in **ACS Sustainable Chemistry & Engineering** (DOI: [10.1021/acssuschemeng.8b01730](https://doi.org/10.1021/acssuschemeng.8b01730)). (DOS SANTOS *et al.*, 2018b) To confirm the cost-effectiveness and viability of scale-up of the ABS-platform for the EGFP purification, the research group performed additional studies in collaboration with other universities, as described in stages 6 and 7.

6. Economic analysis of EGFP purification (collaboration): The sixth stage aimed to confirm the economic feasibility of ABS-based platforms for the purification of EGFP. Through collaborative work with *Tecnológico de Monterrey*, University College London, and University of Aveiro, the joint team carried out an economic analysis of all ABS used for EGFP purification (*i.e.*, experimentally developed in stage 5). The research group recently published this work in **Separation and Purification Technology** (DOI: [10.1016/j.seppur.2020.117595](https://doi.org/10.1016/j.seppur.2020.117595)). (TORRES-ACOSTA *et al.*, 2021)

7. Scaling-up of EGFP purification (collaboration): Stage 7 aimed to confirm the industrial feasibility of large-scale and commercial technologies for the purification of EGFP using ABS. Through collaborative work with University of Aveiro and the University of Porto, it was possible to test the scaling-up of some ABS using Fast Centrifugal Partition Chromatography (FCPC) and Oscillatory Flow-Reactor (OFR), respectively. The FCPC results for the EGFP purification were promising, as detailed in the article published in **Separation and Purification Technology** (DOI: [10.1016/j.seppur.2020.117648](https://doi.org/10.1016/j.seppur.2020.117648)). (SOARES *et al.*, 2021) On the other hand, despite the higher purification yields of EGFP obtained in the OFR, further improvements of the technology are still required for the industrial application of this system (this is still a work in progress and unpublished).

The next subsections of the “Introduction” will provide deeper insights and the current state-of-the-art regarding the following subject: EGFP and other FP properties and applications; the stability of proteins and potential protein stabilizing agents; the purification platforms for proteins and other biomolecules currently available; and novel sustainable and lower-cost alternatives available for extraction and purification of protein-based products. Additionally, envisaging future applications of the technologies developed in this research for biopharmaceuticals, the last section of the introduction will provide an overview on biopharmaceuticals definitions and production, while also designing a prospective regarding imminent opportunities in the field.

The “Objectives” section will list the specific goals for the eight different stages of the project. The “Materials and Methods” will give a detailed account of all the consumables, equipment, and techniques applied during this research (particularly for stages 2, 3, 4, and 5). This section was divided into the subsections “Rights and permissions” (reporting the required authorizations to reproduce the content of the published articles), “Materials” (listing all the consumables, their suppliers, and purities), “Protocols” (describing the methods for stages 2, 3, 4, and 5, individually), and “Instrumental Methods” (detailing technical specifications and procedures for all equipment and analytical methods). Additionally, the “Results and Discussion” will have subdivisions for each of the eight research stages, presenting a summary of the findings from the collaborative works (stages 1, 6, 7, and 8), and the complete results from the published articles or manuscripts in preparation from which Nathalia V. dos Santos is the first author (stages 2, 3, 4, and 5). Lastly, the “Conclusions” section will present the final remarks regarding the overall discoveries and achievements of this research.

As for the layout and references of this Thesis, this work was formatted according to the Brazilian Association of Technical Standards (ABNT) rules in force, particularly NBR 14724:2011 (“Information and documentation – Academic work – Presentation”) and associated norms (listed in the ABNT catalog of reference). (ABNT, 2020)

The next subsection will initiate the literature review by exploring the origins, properties, and applications of FP, while also highlighting the gaps in the current knowledge regarding these proteins and their potential for novel applications.

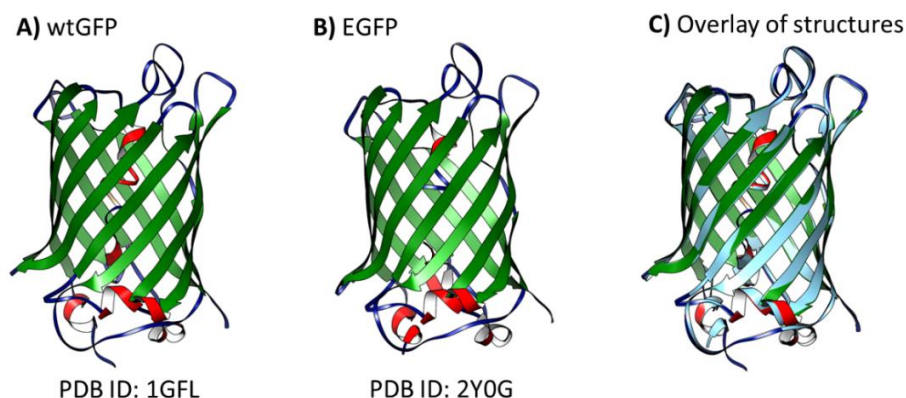
1.3 FLUORESCENT PROTEINS (FP)

FP are naturally fluorescent biomolecules (TSIEN, 1998) with several applications as biomarkers and biosensors (POPPENBORG *et al.*, 1997) such as monitoring of intracellular pH, (NAKABAYASHI *et al.*, 2008) fusion protein in the quantification of cells, chemical reactions, and cellular components, (MARCH *et al.*, 2003) whole-body imaging, (YANG *et al.*, 2000), among others. (MARCH *et al.*, 2003; ZIMMER, 2002) Because of this wide range of functions and the importance of FP for the scientific community, Osamu Shimomura, Marty Chalfie, and Roger Tsien received the Nobel Prize in Chemistry in 2008 for their contributions to FP discovery and development of applications. (NOBEL FOUNDATION, 2008) Although the previous recognition of the importance of FP, further developments in discovering novel research and industrial uses, as well as the understanding of FP origins, properties, structure, and stability is still required.

The first FP, known as wild-type Green Fluorescent Protein (wtGFP), was isolated from the jellyfish *Aequorea victoria* (TSIEN, 1998) and later expressed in a series of organisms [*e.g.*, *Escherichia coli* (*E. coli*), *Caenorhabditis elegans*] after the isolation of its genetic sequence. (POPPENBORG *et al.*, 1997) This scientific discovery initiated a series of works in which wtGFP was used as an intracellular biomarker, propitiated by its intense and natural fluorescence, a low impact on the activity of cells, and the possibility of expression together or fused with endogenous proteins. (ZIMMER, 2002) These properties favored wtGFP biological applications over more traditional synthetic dyes, considering their higher toxicity (LUTTY, 1978) and the difficulties of fusing them with cellular proteins. (ZIMMER, 2002) However, despite the advantages of wtGFP over synthetic compounds, there were aspects of the wtGFP structure that hindered its widespread use in biological systems. (ZIMMER, 2002)

In its native form, the structure of wtGFP comprises a β -barrel scaffold (with 11 β -strands and one central α -helix), which holds the fluorophore inside its frame (ENOKI *et al.*, 2004) as shown in **Figure 3.A**. An autocatalytic cyclization of the tripeptide –Ser65-Tyr66-Gly67 is responsible for forming the fluorophore. This structure confers two peaks of fluorescence with maximum intensity at the excitation wavelengths (λ_{ex}) of 395 nm for the protonated state of the protein (higher intensity peak) and 475 nm for its unprotonated state (lower intensity peak), with a corresponding emission wavelength (λ_{em}) at 508 nm. (YANG *et al.*, 1996) Unfortunately, because of the low fluorescent intensity when excited at higher wavelengths (λ), the range of biological applications of wtGFP as a biomarker and biosensor was limited. This issue is due to longer λ being less energetic than shorter λ , and therefore, causing less damage to biological samples, the reason why they are more appropriate for experiments with organisms. (WARD *et al.*, 1982; ZIMMER, 2002) Additionally, because the wtGFP comes from jellyfish of cold oceans, its folding above 20 °C was inefficient. (WARD *et al.*, 1982) Unfortunately, this temperature is considerably below the optimal temperature for the most common cultures used in research, like *E. coli*, yeast, and mammalian cells (FARRELL; ROSE, 1967; MASTERTON; SMALES, 2014)], and thus, restricting more the wtGFP biomarker applications.

Figure 3 –**A)** Representation of the crystalline structure of the *Aequorea victoria* by *E. coli*. **B)** Representation of the crystalline structure of EGFP expressed by *E. coli*. The images present helices in red, strands in green, and coils in blue. **C)** Overlay of wtGFP structure (color) and EGFP (gray).



Source: PDB ID: 1GFL (YANG *et al.*, 1996) and PDB ID: 2Y0G. (ROYANT; NOIRCLERC-SAVOYE, 2011) The author produced the images with the PDB structures using UCSF Chimera 1.14. (BERMAN *et al.*, 2002; PETTERSEN *et al.*, 2004)

Despite the advantages and potential of wtGFP, the two abovementioned issues barred many of its possible applications as biomarkers and biosensors in biological systems. Hence, to overcome these limitations, researchers developed recombinant variants of GFP with diverse characteristics and properties. One of the main representative variants is the EGFP [mutations F64L/S65T, (CORMACK *et al.*, 1996) structure shown in **Figure 3.B**], which included modifications that improved folding above 20 °C and high fluorescence intensity when excited at higher wavelengths (TSIEN, 1998; ZIMMER, 2002). As depicted in **Figure 3.C**, where it is possible to see the minor differences between the structures of EGFP and wtGFP, despite greater improvements in folding and fluorescence activity, the structural changes mostly comprised small alterations in the position of coils and helix in the extremities of the protein. On the other hand, it is also important to highlight that the isolation of FP from other organisms, such as the *Discosoma* Red Fluorescent Protein (DsRed or RFP) from the *Discosoma* sp., also helped the development of other FP-based biomarkers and biosensors. (CHUDAKOV *et al.*, 2010; MATZ *et al.*, 1999)

After 60 years since Shimomura isolated the first FP (*i.e.*, 1960), (SHIMOMURA, 2009) there are already 737 FP/chromoproteins registered in the FPbase (FP Database), creating a “colorful toolbox” for the development of biological sensors and markers. (LAMBERT, 2020, 2019; RODRIGUEZ *et al.*, 2017) The proteins registered in the FPbase already cover an almost complete range of the ultraviolet-visible (UV-vis) spectra. The importance of these FP can also be confirmed by the plethora of academic studies [1,720,000 results in Google Academic for

“fluorescent protein” (GOOGLE SCHOLAR, 2020)] and patents [29,610 results in Google Patents for “fluorescent protein” (GOOGLE PATENTS, 2020)].

Even considering the large FPbase currently available, EGFP is still one of the most applied FP in research, mainly because of its intense green fluorescence, considerable stability, and compatibility with many biological systems. (ABE *et al.*, 2011; DING *et al.*, 2016; LOPES *et al.*, 2019) Together these advantageous properties turn the EGFP the ideal FP to be studied as a case-of-study in the Ph.D. project. However, despite its widespread use and amount of fundamental studies, at the beginning of this project, it was observed that there are many aspects regarding EGFP properties, structure, and stability that still require additional insights. The next subsection of this Thesis will further explore this topic.

1.3.1 Enhanced Green Fluorescent Protein (EGFP)

Section adapted from the original article published in RSC Advances, v. 9, n. 40, 22853-22858, 2019. (DOS SANTOS et al., 2019)

Despite being one of the most popular FP, there are still many unknown aspects regarding EGFP structure and fluorescence properties. For example, initial reports stated that EGFP presented a single state (deprotonated) and fluorescence peak as a result of the S65T mutation present in this variant that favors this state. (ARPINO *et al.*, 2012; HEIM *et al.*, 1995) However, the determination of its crystalline structure showed the existence of two states for the protein (protonated and deprotonated), (ARPINO *et al.*, 2012; ROYANT; NOIRCLERC-SAVOYE, 2011) creating additional questions regarding the relationship between EGFP structure and spectral characteristics. This controversy also had implications for previous studies regarding EGFP behavior at different pH, considering preceding discussions revolved around the existence of only the deprotonated state or did not address the structural effects of pH on the protein. (CAMPBELL; CHOY, 2001; KRASOWSKA *et al.*, 2010; PATTERSON *et al.*, 1997) Further studies to elucidate EGFP fluorescence properties, structure, and stability are, thus, essential to update the knowledge on this protein and to improve and facilitate its applications as a biosensor and biomarker.

As previously stated, the first fluorescence studies on EGFP suggested that the protein had a single state and fluorescence peak, in contrast to what is known for wtGFP, which presented protonated and unprotonated states with distinct conformations and fluorescence peaks. (JAIN *et al.*, 2004; TSIEN, 1998) Nonetheless, after the experimental determination of the crystalline structure of EGFP, multiple groups reported two different states for the protein.

(ARPINO *et al.*, 2012; ROYANT; NOIRCLERC-SAVOYE, 2011) This new information fomented questions regarding the relationship between the existence of the two protonation states of EGFP and respective spectral characteristics.

Considering fluorescence is usually extremely sensitive to changes in the fluorophore conformation or its microenvironment, (LADOKHIN, 2006) the first study determining the EGFP structure reported that the presence of two crystalline states was an “*apparent contradiction with the single fluorescence lifetime of the protein.*” (ROYANT; NOIRCLERC-SAVOYE, 2011) Royant and Noirclerc-Savoie explained this phenomenon as a stabilizing effect of the Glu22 residue over the chromophore, which supposedly maintained its structure despite the conformational changes of the protein (with Arpino *et al.* also reporting this effect). (ARPINO *et al.*, 2012) However, the multiple fluorescence studies that reported the existence of only a single fluorescence peak for EGFP (ARPINO *et al.*, 2012; CORMACK *et al.*, 1996; PATTERSON *et al.*, 1997; ROYANT; NOIRCLERC-SAVOYE, 2011) only evaluated longer excitation wavelengths (above 350 nm), using two-dimensional (2D) fluorescence spectra and spectrofluorophotometers with resolutions far inferior to what is currently available. Additionally, modern spectrofluorophotometers allow the evaluation of three-dimensional (3D) fluorescence spectra, which analyses both excitation and emission spectra in a single assay across a broader spectral range. Therefore, 3D fluorescence can unravel the complex nature of fluorescent-based compounds and provide further insights into their spectroscopic properties. (BORTOLOTTI *et al.*, 2016) Hence, considering EGFP homogeneous fluorescence spectrum for the two distinct conformations is still a disputed subject, this Thesis project aimed to elucidate this controversy using modern 3D fluorescence analysis at lower wavelengths. This evaluation can provide better insights about EGFP structure and properties, allowing a broader and more mindful application of this protein.

The study of properties and stability of proteins is a multidisciplinary and highly complex field, requiring the analysis of multiple variables and conditions to obtain reliable information on these molecules. The next subsection will supply the properties and indexes already reported for EGFP, in addition to definitions and interpretations of these parameters. The understanding and use of these values will be crucial to the in-depth discussions to explain the stability and partition behavior of EGFP in the fundamental and applied studies of this Thesis.

1.3.1.1 EGFP specific properties and indexes

There are many theoretical and empirical variables necessary for understanding and predicting the behavior of a protein. In **Appendix A**, the most relevant information on EGFP is compiled and will be used in this study to elucidate and explain EGFP properties, stability, and partition behavior.

Appendix A includes EGFP amino acid sequence, (GENBANK, 1996) dimerization (weak dimer, with a binding affinity of $100 \mu\text{mol.L}^{-1}$), (MYATT *et al.*, 2017) maximum fluorescence peak (λ_{ex} 488 nm, λ_{em} 510 nm), quantum yield (QY, 0.6), molar Extinction Coefficient (EC) at peak λ 488 nm ($55,000\text{-}57,000 \text{ L.mol}^{-1}.\text{cm}^{-1}$), (TSIEN, 1998) relative brightness ($33,000 \text{ L.mol}^{-1}.\text{cm}^{-1}$), (TOSELAND, 2013) lifetime (2.5 ns) (SCHLEIFENBAUM *et al.*, 2010), pKa (5.7), (BOMATI *et al.*, 2014) isoelectric point (pI, 6.2), (GURUNATHAN *et al.*, 2014) and ProtParam analysis. (GASTEIGER *et al.*, 2005) With the ProtParam analysis, it was possible to calculate EGFP molar EC at λ 280 nm in water ($21,890 \text{ L.mol}^{-1}.\text{cm}^{-1}$), the average Molecular Weight (MW, 26.9 kDa), the number of atoms (3,764), amino acid composition (**Table A-1**), aliphatic index (79.04), Grand Average of Hydropathicity (GRAVY) (-0.467), and instability index (29.06).

To clarify the meaning and effect of the different parameters and indexes used in the analysis of the properties and behavior of EGFP, they were grouped below into different categories, as follows:

Dimerization: EGFP classification as a weak dimer indicates this protein can be present in solution as a monomer or as a dimer, according to its concentration (namely, its binding affinity of $100 \mu\text{mol.L}^{-1}$) (MYATT *et al.*, 2017) and its environment (*i.e.*, binding affinity will change depending on pH, temperature, salt concentration, and interaction with other molecules present in the medium).

Fluorescence properties: The wavelengths with the highest fluorescence intensity represent the maximum fluorescence peak for an FP (*i.e.*, at λ_{ex} 488 nm, λ_{em} 510 nm for EGFP). The fluorescence QY is the ratio of the number of photons emitted to the number absorbed, in which 1.0 corresponds to the maximum value. (CHRISTOPOULOS; DIAMANDIS, 1996) EGFP QY of 0.6 indicates that 60 % of the photons it absorbs, the protein emits as fluorescence. The extinction coefficient (EC) shows how much light a protein absorbs at specific wavelengths. (GILL; VON HIPPEL, 1989) For FP, it is usually reported the EC at its maximum absorbance (λ) wavelength (*i.e.*, related to the FP chromophore, which for EGFP is at λ 488 nm, 55000-

57000 L.mol⁻¹.cm⁻¹) and at 280 nm [which is associated with the intrinsic absorbance of proteins due to specific amino acids, like tyrosine (Tyr), tryptophan (Trp), and phenylalanine (Phe), (EFTINK, 2002) with a value of 21,890 L.mol⁻¹.cm⁻¹ for EGFP]. Relative brightness is the product of EC and QY divided by 1,000, being of 33,000 L.mol⁻¹.cm⁻¹ for EGFP. (CRANFILL *et al.*, 2016) The lifetime for FP is the time they can remain in their excited state before returning to their grounded state, and for EGFP, it is 2.5 ns. (JAIN *et al.*, 2009)

Acid/base equilibrium constants: The pKa is the pH value at which the concentrations of the acid and conjugate base of a substance are equal; it is also the negative decadic logarithm of the ionization constant (Ka), which is associated with the strength of acids. (ACD/LABS, 2020) The isoelectric point (pI) is the pH value in a titration curve where the overall net surface charge of a protein is zero. (AUDAIN *et al.*, 2016) EGFP pKa of 5.7 and pI of 6.2 is associated with EGFP moderate sensitivity to acid when compared to the wtGFP, which has a pKa of 4.5 (TSIEN, 1998) and pI around 4.8-5.5, (HUGHES *et al.*, 2012) being more favorable to acidic conditions and granting wtGFP more resistance to lower pH.

Stability indexes: The aliphatic index is determined by the relative volume occupied by the aliphatic side chains of proteins (alanine, isoleucine, leucine, and valine), and it is positively associated with the increase of the thermostability of globular proteins. (IKAI, 1980) EGFP aliphatic index of 79.04 is medium to high and similar to different proteins encountered in thermophilic bacteria, indicating a good thermostability. (IKAI, 1980) This index is coherent with the high experimental EGFP thermostability up to 79.5 °C. (SCHEYHING *et al.*, 2002) The instability index is an estimate of the stability of a protein based on statistical analysis regarding the presence of certain dipeptides that are associated with unstable proteins. Proteins with an instability index below 40 are considered stable; hence, the 29.06 value for EGFP indicates a resistant structure. (GURUPRASAD *et al.*, 1990)

Hydropathic indexes: There are many scales to indicate the hydropathic character of proteins, each considering different variables. They are usually a prediction based on the primary structure of the protein; therefore, they are an indication and not a definitive measure of the interactions of proteins with solvents, considering that the spatial conformation of the amino acids of the protein in its tertiary structure can alter its affinities. One very used and simple hydrophobicity plot is the grand average of hydropathicity index (GRAVY) based on the Kyte-Doolittle scale, where lower values indicate more hydrophilic proteins, while higher values represent more lipophilic proteins. (KYTE; DOOLITTLE, 1982) Therefore, a GRAVY of -0.467 indicates that EGFP has a hydrophilic character.

Protein structure: In addition to the different indexes for EGFP properties, it is also crucial to discuss the EGFP structure. EGFP strongly resembles wtGFP, as can be seen in **Figure 3.B** and **C**, with the chromophore located inside a β -barrel maintained by a core helix. The secondary structure of EGFP has 47 % β -strands, 13 % helical forms (3_{10} and α conformations), and 40 % coils (mainly from loops at the two ends of the β -barrel). (ARPINO *et al.*, 2012)

Evaluating multiple parameters and indexes is essential for the comprehension and prediction of the complex properties and behaviors of proteins. However, they only help to represent the protein at ideal conditions (*e.g.*, usually in water, at room temperature, and neutral pH). Thus, experimental evaluation of proteins in stressful environments is fundamental to improve the knowledge regarding these macromolecules. With this in mind, the next section will discuss the state-of-art regarding the behavior and stability of EGFP at different pH and suggest the potential of this protein for the design of pH-biosensors biocompatible with biological systems.

1.3.2 GFP behavior at different pH and its potential for the development of pH-biosensors

Section adapted from the published article at International Journal of Biological Macromolecules, v. 164, p. 3474-3484, 2020. (DOS SANTOS et al., 2020d)

In general, the GFP variants present the same overall structure, with one main β -barrel with a core helix maintaining the chromophore. However, the small modifications in their structures reflect prominently on their physical-chemical properties and stabilities, and this is particularly evident for their pH stabilities. Each GFP variant is stable over a different pH range. For example, wtGFP is stable between pH 5.5 and 12.0, (WARD *et al.*, 1982) with a pKa of 4.5; (TSIEN, 1998) as for EGFP, it has a pKa of 6.0, (CORMACK *et al.*, 1996) is less stable at acidic pH, and more resistant to alkaline pH. The wtGFP has a low sensitivity to acidic pH (*viz.* higher resistance to pH-induced fluorescence quenching), while EGFP and GFP-S65T have moderate sensitivity (PATTERSON *et al.*, 1997) and other GFP variants such as pHluorin and pHluorin2 high sensitivity. (MAHON, 2011) The loss of fluorescence due to pH is not always undesirable for fluorescent proteins, and in the case of pHluorin and pHluorin2, (MAHON, 2011) their low resistance to acidic pH allows their application in the monitoring of intracellular pH. (CAMPBELL; CHOY, 2001; MAHON, 2011) Additionally, changes to the environment of FP can lead to reversible and irreversible changes to their fluorescence activity

(ZIMMER, 2002) – a crucial phenomenon for the development of pH-biosensors. (HANSON *et al.*, 2002; JOHNSON *et al.*, 2009)

Biosensors for medical applications are essential tools to detect and monitor a series of diseases, and there is a great demand for them with the increased focus on prevention over remediation of health issues. (HIGSON, 2012) Because of their high sensitivity, fluorescent compounds are particularly useful in the development of sensors. (BURNWORTH *et al.*, 2007; CARTER *et al.*, 2014; DOMAILLE *et al.*, 2008) However, it is necessary to address particular concerns for the selection of fluorophores for pharmaceutical formulations. (HIGSON, 2012) Although inorganic fluorophores are easier to work with than fluorescent proteins, their toxicity is a barrier for medical use. There are reports of the toxicity of fluorescence dyes from decades ago, (LUTTY, 1978) and recently, from the cytotoxicity at low concentrations of novel fluorophores like quantum dots. (HARDMAN, 2006; SHIOHARA *et al.*, 2004) The FP are already extensively applied for biological studies, including *in vivo* assays, being biocompatible with cells and animals, and presenting very low toxicities, (CHUDAKOV *et al.*, 2010; GERDES; KAETHER, 1996; ZIMMER, 2002) and this makes them well suited for use in pharmaceutical formulations. Additionally, fluorescent proteins are also biodegradable and environmentally-friendly, and therefore, do not poses the same risks and difficulties in disposal as some other organic and heavy metals-based fluorophores. (ALFORD *et al.*, 2009)

Biocompatible pH-biosensors are particularly interesting for the medical field because some diseases alter the pH around the afflicted areas. For example, the region around tumors usually presents a more acidic pH than healthy tissues (with the average pH for tumors around 7.0 and 7.4 for healthy tissues). (TANNOCK; ROTIN, 1989; WIKE-HOOLEY *et al.*, 1984) Similarly, there is a relationship between the acidic pH of synovial fluids and a variety of joint diseases. (TREUHAF; MCCARTY, 1971) However, it is relevant to note that these applications are also dependent on the development of novel technologies, for example, devices capable of subtracting the background autofluorescence of tissues and biological materials that could interfere with GFP fluorescence on *in vivo* experiments. For example, there was a successful technology developed by Wack *et al.* to monitor pancreatic tumors in mice, using GFP in a whole-body imaging system. (WACK *et al.*, 2003) Hence, there is a vast potential for pH-biosensors to diagnose and monitor different diseases, and low toxicity is an essential requirement for their medical applications and commercial use.

There are also other unexplored fields for FP application, which could benefit from more fundamental studies about their structure and behavior in aqueous solutions. Particularly for

EGFP, recent work demonstrated the potential of this GFP variant for developing novel environmentally friendly luminescent solar concentrators, yielding power conversion efficiencies up to 30% higher than the traditional systems commercially available. (FERREIRA *et al.*, 2020)

Considering this information, another goal of this Thesis research was to evaluate the fluorescence and structure of EGFP at different pH, using three-dimensional fluorescence spectroscopy (3D fluorescence), circular dichroism (CD), spectroscopy of the intrinsic fluorescence of proteins (IFP), and Small-Angle X-ray Scattering (SAXS) in well-plates or with Size-Exclusion Chromatography (SEC)-SAXS. The selection of different techniques aimed to elucidate the effects and processes associated with protein unfolding and refolding at different pH. This in-depth study of EGFP stability in varying pH, and the evaluation of the relationship between EGFP fluorescence and pH, provides essential information for the development of biomarker and biosensor applications of this low-toxicity and natural alternative to other fluorophores, such as dyes and quantum dots.

1.4 STABILITY OF GFP

Section adapted from original manuscripts in preparation. (DOS SANTOS et al., 2020b, 2020c)

As previously stated, the instability of many proteins outside their optimal conditions can hinder their commercial use. Thus, the investigation of the stability of proteins and the search for solvents with stabilizing potential is essential to broaden their application on a large scale. This issue is particularly relevant for GFP application, considering its fluorescence is dependent on the maintenance of its protein structure. (POPPENBORG; FRIEHS; FLASCHEL, 1997; TSIEN, 1998) Stability studies are therefore crucial to allow GFP commercial use, especially considering solvents with the ability to protect, increase, or quench GFP fluorescence can be useful in the development and application of biosensors.

As highlighted in the previous section, the chromophore of GFP is easily accessible to a variety of external disturbances, such as pH, temperature, and certain substances. (WARD *et al.*, 1982) Once the FP is denatured, there is a disruption of the cylindrical protein structure that holds the chromophore at its center, and consequently, occurs a fluorescence extinction. Therefore, GFP and its variants emit fluorescence only when they have their protein structure intact, (ENOKI *et al.*, 2004) and each variant has a different resistance to physical and chemical stresses, as related in the previous section regarding their pH stability.

Most GFP mutants are relatively resistant to photobleaching (loss of fluorescence from exposure to light), (CUBITT *et al.*, 1995) and the fluorescence life of GFP seems to be independent of the viscosity of its environment; however, there is a small increase in the decay time of fluorescence as the viscosity increases. (SUHLING *et al.*, 2002; WARD *et al.*, 1982) Additionally, the wtGFP is also considerably resistant to chemical and biological denaturation, remaining fluorescent in the presence of surfactants [solutions of 1 w/v% of sodium dodecyl sulfate (SDS)] and other chaotropic agents, and even resisting exposure for two days to most proteases (*e.g.*, trypsin, chymotrypsin, and papain). (CUBITT *et al.*, 1995) Nonetheless, at acidic pH, wtGFP is very sensitive to denaturation by SDS, urea, and heat, (ALKAABI *et al.*, 2005) with a decrease in their global thermal stability and melting point. (NAGY *et al.*, 2004) However, the acid, alkaline, or guanidine denaturation of wtGFP can be reversible due to the flexibility of its chromophore binding site. (WARD *et al.*, 1982) Besides, there are reports of the instability of other GFP variants in the presence of chlorinated solutions and oxidizing agents such as hydrogen peroxide (H₂O₂). (MAZZOLA *et al.*, 2006a; SANTOS *et al.*, 2007)

Despite many studies on the GFP stability at different conditions, there are still considerable knowledge gaps in this field, particularly regarding the mechanisms of denaturation and solvent-protein interactions. This issue happens because most studies only report the GFP stability range and do not elucidate the behavior and structural dynamics of the protein-solvent systems. The lack of in-depth information hampers the development of novel applications and stabilizing formulations for GFP, delaying their practical use.

Even considering the advantages of GFP over inorganic fluorescent compounds (*e.g.*, their biocompatibility and non-persistence in the environment), (FROMMER *et al.*, 2009) the difficulties in applying GFP under certain stress conditions and its high costs still restrict its application to small-scale research and processes. The use of compounds that can protect the structure of the GFP fluorophore appears as an effective solution to allow the use of the protein in unfavorable conditions. The next subsection will explore the potential candidates of solvents and adjuvants to act as protein stabilizers in pharmaceutical formulations (particularly ILs), which was a key state-of-art for selecting the best compounds for the experimental studies of this Thesis project.

1.4.1 Stabilization of proteins with ionic liquids

Different solvents can be used in protein formulations as stabilizers or even as enhancers and additives for their biological activity. For example, solvents can potentially be associated

with GFP to increase its stability or to quench or dequench its fluorescence for biosensing applications. There are certain classes of compounds already applied as stabilizing agents or additives for proteins, such as polymers, (CARO; MARCÉ; BORRULL; CORMACK *et al.*, 2006; HE; LONG; PAN; LI *et al.*, 2007) organic biocompatible salts, (BLANCHARD; HANCU; BECKMAN; BRENNECKE, 1999; ROGERS; SEDDON, 2003) and ionic liquids (ILs). (KUNZ; HÄCKL, 2016) This subsection presents an overview of the advances in this field and the opportunities and perspectives on the topic, specifically focusing on ILs as protein stabilizers.

ILs are particularly interesting for commercial use in protein-based formulations, considering they are salts with low melting points and can circumvent unwanted properties of organic solvents in industrial processes. (KUMAR *et al.*, 2017) The disadvantages of organic solvents can include high flammability, (ANASTAS; KIRCHHOFF, 2002) harsh working conditions (*e.g.*, high temperatures and pressure), (CHAN; DILL, 1991; DEBENEDICTIS *et al.*, 2015) negative impact on the stability and activity of bioproducts, (BANIK *et al.*, 2003) and environmental risks due to high toxicity and persistence in nature. (ANASTAS; KIRCHHOFF, 2002; CAPELLO *et al.*, 2007) Additionally, in comparison with other solvents that also present low flammability and toxicity (*e.g.*, polymers and certain salts), ILs can be easily tailored to present a vast range of structures and properties, making them extremely diverse and versatile for industrial applications. Besides, different studies already demonstrated the potential of ILs to act as protein stabilizers or activity enhancers. (KUMAR *et al.*, 2017; PATEL *et al.*, 2014) For example, in 2000, Summers and Flowers showed that IL ethylammonium nitrate (EAN) could prevent the aggregation of denatured Hen Egg White Lysozyme (HEWL). (SUMMERS; FLOWERS, 2000) This study also showed it is possible to separate the refolded active protein from EAN with a simple desalination method, which could be useful to allow their application in intermediate processing phases, even if the IL is inadequate for the final formulation. Different research groups have since studied ILs for protein stabilization, particularly considering the current efforts to replace organic solvents with greener alternatives for industrial processes. (KUMAR *et al.*, 2017) Even recent work from the research group have demonstrated the capacity of ILs from the imidazolium (NASCIMENTO *et al.*, 2019a) and cholinium families (MAGRI *et al.*, 2019; NASCIMENTO *et al.*, 2019b) to stabilize or enhance the activity of enzymes (particularly lipase for both families and L-asparaginase for cholinium ILs). There is also work showing the stabilization of cytochrome C (BISHT *et al.*, 2017) and lysozyme (RODRIGUES *et al.*, 2011) with cholinium ILs, and the

increase of Bovine Serum Albumin (BSA) stability in cholinium-based good's buffers ILs (formed by the association of biological buffers and ILs). (TAHA *et al.*, 2015) However, not all substitutions of organic solvents for ILs will be beneficial, and it is necessary to consider both the characteristics and stability of the target biomolecule and the properties of the ILs selected for applications.

Several properties of ILs are responsible for determining their influence on the stability and activity of proteins, including their polarity, hydrophobicity, hydrogen bonding capacity, and characteristics of their cation and anion pair. (PATEL *et al.*, 2014) According to Sedlak *et al.*, there are several charged groups on the surfaces of the dissolved protein in an aqueous solution. (SEDLÁK *et al.*, 2008) These charged groups will be responsible for the interactions between the IL ions in solution and different regions of the protein, and therefore, will determine the potential of the IL to impair or improve the stability and function of the macromolecule. Depending on the properties of the protein and the IL, both cations and anions in ILs can play a central role in stabilizing and destabilizing proteins. (BAKER *et al.*, 2011)

To effectively understand the effects and interactions between ILs and proteins, it is essential to design in-depth studies that consider not only the intrinsic characteristics of macromolecules, but that also evaluate the properties of the ILs and the contributions of both cations and anions. For example, initial works with imidazolium-based ILs and proteins showed each imidazolium cation interacted with three anions, and every anion interacted with three cations, forming a network similar to the three-dimensional arrangement of water. (PATEL *et al.*, 2014) The researchers concluded that when a macromolecule is inside this network, there are protein-IL interactions due to the formation of polar and non-polar regions. (DUPONT, 2004; SCHRÖDER *et al.*, 2000) When the molecule is a protein and interacts with ILs through hydrogen bonding, protein unfolding is often disadvantaged, which can explain the stabilizing effect observed for certain imidazolium ILs in specific proteins. (LOZANO *et al.*, 2005) These were the initial findings to explain the mechanism behind protein-IL synergy; however, more recent works have been showing there are also other interactions between ILs and certain groups in the protein surface that can also influence the stability of the macromolecules. (RESLAN; KAYSER, 2018)

In the last decade, studies using cholinium ILs and salts indicated additional ways from which the protein can interact with them. For example, as demonstrated by Tarannum *et al.*, cholinium-based amino acid ILs (namely, cholinium serinate, threoninate, lysinate, and phenylalaninate) can destabilize collagen by competing with the hydrogen bonds between ILs

and the hydroxyl group on the protein. (TARANNUM *et al.*, 2018) Considering cholinium salts, Weaver *et al.* showed that cholinium dihydrogen phosphate ([Ch][DHP]) can inhibit the denaturation of lysozyme and recombinant human interleukin-2 (rHIL-2) through interactions with the charged residues on the surface of the protein and, depending on the protein, these could even impair or improve the secondary structure of the macromolecule. (WEAVER *et al.*, 2012) In another study, Mazid *et al.* revealed that [Ch][DHP] buffered solution could protect the epidermal growth factor receptor monoclonal antibody (EGFR mAb) against fragmentation by proteinase K by stabilizing its secondary structure (*i.e.*, α -helices). (MAZID *et al.*, 2015) Hence, there is an immense diversity of mechanisms that controls the interactions between cholinium-based salts and ILs and proteins, such as ILs and proteins properties, their concentration, and the physical-chemical conditions of their surrounded medium, among many others that are still under investigation. (RESLAN; KAYSER, 2018)

Another aspect that must be taken into account for commercial applications of ILs as protein stabilizers, particularly in the medical field, is the toxicity and biodegradability of the different IL classes. Although ILs have been called “green solvents” in the last decades because of their negligible vapor pressure (*i.e.*, low atmospheric contamination) and low flammability, certain classes of ILs can still be toxic or have poorly biodegradable constituents (*e.g.*, ILs with fluorinated anions and imidazolium cations). (KUNZ; HÄCKL, 2016) Thus, an appropriate selection of more benign and biocompatible ILs is essential for these uses. For example, cholinium-based ILs ([Ch]X) can be promising options for use as modern solvents, mainly due to their low toxicity and biodegradability. (BOETHLING *et al.*, 2007; KUNZ; HÄCKL, 2016; PEREIRA *et al.*, 2016a; PETKOVIC *et al.*, 2010; SANTOS *et al.*, 2015; VENTURA *et al.*, 2014) Cholinium ILs (or salts) have the additional advantage of being derived from a vitamin (Vitamin B8, a quaternary ammonium cation), and some are even already used as nutritional supplements and pharmaceuticals. (FEEDAP, 2011) A good example is cholinium chloride ([Ch]Cl), which is classified by Food and Drug Administration (FDA, American drug regulatory agency) as “Generally Recognized as Safe” (GRAS, from Generally Recognized As Safe) and approved to be used as a vitamin supplement and medicine. (FEEDAP, 2011)

Regarding the stability of GFP in ILs solutions, there was a recent study using superfolder GFP (sfGFP) and a series of pyrrolidinium and imidazolium salts and ILs (with acetate, chloride, and triflate anions) aqueous solutions (at 1 mol.L⁻¹). (BUI-LE *et al.*, 2020) Interestingly, this study demonstrated that multiple evaluation techniques are necessary to obtain an accurate understanding of the complex protein-ILs interactions, *i.e.*, it is not possible

to draw robust conclusions from individual measurements. Overall, this previous study demonstrated that the ILs impaired sfGFP stability and the anion played a central role in determining the protein-IL interactions. However, the focus was to evaluate the stability of sfGFP at considerably high concentrations of ILs (1 mol.L^{-1}) and, thus, to provide insights into protein-IL systems with lower water content, particularly for catalysis. On the other hand, this Thesis project aimed to evaluate the GFP stability in more diluted ILs aqueous solutions (*i.e.*, IL concentrations $\leq 0.5 \text{ mol.L}^{-1}$), envisaging applications for medical and biological purposes. Higher ILs concentrations are usually more disruptive; hence, the same IL can have different effects on proteins also based on their concentration.

With this in mind, another goal of this Thesis research was to assess the effect of dilute aqueous solutions of ILs and salts on EGFP stability at different stress conditions, intending to find solvents with the ability to protect proteins, act as adjuvants in the development of biosensors, or be applied in protein purification platforms. Different concentrations of imidazolium- and cholinium-based ILs were selected to assess the effects of modification on cations and anions of ILs, respectively, in the solvent-protein interactions. The researcher chose these two ILs families according to previous reports on their capacity to stabilize proteins in aqueous solutions, namely: I) cholinium ILs, due to their favorable properties, such as biocompatibility with cells and macromolecules, biodegradability, and natural and renewable sources; (KUNZ; HÄCKL, 2016; PEREIRA *et al.*, 2016a) and II) imidazolium ILs, because they are the most diverse and studied class of ILs and, despite not being as biocompatible as cholinium ILs, (GOMES *et al.*, 2019) they are good counterparts for them, considering they will allow this study to monitor the effects of both cations and anions on protein stabilization.

1.5 PURIFICATION OF GFP

Section adapted from the original articles published in ACS Sustainable Chemistry & Engineering, v. 6, n. 7, 9383-9393, 2018 (DOS SANTOS et al., 2018b) and Separation and Purification Technology, v. 254, p. 117595, 2021. (TORRES-ACOSTA et al., 2021).

Despite the great potential of GFP to be applied as biosensors and biomarkers, their instability under stress conditions and very high prices still limit their large-scale uses. Currently, there is already some profitable field for GFP in the Reporter Gene Assay Kits market (including FP and enzymes like luciferase), which achieved a market value of USD 1.6 billion in 2019. (MARKETS AND MARKETS, 2019) However, considering the exorbitant current prices of GFP (ranging from around USD 350 to 600 *per* 0.1 mg), (ABCAM, 2020;

ABNOVA, 2020; ASSAY GENIE, 2020; BIOVISION, 2020; MYBIOSOURCE, 2020; NOVUS BIOLOGICALS, 2020) this application is only commercially viable because it uses negligible concentrations of the protein (only around 0.1 mg of GFP *per* kit for 100 assays). (ABCAM, 2020; BIOVISION, 2020; NOVUS BIOLOGICALS, 2020) Moreover, other fields of application are emerging, enclosing innovative products in the medical, (HOCHREITER *et al.*, 2015; STEPANENKO *et al.*, 2008) energy, (CARLOS *et al.*, 2020) and textile (LEE *et al.*, 2020) fields, which are still in their infancy mainly because of the restrictive prices of FP.

Several reasons can explain the exorbitant prices for commercial GFP. As previously stated, assay kits are the central market for FP today, generally produced by pharmaceutical industries. Although very high, the general price *per* kg of GFP (approx. USD 2 billion *per* kg) is similar to other pharmaceutical proteins, like recombinant factor VIII and activated factor VII for hemophilia treatment, and recombinant Hepatitis B surface antigen for vaccine production costing from USD 2 to 10 billion *per* kg. (PUETZ; WURM, 2019) These values are very distinct from the price of industrial proteins like cellulase and β -Glucosidase for ethanol production, which cost ranges from USD 10 to 37 *per* kg. (PUETZ; WURM, 2019)

There are many arguments regarding this gigantic difference in price from industrial and pharmaceutical proteins, like the use of the more costly mammalian cells over bacteria and yeast for the expression of the recombinant proteins, the higher degree of purity required, the strict quality controls for the production of pharmaceutical-related products, in addition to more R&D costs (usually because of preclinical and clinical trials), patenting, and marketing. (PUETZ; WURM, 2019) However, some researchers argue that this is not enough to explain the 8-9 orders of magnitude of difference in price between industrial and pharmaceutical proteins, suggesting there is a lack of transparency in the pharmaceutical industry that prevents an open discussion in the topic to evaluate if the prices are justified. (PUETZ; WURM, 2019) This striking difference is even more evident for GFP, as this protein had no private R&D and patenting costs (because it was developed in academia and not under a patent), and it uses bacterial expression systems. (TSIEN, 1998; ZIMMER, 2002) The unique plausible justification for its exorbitant prices, even if weak, would be related to the high degree of purity required for its commercialization. Hence, simpler and cheaper purification platforms could be an alternative to allow for other industries besides pharmaceutical complexes to produce recombinant proteins with more competitive prices.

In the last decades, some studies aimed to decrease GFP prices by developing new ways to produce it successfully. (FIGUEIRA *et al.*, 2000; PÉREZ-ARELLANO; PÉREZ-MARTÍNEZ, 2003) Mainly two strategies are in place, with the first one focusing on improvements in upstream processes by obtaining high titers using recombinant *E. coli* (the currently available commercial product) (LOPES *et al.*, 2019) or tobacco plants (DONG *et al.*, 2019) to express the protein. There are also studies envisaging to reduce costs by improving the recovery and purification techniques in the downstream process as they can represent a large fraction of the production costs. (HUMMEL *et al.*, 2019; STRAATHOF, 2011) Particularly for biomolecules with medical applications, like *in vivo* GFP-based biomarkers/biosensors, a remarkably high purity degree is required to avoid impurities, which can cause an inappropriate immunogenic reaction and harm the patient. (GUIOCHON; BEAVER, 2011) Therefore, the purification of pharmaceutical bioproducts is usually complex and based on multi-step platforms. (DOS SANTOS *et al.*, 2018a)

The current strategies for GFP purification involve, mainly, the use of multiple chromatographic units, which are intricate and costly. (CHO *et al.*, 2001; DESCHAMPS *et al.*, 1995; DIERYCK *et al.*, 2003; GONZÁLEZ; WARD, 1999; LI *et al.*, 2001; MCRAE *et al.*, 2005; URETSCHLÄGER *et al.*, 2001; ZHUANG *et al.*, 2008) Hence, there is still no efficient purification methodology for this protein that combines selective recovery and affordable costs. (YAKHNIN *et al.*, 1998) Additionally, even promising purification techniques for GFP as elastin-like polypeptide tags (CHOW *et al.*, 2006) are still not commercially available, (YEBOAH *et al.*, 2016) as they would require further tests for pharmaceutical applications of GFP to guarantee the tags (or its residues) are not immunogenic or detrimental for medical use. (LAN *et al.*, 2011; YEBOAH *et al.*, 2016) As a consequence of the high number of costly purification stages required for the acquisition of pure GFP, the price of the final product is steep (*e.g.*, Biovision® commercial EGFP that costs approximately USD 2,000 *per mg*). (BIOVISION, 2020)

These exorbitant prices have driven a search for alternative purification techniques (such as liquid-liquid extraction) to reduce GFP production costs. For example, Samarkina *et al.* and Yakhnin *et al.* already recovered wtGFP from cell lysate using organic solvent extraction, achieving its complete purification by combining the liquid-liquid extraction stages with a further chromatography step. (SAMARKINA *et al.*, 2009; YAKHNIN *et al.*, 1998) However, the process involved multiple extraction steps and the use of organic solvents, which can be toxic, volatile, or flammable, and the current industrial trend is to replace these compounds with

safer and more environmentally-friendly alternatives, (ANASTAS; KIRCHHOFF, 2002) like polymers (CARO *et al.*, 2006; HE *et al.*, 2007) and ILs. (BLANCHARD *et al.*, 1999; ROGERS; SEDDON, 2003)

Polymers such as polypropylene glycol (PPG) and polyethylene glycol (PEG), and salts like cholinium chloride [(2-hydroxyethyl)trimethylammonium] chloride ([Ch]Cl), exhibit excellent properties for industrial use, having low toxicity (ERICKSON; NELSON; WINTERS, 2011; FDA, 2015; QUENTAL *et al.*, 2015) and enabling milder work conditions than organic solvents. (CHAN; DILL, 1991; DEBENEDICTIS *et al.*, 2015; OECD, 2004) Additionally, different groups described the formation of ABS by the combinations of these biocompatible polymers and cholinium-based salts or ILs. (PEREIRA *et al.*, 2013b; QUENTAL *et al.*, 2015) Considering ABS were already successfully applied to develop biocompatible, efficient, cheaper, and easy to scaled-up platforms for the purification of different biomolecules in the past, (ASENJO; ANDREWS, 2011; FREIRE, 2016; FREIRE *et al.*, 2012; MAZZOLA *et al.*, 2008; PANAS *et al.*, 2017; PEREIRA *et al.*, 2013b; QUENTAL *et al.*, 2015; RAJA *et al.*, 2011; ROSA *et al.*, 2010, 2011; SANTOS *et al.*, 2011; SHELDON, 2005; WOLSKI *et al.*, 2011) novel polymer/[Ch]Cl ABS are a promising tool to improve GFP purification and extraction.

There are already some successful studies showing the use of ABS for the extraction of GFP and its variants; (SONG *et al.*, 2018; JOHANSSON *et al.*, 2008a; LOPES *et al.*, 2011) however, higher levels of purification were still not demonstrated and are essential for the commercial success and economic viability of the process. (JOHANSSON *et al.*, 2008a; LOPES *et al.*, 2011) Hence, although ABS present great potential for the purification of biomolecules like EGFP, a thoughtful selection of phase-forming agents and integration in the adequate stage of the downstream process is required to allow the development of sustainable and cost-effective purification of biomolecules. Therefore, the next subsection presents an extensive review of literature on the purification of biomolecules using liquid-liquid extraction (such as ABS), intending to guide the selection of the most suitable systems for the purification of EGFP.

1.5.1 Purification of biomolecules using liquid-liquid extraction

Section adapted from the critical review published in Journal of Chemical Technology & Biotechnology, v. 93, n. 7, p. 1845-1863, 2018. (DOS SANTOS et al., 2018a)

Biomolecules of commercial application, as previously stated, require a high degree of purity, which usually is associated with a multi-step and costly purification process that increases the final cost of bioproducts. (GRONEMEYER *et al.*, 2014) The development of alternative, more sustainable, and lower-cost purification strategies can solve these issues, and liquid-liquid extraction methods are a promising option to help to simplify the production of biomolecules. (MAZZOLA *et al.*, 2008) However, downstream processing is a complex system, and a deep understanding of its many variables is necessary to guarantee the success of novel purification platforms, as will be addressed in this section.

It is essential to have an in-depth knowledge of both the heterologous expression system, the desired biomolecule, and the production medium when aiming to select the most suitable recovery and purification methods for biological products. Most biomolecules of commercial interest are formed by protein core with many substituents, with an amino acid sequence folded into a complex three-dimensional structure, and, therefore, they usually present chemical and physical instability. (TAMIZI; JOUYBAN, 2016) The complex secondary and tertiary structures of proteins are fundamental to their activities, but proteins can unfold, denature, self-associate, aggregate, precipitate or have small alterations in high order structures under stress conditions, and consequently, lose its biological activity. (TAMIZI; JOUYBAN, 2016) Besides, chemical reactions as deamidation, isomerization, hydrolysis, racemization, oxidation, disulfide formation, and β -elimination can also promote protein degradation. However, not only strong chemical or physical conditions can inactivate proteins, but this can also occur due to small changes in temperature, pH, oxidative stress, or ionic strength, which can lead to a weakening of the chemical interactions that maintain the protein structure. (GUIOCHON; BEAVER, 2011; TAMIZI; JOUYBAN, 2016) Thus, to preserve the structure of biomolecules, the downstream processes must consider the characteristics of the target product and the complex composition of the production system, never exceeding pH, temperature, ionic strength, or causing unfavorable chemical reactions leading to denaturation.

In the production of biomolecules through the cultivation of microorganisms (generally referred to as fermentation or bioprocess), the products can be generated in the intracellular or periplasmic space or exported to the extracellular environment. For this last condition, it is possible to recover the biomolecule in the fermented broth, which is a combination of insoluble gelatinous biomass, nutrient fluid, and soluble metabolites. Usually, only 3 to 7 % by weight of the liquid volume is related to the biomass content, and thus, it is imperative to reduce this volume by at least an order of magnitude from the initial broth to the final stages of the

extraction. (DECHOW, 1989) Moreover, the fermented broth is also very unstable, and once it leaves the aseptic environment of the fermenter, the deprivation of oxygen and nutrients can lead to rapid changes in physical properties and further degradation of the bioproducts. Delaying the recovery operations can compromise the integrity of the fermented target products, and therefore, simpler and faster procedures are better for the initial low-resolution stages of downstream processing. (DECHOW, 1989)

It is also essential to account that the metabolic shift caused by induction in genetically modified expression systems, considering they can impose a severe impact on the downstream processing of fermented broth. Although recombinant microorganisms overexpress the target molecule and generate fewer byproducts, the contaminants produced may be very similar to the target molecule, hindering their selective separation and the attainment of high purity. (BERLEC; ŠTRUKELJ, 2013; GONÇALVES *et al.*, 2011) Hence, it is fundamental to evaluate the types of contaminants produced in the upstream stage and not only the degree of purity, given some substances can be harder to remove than others.

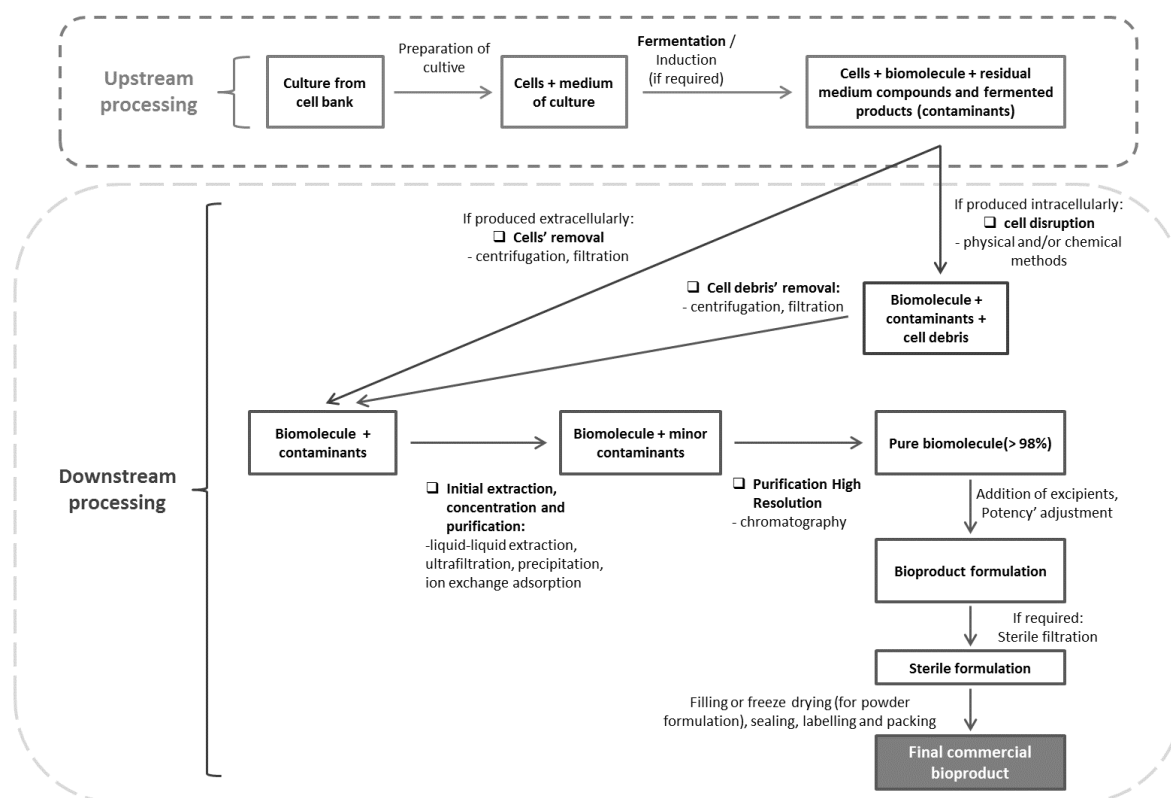
In the case of intracellular biomolecules, it is also necessary to add at least one cell disruption stage to liberate the compounds from the cell interior. (DECHOW, 1989) In addition to the incorporation of an extra step for cell disruption, biomolecules recovered from the intracellular environment present more impurities than their extracellular counterparts due to the release of the different cellular substances (*e.g.*, membrane, cytoplasmic and periplasmatic components) to the recovery medium, increasing the purification concerns and consequently, the time and cost during the downstream processing. (DECHOW, 1989)

Even considering the difficulties in the downstream processing of intracellular compounds, a large number of commercial bioproducts still use microorganisms with intracellular production like *E. coli* for their synthesis because they have high yields and quick fermentations. Moreover, researchers have also genetically modified *E. coli* to induce or increase the secretion of target-biomolecules into the periplasm or extracellular environment, facilitating the next stages of the downstream processing and helping to overcome some drawbacks of this type of expression system. Genetic engineering can also improve the folding and the *in vivo* stability of proteins, potentially improving yields and reducing the production costs. (MERGULHAO *et al.*, 2005)

Despite the advances in the recovery of intracellular bioproducts, this is only one of many steps needed to obtain biomolecules with high purity. For example, it is still always necessary to perform a complex cascade of unitary operations to achieve the standard purity

levels required for pharmaceuticals and food additives. To exemplify this issue, **Figure 4** depicts a schematic conceptualization of the integration of units from production to the final formulation of biomolecules using microorganisms.

Figure 4 – General steps for the production, purification, and formulation of bioproducts employing microbial heterologous expression system.



Source: Produced by the author and published (adapted) in (DOS SANTOS *et al.*, 2018a).

As can be seen in **Figure 4**, the initial stages of the manufacturing of bioproducts mainly focus on bioengineering manipulation and production optimization (upstream processing). After production, if the target molecule is extracellular, simple removal of the cells by applying centrifugation or filtration technique is usually performed. On the other hand, if the bioproduct is intracellular, a cell disruption stage is required to lyse the whole cell and release the target-biomolecule, and only then it is possible to remove the cell debris (commonly by filtration or centrifugation). After the clarification stage (either for the removal of the whole cell or cell debris), a further concentration/initial purification stage is usually applied. This downstream stage incorporates at least one of the following operation units: ultrafiltration, precipitation, ion-exchange chromatography, or liquid-liquid extraction. Then, as the final purification stage, a series of chromatographic units are carefully integrated to achieve purity above 98 %. Finally, after obtaining a pure biomolecule (high-resolution chromatography yields a protein that is 98-

99 % pure), the final polishing stages are performed, where the product goes through formulation, filling, labeling, and packing steps. Thus, these stages will also adjust the potency of bioproducts and add excipients to the formulations to stabilize or enhance their activity. After a proper formulation, if necessary (and particularly for medical formulations), the product can be sterile filtered (through a 0.22 μm absolute filter), aseptically filled, and freeze-dried for powder preparations. (WALSH, 2013)

Figure 4 introduces the manufacturing process of biomolecules in a straightforward approach; nevertheless, it is still necessary to emphasize some fundamental aspects of the system to provide a more accurate picture of the production of bioproducts. The high-resolution stages in bioseparations are still mainly based on a combination of several chromatographic techniques. (PRZYBYCIEN *et al.*, 2004) By chromatography, it is possible to separate even very similar components, ensuring high purity. (HANKE; OTTENS, 2014) However, chromatographic methods exhibit some drawbacks. In general, these methods can only handle small amounts of feed at a time (BANIK *et al.*, 2003) and have high processing costs, mainly due to consumable and columns prices, in addition to lengthy cycle times. (JOZALA *et al.*, 2016; PRZYBYCIEN *et al.*, 2004)

Some of the problems associated with the use of multiple chromatographic stages can be overcome (or at least reduced) by proper integration of the earlier low-resolution downstream processes. On this basis, the pharmaceutical industry already employs different techniques as early-concentration or initial purification platforms (*e.g.*, centrifugation and precipitation), but they also have disadvantages, such as a low degree of resolution and relatively high energy consumption to deal with viscous mixtures. (BANIK *et al.*, 2003) Furthermore, centrifugation and precipitation techniques can promote the aggregation of proteins, denaturing the target biomolecule. Moreover, most of these traditional purification processes involve more than one step, which leads to common problems of the industrial processes, as the reduction of yields, time-consuming operations, and the increase of manufacturing costs. Given the drawbacks of the purification techniques previously mentioned, liquid-liquid extraction (LLE) is still the most attractive platform to combine various steps in a single operation, as well as to allow an integrative and efficient connection between the initial and final downstream processes. (MAZZOLA *et al.*, 2008)

1.5.1.1 Liquid-liquid extraction

LLE is a traditional technique widely used in the chemical industry due to its simplicity, low cost, reduced separation time, and easy scaling-up. (MAZZOLA *et al.*, 2008) It consists of the formation of two immiscible phases by combining components with different physical-chemical properties (traditionally constituted by an aqueous and an organic phase). Depending on the solute affinity, the target-molecule will migrate (partially or completely) to one of the co-existing phases of the system, allowing its concentration and sometimes even its purification. (MAZZOLA *et al.*, 2008)

Chemical and pharmaceutical industrial processes extensively apply traditional LLE (formed by organic solvents); however, their use in the biotechnology field is facing some concerns, mainly due to the impact of organic solvents in the structure and biological activity of several bioproducts, which may cause considerable denaturation of proteins. (BANIK *et al.*, 2003) Besides, the organic solvents commonly used exhibit quite significant environmental hazards, many times presenting elevated toxicity (causing irritation or acute and chronic toxicity), volatility, flammability, and persistence in the environment. (ANASTAS; KIRCHHOFF, 2002; CAPELLO *et al.*, 2007) Thus, considering the environmental concerns, and mainly, the negative effect over biological products, these traditional LLE systems are usually unsuitable for the recovery of biomolecules. (BANIK *et al.*, 2003)

Despite these disadvantages, some research groups have proposed traditional LLE as an intermediate platform for the recovery of biomolecules and their partial purification. However, it is essential to note that probably due to the negative impact of organic solvents on the structural integrity and biological activity of several biomolecules, only a few studies used them in the recovery of proteins. (SAMARKINA *et al.*, 2009; YAKHNIN *et al.*, 1998)

Particularly for the recovery of GFP, which traditionally requires a series of chromatographic steps for its purification, one work was able to extract the protein using an LLE composed of ammonium sulfate, ethanol, and n-butanol. An additional back extraction using chloroform was necessary after the LLE, and it was possible to recover 60 % of the GFP, with a purity of 90 %. (YAKHNIN *et al.*, 1998) The authors also showed that by integrating the LLE process with a single unit of Phenyl-Sepharose Chromatography, a complete GFP purification is obtained, with an overall yield of 36 %. Although this method was successful in the recovery and purification of GFP using organic solvents, the work itself may be indicative of the difficulties of using traditional LLE for the extraction of other less stable biomolecules since the authors have tested three other solvents (isopropanol, acetonitrile, and methanol), which were not suitable for the recovery of target-protein. (YAKHNIN *et al.*, 1998) In addition

to GFP, similar organic solvent-based LLE systems also purified other FP. (SAMARKINA *et al.*, 2009)

To overcome the main concerns of the use of volatile organic solvents, a new type of LLE, which do not require organic solvents to form immiscible phases, is becoming popular among biotechnologists. First introduced by Albertsson in 1958, (ALBERTSSON, 1958) the LLE called aqueous biphasic systems (ABS) or aqueous two-phase systems (ATPS), appeared like the ideal solution for the concentration and separation of biological products, mainly due to the gentle nature of both water-rich immiscible phases. (BANIK *et al.*, 2003; FREIRE *et al.*, 2012)

Different researchers associated the surge in popularity of ABS with its cheaper and more sustainable characteristics, which can reduce production costs and environmental impacts. (BANIK *et al.*, 2003; FREIRE *et al.*, 2012) As previously addressed, ABS form two aqueous-rich phases by the mixture of specific concentrations of two structurally different compounds (*e.g.*, polymers, salts, surfactants, amino acids, and ILs) in an aqueous medium. (FREIRE *et al.*, 2012; PEREIRA *et al.*, 2013a; ROSA *et al.*, 2010) Because ABS usually contain high portions of water, it can provide a biocompatible environment for cells, organelles, and other biologically active substances. (BANIK *et al.*, 2003)

Depending on the phase-forming agents, ABS can promote a suitable environment and different affinities for biomolecules for the co-existing phases. This setting can lead to a selective partitioning of the solutes and allow the isolation and purification of biomolecules. (SANTOS *et al.*, 2011; WOLSKI *et al.*, 2011) Thus, as ABS have low energetic and operational costs, a considerable straightforward scaling-up in comparison with the conventional chromatographic techniques, can have high biocompatibility and stabilizing properties, and could be easily integrated with other downstream methodologies. (FREIRE, 2016) ABS appear as suitable platforms for the concentration and initial purification of bioproducts, particularly for applications that require lower toxicity and more environmentally-friendly processes.

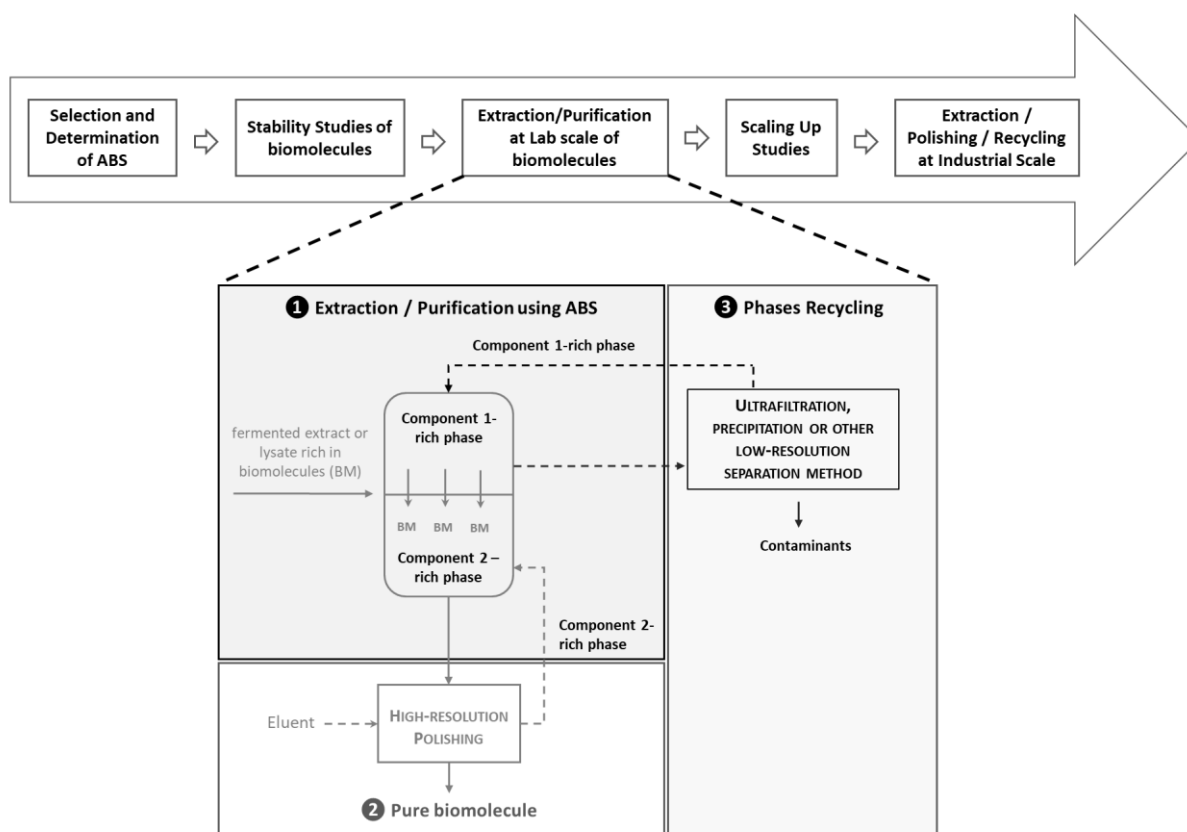
1.5.1.2 ABS for the purification of biomolecules

The use of ABS for the recovery of proteins is already well-established in the literature, not only allowing good results for their extraction and purification, but some formulations can even stabilize specific proteins. (DESAI *et al.*, 2016) Hence, ABS are already successfully applied in the extraction and purification of biomolecules from many sources, including animal and hybridoma cells, plants, human and animal tissues, and fluids, insect cells, and fermented

broth of microorganisms. (ASENJO; ANDREWS, 2011; AZEVEDO *et al.*, 2009; GUPTA *et al.*, 1999; JOZALA *et al.*, 2016; MAZZOLA *et al.*, 2006a; MOLINO *et al.*, 2013; RAJA *et al.*, 2011; ROSA *et al.*, 2010) However, some phase forming components (at specific concentrations) can induce the denaturation of proteins. Thus, preliminary stability studies are always fundamental to determine the most suitable ABS for the recovery of bioproducts. (DESAI *et al.*, 2016)

An intricate step-by-step procedure is required to select adequate ABS for large-scale purification of biomolecules, as schematized in **Figure 5**. First, it is fundamental to define the ABS forming components and determine the respective phase diagrams. Then, it is crucial to evaluate if the biomolecule is stable under the selected conditions. This step is the key to guarantee the structural integrity and biological activity of the biomolecule after the downstream processing.

Figure 5 – General steps for the development and application of aqueous biphasic systems (ABS) for the extraction and purification of biomolecules (BM) at an industrial scale.



Source: Produced by the author and published (adapted) in (DOS SANTOS *et al.*, 2018a).

Among the components usually applied in the formation of ABS, organic and inorganic salts are the most used, and it is possible to divide them into protein stabilizers (kosmotropic) or destabilizers (chaotropic). Both effects were previously well-described by the Hofmeister

series, (DESAI *et al.*, 2016) in which the most suitable ionic composition to stabilize proteins is a formulation containing salts with a kosmotropic anion and a chaotropic cation. (BALDWIN, 1996; COLLINS; WASHABAUGH, 1985; DESAI *et al.*, 2016; ZHAO, 2005) Hence, it is encouraged to take advantage of this characteristic when choosing the ABS forming agents considering the protein stabilizing effect of some salts, since this may confer additional protection for the biomolecule during the downstream processing.

After the stability tests, it is necessary to evaluate the aptitude of different ABS for specific biomolecules. At a laboratory and pilot scale, it is also crucial to perform all partitioning and recycling studies to obtain the biomolecule and recover phase-forming agents. Moreover, this stage should also evaluate the polishing of target molecules. Then, the most promising system can be scaled-up to an industrial scale, validating the viability of the process.

The recycling of the phase-forming components of the ABS is also essential to allow the economic viability of the industrial operations. Thus, as shown in **Figure 5**, the purification process applying ABS should form a cycle, being initiated with the formation of the ABS, followed by the extraction and purification of the biomolecule, then the separation of phase-forming agents and the target molecule. Finally, the phase with the biomolecule goes to a high-resolution polishing stage, and the following extraction cycle re-uses the recycled phase-forming components. In parallel, it is necessary to remove the contaminants of the phase with no biomolecule, usually through ultrafiltration or precipitation (low-resolution separation methods). Next, the recycled phase-forming agents should be used to promote the formation of the next extractive system. (DESAI *et al.*, 2016)

ABS can provide a simple and integrated approach for clarification, concentration, and purification of biomolecules, with the possibility to control their selectivity by adjusting the biphasic environment and enhancing their versatility by using affinity ligands (conjugation of modifying groups to favor the partition of solutes to a particular phase). (SOARES *et al.*, 2015) It is also possible to use “back-extraction” (also called re-extraction, which is the integration of two or more stages of extraction using ABS) to obtain higher purity values. Moreover, ABS accommodate very high loads of biomolecules (up to 50 g.L⁻¹ in a single system), are relatively easy to implement and scale-up, as well as are simple to integrate with other downstream techniques, (SOARES *et al.*, 2015) as exemplified in **Figure 4**. Thus, in a single step, some ABS can integrate the clarification, concentration, and purification stages, reducing the number of downstream units and simplifying the design of the manufacturing process. ABS also require relatively simple materials and equipment, mostly using well-established conventional liquid-

liquid extraction apparatus available in the chemical industry, like mixer-settlers, columns, and centrifugal contactors. (GOMES *et al.*, 2009; ROSA *et al.*, 2010) Furthermore, the performance data is usually retained from laboratory to industrial scale, being the technical feasibility observed up to 100,000 L for the purification of proteins. (ROSA *et al.*, 2010)

Although ABS are promising platforms for the purification of biomolecules, it is imperative to overcome certain drawbacks for their appropriate use on an industrial scale. The molecular understanding of ABS partitioning processes, in particular for proteins, is still insufficient and hinders the ability of researchers to model and optimize the recovery stages. (SOARES *et al.*, 2015) Because the use of ABS involves several variables, it is hard to predict the outcome of partition, and therefore, the study of ABS relies heavily on empirical data. Another difficulty for large scale use in the application of ABS is the long time required for phase separation when using systems composed of viscous components (up to 15 h to obtain a complete separation). (SOARES *et al.*, 2015) On the other hand, although the use of less-viscous compounds (*e.g.*, organic and inorganic salts) favors the separative process, the high ionic concentrations for certain salts could potentially corrode metal pumps and other equipment. (SOARES *et al.*, 2015) It is possible to minimize this issue by reducing salt concentrations or using other forming agents.

Other disadvantages of ABS are their high cost and the environmental impact of some ABS-forming compounds (*e.g.*, polymers, ionic liquids) and, thus, the total recovery and recycling of the phase forming agents are essential to enable their industrial application. (CLÁUDIO *et al.*, 2014; ROSA *et al.*, 2010; SOARES *et al.*, 2015) However, some studies solved most of the ABS drawbacks by completely recovering ABS components, (CLÁUDIO *et al.*, 2014; ROSA *et al.*, 2010; SOARES *et al.*, 2015) or by designing economical, non-corrosive, and sustainable ABS composed of non-ionic compounds or low-cost polymers. (SOARES *et al.*, 2015) Thus, it is still possible to consider ABS as a valid option for the downstream processing of biomolecules.

Depending on the biological source, different bioproducts can be easily recovered and even purified by a proper choice of the ABS. As an example, it is possible to extract monoclonal antibodies, (MUENDGES *et al.*, 2015; MÜNDGES *et al.*, 2015b; OELMEIER *et al.*, 2012; ZIJLSTRA *et al.*, 1996) antibodies, (AZEVEDO *et al.*, 2007, 2008, 2009; FERREIRA *et al.*, 2008; MÜNDGES *et al.*, 2015a; ROSA *et al.*, 2009a, 2009b, 2012) and virus (JACINTO *et al.*, 2015) from CHO cells, in addition to monoclonal antibodies (SULK *et al.*, 1992; ZIJLSTRA *et al.*, 1995, 1996) and antibodies (AZEVEDO *et al.*, 2007, 2009; ROSA *et al.*, 2009a) from

hybridoma cells. From plants and plant cells, it is also possible to recover monoclonal antibodies, (PLATIS; LABROU, 2006) as well as pigments, (CHETHANA *et al.*, 2007; NITSAWANG *et al.*, 2006) enzymes, (CAO *et al.*, 2008; YÜCEKAN; ÖNAL, 2011) and other proteins. (BALASUBRAMANIAM *et al.*, 2003; PLATIS; LABROU, 2006; SELVAKUMAR *et al.*, 2012) ABS can also be used to obtain proteins, (QU *et al.*, 2009; RITO-PALOMARES *et al.*, 2000; WALKER *et al.*, 1996) enzymes, (BOERIS *et al.*, 2009; DELGADO *et al.*, 1990; PÉREZ *et al.*, 2015, 2015) antibodies, (FERREIRA *et al.*, 2016) and hormones (HE *et al.*, 2005; LIU *et al.*, 2006a) from human and animal fluids and tissues. It is even possible to extract proteins from cheese (RITO-PALOMARES; HERNANDEZ, 1998) and fungus, (SELBER *et al.*, 2004) enzymes from fungus (DEIVE *et al.*, 2011; NAGANAGOUDA; MULIMANI, 2008; PRINZ *et al.*, 2014) and virus from insect cells (LUECHAU *et al.*, 2011) using ABS as a downstream process. This vast diversity of bioproducts already successfully extracted with ABS demonstrates the great potential of this platform for the extraction of biomolecules produced by genetic engineering.

As stated before, many biological-based ABS applications are related to the recovery of biomolecules from fermented broth produced by microorganisms, using diverse bacteria and yeast microorganisms. (BERLEC; ŠTRUKELJ, 2013; JOZALA *et al.*, 2016; RITO-PALOMARES, 2004; ROSA *et al.*, 2010) Several studies show the extraction of different biological substances from bacterial fermented broth or cell lysate. For *E. coli*, this includes proteins, (MAZZOLA *et al.*, 2006b) virus, (LIU *et al.*, 1998) plasmid DNA (pDNA), (FRERIX *et al.*, 2005; GOMES *et al.*, 2009; RAHIMPOUR *et al.*, 2006; RIBEIRO *et al.*, 2002; TRINDADE *et al.*, 2005) and enzymes. (AGUILAR *et al.*, 2006; GUAN *et al.*, 1992; JOHANSSON, 1974; KULKARNI *et al.*, 1999; SOUZA *et al.*, 2015b) ABS-based protocols can also be applied to recover toxin A (CAVALCANTI *et al.*, 2006) and enzyme from *Clostridium perfringens*, (PORTO *et al.*, 2008, 2007) virus from *Salmonella typhimurium*, (LIU *et al.*, 1998) pigments from *Spirulina platensis*, (PATIL; RAGHAVARAO, 2007) enzymes from *Penicillium restrictum*, (BARROS *et al.*, 2014) *Penicillium janthinellum* (COSTA *et al.*, 1998, 2000) and *Bacillus* sp., (SINHA *et al.*, 1996, 1996) antibiotics from *Streptomyces aureofaciens* (PEREIRA *et al.*, 2013b) and pharmaceuticals from *Streptomyces clavuligerus*. (PANAS *et al.*, 2017; PEREIRA *et al.*, 2012) Yeast is also suitable for biomolecules synthesis, and ABS studies have shown the recovery of enzymes from *S. cerevisiae* (RIBEIRO *et al.*, 2007) and proteins from *Pichia pastoris* (*P. pastoris*) (DONG *et al.*, 2012) and baker's yeast. (RITO-PALOMARES; LYDDIATT, 2002)

Table 1 presents the application of ABS to the concentration or initial purification of different biomolecules being detailed the information about recovery yields (%) and purity of final product through the purification factor (PF), when available.

Table 1 – Examples of biomolecules extracted from cell lysate* or fermented broth** using ABS.

Microor-ganism	Biomolecules	ABS	Recovery yield (%)	Purity (%)	PF ^a	Reference
<i>E. coli</i>	Apolipoprotein A (Apo A)**	EOPO/Reppal	82	-	2.7	(PERSSON <i>et al.</i> , 1998)
	Green Fluorescent Protein (GFP)*	PEG/C ₆ H ₅ Na ₃ O ₇	91	-	3.3	(LI; BEITLE, 2002)
		PEG/C ₆ H ₅ Na ₃ O ₇ , back extraction with PEG/ Na ₂ SO ₄ + IMAC	71	-	94.0	
	Human growth hormone (hGH)*	EOPO/waxy starch	70	-	5.0	(PERSSON <i>et al.</i> , 2005)
	Human growth hormone antagonist (hGHA)*	PEG/(NH ₄) ₂ SO ₄	83	-	-	(HAYENGA; VALEX, 2002)
	Human recombinant interferon α-1 (rhIFNα-1)*	PEG-phosphate ester/K ₂ HPO ₄ /NaCl	99	-	25.0	(GUAN <i>et al.</i> , 1996)
	Human recombinant interferon-α2b (IFN-α2b)*	2-propanol/(NH ₄) ₂ SO ₄ /NaCl	75	-	16.2	(LIN <i>et al.</i> , 2013)
		1-Propanol/K ₂ HPO ₄	97	-	2.6	
		2-Propanol/K ₂ HPO ₄	92	-	1.4	
		Ethanol/K ₂ HPO ₄	60	-	0.6	
		1-Propanol/(NH ₄) ₂ SO ₄	82	-	7.0	
		2-Propanol/(NH ₄) ₂ SO ₄	78	-	12.8	
		Ethanol/(NH ₄) ₂ SO ₄	35	-	3.2	
		1-Propanol/C ₆ H ₇ NaO ₇	83	-	3.3	
		2-Propanol/C ₆ H ₇ NaO ₇	79	-	5.0	
		Ethanol/C ₆ H ₇ NaO ₇	39	-	1.2	
		PEG/KH ₂ PO ₄ /NaCl	41	-	26.3	
	Human insulin-like growth factor-I (IGF-I)*	PEG/Na ₂ SO ₄	70	97	-	(HART <i>et al.</i> , 1994)
	L1 protein*	PEG/KH ₂ PO ₄	65	-	-	(RITO-PALOMARES; MIDDELBERG, 2002)
<i>P. pastoris</i> **	Recombinant human serum albumin (rHSA)	ethanol/K ₂ HPO ₄	100	-	-	(DONG <i>et al.</i> , 2012)
	GFP*	PEG/C ₆ H ₅ Na ₃ O ₇	91		3.3	(LI; BEITLE, 2002)
		ethanol/Na ₂ CO ₃	91	-	-	(DONG <i>et al.</i> , 2012)
		ethanol/Na ₂ SO ₄	74	-	-	
		ethanol/(NH ₄) ₂ SO ₄	75	-	-	
		ethanol/K ₂ HPO ₄ associate with HIC	75	99	-	

^aPF – Purification Factor. *Source:* Produced by the author and published (adapted) in (DOS SANTOS *et al.*, 2018a).

As depicted in **Table 1**, a great option to recover biomolecules consists of the use of polymer/starch-based ABS. In 1998, Persson *et al.* demonstrated the extraction of Apolipoprotein A-I (Apo A, Milano variant) from *E. coli* broth, using ABS composed of a copolymer of ethylene oxide and propylene oxide (EOPO) and reppal (hydroxypropyl starch) (EOPO/Reppal ABS). (PERSSON *et al.*, 1998) In that work, the group evaluated the effect of polymer concentration, salt composition, and pH in the partition and purification of Apo A, concluding that higher pH values, lower protein concentration, and the addition of salt (at low concentration) and urea improve the protein purification. Additionally, by adding a higher concentration of polymers and a small concentration of salts, the protein solubility decreased, and the precipitation of bulk proteins was favored, increasing Apo A purification yields. However, the recovery yields of Apo A were lower at higher salt concentrations. The authors suggested this occurred because Apo A precipitated together with the contaminants with the increase in ionicity. (PERSSON *et al.*, 1998)

The last parameter evaluated by the authors showed how the addition of urea affects the purification of Apo A. (PERSSON *et al.*, 1998) The researchers found that with the destabilization of the hydrogen bond interactions in the system, the solubility of Apo-1 was increased, reducing the protein aggregation, and consequently, allowing an improvement in the purification. With a complete understanding and control of variables under study (and thus, a consequent increase or decrease of the protein solubility), the authors identified the best extractive system, composed of 17 % Reppal PES200, 12 % EO30PO70, and 2.5 M urea, in which 82 % of ApoA was recovered from a 5 kg scale system, exhibiting a PF of 2.7. (PERSSON *et al.*, 1998)

In 2005, Persson *et al.* have also shown the aptitude of ABS composed of a random copolymer of ethylene oxide and propylene oxide (EOPO) and starch (waxy starch) to extract human growth hormone (hGH) from *E. coli* homogenate, demonstrating better purification levels than clarification by centrifugation. In that work, the researchers found that the systems with higher concentrations of polymer had the best results, but the addition of salt did not have any significant impact on the extraction efficiency. The authors claimed that the increase in the purification yields was a result of the precipitation of proteins when increasing the concentration of polymer, considering there was a reduction in the water content of the system. The best EOPO/waxy starch-based ABS allowed the recovery of hGH with a yield of 70 % and a PF of 5.0. (PERSSON *et al.*, 2005) In that work, the group recovered the protein mainly in the polymer-rich phase; thus, the authors considered that this phase was compatible with the

further downstream chromatography stages (although it would still be necessary to consider the high viscosity of loading material, particularly at low temperatures). (PERSSON *et al.*, 2005) In the end, after running some process calculations, they even concluded that the use of this ABS with high polymer concentration could be a feasible option for the primary recovery of the hGH from a 10,000 L fermentation. (PERSSON *et al.*, 2005) The introduction of this initial extraction stage (although with some overall losses) can increase the purity levels, stabilizing the feed streams, reducing the processing time and raw material requirements, and allowing an increase of the chromatography resin capacity. (PERSSON *et al.*, 2005)

Polymer/salt-based ABS are one the most known and traditional ABS, and several studies showed their application in the extraction of biomolecules. A study from 2002 showed the recovery of 65 % of a recombinant viral coat protein from *E. coli* cell lysate (L1 protein) using an ABS composed of polyethylene glycol (PEG) and potassium hydrogen phosphate (K_2HPO_4). (RITO-PALOMARES; MIDDELBERG, 2002) The group also tested the use of urea as an additive, but in that case, the higher concentrations of urea compromised the polymer/salt ABS formation. As previously demonstrated by Persson *et al.*, (PERSSON *et al.*, 1998, 2005) it was possible to obtain the best partitioning at high compositions of the phase-forming agents (an increase of TLL). (RITO-PALOMARES; MIDDELBERG, 2002) The authors considered that the changes in the free volume (available for protein dissolution in the PEG-rich phase) or the density of the phases caused this partitioning behavior. (RITO-PALOMARES; MIDDELBERG, 2002) However, it is relevant to note that these were only assumptions based on the partition coefficient (*K*) values, with no further studies to evidence the mechanisms that occur at the molecular level.

In another study using a polymer/salt system, but with modified PEG (PEG-phosphate ester), K_2HPO_4 , and inorganic salts [like sodium chloride (NaCl)], it was possible to recover the human recombinant interferon α -1 (rhIFN α -1) from an *E. coli* cell lysate. (GUAN *et al.*, 1996) The best system (PEG-phosphate ester/ K_2HPO_4 /NaCl) has allowed 99.6 % of the rhIFN α -1 recovery, attaining a PF of 25.0. The authors suggested that the mechanism behind the efficient protein extraction was the electrostatic repulsion effect, considering that most of the modified PEG were present in the phosphate-rich bottom phase. (GUAN *et al.*, 1996) Afterward, the researchers introduced a back-extraction step to transfer the partially purified rhIFN α -1 from the PEG-rich top phase to a phosphate buffer, with a proper adjustment of the pH and the concentrations of phase forming-agents. This approach showed that the pH was a very sensitive

parameter, and it was possible to recover around 76 % of the purified rhIFN α -1 in the bottom phase at pH 6.0. (GUAN *et al.*, 1996)

More recently, also using PEG/potassium phosphate monobasic (KH₂PO₄)/NaCl-based ABS, 41 % of a human recombinant interferon- α 2b (IFN- α 2b) was purified (PF = 26.3) from *E. coli* cells. (LIN *et al.*, 2013) Lin *et al.* evaluated the influence of PEG molecular weight, tie-line length, volume ratio, crude stock loading, pH, and NaCl concentration in the partitioning of the biomolecule, achieving the best condition with ABS composed of 4 wt% of PEG-8000, 13 wt% of potassium phosphate, 0.5 wt% of NaCl and 10 wt% of the crude stock, at pH 6.5. (LIN *et al.*, 2013)

The same research group also evaluated the purification of IFN- α 2b, applying alcohol/salts-based ABS. The most promising results demonstrate that the combination of 1-propanol, 2-propanol, or ethanol, with aqueous solutions of K₂HPO₄, ammonium sulfate [(NH₄)₂SO₄] or monosodium citrate (C₆H₇NaO₇) salts, can allow extracting 35-97 % of the biopharmaceutical from bacterial extracts, with variable PF from 0.6 to 12.8. (LIN *et al.*, 2013)

The authors also showed that it was possible to control the IFN- α 2b recovery and PF with a pH adjustment. For example, different salts altered the pH of the ABS [*e.g.*, pH 6.0-6.5 for (NH₄)₂SO₄, pH 8.0–8.3 for C₆H₇NaO₇ and pH 9.0–9.5 for K₂HPO₄], which impacted the purification indexes (*e.g.*, the increase of pH caused higher recovery yields, but lower PF). (LIN *et al.*, 2013) The researchers suggested that the contaminants and IFN- α 2b migrated towards the top phase when the pH increased, as a result of the electrostatic repulsion of the salt-rich bottom-phase over the negatively charged proteins (considering more contaminant proteins have a negative charge at high pH values).

The authors chose the (NH₄)₂SO₄ salt for additional studies considering that the purity of biomolecule was the primary purpose of their downstream processing, and this salt as an additive provided the best purification indexes. To improve the purity yields, the authors have then selected the best overall system [2-propanol/(NH₄)₂SO₄] and added other salts as electrolytes, increasing the PF to 16.2 for a recovery of 75 % of IFN- α 2b when used 2-propanol/(NH₄)₂SO₄/NaCl-based ABS. (LIN *et al.*, 2013) They observed that the addition of NaCl increased the difference of the relative hydrophobicity between both co-existing phases, and thus, intensifying the interaction between IFN- α 2b's hydrophobic surface area and the propanol's hydrophobic chain, allowing higher partitioning and purification yields.

Hart *et al.* also proposed the use of ABS composed of PEG and sodium sulfate (Na_2SO_4) as an effective alternative to purify human insulin-like growth factor-I (IGF-I) produced by *E. coli* cells, achieving yields around 70 % and purity of 97 %. For this system, hydrophobic interactions mainly controlled the partitioning behavior and isolation of IGF-I. (HART *et al.*, 1994)

In 2002, PEG/ $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ -based ABS, using NaCl as an additive, were used to extract 90 % of GFP from the cell lysate of *E. coli*. However, due to low PF 3.3 attained, it was necessary to integrate this particular system to a further back extraction using a PEG/ Na_2SO_4 -based ABS and an additional step with Immobilized Metal Affinity Chromatography (IMAC). (LI; BEITTE, 2002) This integrative downstream approach allowed the recovery of 71 % of recombinant GFP with a PF around 94. The authors have demonstrated the multiple advantages of this integrative system, since the initial use of ABS provided a selective clarification, removing contaminants and concentrating the GFP. Since the IMAC chromatography can easily tolerate the salts applied in the extraction, these are complementary operations. The back extraction using different ABS can allow the increase of PF values, since it is possible to adjust different interactions (such as hydrophobic interactions, salting-out effects, among others) by the manipulation of the type and concentration of the phase forming used, favoring the purification of the biomolecules. (LI; BEITTE, 2002)

Most ABS studies involve the recovery of biomolecules from *E. coli* cell lysate; however, some works demonstrated the use of these platforms to recover them from yeast-fermented broths. In 2012, Dong *et al.* used alcohol/salt-based ABS for the recovery of recombinant human serum albumin (rHSA) from *P. pastoris*. (DONG *et al.*, 2012) In that particular work, ABS composed of ethanol and salts [K_2HPO_4 , sodium carbonate (Na_2CO_3), Na_2SO_4 , and $(\text{NH}_4)_2\text{SO}_4$] were able to recover 75 to 100 % of the protein. However, these values were almost the same for the extraction performed in a temperature range from 4 to 30 °C and at different protein concentrations (1 to 30 g.L^{-1}), indicating that both parameters had little effect on protein recovery. (DONG *et al.*, 2012) On the other hand, an increase of the ethanol concentration or in the salt concentration can boost rHSA recovery yields. (DONG *et al.*, 2012) The authors achieved an increase in recovery of rHSA when the protein was extracted directly from the unclarified fermented broth (without a centrifugation step) since it was possible to recover additional rHSA that persisted between the cells. Additionally, the group was able to demonstrate the integration of ABS with further chromatography. The system with the highest extraction aptitude (ABS composed of ethanol and K_2HPO_4) was associated with a hydrophobic

interaction chromatography (HIC), recovering approximately 75 % of highly pure rHSA (> 99 %) from the fermented broth (up to 73 L). (DONG *et al.*, 2012)

Besides the promising academic studies, the use of ABS to purify biomolecules is catching the attention of some industrial partners, and different patent deposits can confirm this interest. In 1999, there was a deposit of a patent that showed the use of PEG/salt-based ABS as effective platforms for the extraction of hGH and human growth hormone antagonist (hGHA) from *E. coli*. This technique was able to recover 83 % of hGHA with a PEG/(NH₄)₂SO₄-based ABS. (HAYENGA; VALEX, 2002)

1.5.1.3 Trends and prospects for ABS for purification of biomolecules

With the increase of the market for biomolecules, the development of new production and purification technologies is crucial not only to allow higher yields and reduce overall costs but also to ensure safer, greener, and simpler processes. ABS, as previously elucidated, appears like an excellent choice to be used as an initial stage for the purification of bioproducts, recently attracting the interest of academic and industrial researchers. However, it is necessary to guarantee a proper selection of the phase-forming agents for the ABS to ensure the fulfillment of the safety and biocompatibility requirements of modern industrial processes.

In this context, novel substances [namely, ILs and deep eutectic solvents (DES)] are a promising option for ABS formulation, allowing the compliance of the sustainability and biocompatibility requirements of the downstream processing. The use of ILs and DES is being pushed by the growing public and governmental policies for industries to adopt more sustainable and greener technologies. (SHELDON, 2005) There is also an increasing concern with the environment on a global scale, and biotechnology and pharmaceutical industries are already adapting their technologies to reduce the impacts of manufacturing processes on nature and public health. Considering this concern, IL-based ABS appear as a perfect technology to fully accomplish the fundamental requirements of a biotechnological concentration/initial purification platform, (HUDDLESTON *et al.*, 1998) since several ILs are defined as friendly solvents, exhibiting low toxicities, low volatilities, and high biodegradability. (AMDE *et al.*, 2015; DE MORAIS *et al.*, 2015; E SILVA *et al.*, 2014; EGOROVA; ANANIKOV, 2014; FUKAYA *et al.*, 2007; PETKOVIC *et al.*, 2010; SANTOS *et al.*, 2015; VENTURA *et al.*, 2013a; WOOD; STEPHENS, 2010) However, ILs are also a very diverse class of chemical compounds, and not all of them present these advantageous properties.

As previously discussed, not all ILs are biodegradable and biocompatible with organisms and macromolecules, particularly those with fluorinated anions or based on imidazolium cations. Thus, it is only possible to determine the potential of the IL-based ABS as a platform for biopharmaceutical purification after a proper selection of the extractive system. Pereira *et al.* suggested that systems formed by cholinium- or amino acid-based ILs are the most promising for environmentally-friendly applications because these two IL classes can be synthesized from renewable raw materials and are also compounds abundant in nature. (PEREIRA *et al.*, 2016a) Moreover, Kunz and emphasized that modern solvents must fulfill some environmental concerns, such as biodegradability and low toxicity, and also indicated cholinium ILs as more sustainable solvents for industrial applications. (KUNZ; HÄCKL, 2016) Additionally, it is crucial not only to pursue the use of environmentally-friendly ILs in ABS, but to combine them with other biocompatible phase-forming agents, such as (bio)polymers, (bio)surfactants, and biodegradable salts.

The environmentally-friendly character of several ILs is indeed one of their most attractive properties for their incorporation in purification systems. However, ILs have other compelling attributes, such as tunability, easy tailoring, high solvation ability for a wide range of organic and inorganic solutes, negligible vapor pressure, and good chemical and thermal stabilities. (HOLBREY *et al.*, 2008; SEDDON, 1997) These advantageous characteristics have ignited the interest in the use of ILs in industrial processes, particularly in the field of extraction and purification of biomolecules. Thus, in the last decade, several ABS combining ILs and polymers, salts, alcohols, among others as phase-forming agents have been determined and proposed as alternative separation processes. (FREIRE, 2016; FREIRE *et al.*, 2012; PEREIRA *et al.*, 2010, 2013a) Different groups also demonstrated the use of ABS to recover several bioproducts from the fermented broth, such as antibiotics [penicillin G, (LIU *et al.*, 2006b) tetracycline (PEREIRA *et al.*, 2013b), clavulanic acid (PANAS *et al.*, 2017)], lipase, (SOUZA *et al.*, 2015a, 2015b; VENTURA *et al.*, 2012) polysaccharides (YAN *et al.*, 2014) and colorants. (SHEN *et al.*, 2014; TORRES *et al.*, 2018; VENTURA *et al.*, 2013b)

In **Table 2**, there is a compilation of IL-based ABS already applied for the extraction of bioproducts from the fermented broth, providing some details regarding the producer microorganism, the type of the IL-based systems (ILs and co-existing phase forming agents used), and partitioning/purification yields.

Table 2 – Examples of bioproducts extracted and purified from different fermented broths using different IL-based ABS.

(continue)

Bioproduct		Microorganism	IL-based ABS		Partitioning/Purification		Reference
Class	Name		IL	Coexisting phase-forming agent	Recovery Yield or Extraction Efficiency (%)	Purification Factor	
Antibiotics	Penicillin G	<i>Penicillium</i> sp.	[C ₄ mim]Cl	NaH ₂ PO ₄	91.7	-	(LIU <i>et al.</i> , 2006b)
	Tetracycline	<i>Streptomyces aureofaciens</i>	[Ch][Bic]	PEG-600	81.6	-	(PEREIRA <i>et al.</i> , 2013b)
			[Ch]Cl	PEG-600	82.5		
				K ₃ PO ₄	92.4		
	Clavulanic Acid	<i>Streptomyces clavuligerus</i>		PEG-600	44.4	22.7	(PANAS <i>et al.</i> , 2017)
			[Ch]Cl	PEG-550-OMe	45.7	4.7	
			PEG-600/NaPA-8000	55.9	2.4		
Colorants or Pigments	Natural red colorant	<i>Penicillium purpurogenum</i>	[C ₄ mim]Cl	potassium citrate buffer	62.4	-	(VENTURA <i>et al.</i> , 2013b)
			[N _{2,2,2,2}]Br		51.5	-	
			[N _{4,4,4,4}]Br		80.2	-	
	Red <i>Monascus</i> pigment	<i>Talaromyces amestolkiae</i>	[C ₈ mim]Cl	Triton X-114 ^a	(only reported K)	-	(TORRES <i>et al.</i> , 2018)
			[C ₁₀ mim]Cl				
			[C ₁₂ mim]Cl				
	Red <i>Monascus</i> pigment	<i>Monascus anka</i>	[C ₄ mim][PF ₆]	Triton X-100 ^a	86	-	(SHEN <i>et al.</i> , 2014)

Table 2 – Examples of bioproducts extracted and purified from different fermented broths using different IL-based ABS.

(conclusion)

Bioproduct		Microorganism	IL-based ABS		Partitioning/Purification		Reference
Class	Name		IL	Coexisting phase-forming agent	Recovery Yield or Extraction Efficiency (%)	Purification Factor	
Enzymes	Lipase	<i>Bacillus</i> sp. ITP-001	[C ₂ mim]Cl	PEG-1500 + K ₂ HPO ₄ /KH ₂ PO ₄	77.2	175.6	(SOUZA <i>et al.</i> , 2015b)
			[C ₄ mim]Cl		77.5	169.3	
			[C ₆ mim]Cl		76.6	220	
			[C ₈ mim]Cl		90.2	245	
			[C ₂ mim]Cl	PEG-4000 + K ₂ HPO ₄ /KH ₂ PO ₄	60.2	181.3	
			[C ₆ mim]Cl		79.3	254	
			[C ₂ mim]Cl	PEG-8000 + K ₂ HPO ₄ /KH ₂ PO ₄	89.7	160.6	(SOUZA <i>et al.</i> , 2015a)
			[C ₆ mim]Cl		80.9	222.8	
			[Ch][Bit]	Tetrahydrofuran	90	136.8	
			[C ₄ mim][N(CN) ₂]		96.1	26	
			[C ₄ mpyr]Cl		94.2	36.7	
			[C ₄ mim]Cl		90.6	40.8	
			[C ₈ mim]Cl		92.2	51	
Polysaccharides	<i>Cordyceps sinensis</i> polysaccharides	<i>Burkholderia cepacia</i> ST8	[N _{4,4,4,4}][BES]	(NH ₄) ₂ SO ₄	from 93.7 to 98.7	from 8.9 to 22.4	(LEE <i>et al.</i> , 2017)
		<i>Cordyceps sinensis</i>	[C ₄ mim]Cl	K ₃ PO ₄	89.4	-	(YAN <i>et al.</i> , 2014)

^a A particular type of ATPS, generally named as Aqueous Micellar Two-Phase systems (AMTPS).

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018a).

From **Table 2**, it is possible to observe that several IL-based ABS combine ILs with a wide range of phase-forming agents. They include polymers (PEG of different molecular weights and polyethylene glycol methyl ether 550 (PEG-550-OMe)), surfactants (Triton X-114 and Triton X-100), salts (sodium dihydrogen phosphate (NaH_2PO_4), potassium phosphate tribasic (K_3PO_4) and $(\text{NH}_4)_2\text{SO}_4$), buffers (potassium citrate buffer and phosphate buffer $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) and organic compounds (tetrahydrofuran), were already applied in the extraction of bioproducts from the complex fermented broth.

The ILs used in the formation of ABS and employed in the recovery of fermented biomolecules from **Table 2** ranged from the most common imidazolium-based ILs {e.g., 1-ethyl-3-methylimidazolium chloride ($[\text{C}_2\text{mim}]\text{Cl}$), 1-butyl-3-methylimidazolium chloride ($[\text{C}_4\text{mim}]\text{Cl}$), 1-hexyl-3-methylimidazolium chloride ($[\text{C}_6\text{mim}]\text{Cl}$), 1-octyl-3-methylimidazolium chloride ($[\text{C}_8\text{mim}]\text{Cl}$)} to benign ammonium-based ILs {such as, tetraethylammonium bromide ($[\text{N}_{2,2,2,2}]\text{Br}$), tetrabutylammonium bromide ($[\text{N}_{4,4,4,4}]\text{Br}$), cholinium chloride ($[\text{Ch}]\text{Cl}$), cholinium bicarbonate ($[\text{Ch}][\text{Bic}]$), cholinium bitartrate ($[\text{Ch}][\text{Bit}]$)}, among others. **Table 2** also shows the excellent aptitudes of most IL-based ABS to recover and purify a diverse set of fermented biomolecules under study. In general, the recovery yields (or extraction efficiencies) are higher than 80 % (approximately), and in some cases, the PF factors were higher than 100, indicating that IL-based ABS can be effective units to recover proteins from the complex fermented broth. Besides, there are also many examples of successful selective separation and extraction of macromolecules from another medium besides fermented broth, using IL-based ABS to recover hormones, (HE *et al.*, 2005) enzymes, (CAO *et al.*, 2008; DREYER *et al.*, 2009; DREYER; KRAGL, 2008; PEI *et al.*, 2009; SOUZA *et al.*, 2015b) and proteins, (DREYER *et al.*, 2009; DU *et al.*, 2007; PEI *et al.*, 2009; RUIZ-ANGEL *et al.*, 2007) proving the high potential for the purification of biomolecules.

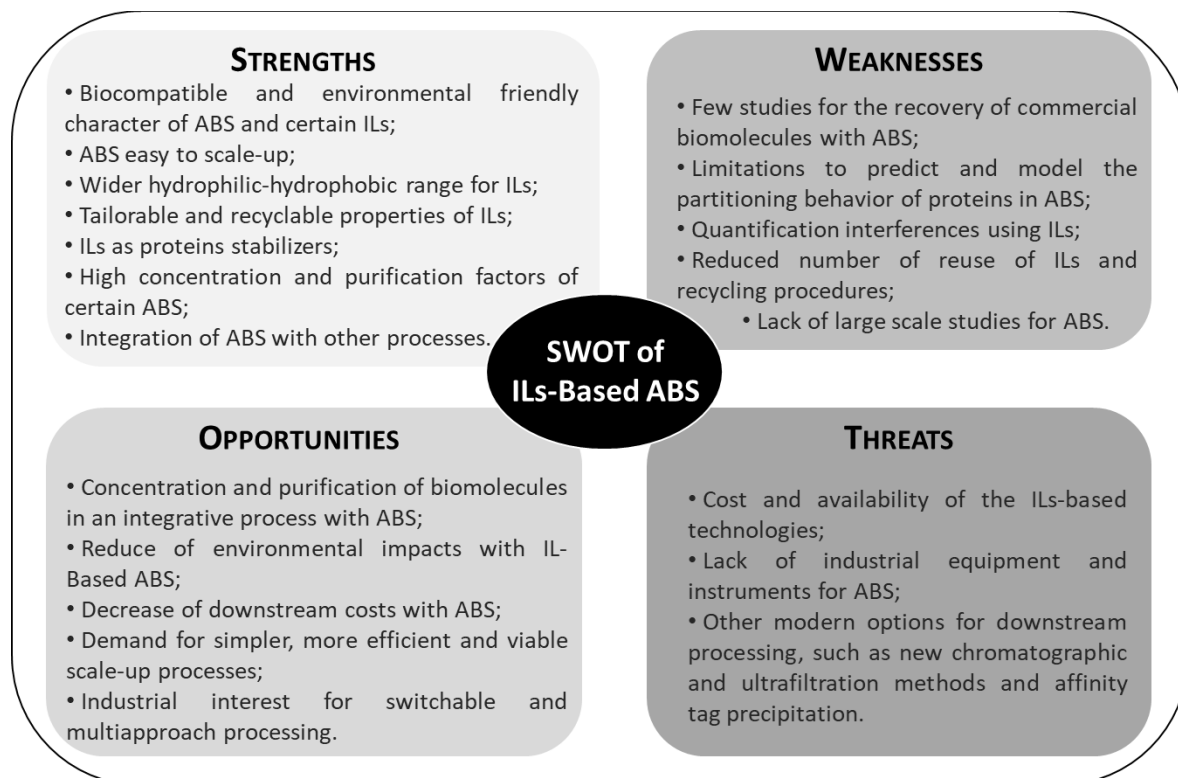
There are also works with applications of IL-based ABS for the recovery of biomolecules of medical use, for example, the purification of the anti-leukemic biopharmaceutical asparaginase produced by *E. coli* cells. (SANTOS *et al.*, 2018) Considering L-asparaginase is used to treat acute lymphoblastic leukemia and lymphosarcoma, this enzyme requires very high purity levels for its commercialization. To increase its purification yields and to reduce its downstream costs, Santos *et al.* proposed the use of polymer-salt ABS composed of PEG and citrate buffer and with ILs as adjuvants. Since the biopharmaceutical was produce intracellularly, they combined IL-based ABS with a previous cell membrane permeabilization stage (using n-dodecane and glycine). This approach was very successful, seeing it was possible

to recover more than 87 % of highly pure (PF around 20) and active (specific activity around 3.61 U.mg^{-1}) from a crude extract (initial specific activity around 0.18 U.mg^{-1}). (SANTOS *et al.*, 2018) The authors obtained this excellent result by adding 5 wt% of 1-butyl-3-methylimidazolium methanesulfonate ($[\text{C}_4\text{mim}][\text{CH}_3\text{SO}_3]$) as an adjuvant to the PEG/citrate buffer ABS. Remarkably, the authors even showed that it is possible to increase the purification yields with the addition of a precipitation step to the downstream processing. Thus, it was possible to obtain better results with the integration of an ammonium sulfate precipitation and the IL-based ABS, achieving PF around 173. (SANTOS *et al.*, 2018) This recent work shows the viability of integrating IL-based ABS and other purification techniques for the successful purification of biopharmaceuticals directly from the complex microbial sources.

Despite many applications, the use of IL-based ABS is still far from an industrial application. For example, the number of scale-up studies of downstream stage integration that includes the recycling and reuse of phase-forming components is still limited; however, they are nonexistent if considering only investigations with real industrial downstream processing. (FREIRE, 2016) This lack of works is mainly a result of the generalization about the price and industrial availability of ILs, which are not always true and quite skewed. (FREIRE, 2016) Thus, it is evident that further economic viability assessments and technological scaling-up are still required to prove if the IL-based ABS can be applied in an industrial setting.

IL-based ABS, as discussed previously for traditional ABS, present good opportunities for the concentration and purification of biopharmaceuticals in an alternative and integrative setting, with simpler and easier scale-up processes, reduced costs, and environmental impacts. (FREIRE, 2016; SOARES *et al.*, 2015) To elucidate the whole picture of the actual stage of IL-based ABS, in **Figure 6**, the elements of these systems are described by a general SWOT analysis (strengths, weaknesses, opportunities, and threats). Certain SWOT presented in **Figure 6** are general characteristics of ABS, while others are specific for IL-based ABS, as will be described in the following paragraphs. As stated before, the major strengths of IL-based ABS comprise the possibility of tailoring, designing, and tuning the properties of the phases, which can allow obtaining a wide hydrophilic-hydrophobic range, and consequently, a selectivity aptitude of different biopharmaceuticals.

Figure 6 – General SWOT analysis (strengths, weaknesses, opportunities, and threats) of IL-based ABS for the extraction and purification of biomolecules.



Source: Produced by the author and published (adapted) in (DOS SANTOS *et al.*, 2018a).

Proper control of these properties can allow the concentration and complete extraction and purification of several biomolecules. These high concentration and purification factors are a result of other ABS strengths, namely, their biocompatible and environmentally friendly characteristics, and protein stabilizer effects. As shown in **Figure 6**, these strengths fully accomplish several opportunities of downstream processing, since the biotechnological industry is searching for integrative processes that allow the obtaining of high concentration and purification yields, reducing at the same time the environmental impacts, and overall downstream costs. Thus, IL-based ABS are a promising platform for the recovery of quite unstable bioproducts.

Like other ABS, the easy scaling-up and recycling processing seems to be a strength for a future industrial application. However, there are still only a few studies on the reuse and recyclability of ILs, which could complicate this scaling-up process. Moreover, there is also a lack of large-scale studies using even more traditional ABS, and they are fewer when only considering the recovery of biomolecules.

Other weaknesses for the large-scale use of IL-based ABS are related to the limitations to predict and model the partitioning behavior of proteins and the difficulties in the analytical quantification of biomolecules in the presence of ILs. With a wide range of ILs able to form ABS and the complex nature of proteins, it is problematic to generalize and predict the general behavior of protein using these systems, and thus, it is crucial to establish well-known models that will help on the understanding about those mechanisms.

Currently, there are already mathematical and computational models that can help to predict some aspects of the formation and partitioning process of ABS, such as regression analysis, molecular dynamics (MD) simulations, conductor like screening model for real solvents (COSMO-RS), and perturbed chain-statistical associating fluid theory (PC-SAFT). (BANERJEE *et al.*, 2008; CHAUMONT *et al.*, 2005; CHEVROT *et al.*, 2006; DISMER *et al.*, 2013; KING *et al.*, 1988; KRESS; BRANDENBUSCH, 2015; KURNIA; COUTINHO, 2013; MOHAMMAD *et al.*, 2016; OELMEIER *et al.*, 2012; PADUSZYŃSKI *et al.*, 2015; PEREIRA *et al.*, 2014; SPIESS *et al.*, 2008; TOMÉ *et al.*, 2014; ZAFARANI-MOATTAR; HAMZEHZADEH, 2016) As an example, regression analysis with the information of ABS characteristics and quantitative structure-activity relationship (QSAR) of three other amino acids (tryptophan, phenylalanine, and valine) was successful in predicting the partitioning behavior of two amino acids (tyrosine and leucine) using IL-based ABS. (ZAFARANI-MOATTAR; HAMZEHZADEH, 2016)

An alternative type of modeling used the osmotic virial coefficients as access to the protein (immunoglobulin G, IgG) partitioning in PEG-phosphate ABS (without IL as phase forming agent). (KRESS; BRANDENBUSCH, 2015) Although this approach was still not used for IL-based ABS, it seems to be a feasible option to give further insights about the mechanisms of the protein partitioning in systems with ILs. MD simulations are also applied in the study of IL-based ABS formation, providing more insights about the mutual solubility of ILs, salts, and polymers, and then the main interactions governing the phase separation at the molecular level. (CHAUMONT *et al.*, 2005; CHEVROT *et al.*, 2006; DISMER *et al.*, 2013; KING *et al.*, 1988; OELMEIER *et al.*, 2012; TOMÉ *et al.*, 2014)

Other powerful tools successfully applied to predict and evaluate ABS are, for example, COSMO-RS (BANERJEE *et al.*, 2008; KURNIA; COUTINHO, 2013; MOHAMMAD *et al.*, 2016; PEREIRA *et al.*, 2014; SPIESS *et al.*, 2008) and PC-SAFT. (MOHAMMAD *et al.*, 2016; PADUSZYŃSKI *et al.*, 2015) Additionally, it is possible to use the design of experiments

(DOE), such as factorial or central composite designs, as powerful tools to analyze different parameters involved in the partitioning process, allowing an assessment of optimal conditions without having to change one parameter at a time. (GOMES *et al.*, 2009) Thus, the combination of these techniques can offer valuable information to predict the protein partitioning behavior in IL-based ABS, facilitating their development, optimization, and integration at an industrial scale.

Finally, it is necessary to overcome some threats, especially those related to the high cost and availability of IL-based technologies. Further improvements on the development of industrial equipment and instrumenting are essential to allow complete integration of IL-based ABS as a real industrial platform. These systems are not alone in the industrial and academic R&D, and researchers have developed other modern options for downstream processing in the last years, such as new chromatographic and ultrafiltration methods and affinity tag precipitation procedures, which can compete with ABS-based technologies. (FREIRE, 2016; ROSA *et al.*, 2010; SOARES *et al.*, 2015)

Based on the aptitude of some IL-based ABS to selectively separate proteins from contaminants while also maintaining their structural integrity, they appear as a promising platform for the recovery and purification of biomolecules directly from the fermented broth. However, more studies are essential to prove if IL-based ABS are effectively the next trend for the extraction of bioproducts. It is necessary to seek the integration of the purification processes, where the selected IL-based ABS should act as a cornerstone platform between the upstream processing and the high-resolution chromatographic stages. Hence, the use of IL-based ABS can increase the extraction and purification levels of many biomolecules, providing a safer, simpler, and biocompatible downstream processing. However, this is still an in-development technology, and more studies are necessary to permit the application of these techniques industrially for the purification of biomolecules.

In this context, this Thesis aimed to address some of the drawbacks for the application of ABS by developing an integrative platform with ABS and ultrafiltration for the purification of EGFP, to reduce the number of steps required to obtain pure EGFP and add the recycling of phase-forming agents to maintain the process sustainable and with a low cost. Additionally, considering the biological e medical applications of EGFP, the goal of the study was also to apply more biocompatible and greener phase-forming agents.

Considering the extensive literature review on LLE for the extraction of biomolecules aiming for more cost-effective and environmentally-friendly purification methods, the low-toxicity cholinium-based ABS were selected in this study to develop simpler, cheaper, and more sustainable platforms for the purification of EGFP. The researcher also chose this type of system because it was already successfully applied for the purification of pharmaceuticals (PEREIRA *et al.*, 2013b; SHAHRIARI *et al.*, 2013) and proteins. (QUENTAL *et al.*, 2015; SOUZA *et al.*, 2015a) Additionally, seeing the goal of this work of applying the technologies developed in this Thesis for biopharmaceuticals, the following section will provide essential concepts and definitions regarding biopharmaceuticals and their upstream and downstream processing. Besides, the section will also address the opportunities to increase the uses and access to biopharmaceuticals by improving their production, purification, and stability.

1.6 BIOPHARMACEUTICALS: DEFINITIONS, PRODUCTION AND FUTURE OPPORTUNITIES

*Section adapted from the critical review published in **Journal of Chemical Technology & Biotechnology**, v. 93, n. 7, p. 1845-1863, 2018. (DOS SANTOS *et al.*, 2018a)*

1.6.1 Biopharmaceuticals overview

Biopharmaceuticals are outstanding products from biotechnology, allowing efficient and safe therapeutic and medical solutions for previously untreatable diseases and pathological conditions. Despite new, biopharmaceuticals already represent almost one-quarter of all pharmaceutical sales and are the fastest-growing sector in the pharmaceutical industry. (HARDMAN & CO, 2017) However, the biosynthesis of these biomolecules is still very complex, and high purity levels are required for their application, resulting in exceedingly expensive final products. (DAS, 2014; YANG, 2011) Hence, the development of new, simpler, and cheaper production and purification techniques is essential to reduce manufacturing costs and allow broader use of biopharmaceuticals. This project Thesis is fully aligned with this interest, considering the goals of creating new, sustainable, and cost-effective platforms for purification and stabilization of biomolecules, envisaging future applications in the medical field.

In 2019, the pharmaceutical market generated USD 880 billion, with biotechnological drugs accounting for 28% of that value (USD 258 billion). (EVALUATEPHARMA, 2019) These are impressive numbers considering that in 2010 biopharmaceuticals accounted for only

18% of pharmaceutical sales and, even today, they have the highest annual growth in the sector, with an expansion forecast of 13.8% in the next six years (according to the Compound Annual Growth Rate, CAGR), estimated to reach USD 526 billion of annual sales by 2025. (SHAIKH; JAISWAL, 2018) The optimism of the pharmaceutical industries regarding biopharmaceuticals is confirmed seeing the best-selling drug in 2019 was Humira® (AbbVie), a monoclonal antibody to treat rheumatoid arthritis, reaching revenues of USD 19.7 billion. (PHARMA BOARDROOM, 2020; URQUHART, 2020)

By observing these remarkable numbers of market share, it is clear why the pharmaceutical industry is so focused on developing new biotechnological products. Until 1994, the United States of America (USA) or the European Union (EU) only had 25 biopharmaceuticals approved for human use, but they accepted another 241 from 1995 to 2016. (HARDMAN & CO, 2017; WALSH, 2014) Furthermore, more than 600 of the 2,950 medicines in development in 2010 were from biotechnology, confirming the importance of biotechnological products not only in the economy but also in Research and Development (R&D) pharmaceutical efforts. (KARAMEHIC *et al.*, 2013) Thus, the massive rise of the biopharmaceuticals market and research is a demonstration of their potential and impact in the medical and biotechnological fields.

Due to the increase of biological products for medical purposes and the growing importance of the sector, it is crucial to establish precise definitions for pharmaceutical products, biologics (biological products), biotechnology medicines (products of pharmaceutical biotechnology), and biopharmaceuticals. Clear and well-defined terms can facilitate the creation of comprehensive legislation and allow better communication and understanding among professionals in the field.

The FDA defines pharmaceutical products (also called drugs or medicines) (WHO, 2017) as substances intended for use in the diagnosis, cure, mitigation, treatment, or prevention of diseases and recognized by an official pharmacopeia or formulary. (FDA, 2016a) Likewise, biological products (or biologics) are within the scope of pharmaceutical products, and the FDA defines them as medical compounds from a variety of natural sources (human, animal, plant, or microorganism) used to treat, prevent, or diagnose diseases and medical conditions. (FDA, 2016b) As such, biological products include a wide range of compounds that varies from vaccines, gene, and cellular therapies, blood and blood products for transfusion and manufacturing into other products like human cells and tissues for transplantation, allergenic

extracts for diagnoses or treatment, and even tests to screen potential blood donors for infectious agents.

For biotechnology medicines or products of pharmaceutical biotechnology, in 2002, Walsh proposed that these and other associated terms can be “used to describe all products produced in part or in full by biotechnological means, either traditional or modern.” (WALSH, 2002) One of the most controversial definitions refers to biopharmaceuticals, with the most accepted interpretation written by Walsh as “a protein or nucleic acid-based pharmaceutical substance used for therapeutic or *in vivo* diagnostic purposes, which is produced by means other than direct extraction from a native (non-engineered) biological source.” (WALSH, 2002) Thus, biopharmaceuticals include recombinant proteins (enzymes, hormones, interleukins, interferons, growth factors, and blood factors), peptides recombinant and engineered synthetic, monoclonal antibodies (mAb), cell and tissue therapies, genetic therapies (genes and antisense and inhibitory RNA fragments) and vaccines recombinant and molecularly engineered. (EVENS; KAITIN, 2014) As can be seen, biopharmaceuticals include a diversity of biomolecules, and therefore, the production of these compounds can involve a wide range of methods, ranging from microorganisms to transgenic animals used as heterologous expression systems.

1.6.2 Upstream processing of biopharmaceuticals

Several heterologous expression systems are currently available for the production of biopharmaceuticals, including bacteria, yeast, and filamentous fungi, mammalian, plant and insect cells, transgenic animals, and even microalgae. (BOROWITZKA, 1995; DEMAINE; VAISHNAV, 2009; SCHMIDT, 2004) Each system presents its characteristics regarding production capacity, costs, safety, complexity, and downstream processing impact. Hence, the choice of the best heterologous expression system depends mainly on the target biopharmaceutical itself.

Other features to consider in the choice of a host system for biopharmaceutical production are their size, production costs, intracellular localization or secretion, glycosylation pattern, and proper folding. (DESAI *et al.*, 2010) Among the wide range of biopharmaceutical production systems on the market today, bacterial expression models are the most used non-mammalian cell systems (**Table 3**). They have attractive characteristics for the industrial production of biopharmaceuticals, such as rapid growth and easy cultivation, which allow high yields of recombinant protein (up to 80 % of its dry weight) even using very low-cost culture

media. (MARTÍNEZ *et al.*, 2012) Additionally, molecular tools for genetic manipulation of bacteria are better developed and established than for other expression systems. For example, their genomes and metabolic pathways are well-known, which makes the manipulation of bacteria for biopharmaceuticals production an easier and quicker task than the use of mammalian cells. (MARTÍNEZ *et al.*, 2012)

Table 3 – Examples of biopharmaceuticals approved by the FDA, obtained from bacteria as the heterologous expression system (Biopharma database). (BIOPHARMA, 2017) (continue)

Microorganism	Medical use	Biopharmaceutical	Commercial names
<i>E. coli</i>	Acromegaly treatment	pegvisomant (modified hGH, hGH antagonist)	Somavert®
	Acute decompensated heart failure	nesiritide (B-type natriuretic peptide)	Natrecor®
	Acute lymphoblastic leukemia	Pegaspargase	Oncaspar®
	Acute myocardial infarction	reteplase (plasminogen activator)	Rapilysi®
	Additional nutrition to short bowel syndrome patients	teduglutide (glucagon-like peptide 2)	Gattex®
	Age-related macular degeneration	ranibizumab (mAb fragment)	Lucentis®
	Chronic gout	pegloticase (urate oxidase, PEGylated)	Krystexxa™
	Chronic hepatitis C and B, certain cancers	interferon- α 2a	Roferon®-A
		interferon- α 2b	Intron A®
		interferon- α 2b pegylated	Peg-intron®
	Chronic hepatitis C, certain liver problems	interferon alfacon-1 (recombinant type-1 interferon)	Infergen®
	Chronic immune thrombocytopenic purpura	romiplostim (thrombopoietin peptibody)	Nplate®
	<i>Diabetes Mellitus</i>	insulin (inhalation)	Exubera®
		insulin (fast-acting lispro)	Humalog®
		insulin (zinc extended)	Humulin® L, Humulin® U
		insulin glargine (long-acting basal insulin analog)	Lantus®, Basaglar®
		glulisine (insulin analog)	Apidra®
		isophane insulin (insulin analog)	Humulin® N
		lixisenatide (glucagon-like peptide-1 receptor agonist)	Adlyxin®
	hGH and IGF-1 deficiencies	mecasermin (IGF-1)	Increlex®
		mecasermin rinfabate (IGF-1 binded to Insulin-like growth factor-binding protein-3)	iPlex™
	hGH deficiencies	somatropin (recombinant hGH)	Accretropin™, Humatrope®, Omnitrope®, Genotropin®, Norditropin®, Nutropin®
		somatrem (analog to hGH)	Protropin®
	Hypoglycemia	glucagon (recombinant)	Glucagon Human®

Table 3 – Examples of biopharmaceuticals approved by the FDA, obtained from bacteria as the heterologous expression system (Biopharma database). (BIOPHARMA, 2017) (conclusion)

Microorganism	Medical use	Biopharmaceutical	Commercial names
<i>E. coli</i>	Infectious diseases	opebacan (bactericidal/permeability-increasing protein)	Neuprex®
	Lyme disease vaccine	OspA (Outer surface protein A fragment from <i>Borrelia burgdorferi</i>)	LYMErix™
	Melanoma and renal cancer	interleukin-2	Proleukin®
	Methotrexate toxicity	glucarpidase (carboxypeptidase G2)	Voraxaze®
	Multiple sclerosis	interferon-β1b	Betaseron®, Extavia®
	Neutropenia	filgrastim (analog to the granulocyte colony-stimulating factor)	Neupogen®
	Oral mucositis	filgrastim pegylated	Neulasta®
		palifermin (truncade keratinocyte growth factor)	Kepivance®
	Osteoporosis	teriparatide (parathyroid hormone)	Forteo®
	Osteoporosis (postmenopausal)	calcitonin (salmon calcitonin)	Fortical®
	Osteoporosis, hypoparathyroidism	parathyroid hormone (1-84)	Preotact®, Natpara®
	Osteopetrosis, chronic granulomatous disease	interferon-γ1b	Actimmune®
	Prevention of severe thrombocytopenia (cancer patients)	oprelvekin (interleukin-11)	Neumega®
	Refractory Crohn's disease	TNF mAb Fab' (tumor necrosis factor (TFN) monoclonal antibody Fab' fragment–PEG conjugate)	Cimzia®
	Fat and glucose metabolism, bodyweight control.	metreleptin (leptin analog)	Myalept®
	Rheumatoid arthritis	anankira (interleukin-1 receptor antagonist)	Kineret®
	Soft tissue sarcoma	tasonermin (TNF-α1a)	Beromun®
	Surgical sutures	poly-4-hydroxybutyrate (biopolymer)	TephaFLEX®
	Systemic candidiasis	efungumab (heat shock protein mAb)	Mycograb®
	T-cell lymphoma	denileukin diftitox (fusion of interleukin-2 and diphtheria toxin)	Ontak®
<i>Neisseria meningitidis</i>	Meningitidis B vaccine	4CMenB	Bexsero®

Source: Produced by the author using data obtained from <http://www.biopharma.com> and published (adapted) in (DOS SANTOS *et al.*, 2018a).

As shown in **Table 3**, the majority of FDA-approved biopharmaceuticals from bacterial cell systems are produced by *E. coli*, except for a meningitis B vaccine approved in 2015, which is expressed by a modified *Neisseria meningitidis*. (BIOPHARMA, 2017) *E. coli* is the most commonly used host for the production of heterologous protein in bacterial cells due to its well-defined metabolism and established large-scale production system. The use of *E. coli* in the production of biomolecules also have other relevant advantages, such as high production yields, rapid grow, reasonable low cost and simplicity of cultivation. (DEMAIN; VAISHNAV, 2009) However, this microorganism has different disadvantages that limit its use, such as accumulation of lipopolysaccharides (LPS) (also called endotoxins, an immunogenic substance) in their cell membrane, usually intracellular production of bioproducts (which makes their recovery more difficult and expensive), in addition to a limited capacity for post-translational modifications when compared to eukaryotic organisms. Because of these issues, there is a search for other expression systems to overcome these drawbacks.

Some biopharmaceuticals require post-translational modifications for their appropriate expression (*e.g.*, for complete protein glycosylation or suitable production of SS-rich proteins). Therefore, the absence of such abilities in *E. coli* represents a significant limitation for their synthesis of biomolecules. (DESAI *et al.*, 2010) Among prokaryotic hosts, there are studies with *Bacillus* spp., (MELLAERT; ANNÉ, 2002) *Staphylococcus carnosus*, (HANSSON *et al.*, 2002) *Pseudomonas fluorescens*, (JIN *et al.*, 2011) and *Ralstonia eutropha*. (BARNARD *et al.*, 2004) Although these strains present fewer disadvantages compared to *E. coli*, their use to produce heterologous protein is not well established, and there were several efforts to improve their production process.

Considering this issue, eukaryotic life forms such as yeasts have been attracting the interest of the pharmaceutical research community. These microorganisms, atop of possessing high productivity and rapid growth in cheap medium, can also promote the glycosylation of proteins and induce the formation of SS-bonds. (DEMAIN; VAISHNAV, 2009) There are several yeast species already reported in the literature capable of producing biopharmaceuticals. Among them, the most promising systems are *P. pastoris*, (GURRAMKONDA *et al.*, 2009; KOBAYASHI *et al.*, 2000; NING *et al.*, 2003; XIE *et al.*, 2003) *Saccharomyces cerevisiae*, (KIM *et al.*, 2004; LIU *et al.*, 2012) *Ogataea polymorpha* (also known as *Hansenula polymorpha* and *Pichia angusta*) (AVGERINOS *et al.*, 2001; DEGELMANN *et al.*, 2002) and *Kluyveromyces lactis*. (FLEER *et al.*, 1991) *S. cerevisiae* and *P. pastoris* are even already used for commercial production of biopharmaceuticals, as seen in **Table 4**. (BIOPHARMA, 2017)

There are also recent results using other kinds of microorganisms for biopharmaceutical synthesis, like microalgae from the species *Chamydomonas reinhardtii*, (RASALA *et al.*, 2010) *Phaeodactylum tricornutum*, (HEMPEL *et al.*, 2011) and *Nannochloropsis* spp. (MATHIEU-RIVET *et al.*, 2014) However, most of the studies are only academic or at an early stage of industrial development.

Table 4 – Examples of biopharmaceuticals approved by FDA obtained from yeast as heterologous expression system (Biopharma database). (BIOPHARMA, 2017)

Microorganism	Medical use	Biopharmaceutical	Commercial names
<i>S. cerevisiae</i>	Anticoagulant	Hirudine	Refludan [®] , Revasc [®]
	<i>Diabetes mellitus</i>	liraglutide (glucagon like peptide-1)	Victoza [®]
		Insulin	Novolin [®] R, Protaphane [®] , Mixtard [®] , Insulatard [®] , Actrapid [®] , Actraphane [®]
		insulin detemir (insulin analog)	Levemir [®]
		insulin aspart (insulin analog)	NovoLog [®] , NovoMix [®] , NovoRapid [®]
		insulin degludec (insulin analog)	Tresiba [®]
	Diphtheria-Tetanus-Pertussis-Hepatitis B- <i>Haemophilus influenza</i> type B vaccine	various antigens	Tritanrix [®] HepB
	Diphtheria-Tetanus-Pertussis-Hepatitis B-Poliomyelitis vaccine	various antigens	Infanrix [®] Penta
	Diphtheria-Tetanus-Pertussis-Hepatitis B-Poliomyelitis- <i>Haemophilus influenza</i> type B vaccine	various antigens	Infarix [®] Hexa
	Hepatitis A and B vaccine	hepatitis B surface antigen and hepatitis A virus inactivated	Ambirix [®] , Twinrix [®] ,
	Hepatitis B vaccine	hepatitis B surface antigen	Engeri [™] x-B, Fendrix [®] , Recombivax HB [®] , HBVAxPro [®]
	hGH deficiencies	somatropin (recombinant hGH)	Valtropin [®]
	<i>Human papillomavirus</i> (HPV) vaccine	HPV vaccine	Gardasil [®]
		HPV surface antigens	Silgard [®]
	Hyperuricaemia	Rasburicase	Fasturtec [®]
<i>P. pastoris</i>	Neuropathic, chronic and diabetic ulcer	platelet-derived growth factor-BB	Regranex [®]
	Neutropenia	Sargramostim	Leukine [®]
	Production of human therapeutics	Albumin	Recombumin [®]
	Symptomatic vitreomacular adhesion	ocriplasmin (microplasmin)	Jetrea [®]
	Hereditary angioedema	ecallantide (kallikrein inhibitor)	Kalbitor [®]

Source: Produced by the author using data obtained from <http://www.biopharma.com> and published (adapted) in (DOS SANTOS *et al.*, 2018a).

As cited before, yeast and microalgae heterologous systems are usually considered efficient options for the low-cost production of biopharmaceuticals. However, despite being eukaryotic, these microorganisms sometimes cannot permit a perfect replication of the mammalian protein synthesis, resulting in improper protein folding and biologically inactive molecules. (DE POURCQ *et al.*, 2010) For this reason, complex eukaryotic systems, such as mammalian cell cultures, are still widely used for the industrial production of biopharmaceuticals (mammalian cells represented 79 % of production between 2015 and 2019), (WALSH, 2018) even considering their higher cultivation costs and usually lower production yields.

Mammalian cells host for biopharmaceuticals production includes Chinese hamster ovary cells (CHO), baby hamster kidney (BHK), murine hybridomas (RFT5 and SP2/0), murine myeloma (NS0), Madin-Darby canine kidney (MDCK), and human embryonic kidney cells (HEK293). (GRILLBERGER *et al.*, 2009; WERNER *et al.*, 1998) There are also reports for the use of transgenic animals, (BRINK *et al.*, 2000; RUDOLPH, 1999) plant cells (like tobacco chloroplasts), (DANIELL *et al.*, 2001b, 2001a; GOLDSTEIN; THOMAS, 2004) and insect cells (CHENG *et al.*, 2013; MONIE *et al.*, 2008; PYLE *et al.*, 1995) as possible recombinant systems for the production of biopharmaceuticals. Nevertheless, complex eukaryotic systems still exhibit high production costs and are difficult to maintain. Thus, there are continuous efforts to overcome these difficulties, especially using post-translational modifications in microorganisms, like the humanization of yeast and bacteria. (CHIBA; JIGAMI, 2007; HAMILTON; GERNGROSS, 2007) Different researchers attained promising results with *E. coli* (CARTER *et al.*, 1992; ZHU *et al.*, 1996) and *P. pastoris*, (WILDT; GERNGROSS, 2005) and present encouraging prospects for an increase in the use of microorganisms as effective platforms for the industrial production of biopharmaceuticals.

Finally, the development of new bioreactors and less costly processes can improve the commercial viability of these biomolecules. Thus, proper design and integration of upstream and downstream processing will be the key to enabling an efficient industrial manufacturing process, particularly for biopharmaceuticals that need to conform to high purity levels and strict legislation.

1.6.1 Future opportunities for biopharmaceuticals

As demonstrated during this section, biopharmaceuticals are the fastest-growing pharmaceutical field today. However, even though they already represent one-quarter of the

revenue generated in medicine sales, biopharmaceuticals only account for a small fraction of units sold. (EVALUATEPHARMA, 2019) For example, biopharmaceuticals represent 60 % of the public health expenditure on medicines in Brazil, although they are only 10 % of units bought by the Brazilian national health system. (SALERNO *et al.*, 2018) This situation only happens because of their egregious prices, reaching more than USD 1 billion *per kg*. (PUETZ; WURM, 2019) Besides, their instability at stress conditions limits their range of application and hinders their distribution, storage, and handling. (PATEL *et al.*, 2011) Therefore, it is essential to develop new technologies to solve these issues to change this setting and increase the access of biopharmaceuticals to disadvantaged communities.

The instability of biopharmaceuticals in stress conditions (particularly thermal) imposes several problems for their production, application, and distribution. (PATEL *et al.*, 2011) Because of their low stability, there are many losses during their manufacturing process. (RATHORE; RAJAN, 2008) Hence, there is a reduction in their yields and, consequently, the process gets more expensive. Besides, their low stability also limits their applications and routes of administration. This issue is one of the main reasons why almost all biopharmaceuticals are intravenous because, in addition to absorption difficulties, the extreme pH and proteolytic enzymes from the digestive system can degrade them in an oral administration. (GUPTA *et al.*, 2013) The transportation of biopharmaceuticals is also vastly limited by their instability at room temperature. For example, there is only one vaccine currently approved for partial use outside the cold chain. (KRISTIANSEN *et al.*, 2012) The partial relaxation of the cold chain for this biopharmaceutical halved the costs of the vaccination campaign carried in the African meningitis belt, showing that improving their transport and storage and removing the need for a cold-chain for their preservation is not only a matter of cost but access to health. (MÉDICOS SEM FRONTEIRAS, 2014) Thus, through increasing the stability of biopharmaceuticals, it will be possible to improve their range of application, manufacturing, and supply chain.

The steep prices from biopharmaceuticals are another cause for their low access in peripheral communities and developed countries. Their high costs are deeply associated with their downstream processing, which can account for 80 % of their production expenses. (WALSH, 2010) The reason why the purification and formulation stages represent so much of the manufacturing efforts for biopharmaceuticals is because of the very rigorous standards for their purification, which usually required purity above 99 %, removal of all immunogenic and pyrogenic substances, and maintenance of aseptic processing during most downstream phases. (KALYANPUR, 2002; LIM; SUH, 2015) However, many researchers believe the immense gap

(around 8-9 orders of magnitude) in price between industrial and biopharmaceutical proteins cannot be justified solely by additional production and R&D costs of medical products, and that the pharmaceutical industry is not fully disclosing the details for their pricing system. (PUETZ; WURM, 2019) Hence, it is necessary to pressure the industry (by market competition or legislation) to make efforts to reduce the costs of these life-saving biomolecules. By creating simple processes for the production and purification of biological products, it is also possible to allow start-ups and other smaller pharmaceutical industries to produce pure biomolecules outside of multinational conglomerates and bring more transparency and competition to the field.

With this in mind, this Thesis project aimed to address these topics by using EGFP as a proof-of-concept to design novel platforms for purification and stabilization of proteins, particularly envisaging their future applications for biopharmaceuticals. These new platforms have great potential to maintain the integrity of biopharmaceuticals at room temperature (and thus, to decrease costs and facilitate their transport, storage, and application) and to reduce their manufacturing costs by simplifying their purification (and hence, lowering their overall price and allowing a fairer competition between small industries and the pharmaceutical conglomerates). Additionally, by developing innovative solutions that could be applied to alleviate the two main drawbacks of biopharmaceuticals, this work intended to help in the democratization of access to these essential products.

2 OBJECTIVES

2.1 GENERAL OBJECTIVES

This work aimed to develop novel sustainable platforms for the stabilization and purification of EGFP using aqueous systems of ILs, organic salts, and polymers, with the major goal of improving and broadening its commercial applications in the areas of biotechnology, pharmacy, and medicine and envisaging future applications for other biomolecules.

2.2 SPECIFIC OBJECTIVES

Each phase of the research had specific objectives, as follows:

- 1. EGFP production (collaboration):** To enhance EGFP expression using bacteria *E. coli* BL21 (DE3) [pLysS; pET28 (a)] by improving production variables such as agitation rate, induction time, and concentration of inducer to increase EGFP production yields and guarantee more sustainability for the process.
- 2. EGFP fluorescence properties and structure:** To understand in-depth the fluorescence properties and structure of EGFP.
- 3. EGFP structure and activity at different pH:** To screen and characterize the stability of EGFP in different pH using modern characterization techniques, such as fluorescence spectroscopy (including 3D fluorescence spectroscopy), Circular Dichroism (CD), and Small Angle X-ray Scattering (SAXS).
- 4. Effect of ionic liquids on EGFP activity:** To evaluate the potential of cholinium- and imidazolium-based ILs in maintaining the EGFP fluorescence activity under stress conditions (presence of denaturing agents) and at room temperature (short and long-term studies).
- 5. Purification of EGFP using aqueous biphasic systems:** To develop simpler, more efficient, sustainable, and cost-effective purification platforms for EGFP using ABS (composed of polymers and organic salts) and ultrafiltration.
- 6. Economic analysis of EGFP production and purification with ABS (collaboration):** To perform an economic evaluation using bioprocess modeling on the production and purification of EGFP through the application of ABS.

7. Scale-up of EGFP purification with ABS (collaboration): To perform the scale-up of the EGFP purification ABS-based platform using fast centrifugal partition chromatography and oscillatory flow reactor.

8. EGFP encapsulation (collaboration): Development and characterization of polymeric hydrogels as support for biosensing applications of EGFP.

3 MATERIALS AND METHODS

3.1 RIGHTS AND PERMISSIONS

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3.2 MATERIALS

3.2.1 Buffers

The researcher used Milli-Q[®] water (ultrapure water of Type 1) to prepare all solutions and experiments.

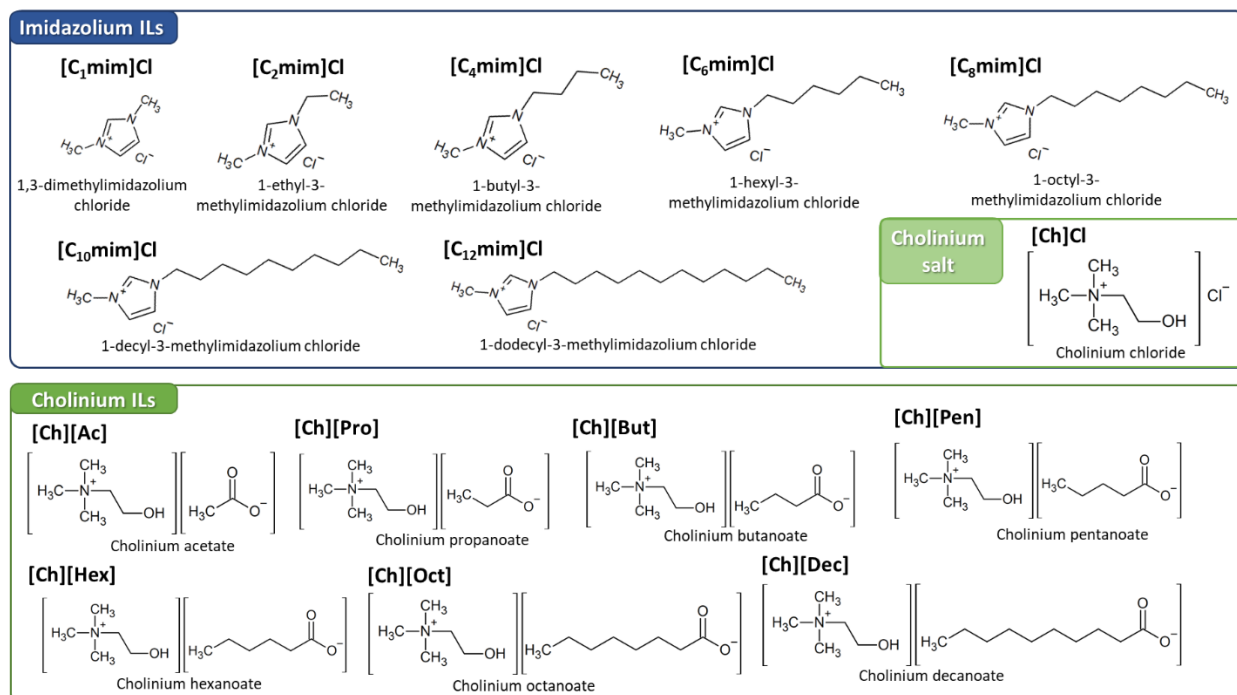
The Tris-HCl buffer at pH 8.0 used in EGFP expression protocol was prepared with 50 mmol.L⁻¹ of tris(hydroxymethyl)aminomethane in water (Synth, > 99 %) and with pH adjustment with hydrochloric acid 37 % (HCl, Neon), allowing a maximum variation of 0.1 in pH.

Potassium Phosphate Buffer (PB) 10.0 mmol.L⁻¹ pH 7.4 and Phosphate-Saline Buffer (PBS) pH 7.4 [137.0 mmol.L⁻¹ NaCl, 2.7 mmol.L⁻¹ potassium chloride (KCl), 8.0 mmol.L⁻¹ sodium hydrogen phosphate (Na₂HPO₄), and 2.0 mmol.L⁻¹ potassium phosphate monobasic (KH₂PO₄)] were selected to simulate physiological conditions, considering that the main use of EGFP is in the medical and biological fields. The PB 10.0 mmol.L⁻¹ pH 7.4 contained Milli-Q[®] water, anhydrous dipotassium hydrogen phosphate (K₂HPO₄, 98-100.5 %) from QHEMIS, and anhydrous KH₂PO₄ (99 %) from Synth (Brazil). PBS was prepared with tablets from Sigma-Aldrich[®] and Milli-Q[®] water. The pH of the PB buffer was corrected using 3 mol.L⁻¹ solutions of phosphoric acid (H₃PO₄, 85 wt% in H₂O) and potassium hydroxide (KOH, 45 wt% in H₂O),

and the pH of the PBS buffer corrected with 3 mol.L⁻¹ solutions of sodium hydroxide (NaOH) and HCl, prepared from NaOH $\geq$ 98.0 % and HCl 37 wt%, allowing a maximum variation of 0.1 in pH, both using reactants from Sigma-Aldrich®. The selection of PB in addition to PBS buffer is due to the use of PB in CD analysis because although PBS is better to simulate physiological conditions, it contains chlorine ions which interfere with CD.

3.2.2 ILs and salts

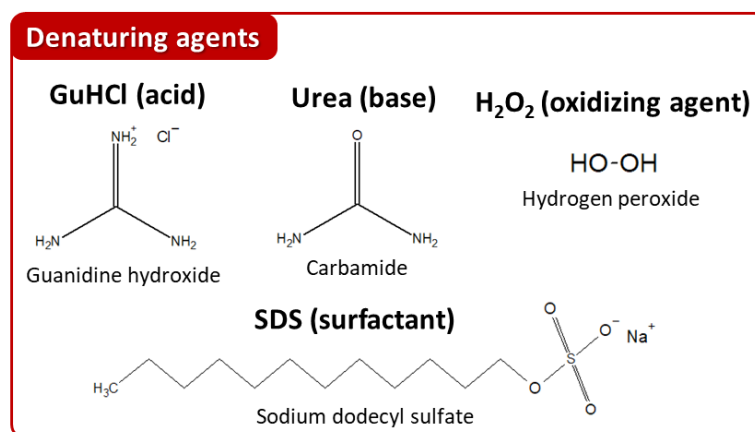
ILs from the 1-*n*-alkyl-3-methylimidazolium chloride ([C_{*n*}mim]Cl) and cholinium ([Ch]X) families were selected for the EGFP stability assays and their chemical structures are presented in **Figure 7**. The [C_{*n*}mim]Cl, 1,3-dimethylimidazolium chloride ([C₁mim]Cl), 1-ethyl-3-methylimidazolium chloride ([C₂mim]Cl), 1-butyl-3-methylimidazolium chloride ([C₄mim]Cl), 1-hexyl-3-methylimidazolium chloride ([C₆mim]Cl), 1-octyl-3-methylimidazolium chloride ([C₈mim]Cl), 1-decyl-3-methylimidazolium chloride ([C₁₀mim]Cl) and 1-dodecyl-3-methylimidazolium chloride ([C₁₂mim]Cl), were acquired from Sigma-Aldrich® or Iolitec®, with purity above 98 %. Cholinium chloride ([Ch]Cl, > 98 %) was acquired from Sigma-Aldrich®. The other [Ch]X ILs, cholinium acetate ([Ch][Ac]), cholinium propanoate ([Ch][Pro]), cholinium butanoate ([Ch][But]), cholinium pentanoate ([Ch][Pen]), cholinium hexanoate ([Ch][Hex]), cholinium octanoate ([Ch][Oct]) and cholinium decanoate ([Ch][Dec]) were synthesized using cholinium hydroxide ([Ch][OH] 45 wt% in methanol) and respective carboxylic acids (acetic acid $\geq$ 99.5 %, propionic acid $\geq$ 99.5 %, butanoic acid $\geq$ 99 %, pentanoic acid $\geq$ 99 %, hexanoic acid $\geq$ 98 %, octanoic acid $\geq$ 98 %, decanoic acid $\geq$ 98 %, lactic acid $\geq$ 98 % and glycolic acid $\geq$ 98%) from Sigma-Aldrich®, following the protocol of Muhammad *et al.* (MUHAMMAD *et al.*, 2012) and resulting in ILs with purity above 97 % (assessed by proton nuclear magnetic resonance, ¹H NMR).

Figure 7 – Chemical structure of studied ILs and salts.

Source: Produced by the author using ACD/ChemSketch® Freeware.

3.2.3 Denaturing agents

The researcher selected the denaturing agents sodium dodecyl sulfate (SDS, $\geq 99.0\%$), carbamide (Urea, 99.0-100.5 %), guanidine hydrochloride (GuHCl, $\geq 99\%$), and hydrogen peroxide (H_2O_2 , 35 wt% in water) for the studies of protection of EGFP fluorescence using ILs, acquired from Sigma-Aldrich®. **Figure 8** presents the chemical structure of the denaturing agents.

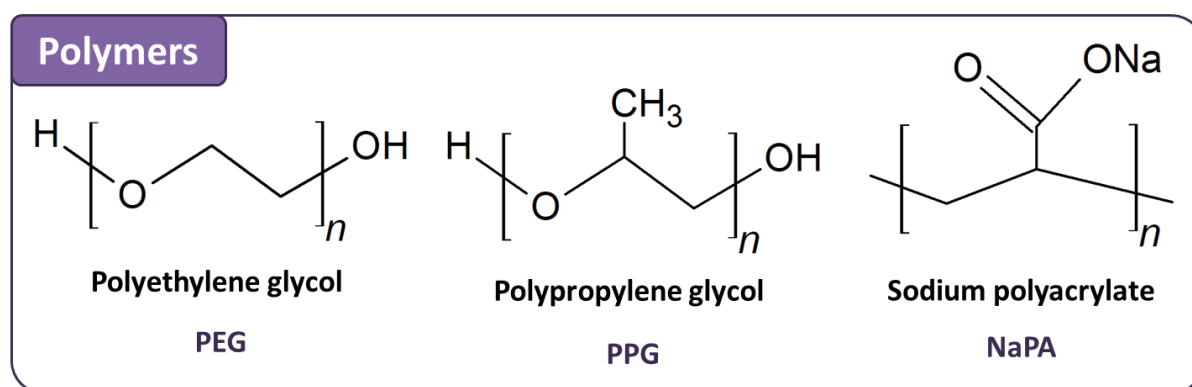
Figure 8 – Chemical structure of studied denaturing agents.

Source: Produced by the author using ACD/ChemSketch® Freeware.

3.2.4 Polymers

Polyethylene glycol with average MW of 600 g.mol⁻¹ (PEG-600, pure), 1500 g.mol⁻¹ (PEG-1500, pure) and 2000 g.mol⁻¹ (PEG-2000, pure), polypropylene glycol with an average MW of 400 g.mol⁻¹ (PPG-400, pure) and sodium polyacrylate with average MW of 8000 g.mol⁻¹ (NaPA-8000, 45 wt% in water) were acquired from Sigma-Aldrich® (Brazil) and used as received. **Figure 9** shows the chemical structures of the polymers.

Figure 9 – Chemical structure of studied polymers.



Source: Produced by the author using ACD/ChemSketch® Freeware and published in (DOS SANTOS *et al.*, 2018b).

3.2.5 Proteins and protein standards

The group produced EGFP raw extract according to the EGFP expression protocol presented in section “3.3.4.1 EGFP expression”.

The researcher produced and purified EGFP with > 97.5 % for the property and stability studies using the method previously developed by the group and published by Dos Santos *et al.* (DOS SANTOS *et al.*, 2018b).

E. coli TOP10 expressed the other recombinant fluorescent proteins studied (Fezziwig Yellow Fluorescent Protein - YFP and Red Fluorescent Protein - RFP) and kindly provided by the research group of Prof. Dr. Danielle Biscaro Pedrolli from the Department of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences at São Paulo State University (UNESP).

The researcher acquired the BSA (> 98%) from Sigma-Aldrich®.

The standard from EGFP was from the EGFP Quantitation Kit K815-100 from Biovision® (purity ≥ 97%).

3.2.6 Other materials

The culture medium Luria Bertani broth (LB) from Difco™ and the inductor β -D-1-thiogalactopyranoside (IPTG) from Sigma-Aldrich® were used in the fermentative process to produce EGFP. The group obtained other reactants and materials from Sigma-Aldrich®.

3.3 PROTOCOLS

3.3.1 Protocols for the “EGFP fluorescence properties and structure” study

3.3.1.1 EGFP absorbance and fluorescence analysis

The 3D fluorescence spectra were acquired with a spectrofluorophotometer (instrument configuration in section “**3.4.1 Fluorescence analysis**”) to evaluate the fluorescence properties of EGFP. The researcher prepared the sample 30 ($\pm$ 5) min before each reading. From the 3D spectra, it was possible to extract excitation and emission spectra for EGFP 5.4 $\mu\text{g.mL}^{-1}$ at PB pH 7.4. Fluorescence Intensity [FI, in unities of fluorescence (UF)] single points and the absorbance spectrum (λ 250-800 nm, EGFP 0.216 mg.mL^{-1}) were obtained in a plate reader. The 3D spectra are depicted in the figures as a representative of the triplicate. The excitation, emission, and absorbance spectra are the average of the three samples.

3.3.1.2 3D spectra of EGFP standard and BSA

A standard from EGFP Quantitation Kit K815-100 from Biovision® (10 $\mu\text{g.mL}^{-1}$ in assay buffer pH 8.0, formulation not disclosed, a property of Biovision®) and BSA (10 $\mu\text{g.mL}^{-1}$ and 3 mg.mL^{-1}) were also evaluated using 3D spectra (instrument configuration in section “**3.4.1 Fluorescence analysis**”) to confirm the presence of the second peak of EGFP and discard the possibility of a reflection from other fluorophores

3.3.1.3 Effect of concentration, pH, and high temperature in EGFP peaks

To assess the behavior of EGFP fluorescence peaks, 3D spectra were also acquired at different EGFP concentrations (at 21.6, 10.8, 7.2, and 5.4 $\mu\text{g.mL}^{-1}$), pH (7.4 and 6.0, EGFP 5.4 $\mu\text{g.mL}^{-1}$), and after 3 h at 90 °C (temperature and time that guarantee to denature, as can be seen in **Figure 28**, EGFP 5.4 $\mu\text{g.mL}^{-1}$). Instrument details are presented in section “**3.4.1 Fluorescence analysis**”. The calibration curve of EGFP was acquired at F1 (λ_{ex} 488, λ_{em} 510 nm) and F2 (λ_{ex} 278 nm, λ_{em} 510 nm) with point fluorescence analysis in the spectrofluorometer.

The test at high temperature was performed in a thermoblock at 90 °C for 3 h. The samples were maintained for 30 min at 25 °C after their removal from the thermoblock; afterward, the researcher measured their fluorescence. All the pH, high temperature, and calibration experiments were carried out in triplicate with corresponding blanks; the 3D spectra are presented as a representative of the triplicate and the calibration curve is the average of three independent samples with the respective standard deviations.

3.3.1.4 Calibration curves and analytical parameters for F1 and F2

To compare the range and precision of the calibration curves of EGFP for both peaks F1 and F2, calibration curves for each peak were produced (as presented in **Figure 13**, **Figure B-1**, and **Figure B-2**) and different analytical parameters were evaluated and are presented at **Table 7**. The coefficient of determination (R^2) was obtained for the linear part of the calibration curves and the upper limit of the curve was defined as the concentration of EGFP at the last point in the linear part of the curve. To determine the minimal concentrations for detection and quantification for each curve, the limit of detection (LOD) and limit of quantification (LOQ) were calculated for confidence levels of 3 and 9 standard deviations (SD) respectively, as suggested by Mocak *et al.* (MOCÁK *et al.*, 1997) in a review of analytical chemistry procedures from ACS and International Union of Pure and Applied Chemistry (IUPAC), following the equation (1):

$$y_{FI} = \mu_b + \kappa\sigma_b \quad (1)$$

where y_{FI} is the fluorescence intensity at LOD or LOQ, μ_b is the average of the blank signal, κ is the confidence level (3 for LOD and 9 for LOQ) and σ_b is the standard deviation of the blank. LOD and LOQ were calculated as the concentration (x) of the determined equation, following the calibration curves equations presented in **Figure 13** and **Figure B-2**.

The relative standard deviation (RSD) was calculated for each point of the curve (RSD_n), following the equation (2): (WILKINSON; MCNAUGHT, 1997)

$$RSD_n = \frac{\sigma_n}{\mu_x} \times 100 \quad (2)$$

where σ_n is the standard deviation of the point of the curve and μ_x is the average of the points of the curve. The RSD for each curve was calculated as the average of RSD_n for all the points of the curve.

3.3.2 Protocols for the “EGFP activity and structure at different pH” study

3.3.2.1 EGFP fluorescence activity in pH over time

This set of experiments evaluated the FI of EGFP ($5.4 \mu\text{g.mL}^{-1}$) in PBS at different pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 7.4, 8.0, 9.0, 10.0, 11.0, 12.0, and 13.0) over time (0, 0.5, 1, 2, 3, 4, 5, 6, 24, and 48 h) in a plate reader. Results are presented as the average of three samples with respective standard deviations.

3.3.2.2 EGFP fluorescence activity and structural analysis at different pH

EGFP in PB solution was exposed to different pH (from 2.0 to 9.0) for 30 min (time required for fluorescence stabilization at the new pH conditions) and evaluated by CD (EGFP 0.3 mg.mL^{-1}) and fluorescence spectroscopy (3D and the single point for EGFP fluorophore and 2D for intrinsic fluorescence of proteins, EGFP $5.4 \mu\text{g.mL}^{-1}$). Then, the pH of each sample was adjusted until the solution reached neutrality (pH 7.4), and after 60 min, EGFP was again assessed by CD and fluorescence analysis.

For the SAXS experiments, an initial concentration test described in section “**3.4.4 Small-Angle X-Ray Scattering (SAXS)**” determined 2.5 mg.mL^{-1} of EGFP for the well-plate SAXS run and 5.0 mg.mL^{-1} for the SEC-SAXS run. Details for the SAXS instruments and methods are described in the aforementioned section.

For the well-plate SAXS protocol, samples were prepared in PB with pH adjusted to pH 4.0, 5.0, 6.0, and 7.4 and after 30 min, scattering in SAXS was measured. Then, the pH of the samples was adjusted to 7.4 and after 60 min, were evaluated by SAXS again. Experiments were performed with a blank and 15 measurements were recorded for each sample. The scattering vector (q , sometimes called momentum transfer) range was 0.006 to $0.53 \text{ \AA}^{-1}$. Results are presented as the average of measurements after blank subtraction of the corresponding solvent.

For SEC-SAXS protocol, PB of pH 7.4, 6.5, and 5.5 were used as eluent for the HPLC column and filtered with an $0.5 \mu\text{m}$ membrane. This pH range was selected because below pH 5.5 (due to loss of structure) and around pH 6 (due to it being close to the pI of the protein) there is precipitation, making the sample not suitable for SEC-SAXS. $50 \mu\text{L}$ of the sample was injected per run, with a flow rate of 0.4 mL.min^{-1} . The HPLC column was equilibrated for 15 min for each eluent and the run was recorded for UV-vis (wavelengths 214, 260, 280, and

450 nm) and SAXS for 15 min per sample. The samples were prepared with PB with the same pH as the eluents and injected in SEC-SAXS after 30 min. For the presentation of the results, time was manually adjusted so the SEC-SAXS peaks from the samples at different pH matched, considering the time of injection varied for each condition. The offset used for the SEC-SAXS results was then applied for the time adjustment for the Radius of Gyration (Rg), Molecular Weight (MW), and UV-vis results.

3.3.2.3 EGFP fluorescence activity relationship with pH

The relationship between the FI of EGFP and pH was also evaluated. FI (in UF) single points were obtained for 16 different pH from 3.0 to 9.0 at F1 (λ_{ex} 488 nm, λ_{em} 510 nm), in triplicate with a blank using an EnSpire® Multimode Plate Reader, from PerkinElmer®. Results were fitted to a growth/sigmoidal dose-response model to calculate pKa.

3.3.3 Protocols for the “Effect of ionic liquids on EGFP activity” study

3.3.3.1. Effect of different concentrations of aqueous solutions of salts and ILs on EGFP fluorescence

In this set of experiments, the effect of ionic force on EGFP ($5.4 \mu\text{g.mL}^{-1}$) fluorescence was evaluated for different concentrations (0.025 , 0.050 , 0.100 , 0.250 and 0.500 mol.L^{-1}) of NaCl in PBS (pH adjusted to 7.4 ± 0.1) using FI analysis over time (0, 0.5, 1, 2, 3, 4, 5, 6, 24 and 48 h) at 25°C . Then, EGFP fluorescence was assessed for solutions of [Ch]X and $[\text{C}_n\text{mim}]\text{Cl}$ in PBS (pH corrected for 7.4 ± 0.1), as listed in **Figure 7**, for the same concentrations, times, and at 25°C (short-term effect of ILs on EGFP activity). Later, the concentration of 0.100 mol.L^{-1} was selected to evaluate the long-term effect of the IL on EGFP fluorescence activity at room temperature (maintained without light exposition, between 22 and 25°C), for 7, 30, 60 and 90 days (long-term effect of ILs on EGFP activity). In this study, FI of EGFP $5.4 \mu\text{g.mL}^{-1}$ in PBS at 0 h and 25°C was the reference for 100 %.

3.3.3.2. Effect of physical and chemical stress on EGFP fluorescence

EGFP fluorescence was also evaluated under physical and chemical stress (temperature and denaturing agents, respectively). EGFP FI in PBS pH 7.4 ($5.4 \mu\text{g.mL}^{-1}$) was measured for the temperatures of 25 , 30 , 40 , 50 , 60 , 70 , 80 and 90°C at 0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h. For the chemical stress evaluation, different classes of denaturing agents were selected, as presented

in **Figure 8** (GuHCl as acid, urea as a base, H₂O₂ as an oxidizing agent, and SDS as a surfactant). The chemical stress was evaluated after 0, 0.5, 1, 2, 3, 4, 5, 6, and 30 h of exposure, at 25 °C and following concentrations of denaturing agent: GuHCl – 1.000, 4.000, 5.000, 6.000, and 8.000 mol.L⁻¹; urea – 4.000 and 8.000 mol.L⁻¹; H₂O₂ – 0.030, 0.300, 1.500, 3.000, 6.000 mol.L⁻¹; SDS – 0.003, 0.035, 0.100 and 0.175 mol.L⁻¹.

3.3.3.3. *Protective/destabilizing effect of ILs on EGFP fluorescence under denaturation by chemical agents*

Moreover, to determine the potential of ILs and salts to act as stabilizing agents for EGFP under stress conditions, EGFP fluorescence (5.4 µg.mL⁻¹) was also evaluated for solutions of 0.100 mol.L⁻¹ of ILs and salts (in PBS, pH corrected to 7.4 ± 0.1) + denaturing agents at 25 °C and 2 h. Denaturing agents, concentrations (SDS 0.175 mol.L⁻¹, GuHCl 4.000 mol.L⁻¹, and H₂O₂ 1.500 mol.L⁻¹) and the time were determined considering the results of the previous experiments, selecting conditions that cause a moderate drop in FI (80-40 % decrease of FI in 2 h). In addition to the ILs/salts + denaturing agents' samples, negative and positive controls (ILs/salts and denaturing agents isolated, respectively) were also evaluated for comparison.

3.3.4 Protocols for the “Purification of EGFP using aqueous biphasic systems” study

3.3.4.1 *EGFP expression*

The expression protocols were slightly adapted from Lopes *et al.* (LOPES *et al.*, 2019), as follows. The plasmid pET28(a) containing the gene for expression of recombinant EGFP was used to transform the host *E. coli* BL21 (DE3) pLysS. The recombinant *E. coli* BL21 (DE3) pLysS was kindly provided by the research group of Prof. Sandro R. Valentini. Initially, the *E. coli* strain was transferred to a Petri dish containing LB agar and IPTG to a final concentration of 5 x 10⁻⁴ mol.L⁻¹. The antibiotics kanamycin and chloramphenicol (both at 50 µg.mL⁻¹) were also added. The plate containing the microorganism was maintained at 37 °C for 24 h. Then, a colony was transferred to Erlenmeyer flasks (100 mL) containing 25 mL of LB medium with the antibiotic kanamycin (50 µg.mL⁻¹) and chloramphenicol (50 µg.mL⁻¹) and incubated overnight at 150 rpm and 37 °C, in a refrigerated incubator with an orbital shake (TE-421 from Technal, Brazil). Following, Erlenmeyer flasks (500 mL) with 250 mL of fresh LB medium were inoculated with 2.5 mL of overnight fermented inoculum [optical density at 600 nm

(OD₆₀₀) of 0.1 Units of Absorbance (UA)] The induction with IPTG (5×10^{-4} mol.L⁻¹) occurred after 6 h of incubation and the fermentation process was carried out for more 12 h. The bright green cells (EGFP was produced intracellularly) were harvested via centrifugation at $2360 \times g$ for 15 min at 4 °C. The pellet containing the cells of *E. coli* was resuspended in 250 mL of cell lysis buffer (Tris-HCl, pH 8.0). The cells were disrupted by the application of four cycles of freezing (-80 °C)/thawing (40 °C). Then, the insoluble cell components were removed from the supernatant with EGFP by centrifugation (15 min at 4 °C and $2360 \times g$). The supernatant containing EGFP (herein considered as EGFP raw extract) was stocked at -20 °C before being used in the extraction and purification experiments. All media were autoclaved at 121 °C for 15 min.

3.3.4.2 Quantification of EGFP and total protein

Initially, to find the optimum fluorescence excitation and emission wavelengths from EGFP and Total Protein (TP) of both standards and EGFP samples, full 3D fluorescence spectra were acquired at 25 °C (instrument details described in the section “**3.4.1 Fluorescence analysis**”). With the 3D spectra, it was possible to observe that both the recombinant EGFP samples and the commercial standard [EGFP quantification Kit K815-100 (Biovision®)] exhibited similar 3D spectra, in which the maximum EGFP fluorescence was obtained at λ_{ex} 488 nm, λ_{em} 510 nm. The maximum fluorescence excitation and emission wavelengths of the protein standard (BSA) were also evaluated and maximum fluorescence was attained at λ_{ex} 276 nm, λ_{em} 336 nm. The peak of fluorescence around 280 nm (indicative of TP) in BSA matched the pattern obtained in the 3D spectra for the recombinant EGFP samples regarding TP, suggesting that BSA can be used as a suitable TP standard for EGFP, as can be seen in **Figure D-1**. Both EGFP and TP fluorescence spectra were not significantly changed neither by the addition of different concentrations nor by the type of phase-forming agents under study, with all the optimum fluorescence excitation and emission wavelengths stable in all the conditions studied.

Thus, according to the previous 3D fluorescence tests, the calibration curve of EGFP concentration *versus* FI at λ_{ex} 488 nm and λ_{em} 510 nm using the commercial standard EGFP was determined at 25 °C and used to determine the EGFP concentration of the samples prior and after purification extraction tests. The concentration of TP was also quantified by fluorescence using a calibration curve with BSA as standard, with the TP concentration calibration curve at

λ_{ex} 276 nm, λ_{em} 336 nm at 25 °C used to determine the TP composition of each sample. The calibration curves, linear equations, and R^2 are presented in **Figure B-3**.

Furthermore, to compare the proportion of EGFP over total protein and assess the purity of the EGFP, a method using the full 3D fluorescence spectra of purified (phases with high EGFP content) and non-purified (EGFP raw extract after lysis) samples was developed, comparing the 3D fluorescence spectra of diluted solutions of EGFP (EGFP concentration equal to $12.7 \pm 0.2 \text{ mg.L}^{-1}$). With that analysis, it was possible to determine the relationship between the FI of EGFP (λ_{ex} 488 nm, λ_{em} 510 nm) by the FI of TP (λ_{ex} 276 nm, λ_{em} 336 nm), described as EGFP/PT (directly proportional to the EGFP purity).

3.3.4.3 Extraction and purification of EGFP

The different mixtures for each ABS (compositions presented in **Table 5** with their references) were prepared to add gravimetrically ($\pm 10^{-4} \text{ g}$) distinct amounts of the respective phase-forming agents in 15 mL graduated glass tubes. Then, 10 wt% of EGFP raw extract was added, and to achieve 5 g (total mass), each system was filled with Milli-Q[®] water. All systems were equilibrated at 25 (± 1) °C for 2 h. The co-existing phases were then carefully separated and characterized for pH, conductivity, viscosity, water content (**Table 8**) and Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) analysis (**Figures E.1 to E.4**). All the extraction systems were prepared in triplicate with the respective blank assays carried out to eliminate the interferences of the phase-forming agents or contaminants in the quantification of EGFP and TP content. The respective EGFP and TP concentrations were measured, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm EGFP purification.

The extraction and purification of EGFP in each series of ABS were assessed in terms of EGFP extraction efficiency (EE_{EGFP}), EGFP mass balance (MB_{EGFP}), and relative concentration of EGFP by total protein ($[EGFP]_{\text{rel}}$), according to equations 5 to 7:

$$EE_{\text{EGFP}}(\%) = \frac{[EGFP]_{\text{EGFP-rich phase}} \cdot V_{\text{EGFP-rich phase}}}{[EGFP]_{\text{T}} \cdot V_{\text{T}} + [EGFP]_{\text{B}} \cdot V_{\text{B}}} \times 100 \quad (5)$$

where $V_{\text{GFP-rich phase}}$, V_0 , V_{T} and V_{B} are the EGFP-rich phase, initial, top and bottom volumes (mL), and $[EGFP]_{\text{GFP-rich phase}}$, $[EGFP]_0$, $[EGFP]_{\text{T}}$ and $[EGFP]_{\text{B}}$ represents the EGFP-rich phase, initial, top, and bottom concentration of EGFP (g.L^{-1}).

$$MB_{\text{EGFP}}(\%) = \frac{[EGFP]_{\text{T}} \cdot V_{\text{T}} + [EGFP]_{\text{B}} \cdot V_{\text{B}}}{[EGFP]_0 \cdot V_0} \times 100 \quad (6)$$

where V_T , V_B and V_0 are the top, bottom, and initial volumes (mL), and $[EGFP]_T$, $[EGFP]_B$ and $[EGFP]_0$ represent the top, bottom, and initial concentration of EGFP (g.L^{-1}).

$$[EGFP]_{\text{rel}}(\%) = \frac{[EGFP]_{\text{EGFP-rich phase}}}{[TP]_{\text{EGFP-rich phase}}} \times 100 \quad (7)$$

where $[EGFP]_{\text{EGFP-rich phase}}$ is the concentration of EGFP in the EGFP rich-phase (g.L^{-1}) and $[TP]_{\text{EGFP-rich phase}}$ is the concentration of TP in the EGFP rich-phase (g.L^{-1}).

Table 5 – Weight fraction (wt%) composition of the components of each ABS. The references where the phase diagrams are described are also defined.

Type of ABS	Weight fraction (wt%)			Reference
	Polymer 1	Polymer 2	Buffer or [Ch]Cl	
Polymer/Buffer				
PEG-1500 + K ₂ HPO ₄ /KH ₂ PO ₄	PEG-1500		K ₂ HPO ₄ /KH ₂ PO ₄	(DE SOUZA <i>et al.</i> , 2014)
	15		15	
PEG-2000 + K ₂ HPO ₄ /KH ₂ PO ₄	PEG-2000		K ₂ HPO ₄ /KH ₂ PO ₄	Annex I
	15		15	
Polymer/Polymer + [Ch]Cl as adjuvant				
PEG-600/NaPA-8000 + [Ch]Cl	PEG-600	NaPA-8000	[Ch]Cl	Unpublished data
	15	15	1	
	15	15	3	
	15	15	5	
	20	15	1	
	20	15	3	
	20	15	5	
Polymer/[Ch]Cl ABS				
PEG-600/[Ch]Cl	PEG-600		[Ch]Cl	(PEREIRA <i>et al.</i> , 2013b, p.)
	38		38	
	40		40	
	42		42	
PPG-400/[Ch]Cl	PPG-400		[Ch]Cl	(QUENTAL <i>et al.</i> , 2015)
	40		10	
	45		12	
	50		14	

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

3.3.4.4 Determination of relative hydrophobicity of proteins

The Grand Average of Hydropathicity (GRAVY) was calculated using the protein sequence of EGFP (GENBANK, 1996) and the software ProtScale from ExPASy with the Kyte-Doolittle Hydrophilicity plot, (ARTIMO *et al.*, 2012; SIB, 2020), following equation (8), described by Ishihama *et al.*, (ISHIHAMA *et al.*, 2008) defined as:

$$\text{GRAVY} = \frac{\sum_{i=1}^n H_i}{n} \quad (8)$$

where H_i denoted the hydrophobicity value of the amino acid at position i of a protein of n amino acids.

3.3.4.5 Back-extraction of EGFP

Back-extraction was applied to possibly increase the purification capacity of the systems by integrating two ABS that exhibited good extraction efficiencies but low EGFP purification aptitudes. Thus, the PEG-rich phase (containing the EGFP) recovered after a first extraction using ABS composed of 15 wt% of PEG-600, 15 wt% of NaPA-8000, and 3 wt% of [Ch]Cl was introduced in a second extraction unit based on a PEG-600/[Ch]Cl ABS (additional content of PEG-600 and [Ch]Cl were added to form a biphasic system with 5 g). After the second extraction stage, the phases were carefully separated and the extraction and purification parameters calculated as previously described in section “**3.3.4.3 Extraction and purification of EGFP**”. The back-extraction procedures were performed in triplicate with the respective blanks to eliminate errors in the quantification.

3.3.4.6 Extraction and purification of other recombinant fluorescent proteins produced by *E. coli* using PPG-400/[Ch]Cl ABS

To evaluate if the purification aptitudes of the ABS with the highest purification yield could be applied to the extraction of other proteins, two other fluorescent proteins (FP) produced by recombinant *E. coli* TOP10 (Fezziwig Yellow Fluorescent Protein - YFP and Red Fluorescent Protein - RFP) were also analyzed. The ABS composed of 45 wt% of PPG-400 and 12 wt% of [Ch]Cl was prepared as described in section “**3.3.4.3 Extraction and purification of EGFP**” (in triplicate with a blank, to subtract interferences) and evaluated by complete GFP 3D fluorescence spectra and analysis of FI of FP (YFP - λ_{ex} 522 nm, λ_{em} 534 nm; RFP - λ_{ex} 584 nm, λ_{em} 610 nm) and TP (λ_{ex} 276 nm, λ_{em} 336 nm), as described in the section “**3.3.4.2**

Quantification of EGFP and total protein". The determination of the Relative Extraction for the FP [RE_{FP} (%)] and FP/TP relation (FP/TP) were given by the following equations:

$$RE_{FP}(\%) = \frac{FP_{FP-rich\ phase}}{FP_{FP-rich\ phase} + FP_{FP-poor\ phase}} \times 100 \quad (9)$$

where $FP_{FP-rich\ phase}$ is the intensity of fluorescence of the FP in the FP rich-phase and $FP_{FP-poor\ phase}$ is the intensity of fluorescence of the FP in the FP poor-phase;

$$FP/TP = \frac{FP_{FP-rich\ phase}}{TP_{FP-rich\ phase}} \quad (10)$$

where $FP_{FP-rich\ phase}$ is the intensity of fluorescence of the FP in the FP-rich phase and $TP_{FP-rich\ phase}$ is the intensity of fluorescence of the TP in the FP-rich phase.

3.3.4.7 Solvent recycling and EGFP polishing

The ABS allowing the highest purification yield, namely PPG-400/[Ch]Cl-based ABS, was chosen to be integrated with an ultrafiltration unit to attempt at solvent recycling and EGFP polishing. Thus, a system (10 g of total mass) composed of 45 wt% of PPG-400 and 12 wt% of [Ch]Cl was prepared as previously described in section “**3.3.4.3 Extraction and purification of EGFP**” (in triplicate with a blank, to subtract interferences). Similarly, after the extraction and separation of the two co-existing phases, the EGFP and TP concentration were measured and the corresponding extraction and purification parameters were properly determined. Then, each of the co-existing phases was subjected to further ultrafiltration to remove the contaminants, EGFP polishing, and solvent recycling. The ultrafiltration using Vivaspin® 20 10 kDa centrifugal concentrators (from GE Healthcare, Brazil) associated with a centrifugation process at 4 °C and $8000 \times g$ for 45 min was applied to separate the protein contaminants (molecular weight above 10 kDa) from the top PPG-rich phase and to allow the recovery of EGFP from the [Ch]Cl-rich aqueous rich phase (EGFP polishing). The Vivaspin® 20 10 kDa contains up to 15 mL of sample and, after the centrifugation, 1 mL of a concentrated aqueous solution containing the proteins (EGFP or contaminants) was recovered in the retentate (in the concentrator tube), while the solvents were recovered in the permeate (in the collector tube coupled below the concentrator).

Each of the co-existing phases was added to the centrifugal concentrators, which were filled with 1 mL of Milli-Q® water, and then subjected to centrifugation. After that, for the tubes containing the PPG-400-rich phase (top phase), the retentate solution containing the

contaminant proteins was discarded, and the permeate aqueous solution rich in PPG-400 was recycled. Additionally, both retentate and permeate aqueous solutions of the [Ch]Cl-rich phase (bottom phase containing the pure EGFP) were carefully recovered. The aqueous solution containing the concentrated and pure EGFP (retentate) was used for the analysis of extraction and purity yields, while the diluted [Ch]Cl-rich phase, without EGFP and recovered as permeate, was recycled in the extraction unit. The recycled phases were then weighted, with the adjustment of the concentration of the phase-forming agents, and then mixed at 25 °C with 10 wt% of EGFP raw extract for the formation of a new ABS (details related to the weight of the phases recovered are compiled in **Table 8**). Again, the phases were separated and the corresponding EGFP extraction and purification parameters were determined.

3.3.4.8 Physicochemical characterization of ABS coexisting phases

After the separation of the coexisting phases of each ABS and considering that the relative densities of both phases were not constant, and thus an inversion of the top and bottom nature phases can occur, their densities were determined. In addition to the densities determination, and to obtain further insights about the characteristics of all systems, pH, conductivity, viscosity, and water content of each coexisting phase were measured (**Table 8**).

The pH values and conductivity of top and bottom phases for all series of ABS were measured using a portable pH-meter. Density and viscosity were measured with an automatic viscosimeter-densimeter. The water content of the top and bottom phases was measured by volumetric Karl-Fischer titration, (except for the NaPA-8000-based ABS, which is incompatible with Karl-Fischer reagents and was measured by a gravimetric method). Finally, to further confirm the nature of each phase in equilibrium, a Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) analysis of each phase was performed (**Figures E.1 to E.4**) and compared with the spectra of pure phase-forming agents (polymers, potassium phosphate buffer or [Ch]Cl). Details for the instruments and analysis are presented in sections “**3.4.2 Physical-chemical analysis**” and “**3.4.3 FTIR-ATR analysis**”.

3.3.4.9 Evaluation of EGFP purification by SDS-PAGE

To further confirm the EGFP purification obtained after each step of the integrated process developed in this work, the researcher performed an SDS-PAGE. Some of the phase-forming agents used in the ABS interfere with the electrophoresis run, and therefore, those were removed using the Vivaspın® 20 10 kDa centrifugal concentrators. All samples, including the

EGFP raw extract, were subjected to the removal of solvents using the centrifugal concentrators and centrifugation at 4 °C and $8,000 \times g$ for 45 min. It is important to note that only components with a molecular weight lower than 10 kDa were removed in the collector, and thus, all the protein content (EGFP and contaminants) of the samples were recovered in the concentrator (the ladder standard for SDS-PAGE starts from 10 kDa). The SDS-PAGE method is detailed in section “**3.4.6 SDS-PAGE**”.

3.4 INSTRUMENTAL METHODS

3.4.1 Fluorescence analysis

3.4.1.1 3D fluorescence spectroscopy

The 3D fluorescence spectra were acquired at 25 °C (maintained with a thermostatic water bath) with a spectrofluorophotometer RF-6000 SHIMADZU®, with an interval of 2.0 nm for λ_{ex} and 1.0 nm for λ_{em} , scan speed $6000 \text{ nm} \cdot \text{min}^{-1}$, λ_{ex} bandwidth 10.0 nm and λ_{em} bandwidth 1.0 nm. The emission and excitation wavelength interval for each analysis varied depending on the fluorophores in analysis, being specified in the caption for each figure with 3D fluorescence spectra.

3.4.1.2 Single point fluorescence

For the fluorescence properties and effect of IL solutions on EGFP, the FI of EGFP solutions was assessed using the EnSpire® multimode plate reader from PerkinElmer® in the point of maximum fluorescence of the protein (F1 λ_{ex} 488, λ_{em} 510 nm), with exception of the EGFP protection studies with ILs, that used the EnSight™ Multimode Plate Reader, also from PerkinElmer®. Experiments were performed in triplicate with a blank and results are presented as average $\pm$ standard deviation. For the stability assays, pure EGFP ($5.4 \mu\text{g} \cdot \text{mL}^{-1}$) in PBS or PB was also evaluated and its FI at 0 h was used as the reference for 100 % to calculate Relative FI (%).

For the calibration curve of EGFP in the purification study, the points were acquired at F1 (λ_{ex} 488, λ_{em} 510 nm) and F2 (λ_{ex} 278, λ_{em} 510 nm) with point fluorescence analysis in the RF-6000 Shimadzu®, 10 ms accumulation time, λ_{ex} bandwidth 10.0 nm and λ_{em} bandwidth 1.0 nm. Considering EGFP bleaching half-life of 174 s (SHANER *et al.*, 2005) and the low accumulation time (10 ms), the experiments were designed to prevent photobleaching in the single point analysis.

3.4.1.3 Intrinsic fluorescence of proteins (IFP)

The intrinsic fluorescence of proteins (IFP, given by the presence of the amino acids Tyr, Trp, and phenylalanine, Phe) (LAKOWICZ, 1999) can also indicate variations in the conformation of proteins. Their contribution was evaluated by emission spectra in the λ_{ex} 280 nm and λ_{em} range from 290 to 410 nm (Tyr and Trp residues), with a scan speed of 200 nm.min⁻¹, λ_{ex} bandwidth of 3.0 nm and λ_{em} bandwidth of 5.0 nm. For the emission spectra of the intrinsic fluorescence of proteins, the data was adjusted so that the FI of the last emission point (at λ_{em} 410 nm) of each sample was 0 UF after subtraction of the blank (spectra of PB). Experiments were performed in triplicate with a blank.

3.4.2 Physical-chemical analysis

3.4.2.1 pH and conductivity calibration and determination

The pH (± 0.003) and conductivity ($\pm 1.0\%$ mS.cm⁻¹) at 25 (± 1) °C of the samples were measured using a portable pH meter and conductometer Metrohm®/Model 914, calibrated with standard buffers for pH (pH values of 7.00 and 4.01) and standard solution of KCl (0.1 mol.L⁻¹) for conductivity. The pH of samples was adjusted using 3 mol.L⁻¹ solutions of H₃PO₄ and KOH for samples in PB and HCL and NaOH for samples in PBS, allowing a maximum variation of 0.1 in pH.

3.4.2.2 Density and viscosity determination

Density and viscosity measurements were performed at atmospheric pressure and 25.00 (± 0.02) °C, using an automated SVM 3000 Anton Paar rotational Stabinger viscometer-densimeter. The dynamic viscosities have a relative uncertainty of $\pm 0.35\%$, while the absolute uncertainty for the density is 5×10^{-4} g.cm⁻³. The viscosimeter was previously calibrated using standard solutions.

3.4.2.3 Water content measurement with Karl-Fischer titration

Volumetric Karl-Fischer titration at 25 °C was performed using a Karl-Fischer 852 Titrando from Metrohm® ($\pm 0.5\%$), HYDRANAL-Methanol Rapid (reagent for accelerated volumetric one-component KF titration), and HYDRANAL-Composite 5 (reagent for volumetric one-component Karl Fischer titration methanol free), both reagents supplied by Sigma-Aldrich® as titrants.

3.4.2.4 Water content measurement with a gravimetric method

For the systems containing NaPA-8000, the water content was determined by freeze-drying. Thus, approximately 1 g of each phase of the NaPA-8000-based ABS was freeze-dried for 48 h using the Thermo Scientific ModulyoD Freeze Dryer. After all the water removal, the samples were re-weighted, and the water content was calculated as the percentage of water removed during the freeze-drying process.

3.4.3 Circular Dichroism (CD) analysis

The secondary structure of EGFP was obtained using CD with a J-1500 spectrometer from Jasco®. The spectra were acquired at 25 °C in triplicate with a blank, using a cell of 1 mm, scan speed 100 nm.min⁻¹, wavelength from 300 to 190 nm, an interval of 1.0 nm, 6 accumulations, Digital Integration Time (DIT) of 4 s, a bandwidth of 1.00 nm, and a concentration of 0.3 mg.mL⁻¹ of EGFP. The High Tension (HT) was maintained below 700 to obtain a reliable CD signal. Experiments were performed in triplicate with a blank. CD analysis was only conducted using the PB buffer since the chlorine ions present in PB solution are incompatible with CD.

CD results are presented as mean residue ellipticity ([θ]). The [θ] was calculated using the following equation (3): (GREENFIELD, 2006)

$$[\theta] = \frac{(\theta \times \text{MRW})}{(P \times C_{PT})} \quad (3)$$

where θ is the CD value (mdeg), MRW is the Mean Residue Weight of the protein, P is the path length of the CD cell in mm and C_{PT} is the concentration of the protein in mg.mL⁻¹.

MRW was calculated for EGFP following the equation (4): (GREENFIELD, 2006)

$$\text{MRW} = \frac{\text{MW protein (Da)}}{(n \text{ of amino acid residues} - 1)} \quad (4)$$

EGFP has an MW of 26.9 kDa and 239 amino acid residues (res), with an MRW of 113.025 (Da.res⁻¹). P was 1 mm and C was 0.3 mg.mL⁻¹.

For the data processing, according to the critical review on CD from Greenfield, (GREENFIELD, 2006) most CD analysis software have difficulties in predicting the secondary structure of proteins which are majorly formed by β-strands, like EGFP. The most accurate results were obtained with K2D and CONTIN programs. Considering K2D only predicts the percentage of β-strands and α-helix, while CONTIN can also calculate turns and make

distinctions between regular and distorted β -strands and α -helix, Dichroweb with CONTIN (WHITMORE; WALLACE, 2004, 2008) set SP175 (optimized for the range of 190-240 nm) (LEES *et al.*, 2006) was used for the CD data analysis.

3.4.4 Small-Angle X-Ray Scattering (SAXS)

The SAXS experiments were performed at the SAXS/WAXS beamline of the Australian Synchrotron (ANSTO), Melbourne, Australia, using two techniques that provided distinct information regarding EGFP behavior at different pH: well-plate SAXS and Size Exclusion Chromatography SAXS (SEC-SAXS). The SEC-SAXS is a newly developed SAXS technique for the analysis of biomolecules (BIOSAXS), which combines SAXS with size-exclusion chromatography (SEC) and a co-flow system, allowing scattering patterns to be acquired continuously on the different fractions of the protein sample. (RYAN *et al.*, 2018) Considering EGFP is a weak dimer, (MYATT *et al.*, 2017) the protein can be found as a monomer, dimer, or even as aggregates; hence, the SEC-SAXS can provide information regarding the structure of the protein and the aggregates at different speciation. The well-plate SAXS was also evaluated because, although it is from scattering from all the EGFP monomers and aggregates, and hence lower quality data than SEC-SAXS, it can give information about samples that are not suitable for SEC-SAXS, including samples with precipitates (for example, denatured proteins or protein solutions with pH close to its pI).

For SEC-SAXS, patterns were acquired while the sample was flowed through a capillary, to minimize protein degradation. The protein solution was under constant flow surrounded by a sheath of the corresponding solvent, and is a technique suitable for weakly-scattering protein samples, minimizing protein denaturation from prolonged X-ray radiation. It has been designed to enable high-quality data with excellent background subtraction, which is suitable for comparison with protein models to obtain information about the tertiary structure of the protein. (RYAN *et al.*, 2018; TREWHELLA *et al.*, 2017) The capillary was washed between samples, and the same location on the capillary was used for all measurements to maximize accuracy in subtracting the solvent. Details regarding sample preparation, data collection parameters, use of software, and structural parameters can be found in **Table C-1**. Advanced technical specifications for the equipment can be found on the ANSTO website. (ANSTO, 2020a) SAXS software scatterbrain 2.82 from ANSTO, (ANSTO, 2020b) ATSAS (FRANKE *et al.*, 2017), and SREFELX (PANJKOVICH; SVERGUN, 2016) were used for

SAXS data analysis. The EGFP PDB ID 2Y0G structure was used as a model for the SAXS fittings. (BERMAN *et al.*, 2002; ROYANT; NOIRCLERC-SAVOYE, 2011)

For the well-plate SAXS, an initial test for concentrations 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, and 5.0 mg.mL⁻¹ of EGFP were evaluated to find the lowest concentration which provided a strong and clear scattering signal. The concentration of 2.5 mg.mL⁻¹ of EGFP was selected.

The SEC-SAXS associates the SAXS technique with High-Performance Liquid Chromatography (HPLC), using a size-exclusion column GE Healthcare® Superdex s200. Equipment and procedures following Ryan *et al.* specifications (RYAN *et al.*, 2018). A concentration test with samples with 2.0, 2.5, 3.0, 5.0, and 10 mg.mL⁻¹ of EGFP was performed to find the lowest concentration which provided a strong and clear scattering signal. The concentration of 5.0 mg.mL⁻¹ of EGFP was selected.

3.4.5 FTIR-ATR analysis

The Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) analysis was performed at 25 (± 1) °C using an ALPHA FTIR spectrometer with Platinum ATR single reflection diamond module from Bruker®. The spectra were obtained in the wavelength range from 4000 to 400 cm⁻¹. For the FTIR-ATR, the first step of the analysis was to run a “blank” to subtract the scattering of the environment. Then, one drop of the liquid sample was deposited in the diamond window of the equipment and the analysis was executed again, providing the infrared spectra of the sample.

3.4.6 SDS-PAGE

SDS-PAGE was performed using a Mini-PROTEAN® III (Bio-Rad®) System (BioRad, Brazil). The standard for SDS-PAGE was the PageRuler Prestained Protein Ladder 10 to 180 kDa (Thermo Fisher®). All reagents for the electrophoresis were obtained from Bio-Rad® or Sigma-Aldrich®. Protein SDS-PAGE was performed as described by Sambrook and Russell. (SAMBROOK; RUSSELL, 2001) The standard (4 µL) was loaded in one well of the gel [10 % resolving gel and 5 % stacking gel (wt/v%), 1 mm thickness], and in the other wells, samples with 15 µg of EGFP were added after 10 min incubation at 96 °C with LaemLi Buffer 2X with 0.2 mol.L⁻¹ dithiothreitol (DTT). The initial voltage of the electrophoresis was 80 V (during stacking) and 120 V (during the resolving phase). The gel was revealed with the Coomassie Blue R solution.

4 RESULTS AND DISCUSSION

4.1 EGFP PRODUCTION

As previously mentioned, due to EGFP exorbitant prices (mainly caused by its expensive production and purification processes), its commercial applications as a biosensor and biomarker are still limited. Considering its production stage, one of the main factors that increase its processing costs is the use of very expensive inducers like isopropyl- β -D-1-thiogalactopyranoside (IPTG). Hence, this work studied the production EGFP using *E. coli* BL21 (DE3) [pLysS; pET28(a)], aiming to increase its yield and reduce costs. First, the group evaluated the influence of agitation rate, induction time, and concentration of IPTG in the production of EGFP, but only the first two parameters were significant. Subsequently, aiming to reduce costs related to the use of inducer, the researchers reduced the IPTG concentration (0.005, 0.010, and 0.025 mmol.L⁻¹) and, interestingly, the production levels were maintained or increased. These results show that a proper choice of production conditions (notably through the decrease of inducer concentration) is effective in reducing the upstream production costs and guarantee high EGFP expression.

Table 6 presents an overview of the main findings of the work:

Table 6 – Overview of the main effects of IPTG concentration ([IPTG], agitation rate, and induction time over the bacterial growth (represented by optical density at 600 nm, OD_{600nm}) and EGFP production (indicated by EGFP concentration, [EGFP]) by *E. coli* BL21 (DE3) [pLysS; pET28(a)] at 30 °C.

Parameter	OD _{600nm}	[EGFP]
[IPTG]	No impact or slight $\uparrow$ OD _{600nm} with the addition of IPTG	No impact or slight $\uparrow$ [EGFP] at lowest [IPTG]
Agitation	$\uparrow$ agitation = $\uparrow$ OD _{600nm} $\downarrow$ agitation = $\downarrow$ OD _{600nm}	100 < 200 $\approx$ 250 < 150 rpm
Induction time		
Beginning of exponential phase	$\downarrow$	$\downarrow$
Middle of exponential phase	No impact or $\uparrow$ OD _{600nm}	$\uparrow$
End of exponential phase	No impact or $\uparrow$ OD _{600nm}	$\uparrow$

Source: Produced by the author with results from the collaboration article (LOPES *et al.*, 2019).

As can be seen in **Table 6**, changes in agitation rate and induction time significantly affected the production of EGFP by recombinant *E. coli* BL21 (DE3) [pLysS; pET28(a)], but IPTG concentration did not affect the productivity. Although the group obtained the best results for bacterial growth at an agitation rate of 250 rpm, it was possible to achieve the highest

production of the protein at 150 rpm. Additionally, changing the time of induction also affected EGFP concentration, with the best results obtained with inductions in the middle or at the end of the log phase. Finally, this study also demonstrated that it is possible to maintain productivity levels of EGFP by *E. coli* BL21 (DE3) while using minimal concentrations of inducer, such as 0.005 mmol.L⁻¹.

These results show that a proper choice of production conditions (notably through the decrease of inducer concentration) is effective in reducing the upstream production costs and guarantee high EGFP.

The complete work, entitled “Improving the cost-effectiveness of enhanced green fluorescent protein production using recombinant *Escherichia coli* BL21 (DE3): Decreasing the expression inducer concentration”, was published in **Biotechnology and Applied Biochemistry**, v. 66, n. 4, 527-536, 2019, DOI: [10.1002/bab.1749](https://doi.org/10.1002/bab.1749). (LOPES *et al.*, 2019)

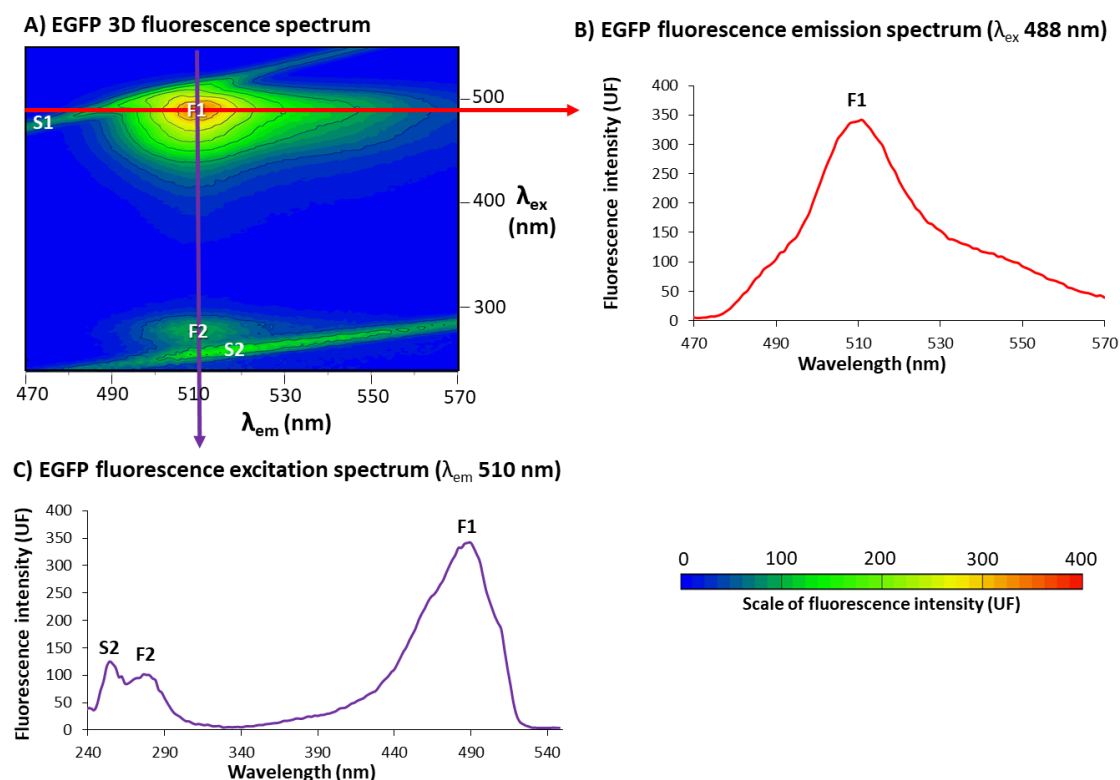
4.2 EGFP FLUORESCENCE PROPERTIES AND STRUCTURE

Section adapted from the published original article in **RSC Advances**, v. 9, n. 40, 22853-22858, 2019. (DOS SANTOS *et al.*, 2019)

This study evaluated EGFP 3D fluorescence spectra at lower wavelengths and under different conditions to clarify the controversy regarding the EGFP homogeneous fluorescence spectrum for the two distinct conformations.

The 3D fluorescence spectra (also called total fluorescence or fluorescence excitation-emission matrix) are depicted in a 2D view, where the λ_{ex} is shown on the Y-axis, the λ_{em} on the X-axis, and the fluorescence intensities are given by the scale from blue to red in UF. **Figure 10** demonstrates the relationship between the 3D fluorescence spectra and their respective excitation and emission spectra. In **Figure 10.A**, the red horizontal line across the 3D spectra represents an emission spectrum at a specific excitation (in this case, λ_{ex} 488 nm), and the purple vertical line is an excitation spectrum at certain emission (in this example, λ_{em} 510 nm). The respective excitation and emission spectra for the red and purple lines of the 3D analysis in **Figure 10.A** are presented in **Figure 10.B** and **Figure 10.C**. The first and second-order Rayleigh light scattering (noted as S1 and S2, respectively) (BORTOLOTTI *et al.*, 2016; WU *et al.*, 2015) are also presented in **Figure 10.1** and **Figure 11**. S1 occurs as a result of the elastic scattering of the light that strikes small particles, and because there is no loss or gain of energy in the interaction between matter and the Rayleigh light, λ_{ex} and λ_{em} are the same in S1 ($\lambda_{\text{ex}} = \lambda_{\text{em}}$) and present a linear aspect. S2 is an artifact generated by grating monochromators, where selecting light at a specific wavelength (λ_{ex}) also allows the transmission of light with half the wavelength ($\lambda_{\text{ex}}/2$), and again causes Rayleigh scattering from small particles when $2\lambda_{\text{ex}} = \lambda_{\text{em}}$. (BORTOLOTTI *et al.*, 2016) For example, S1 is observed at λ_{ex} 500 nm, λ_{em} 500 nm, and S2 at λ_{ex} 500 nm, λ_{em} 250 nm in the 3D spectrum presented in **Figure 10. A**. Fluorophores present a more spherical or oval shape, with the highest fluorescence intensity at their central region and decreasing intensity in their extremities, as can be seen in **Figure 10.A** for EGFP ($5.4 \mu\text{g.mL}^{-1}$).

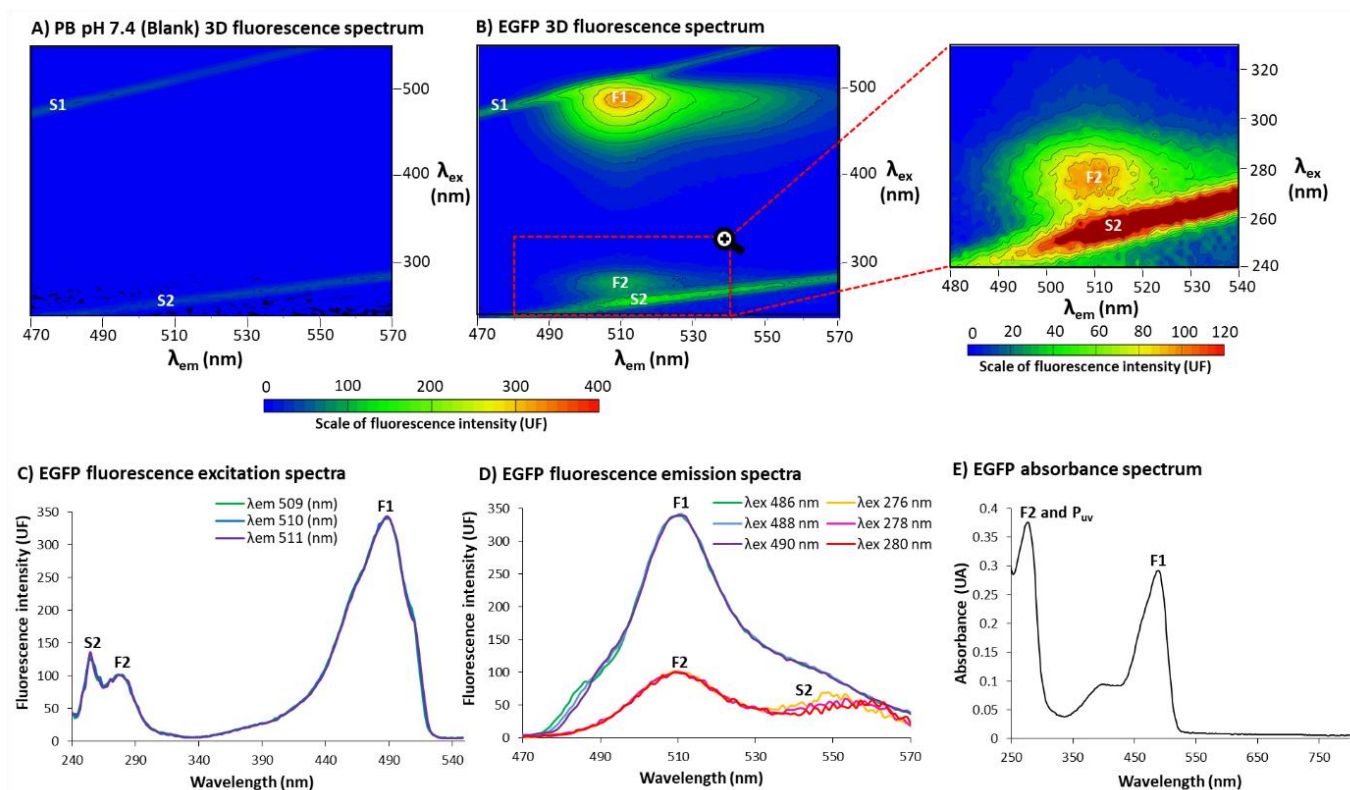
Figure 10 – **A)** 3D fluorescence spectrum, **B)** emission spectrum (λ_{ex} 488 nm) and **C)** excitation spectrum (λ_{em} 510 nm) of EGFP $5.4 \mu\text{g.mL}^{-1}$ 25 °C in phosphate buffer (PB) pH 7.4. The emission spectrum in **B)** is equivalent to the red horizontal line in the 3D fluorescence spectrum in **A)**, and the excitation spectrum in **C)** comprises the purple line in **A)**. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (unit of fluorescence, UF) according to the color scale from blue to red. F1 (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (around λ_{ex} 278 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity; S1 and S2 are first and second-order light scattering, respectively.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2019).

In **Figure 11**, EGFP in phosphate buffer (PB) pH 7.4 was evaluated by 3D fluorescence spectra and by absorbance at 25 °C to provide insights into EGFP fluorescence and absorbance properties.

Figure 11 – 3D fluorescence spectra at 25 °C in phosphate buffer (PB) pH 7.4 of **A)** Blank and **B)** EGFP 5.4 $\mu\text{g.mL}^{-1}$, range λ_{ex} 240-550 nm and λ_{em} 470-570 nm, scale of fluorescence intensity 0-400 units of fluorescence (UF) and corresponding zoomed region of EGFP in a range of λ_{ex} 240-330 nm and λ_{em} 480-540 nm, scale of fluorescence intensity 0-120 UF. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (UF) according to the color scale from blue to red. F1 (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (around λ_{ex} 278 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity; S1 and S2 are first and second-order light scattering, respectively. **C)** EGFP (5.4 $\mu\text{g.mL}^{-1}$) fluorescence excitation spectra (λ_{em} 240-550 nm) for emissions 509 (green), 510 (blue) and 511 nm (purple) and **D)** EGFP (5.4 $\mu\text{g.mL}^{-1}$) fluorescence emission spectra (λ_{em} 470-570 nm) for excitations 276 nm (yellow), 278 nm (pink), 280 nm (red), 486 nm (green), 488 nm (blue) and 490 nm (purple). **E)** EGFP (0.216 mg.mL^{-1}) absorbance spectrum (λ 250-800 nm) in units of absorbance (UA); F2 and protein intrinsic absorbance in UV (P_{uv}) have an overlapping peak at 276 nm and F1 a peak at 488 nm. The results were obtained in triplicate with a blank. A representative of the triplicate is presented for the 3D spectra, and the average of the results is shown for excitation, emission, and absorbance spectra.

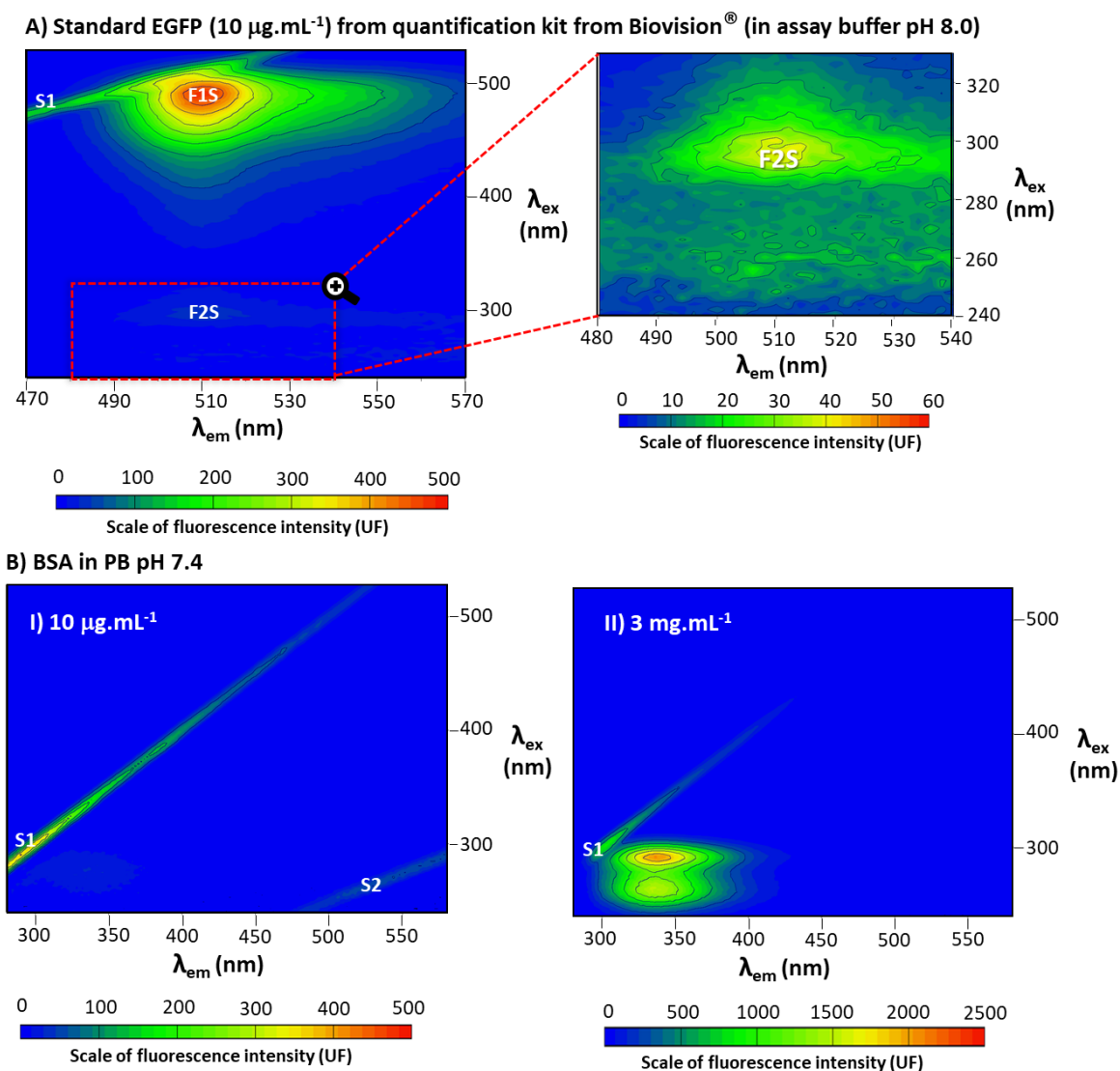


Source: Produced by the author and published in (DOS SANTOS *et al.*, 2019).

In **Figure 11.A**, the 3D spectra of PB pH 7.4 (blank) showed no evidence of fluorophores, with detection of only the first and second-order Rayleigh light scattering (S1 and S2, respectively). In **Figure 11.B**, the EGFP 3D spectrum contained two distinct non-overlapping peaks, labeled as F1 and F2. At F1, there was a higher and sharper peak at λ_{ex} 488 nm and λ_{em} 510 nm, while at F2 (highlighted in the zoomed region), there was a smaller and less distinct peak λ_{ex} 278 nm and λ_{em} 510 nm. The 2D fluorescence spectra of excitation (**Figure 11.C**) and emission (**Figure 11.D**) for EGFP also depict F1, F2, and S2 (wavelengths (λ) selected to be close to the points of highest FI). Besides, **Figure 11.E** shows the absorbance spectrum for EGFP from ultraviolet (UV) to visible light (250-800 nm), with the peaks corresponding to F1 and F2 labeled. Together with F2, **Figure 11.E** also presents the intrinsic absorbance in UV light of proteins that contain the amino acids Tyr, Trp, or Phe, labeled as P_{uv} . The absorbance and fluorescence of proteins with these amino acids around λ 280 nm is a well-established phenomenon, (BORTOLOTTI *et al.*, 2016; WHITAKER; GRANUM, 1980) and considering EGFP presents both Tyr, Trp, and Phe in its structure, (ROYANT; NOIRCLERC-SAVOYE, 2011) the highest peak at 276 nm is likely the result of an overlap of F2 and P_{uv} . The other band observed around 350-450 nm in the absorbance spectrum corresponds to the protonation state of the chromophore, as previously suggested. (SEWARD; BAGSHAW, 2009) Although this band appeared in the absorbance spectrum, it is absent in the fluorescence spectrum. This issue is likely due to fluorescence needing to fulfill more conditions to its manifestation when compared to colors, and these requirements are not satisfied in EGFP protonated state.

A standard from a quantification kit from Biovision[®] was also measured by 3D fluorescence (EGFP standard in assay buffer pH 8.0) in **Figure 12** to confirm the presence of the second peak in EGFP, and that this was not a result of modifications of the protein during expression and purification.

Figure 12 – A) 3D fluorescence spectra at 25 °C of standard EGFP ($10 \mu\text{g.mL}^{-1}$) from quantification kit from Biovision® (in assay buffer pH 8.0), range λ_{ex} 240-530 nm and λ_{em} 470-570 nm, scale of fluorescence intensity 0-500 units of fluorescence (UF) and corresponding zoomed region of EGFP in a range of λ_{ex} 240-330 nm and λ_{em} 480-540 nm, scale of fluorescence intensity 0-60 UF. **B)** 3D fluorescence spectra of BSA in PB pH 7.4, range λ_{ex} 240-530 nm and λ_{em} 280-580 nm (EGFP concentration $10 \mu\text{g.mL}^{-1}$ and scale of fluorescence intensity 0-500 UF in **B.I** and 3 mg.mL^{-1} in 0-2500 UF in **B.II**. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (UF) according to the color scale from blue to red. F1S (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2S (around λ_{ex} 296 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity, and S1 and S2 are the first and second-order light scattering, respectively.



Source: Produced by the author and published (adapted) in (DOS SANTOS *et al.*, 2019)

The 3D fluorescence spectrum of the EGFP standard, depicted in **Figure 12.A**, shows that it is possible to discern two distinct peaks labeled as F1S and F2S (the latter highlighted in the zoomed region) for the EGFP produced in the laboratory. The peak from the standard (marked as F1S) is identical to F1 for the EGFP from the group. Besides, the emission for F2S is maintained λ_{em} 510 nm. However, there is a considerable shift for the excitation wavelength

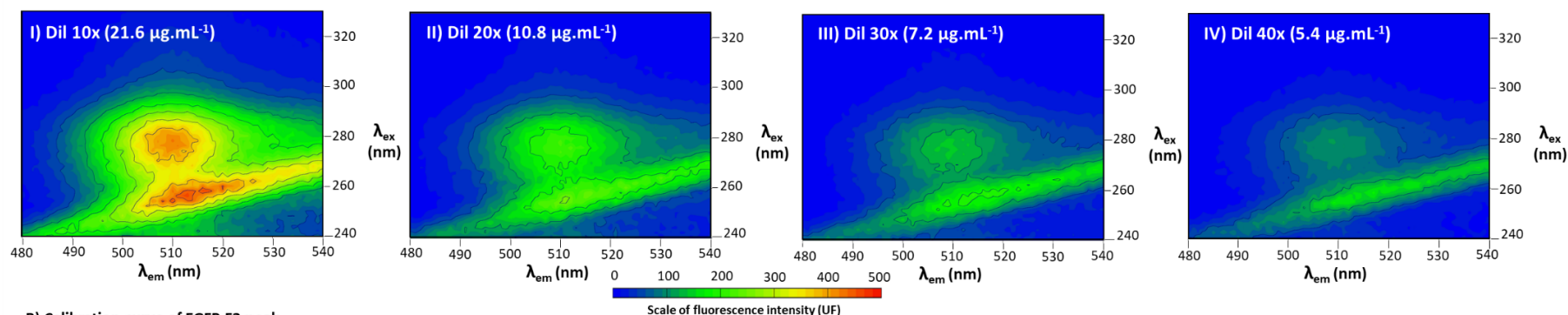
of F2S (highest fluorescence intensity at λ_{ex} 278 nm for EGFP F2 to λ_{ex} 296 nm for EGFP standard F2S). The redshift in F2S is likely due to a change of the environment around the fluorophore, caused by the difference in buffer pH and composition (a well-known effect for fluorophores). (MATAKA *et al.*, 1956) Nevertheless, even considering the shift in λ_{ex} , the presence of two distinct and not overlapping peaks for EGFP is evident in **Figure 12.A**.

To confirm peak F2 was related to EGFP fluorophore, and not only a reflection or residue of the intrinsic fluorescence of proteins (characteristic of proteins which contain the amino acids Tyr, Trp, or Phe in their structure, with peaks around λ_{ex} 280 nm and λ_{em} 300-400 nm), (BORTOLOTTI *et al.*, 2016) BSA (rich in Tyr, Trp, and Phe) (BUJACZ, 2012) was characterized by 3D fluorescence spectra (**Figure 12.B**). As can be seen in **Figure 12.B.I**, there was no detectable fluorescence above λ_{em} 450 nm for BSA at the same concentration of the EGFP standard (10 $\mu\text{g.mL}^{-1}$). This result confirms there is no reflection or residue of the fluorescence of amino acids at the position of F2S. Even at high BSA concentration (3 mg.mL^{-1}), shown in **Figure 12.B.II**, where the fluorescence intensity between λ_{em} 300-400 nm surpassing 2000 UF, there was still no fluorescence around the F2 position, fully confirming that the amino acids do not contribute to EGFP fluorescence at F2S.

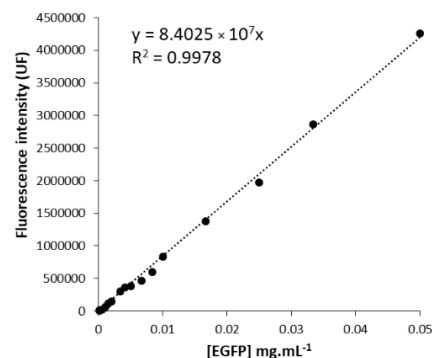
Finally, to understand the behavior of EGFP fluorescent peaks under different conditions, 3D spectra were also acquired for EGFP in a series of dilutions (**Figure 13.A**, with a calibration curve for peak F2 in **Figure 13.B**), at different pH (pH 7.4 in **Figure 13.C.I** and pH 6.0 in **Figure 13.C.II**) and after denaturation at high temperature (3 h at 90 °C, **Figure 13.D**).

Figure 13 – A) 3D fluorescence spectra at PB pH 7.4 at 25 °C of different concentrations of EGFP (**A.I** – dil 10x - 21.6 $\mu\text{g.mL}^{-1}$, **A.II** – dil 20x - 10.8 $\mu\text{g.mL}^{-1}$, **A.III** – dil 30x - 7.2 $\mu\text{g.mL}^{-1}$, **A.IV** – dil 40x - 5.4 $\mu\text{g.mL}^{-1}$), range λ_{ex} 240-330 nm and λ_{em} 480-540 nm, scale of fluorescence intensity 0-500 UF. **B)** Calibration curve for EGFP F2 peak (λ_{ex} 278 nm, λ_{em} 510 nm), concentration of EGFP [EGFP] (mg.mL^{-1}) in the X-axis and fluorescence intensity (UF) in the Y-axis. **C)** 3D fluorescence spectra of EGFP (5.4 $\mu\text{g.mL}^{-1}$) in PB of different pH (**C.I** – pH 6.0 and **C.II** – pH 7.4). **D)** after denaturation by temperature in PB pH 7.4 (30 min at 90 °C), range λ_{ex} 240-550 nm and λ_{em} 470-570 nm, scale of fluorescence intensity (UF) 0-400. 3D fluorescence spectra with the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the scale of fluorescence intensity (UF) according to the color scale from blue to red. F1 (around λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (around λ_{ex} 296 nm, λ_{em} 510 nm) are the two fluorescence points with the highest intensity. The results correspond to triplicate measurements with corresponding blank assays for the pH, calibration curve, and high-temperature experiments. The 3D spectra are shown as a representative of the triplicate, and the calibration curve as the average of the experiments.

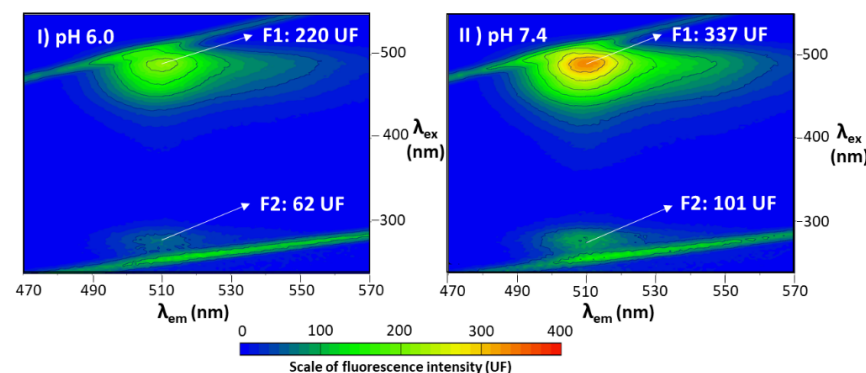
A) Effect of dilution on EGFP F2 peak



B) Calibration curve of EGFP F2 peak
(λ_{ex} 278 nm, λ_{em} 510 nm)



C) Effect of pH on EGFP peaks



D) Effect of denaturation by temperature (30 min at 90 °C) on EGFP peaks

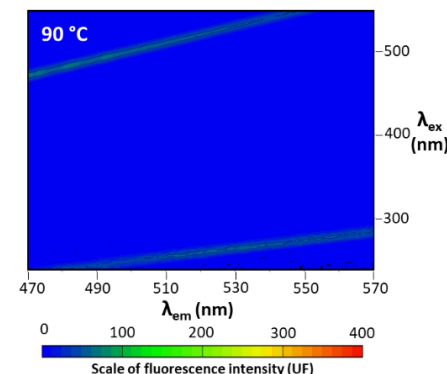


Figure 13.A and **Figure 13.B** indicate that the fluorescence of F2 is highly dependent on protein concentration. The effect of pH was also explored through decreasing pH from 7.4 to 6, as presented in **Figure 13.C.I** and **Figure 13.C.II**, respectively. It is possible to conclude that both peaks are pH-sensitive, considering there was a decrease of 46% of FI for F1 and 45% for F2 (average of a triplicate) from pH 7.4 to 6.0. This pH-dependence is analogous to what is seen for wtGFP, which presents one peak for its protonated form (favored by acidic pH) and another for its unprotonated form (favored by alkaline pH).¹¹ This result is also aligned with reports by Arpino *et al.* 2012 (ARPINO *et al.*, 2012) about the relationship between the two EGFP forms and the charged state of the fluorophore. Furthermore, when EGFP was denatured after exposure to 90 °C for 3 h (**Figure 13.D**), the fluorescence disappeared, showing both peaks are sensitive to high temperatures. This effect suggests F2 fluorescence is intrinsically dependent on the maintenance of the protein structure, and not only a result of the amino acids fluorescence (which can maintain its fluorescence even in their free form and in denatured proteins) (BORTOLOTTI *et al.*, 2016) or other independent fluorophore groups. Hence, the results confirm a direct dependence of the protein structure for the fluorescence manifestation of peak F2.

In a comparison of the calibration curves for F1 and F2 (**Table 7**), it is possible to see many similarities between the properties of the two peaks. For example, both R^2 and RSD for F1 and F2 are very similar: R^2 of 0.9981 and 0.9978, and RSD of 2.1% and 2.8% for F1 and F2, respectively. These parameters are associated with the precision and repeatability of quantification, indicating F2 can be as precise as F1 for analytical procedures. The curves for F1 and F2 presented the same upper limit (0.05 mg.mL⁻¹) but different lower limits of detection and quantification (LOD and LOQ for F1 were 25 and 33 ng.mL⁻¹ and 462 and 633 ng.mL⁻¹ for F2), showing F1 is indeed one magnitude more sensitive than F2. This increased sensitivity of F1 likely due to its higher fluorescence intensity when compared to F2. Despite presenting lower quantification limits, F2 still can be used to quantify EGFP with as much precision as F1 for concentrations as low as 0.6 µg.mL⁻¹, indicating it can be also applied for analytical purposes like F1. Besides, F2 was detected in three different fluorophotometers, namely EnSpire® and EnSight™ Multimode Plate Readers from PerkinElmer® and RF-6000 SHIMADZU spectrofluorophotometer, confirming F2 was not a fluorescence artifact from specific equipment.

Table 7 – Comparison of range and precision of EGFP calibration curves at fluorescence peaks F1 (λ_{ex} 488 nm, λ_{em} 510 nm) and F2 (λ_{ex} 278 nm, λ_{em} 510 nm). Parameters presented are the upper limit of the curve, coefficient of determination (R^2), the limit of detection (LOD), the limit of quantification (LOQ), and relative standard deviation (RSD).

	F1 (λ_{ex} 488 nm, λ_{em} 510 nm)	F2 (λ_{ex} 278 nm, λ_{em} 510 nm)
Upper limit of the curve (mg.mL⁻¹)	0.05	0.05
R²	0.9981	0.9978
LOD (ng.mL⁻¹)	25	462
LOQ (ng.mL⁻¹)	33	633
RSD (%)	2.1	2.8

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2019).

From this study, it was revealed that EGFP presents two, and not one, fluorescence peaks (around λ_{ex} 488 nm, λ_{em} 510 nm and λ_{ex} 278 nm, λ_{em} 510 nm), which is consistent with the two conformations observed in its crystalline structure by different independent reports. (ARPINO *et al.*, 2012; ROYANT; NOIRCLERC-SAVOYE, 2011) This property is analogous to what is seen for wtGFP, which displays two fluorescence peaks in the visible range (a major peak around λ_{ex} 400 nm for the protonated form and a minor peak at λ_{ex} 475 nm for the unprotonated state). (TSIEN, 1998) Although the EGFP mutations (F64L/S65T) favor the unprotonated form (peak at λ_{ex} 488 nm, λ_{em} 510 nm), the protonated state is still present in equilibrium, but at significantly lower λ_{ex} when compared to the wtGFP (λ_{ex} 278 nm for EGFP and λ_{ex} 395 nm for wtGFP). This greater difference between the excitation wavelengths of the protonated and unprotonated forms also explains the increase in the fluorescence intensity of EGFP compared to wtGFP.

Considering the excitation spectra for the two conformations of EGFP do not overlap like in wtGFP, at λ_{ex} 488 nm, all energy is directed to excite the fluorophore of the unprotonated form. This lack of overlap also explains why previous studies only found one lifetime for EGFP, considering the excitation wavelength of the experiments was well above F2 [at 365 nm, (STEPANENKO *et al.*, 2004) 440 nm (ROYANT *et al.*, 2007; ROYANT; NOIRCLERC-SAVOYE, 2011) and 488 nm (ARPINO *et al.*, 2012)]. Another factor that complicated the detection of the EGFP second peak in previous studies was that both peak F2 of EGFP and the amino acids Tyr, Phe, or Trp (also present in EGFP structure) present an absorbance peak around 280 nm. (SEWARD; BAGSHAW, 2009) This phenomenon could have led previous groups to conclude that the absorbance of EGFP in UV light was only due to the presence of these amino acids.

As can be seen in **Figure 11.C** and **Figure 11.D**, it can be challenging to discern fluorophores from fluorescence artifacts like Rayleigh scatterings using only 2D fluorescence analysis, and this can be even more arduous considering the possibility of other types of scattering like Raman, (BORTOLOTTI *et al.*, 2016) the presence of impurities in the sample or the evaluation of mixtures with multiple fluorophores. Thus, using 3D fluorescence spectroscopy, it is possible to evaluate the shape of the fluorophores and distinguish them from the different fluorescence artifacts, while also being able to extract the 2D spectra. These characteristics make 3D fluorescence spectroscopy a powerful tool in the analysis of fluorescent samples.

Another information that reinforces the presence of F2 in EGFP is a study by Voityuk *et al.* (VOITYUK *et al.*, 2001) with the free chromophore of GFP. Voityuk *et al.* (VOITYUK *et al.*, 2001) confirmed that GFP chromophore could present an intrinsic absorbance at 278 nm in certain forms of protonation, with a pH-dependent behavior. The GFP chromophore absorbance peak at 278 nm appeared with the addition of HCl solution and disappeared with the addition of NaOH, in agreement with our results that this peak is associated with protonated forms of EGFP. Voityuk *et al.* (VOITYUK *et al.*, 2001) studied eight theoretical forms of the GFP chromophore due to protonation/deprotonation. However, it must be noted that the possibility of certain conformations of chromophores in fluorescent proteins will also rely on the FP variant structure because the chromophore is restrained by its links and interaction with the structure of the protein. That is why, in practice, there are only two conformations for wtGFP and EGFP detected by crystallography. (ARPINO *et al.*, 2012; YANG *et al.*, 1996) Hence, this suggests that the protonated form of EGFP presents the protonated GFP chromophore of Voityuk *et al.* study (VOITYUK *et al.*, 2001), which conferred an absorbance when excited at λ 278 nm.

The discovery of a second EGFP peak can be useful in the analysis of complex biological samples that present endogenous fluorophores with overlapping peaks with F1 (*i.e.*, fluorophores that exhibit fluorescence at the same λ as EGFP F1). For example, this included biomolecules such as lipofuscin (range λ_{ex} 410-470 nm, λ_{em} 500-695 nm) - a yellow-brown pigment granule present in many long-lived eukaryotic cells due to aging, (CROCE; BOTTIROLI, 2014; SCHONENBRUCHER *et al.*, 2008) other lipofuscin like-lipopigments/ceroids (range λ_{ex} 400-500 nm, λ_{em} 480-700 nm), (CROCE; BOTTIROLI, 2014) and many plants-derived biomolecules like lignin (maximum peaks at λ_{ex} 335 nm, λ_{em} 455 nm and λ_{ex} 488 nm, λ_{em} 535 nm) and flavonoids (maximum λ_{ex} 488 nm, λ_{em} 535 nm).

(DONALDSON; WILLIAMS, 2018) As both F1 and F2 can be quantified by fluorescence, F2 can be used to either confirm the values from F1 or to analyze samples that exhibit interference in the F1 region.

In this study, a new peak for EGFP was detected, clarifying a controversy regarding its two distinct conformations in what was previously reported as a homogeneous fluorescence spectrum. Based on the new report regarding the structure of this broadly used protein, we suggest that researchers re-evaluate the fluorescence spectra of other fluorophores using more modern spectrofluorophotometers, which allow assessments at lower wavelengths and with higher resolution. Considering the importance of fluorescent proteins and other fluorophores as biomarkers and biosensors to thousands of studies in the biological, bioengineering, and medical fields, the discovery of new peaks of fluorescence can improve and broaden the application of many techniques involving this property.

4.3 EGFP ACTIVITY AND STRUCTURE AT DIFFERENT PH

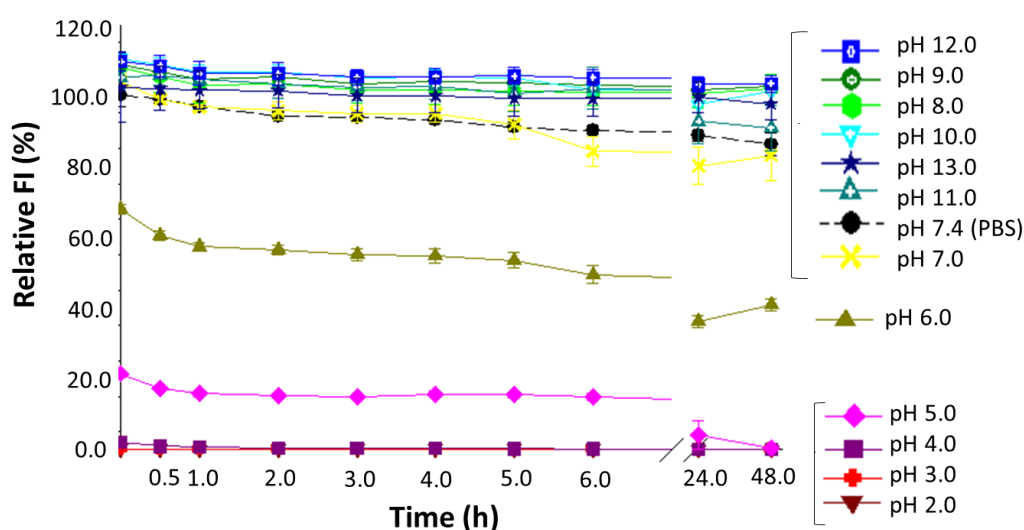
Section adapted from the published article at *International Journal of Biological Macromolecules*, v. 164, p. 3474-3484, 2020. (DOS SANTOS *et al.*, 2020d)

In the present section, the fluorescence and structure of EGFP at different pH were evaluated using 3D fluorescence, CD, IFP, well-plate SAXS, and SEC-SAXS. All techniques were applied to elucidate the effects and processes associated with EGFP structure and activity at different pH. This in-depth study of EGFP stability in varying pH, and the evaluation of the relationship between EGFP fluorescence and pH, provides important information for the development of acidic-to-basic pH-biosensors for medical applications.

4.3.1 Fluorescence activity of EGFP in the function of pH and time

The first set of assays evaluated the fluorescence activity from pH 2.0 to 13.0 over 48 h for the highest intensity peak of EGFP (F1, λ_{ex} 488 nm, λ_{em} 510 nm). **Table C-1** from **Appendix C** presents the real pH and conductivity measurements of the samples from **Figure 14**. As shown in **Figure 14**, the fluorescence of EGFP was maintained in aqueous solutions with pH between 7.0 and 13.0. When the pH was 6.0 and below, the fluorescence was significantly reduced. At pH 6.0, EGFP lost around half of its relative FI when compared to EGFP at pH 7.4. At pH 5.0, there was a decrease of more than 80 % of EGFP fluorescence, and at pH 4.0 and below, fluorescence was fully suppressed.

Figure 14 – Effect of pH in EGFP relative fluorescence intensity (FI) (%) over time (48 h). EGFP ($5.4 \mu\text{g.mL}^{-1}$) in PBS 0 h is the reference for 100%. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation.



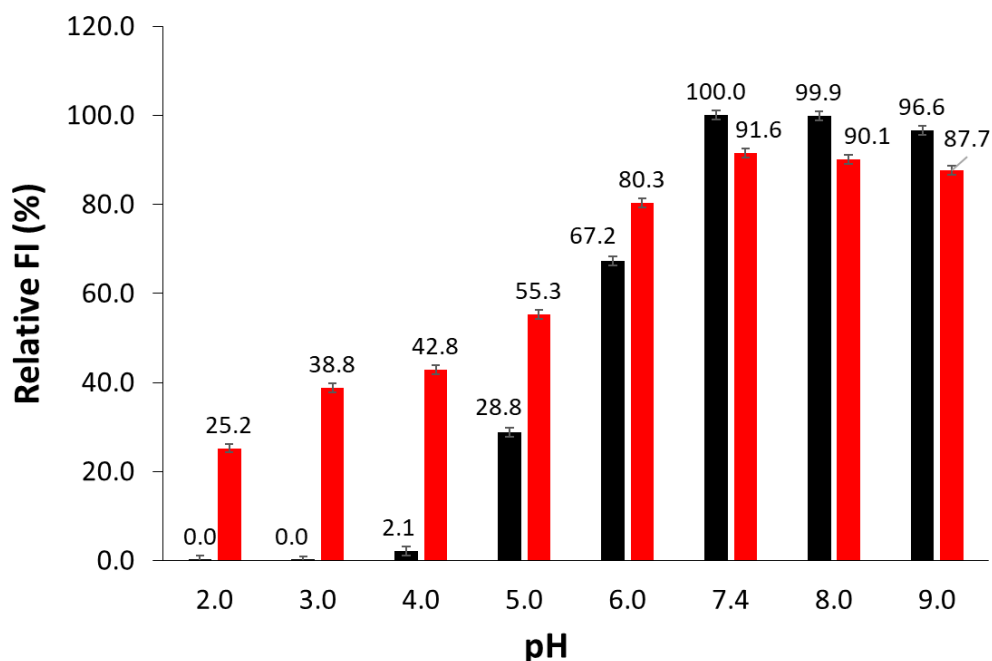
Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

4.3.2 Study of the reversibility of fluorescence activity of EGFP in pH

The second set of experiments aimed to infer if the fluorescence quenching of EGFP in neutral to acidic pH was reversible. For that purpose, the EGFP was exposed to different pH for 30 min, followed by 60 min at pH 7.4. The exposure time of 30 min to the different pH was selected since the fluorescence of EGFP was mainly stabilized after this period (as can be seen in **Figure 14**). Additionally, the goal was to evaluate properties for the development of biosensors with quick responses. The pH 7.4 was selected as the control (reference for neutral pH), pH 8.0 and 9.0 were chosen to evaluate the effect of alkalinity (considering there was almost no difference in fluorescence from pH 8.0 to 13.0). Furthermore, the pH of 2.0, 3.0, 4.0, 5.0, and 6.0 were selected to assess the impact of acidity on the EGFP structure, given the greater sensitivity of the protein to lower pH values. Firstly, EGFP in potassium phosphate buffer (PB) solutions were exposed to the different pH for 30 min and the point fluorescence and 3D fluorescence spectra were acquired. Secondly, the pH of each EGFP solution was adjusted to reach neutrality (pH 7.4), and after 60 min of stabilization, the EGFP fluorescence activity was assessed again.

The reversibility of the pH-induced fluorescence loss for the highest FI point of EGFP (F1) is shown in **Figure 15**, where the black bars represent the relative FI (%) after 30 min at the different pH, and the red bars indicate the intensity after returning to pH 7.4. The relative FI (%) of EGFP decreased around 30 % after 30 min at pH 6.0 (reaching 67.2 ± 0.9 %); however, 60 min after returning to pH 7.4 the relative FI returned to 80.3 ± 0.5 %. In contrast, the fluorescence of EGFP after 30 min at pH 5.0 decreased to 28.8 ± 0.1 %, and only 55.3 ± 0.5 % of relative FI was regained after 60 min at pH 7.4. The fluorescence of EGFP was effectively quenched after 30 min exposure to pH 4.0, 3.0 or 2.0, and the fluorescence was restored up to 42.8% after 60 min returning to pH 7.4 (42.8 ± 0.4 %, 38.8 ± 0.4 % and 25.2 ± 0.3 % of relative FI for pH 4.0, 3.0 and 2.0, respectively).

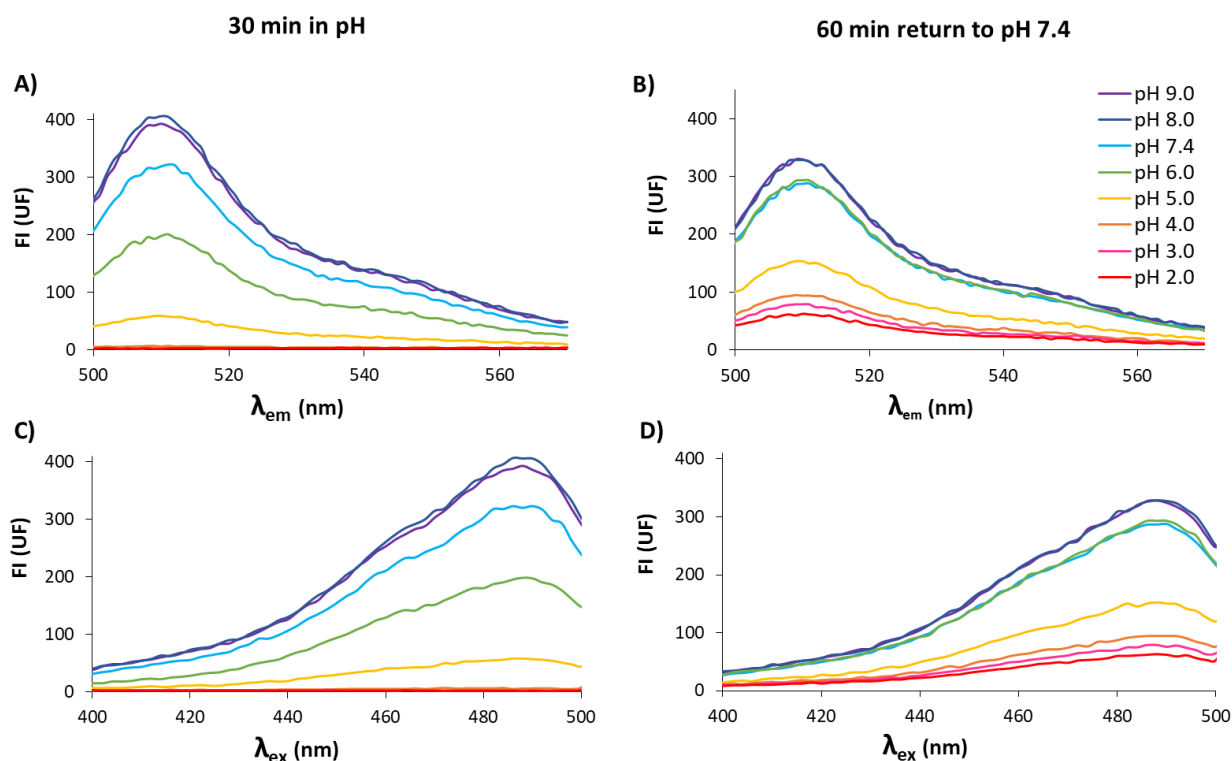
Figure 15 – Effect of pH in EGFP relative fluorescence intensity (FI) (%) after 30 min exposure to different pH (black bars ■), followed by return to pH 7.4 for 60 min (red bars ■). EGFP ($5.4 \mu\text{g.mL}^{-1}$) in PBS 0 h is the reference for 100%. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

Considering wtGFP presents a pH-dependent fluorescence for its protonated and deprotonated states, [2] the 3D spectra of EGFP was also evaluated to verify if this phenomenon was also present for the enhanced variant. The 3D spectra can simultaneously assess excitation and emission curves for the protein and can indicate if blue or redshifts are occurring. The 2D spectra (emission and excitation) are shown in **Figure 16**, and the complete 3D spectra in **Figures F-1** and **F-2**, which overall provide a more complete representation of the effects of pH on EGFP fluorescence.

Figure 16 – Emission spectra at λ_{ex} 488 nm **A)** after 30 min of EGFP ($5.4 \mu\text{g.mL}^{-1}$) in different pH and **B)** after 60 min return to pH 7.4. Excitation spectra at λ_{ex} 510 nm **C)** after 30 min of EGFP ($5.4 \mu\text{g.mL}^{-1}$) in different pH and **D)** after 60 min return to pH 7.4. Data presented in fluorescence intensity (FI) in unities of fluorescence (UF).



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

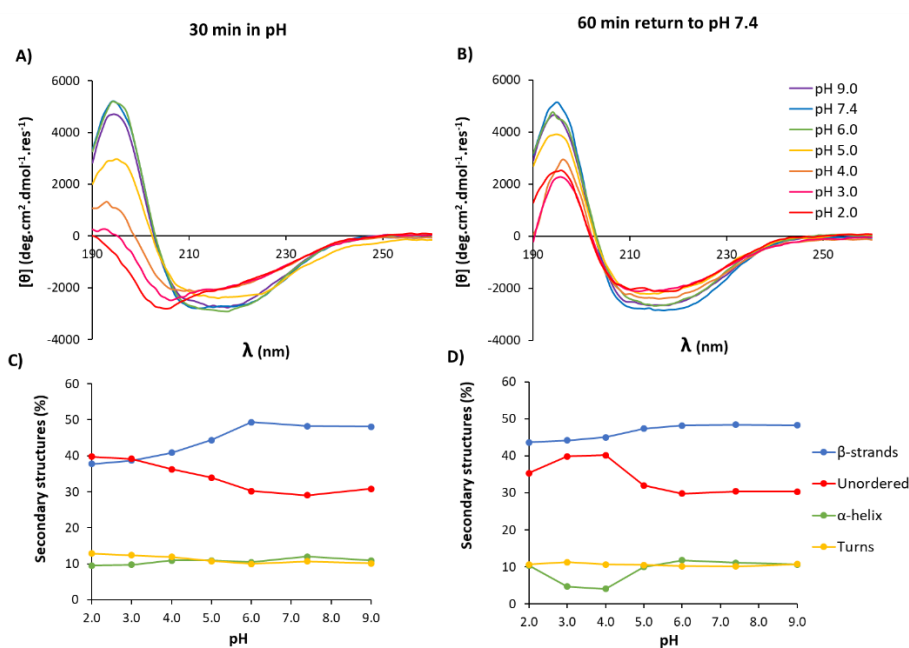
The results presented in **Figure 16**, **Figures F-1** and **F-2** are consistent with those shown in **Figure 15**, with fluorescence quenching after 30 min of exposure to pH below 7.4. This again was followed by a small return of fluorescence for pH 2.0 to 5.0, and an almost complete return of fluorescence for pH 6.0 after the pH was adjusted to neutrality for 60 min. The maximum FI of EGFP was achieved at pH 8.0 for F1, as shown in **Figure 16.A**, **Figure 16.C**, and **Figure F-1**. No changes or shifts in the shape of the spectra, or the maximum excitation and emission wavelengths were observed at different pH, though the FI varied as previously shown in **Figure 14** and **Figure 15**. Relative to EGFP at pH 7.4, **Figure 16.A** and **Figure 16.C** show there was a slight increase of fluorescence at pH 8.0 and 9.0, while a fluorescence quenching was observed at pH 6.0 and below (with greater fluorescence decrease for lower pH values). After the return to pH 7.4, as shown in **Figure 16.B** and **Figure 16.D**, the samples previously kept at pH 6.0 had their fluorescence restored, while the ones at pH 5.0 presented only a partial recovery of fluorescence. On the other hand, the samples kept at $\text{pH} \leq 4.0$ had a very low return of FI, as proven by both the EGFP emission and excitation spectra.

4.3.3 Structural alterations of EGFP in the function of pH

To obtain further insights behind the EGFP fluorescence reversibility with pH, the effect of pH on the secondary and tertiary structure of EGFP was analyzed, using CD, IFP, well-plate SAXS, and SEC-SAXS. As in the previous experiments, EGFP in PB solutions were exposed to different pH for 30 min and CD, IFP, well-plate SAXS, and SEC-SAXS were evaluated. Then, the pH of each EGFP solution was adjusted to reach neutrality (pH 7.4), and after 60 min, EGFP was again assessed with the aforementioned techniques.

In CD spectroscopy, α -helix presents negative bands at 222 nm and 208 nm, and a positive band at 193 nm, while β -helices have a negative band at 218 nm and a positive band at 195 nm (GREENFIELD, 2006). EGFP secondary structure at neutral pH is already reported in the literature, (ARPINO *et al.*, 2012) being comprised of 47 % of β -strands (11 strands forming a β -barrel core, with the chromophore located in its center), 13 % of helical forms (3_{10} and α conformations, with the core helix containing the chromophore) and 40 % of coils (mostly from loops located at the two ends of the β -barrel structure). Considering the applications of CD spectroscopy, this technique was used to assess the effects of pH on the EGFP secondary structure and results are presented in **Figure 17**.

Figure 17 – Mean residue ellipticity ($[\theta]$) from the CD analysis of EGFP (0.3 mg.mL⁻¹, 1 mm cell) in PB of different pH after **A)** 30 min exposure to different pH and **B)** 30 min exposure to different pH + 60 min return to pH 7.4. Percentage (%) of secondary structures of EGFP (CD analysis in Dichroweb CONTIN-LL, set SP175) after **C)** 30 min exposure to different pH and **D)** 30 min exposure to different pH + 60 min return to pH 7.4. Samples were prepared in triplicate with a blank and presented as the average of the results.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

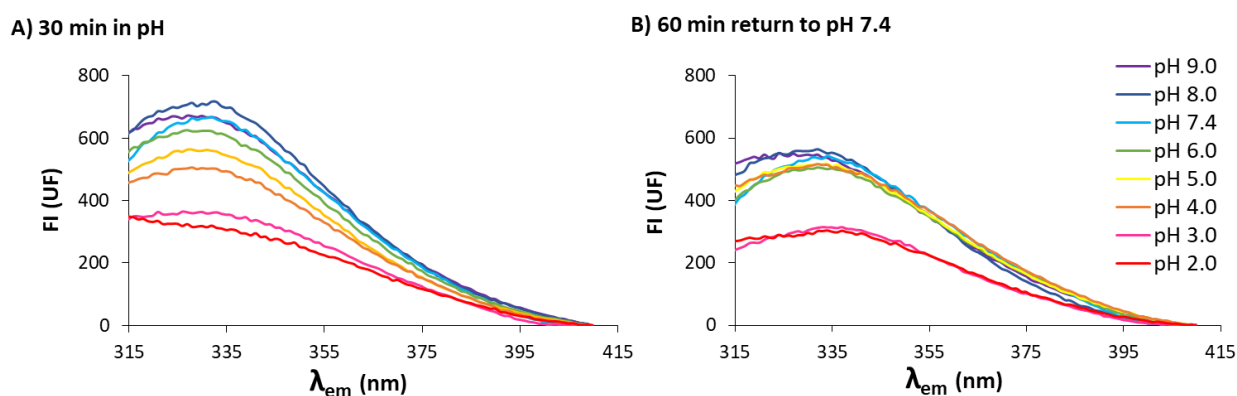
As can be seen in **Figure 17.A**, from pH 5.0 to 9.0, there is a negative ellipticity observed from around wavelength 205 to 240 nm (a negative band for α -helix at 222 nm and 208 nm, a negative band for β -strands at 218 nm) and positive ellipticity from 190 to 205 nm (a positive band for α -helix at 193 nm and a positive band for β -strands at 195 nm). The CD spectra in **Figure 17.A** for pH of 5.0 to 9.0 matches other CD studies for EGFP (POSTUPALENKO *et al.*, 2014; ZHANG *et al.*, 2017). However, at pH 5.0, there was a decrease in both positive and negative ellipticities, suggesting that the protein suffered secondary structural changes in this condition. From pH 2.0 to 4.0, there are further alterations, not only in the intensity but in the shape of the spectra, consistent with modifications to the secondary structure of EGFP. The CD spectra of the samples after returning to the neutral pH of 7.4 for 60 min are shown in **Figure 17.B**. The EGFP which was exposed to pH 5.0 partially recovered its ellipticities, and similarly, EGFP exposed to pH 2.0, 3.0, or 4.0 had their positive and negative ellipticities and intensities partially restored. However, the samples exposed to these lower pH values had lower intensities relative to the EGFP maintained at pH 7.4, with EGFP exposed to pH 5.0 being closest in intensity and shape to EGFP in pH 7.4.

In **Figure 17.C** and **Figure 17.D**, and **Table G-1** from **Appendix G**, the results for the CD analysis with Dichroweb CONTIN-LL (set SP175) are presented. As can be seen in **Figure 17.C** and **Table G-1**, the results for EGFP at pH 7.4 match very closely with the previous reports for the crystalline structure of EGFP neutral environments, (ARPINO *et al.*, 2012) as confirmed by the 1 % variation for the calculated β -strands and helical forms, respectively 48 % and 12% for this study, versus 47 % and 13% for EGFP crystalline structure described in the literature, (ARPINO *et al.*, 2012) confirming the accuracy of our CD data analysis. This set was then selected for subsequent studies of the effect of pH on EGFP secondary structure and recovery after pH neutralization. In **Figure 17.C**, it is possible to see there are no modifications to the secondary structure of EGFP from pH 6.0 to 9.0. On the other hand, from pH 5.0 to 2.0, there is an incremental decrease in β -strands and an increase in unordered structures of EGFP. Although the modifications on overall helical forms are small (**Figure 17.C**), **Table G-1** shows that with the decrease of pH, the percentage of distorted α -helix is higher than at neutral and alkaline pH. From **Figure 17.D**, with the return of pH to neutrality, it is possible to observe that EGFP almost, but not completely, recovers its secondary forms after being exposed to pH 5.0, while only a partial recovery occurs for pH values ≤ 4.0 . At acidic pH values (≤ 4.0), EGFP still has a higher proportion of unordered forms and distorted α -helix, with only a partial

recovery of β -strands. The CD data helps understand why less than 50 % of EGFP FI returns after the pH adjustment to neutrality.

The IFP was also evaluated for information on the effect of pH on EGFP, as presented in **Figure 18**. The IFP provides information about the conformation and stability of proteins because the fluorescence of certain amino acids (namely Trp, Tyr, and Phe) are strongly influenced by their surrounding environment and position in the protein structure (EFTINK, 2002). The emission of the Tyr residues can be affected by proton loss, rotational motions in the protein, and the presence of quenching groups close to the protein. Hence, these alterations in fluorescence can provide information about structural changes in the protein surface (EFTINK, 2002).

Figure 18 – Emission spectra of fluorescence of tyrosine and tryptophan of EGFP (5.4 $\mu\text{g.mL}^{-1}$ of EGFP) at the excitation wavelength of 280 nm after **A)** 30 min exposure to different pH and **B)** 30 min exposure to different pH + 60 min return to pH 7.4. Data presented as fluorescence intensity (FI) in unities of fluorescence (UF).



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

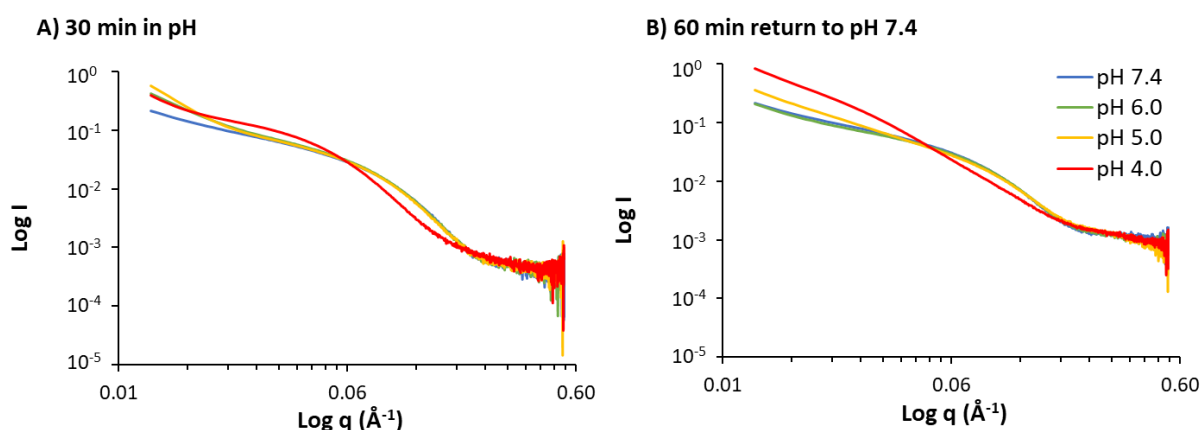
As can be seen in **Figure 18.A**, in general, the shape of the spectra and the maximum peaks for the IFP of EGFP after 30 min at different pH were not changed. The exception was the EGFP exposed to a pH of 2.0, where the shape of the emission spectra was altered. The FI of the IFP changed according to the pH, following the trend previously observed in the fluorescence and CD studies, with a decrease of intensity with decreasing pH. This effect was expected considering a change in the surrounding environment can alter the IFP because it modifies the exposure of the fluorescent amino acids. However, on returning all samples to pH 7.4 in **Figure 18.B**, the intensity of the IFP should return if the structure of the protein goes back to its usual form. This return is observed for pH 4.0 to 9.0, but not for pH 2.0 and 3.0. This suggests the alterations for pH 2.0 and 3.0 were too severe, and even with the neutralization of pH, the arrangement of the protein and the fluorescent amino acid residues did not return to the same position as in the properly folded EGFP. The results suggest there is partial maintenance

of the secondary structure of EGFP, which allows at least an incomplete recovery of amino acid positioning, even after exposure to very acidic pH (pH 3.0 and 2.0). Additionally, although there is only a partial return of activity for EGFP after exposure to pH 4.0, there is enough structural restoration to recover amino acid positioning. However, as observed in CD, there are permanent alterations in secondary structure elements, which in turn, reduce the capacity of the chromophore to emit fluorescence.

To evaluate the effect of pH on the EGFP tertiary structure, two SAXS techniques were applied: well-plate SAXS and SEC-SAXS. SAXS can provide data regarding the structure and structural transition of biomolecules in solution, but for accurate determination, monodisperse and dilute samples are required (RYAN *et al.*, 2018). In the SEC-SAXS technique, the use of chromatography to separate different species from polydisperse solutions allows the evaluation of complex biological samples, for example, proteins in different solvents or solutions with distinct pH (RYAN *et al.*, 2018). However, although SEC-SAXS can provide better resolution and more accurate information on EGFP as a monomer, this method is not appropriate for samples with large aggregates, as those with pH close to the pI, or denatured proteins. Hence, to obtain broader information on the behavior of EGFP after 30 min at different pH, both methods were used. Additionally, EGFP after 60 min return to pH 7.4 was evaluated on the well-plate SAXS.

The results for the well-plate SAXS are shown in **Figure 19** below.

Figure 19 – Well-plate SAXS of EGFP (2.5 mg.mL⁻¹) after **A)** 30 min exposure to different pH and **B)** 30 min exposure to different pH + 60 min return to pH 7.4. Results are presented in the log of intensity (Log I) and log of q (Å⁻¹).



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

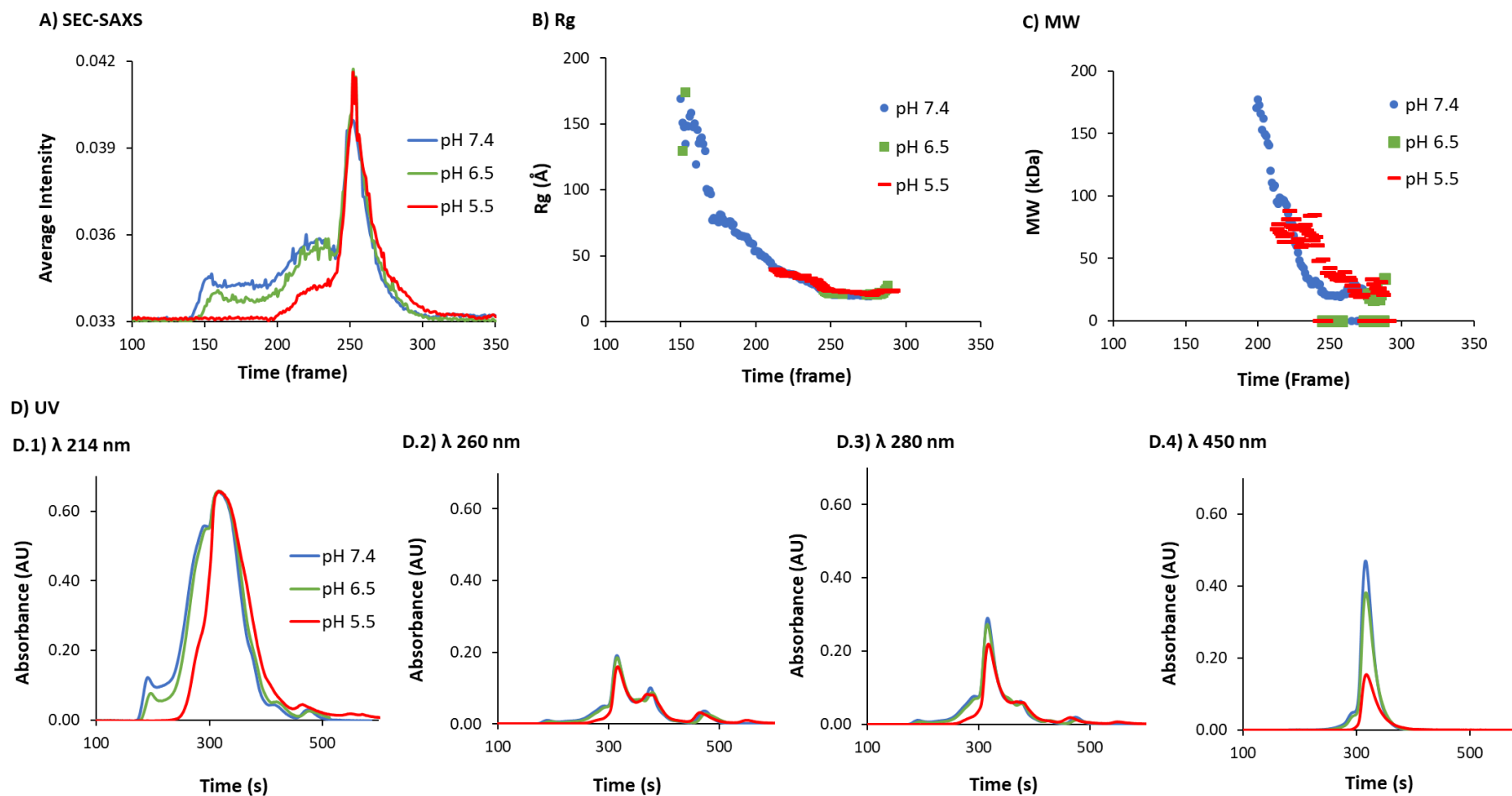
Since q is inversely proportional to real space, scattering at lower q is associated with larger specimens in the samples, while higher q is related to a scattering of smaller species. The

inclination at low q (below $\log q$ 0.06 $\text{\AA}^{-1}$) in **Figure 19** for all conditions indicates the presence of aggregates for all the samples in the well-plate SAXS. Considering EGFP is a weak dimer prone to aggregation (KRASOWSKA *et al.*, 2010; MYATT *et al.*, 2017) and the concentration is high (2.5 mg.mL^{-1}), the presence of species with different sizes was expected and was the reason why SEC-SAXS was also evaluated for clearer data on the structure and distribution of the different formations where possible. Because of the heterogeneity of the species in **Figure 19**, it was not possible to fit the scattering to models, but the behavior of EGFP at different pH could still be assessed.

In **Figure 19.A**, it can be seen that the EGFP at pH 5.0, 6.0, and 7.4 had effectively identical scattering, except for variation in the larger aggregates at low q , where it appears that the proportion of aggregates present increased with decreasing pH. This indicates that, at pH 5.0 and 6.0, the overall structure of EGFP is maintained, but the formation of associations and aggregates is favored. For EGFP at pH 6.0, the aggregation could be due to the proximity to the pI of the protein (6.2) (GURUNATHAN *et al.*, 2014), and not necessarily because of structural alterations. For EGFP at pH 4.0, there is not only the formation of aggregates at lower q , but the shape of the scattering at higher q was also altered, confirming structural changes of EGFP. After the return to pH 7.4 (**Figure 19.B**), there was a decrease in larger formations for both pH 5.0 and 6.0, with the latter presenting a scattering almost identical to EGFP at pH 7.4. However, for EGFP at pH 4.0, the return to pH 7.4 led to greater differences between this sample and the EGFP maintained at pH 7.4. This suggests that, at pH 5.0 and 6.0, EGFP mostly retains its tertiary structure with aggregation more favorable than at pH 7.4, while at pH 4.0, there were extensive structural modifications in the EGFP structure. This is in accordance with the previous fluorescence and CD studies and can explain why EGFP can fully recover its fluorescence activity after exposure to pH 6.0, while there is only a partial recovery for pH 5.0 and even lower for pH 4.0.

The SEC-SAXS were acquired continuously on all fractions through the HPLC as a function of time, and results are presented in **Figure 20** and **Figure 21**. For these experiments, EGFP at pH 7.4 was chosen as the neutral control, and pH 6.5 was selected instead of pH 6.0 to avoid the pI of EGFP (pH 6.2), (GURUNATHAN *et al.*, 2014) because proteins precipitate at the pI and could clog the HPLC column. The pH 5.5 was selected instead of 5.0 and 4.0 because EGFP starts precipitating at these lower pH values. The average intensity of each SAXS pattern over time is provided in **Figure 20**.

Figure 20 – SEC-SAXS results of all EGFP (5 mg.mL⁻¹) fractions from HPLC in function of time after 30 min at different pH. **A)** SEC-SAXS average intensity. **B)** Radius of gyration (Rg). **C)** Molecular weight (MW). **D)** UV from HPLC for SEC-SAXS at wavelengths (λ) **D.1)** 214 nm, **D.2)** 260 nm, **D.3)** 280 nm and **D.4)** 450 nm; results are presented as Absorbance (Absorbance unities, AU). Time was adjusted so the SEC-SAXS highest intensity peaks for EGFP at different pH matched, and the offset was applied for the other results.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

As was previously stated, EGFP is a weak dimer, (MYATT *et al.*, 2017) and in addition to its monomeric form, it can appear as dimers or other associations at higher concentrations. The highest average intensity of the SAXS patterns around frame 250 in **Figure 20.A**, represents the monomeric EGFP, the peak between frames 200 and 250 is from the EGFP dimer, and the plateau between frames 150 and 200 is attributed to larger associations or aggregates. The scattering in **Figure 20.A** for pH 7.4 and 6.5 is very similar, with a slight decrease in dimers for pH 6.5. However, there is a large decrease in the proportion of dimers and aggregates for pH 5.5, and there is also an alteration to the shape of the monomeric EGFP. Although usually higher concentrations of aggregates are associated with lower protein stability, (CHI *et al.*, 2003) considering the alterations to EGFP monomer, this could represent intense stress at pH 5.5, which dissociated aggregates and also altered the structure of the protein.

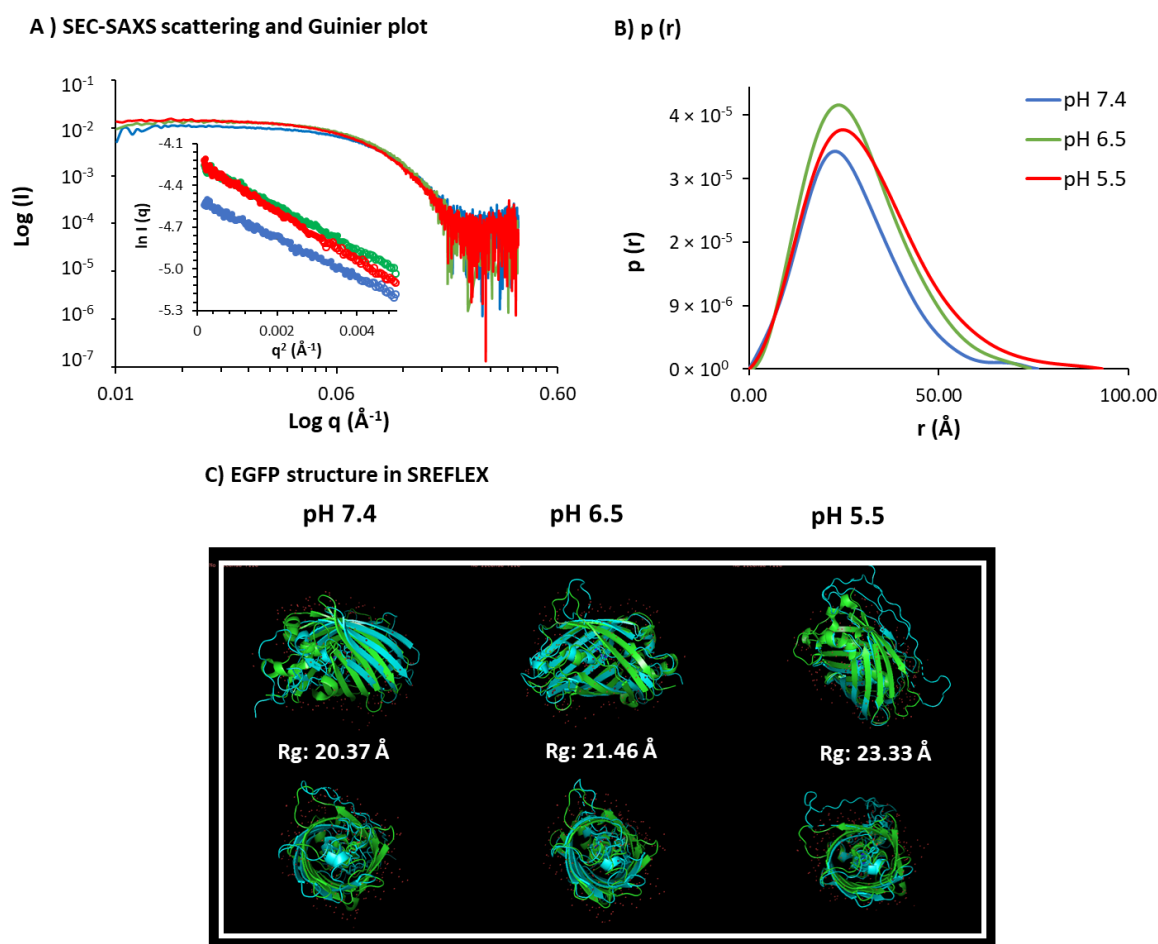
The R_g and MW were obtained for each SAXS pattern and are provided in **Figure 20.B** and **Figure 20.C**, respectively, for the EGFP sample after going through the HPLC column. The x-axis is the same for **Figure 20.A**, **Figure 20.B**, and **Figure 20.C**, to allow comparison. The broad range of R_g and MW values for EGFP at pH 7.4 indicates heterogeneity in species sizes in the sample, with the larger species eluting first. Although the EGFP at pH 6.5 generally matches EGFP at pH 7.4 for frames between 250 and 300, sections are missing for this pH. The pH of 6.5 was chosen in an attempt to minimize precipitation near the pI of EGFP of 6.2 (GURUNATHAN *et al.*, 2014). However, this data suggests the difference of 0.3 in pH was not enough to avoid the precipitation. Therefore, the missing sections for R_g and MW for EGFP at pH 6.5 are attributed to aggregation due to pI proximity. For EGFP at pH 5.5, the R_g and MW do not match those seen for EGFP at pH 7.5. There is a narrower dispersion of R_g values than at 6.5, indicating fewer aggregates are present. The MW of EGFP is 26.9 kDa (ARPINO *et al.*, 2012), which is consistent with the plateau around frames 250 and 200 for EGFP monomer at pH 7.4 and 6.5, but there is no clear plateau in MW for EGFP at pH 5.5.

The HPLC allows the monitoring of the UV absorbance (A) of the samples simultaneously to the SEC-SAXS acquisition. Similar to the IFP, amino acids also have absorbance spectra and these can provide insight about alterations to the protein structure or its environment (MELO *et al.*, 1997; PACE *et al.*, 1995). The absorbances at λ 214 nm, 260 nm, 280 nm, and 450 nm are shown in **Figure 20.D**. The λ of 214 nm is associated with Phe absorbance, while 260 nm and 280 nm are related to Tyr and Trp absorbances. The λ of 450 nm was chosen to detect EGFP absorbance. As can be seen in **Figure 20.D.1**, **Figure 20.D.2**, and

Figure 20.D.3, pH 7.4 and pH 6.5 exhibited similar absorbance as a function of time, with some reduction in the intensity of pH 6.5 for the 450 nm absorbances (as shown in **Figure 20.D.4**). In contrast, EGFP at pH 5.5 had an absence of the first two peaks at 214 nm, and the absence of the first peak for 260 nm, 280 nm, and 450 nm (**Figure 20.D**). These alterations indicate changes to the chromophore (due to the 450 nm spectra) and for amino acid environment/positioning (due to changes of lower wavelengths spectra) at pH 5.5, like the previous results observed in this study.

The SAXS scattering, structure of EGFP, the pair distance distribution function $[p(r)]$, and R_g for the monomeric fraction of EGFP at different pH after SEC-SAXS are presented in **Figure 21**.

Figure 21 – Analysis of the monomeric fraction of EGFP (5 mg.mL⁻¹) from SEC-SAXS. **A)** SEC-SAXS and Guinier plot of EGFP after 30 min exposure to different pH. Results are presented in the log of intensity (Log I) and log of q ($\text{\AA}^{-1}$) for SAXS and the natural log of the intensity of q [$\ln I(q)$] and q^2 ($\text{\AA}^{-1}$) for the Guinier plot. **B)** $csp(r)$ of EGFP after 30 min at different pH. **C)** Radius of gyration (R_g) and images of monomeric EGFP structure after 30 min at different pH. Structures generated in SREFLEX using SEC-SAXS scattering and fittings to the EGFP PDB ID 2Y0G file. The top images are the lateral view of the EGFP structure and the bottom images are the overview. EGFP PDB ID 2y0G is shown in Green and refined fit to data shown in Cyan.



The SAXS scattering for the monomeric EGFP fraction of samples exposed for 30 min to pH 7.4, 6.5, and 5.5 are shown in **Figure 21.A**. The corresponding $p(r)$ are shown in **Figure 21.B**, which shows similar size distributions for EGFP at pH 7.4 and 6.5. However, on reducing the pH to 5.5, the distribution is skewed to larger sizes. The SAXS patterns were fitted to EGFP using PDB ID 2Y0G (ROYANT; NOIRCLERC-SAVOYE, 2011), and refined using SREFLEX (PANJKOVICH; SVERGUN, 2016). Snapshots of these structures are provided in

.C, comparing the refined structures (cyan) to the 2Y0g PDB structure (green). The EGFP structure at pH 7.4 overall matched well to the PDB file and was similar to the PDB file at pH 6.5. However, at pH 5.5, strands around the β -barrel are loosened and the barrel appears to be slightly “squashed”. Additionally, the R_g for the EGFP at pH 7.4 (20.37 Å) (MYATT *et al.*, 2017) is very similar to reports for the monomeric variation of EGFP (m-EGFP, R_g of 20.29 ± 1.00 Å), but there is an increase on the R_g at pH 6.5 (21.46 Å) and pH 5.5 (23.32 Å). This suggests there were modifications to EGFP tertiary structure after 30 min at pH 5.5, which can be responsible for the fluorescence activity decrease (in comparison with pH 7.4).

4.3.4 Impacts of pH on the activity and structure of EGFP

Allying the results from the fluorescence activity with the structural studies, it is possible to infer that EGFP is highly stable towards alkaline pH (7.4 to 13.0), lightly quenched from neutral to weakly acidic pH (8.0 to 5.0, with pK_a 5.69), and heavily quenched below pH 5.0 (5.0 to 2.0, **Figure 14**). For pH 6.0, the fluorescence was almost completely recovered with the return of pH to neutral, but only partially recovered for pH 5.0 to 2.0 (**Figure 15** and **Figure 16**). As confirmed by CD (**Figure 17**) and SEC-SAXS (**Figure 21**), this occurred because there was no modification in the secondary and tertiary structure of EGFP at pH 6.0, hence, this effect was likely due to changes caused by a reversible protonation. However, there was a structural modification, even at the secondary structure level, from pH 5.0 to 2.0 (as can be seen by CD in **Figure 17** for pH 5.0 and below, in IFP in **Figure 18** for EGFP at pH 3.0 and 2.0, and pH 5.5 and lower in well-plate SAXS and SEC-SAXS (**Figure 19****Figure 19** below).

Figure 19, **Figure 20** and **Figure 21**. This can explain why the fluorescence dequenching was only partial below pH 6.0.

This observation is consistent with what has previously been observed for wtGFP, which has two protonated states and reversible fluorescence quenching from pH 5.5 to 12.0 due to an equilibrium between protonated and deprotonated states. (WARD *et al.*, 1982) The results

indicate EGFP presents the same behavior from pH 6.0 to 13.0, with state changes due to protonation causing reversible fluorescence quenching.

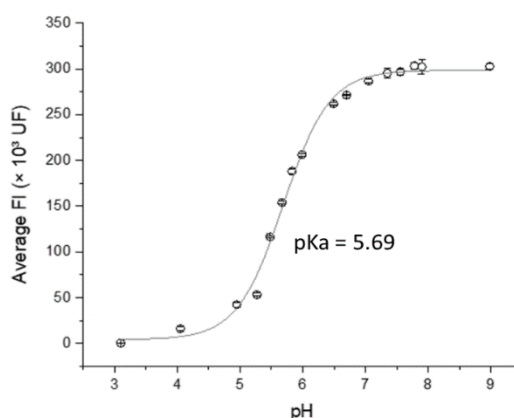
The irreversible fluorescence loss of another GFP variant (sfGFP) was previously studied using Nuclear Magnetic Resonance (NMR) by Andrews *et al.* (ANDREWS *et al.*, 2009). The study presented experimental evidence that sfGFP chromophore flexibility is associated with the mispacking of intermediate states, where sfGFP folding under certain conditions can lead to a loosely packed or incorrect barrel. This effect can decrease the rigidity of the structure necessary for fluorescence, (ANDREWS *et al.*, 2009) supporting why a fluorescent protein can recover its overall structure but still present irreversible fluorescence loss.

With this study, it was possible to update the knowledge regarding the fluorescence activity and structure of EGFP at different pH considering its two states, confirming EGFP is more similar to wtGFP than originally reported.

4.3.5 EGFP as analytical neutral to acidic pH-biosensors

Envisaging a further development of pH-biosensors, the relationship between EGFP fluorescence and pH was determined, assessing the viability of using EGFP as an analytical tool. For that purpose, EGFP FI in the pH range from 3 to 9 was further evaluated. **Figure 22** shows the fitting of the sigmoidal curve to a growth/sigmoidal dose-response model. (BOMATI *et al.*, 2014) From this sigmoidal curve, it was possible to determine the pKa of 5.69 for EGFP, identical to the pKa of 5.7 previously found by Bomati *et al.* (BOMATI *et al.*, 2014) for the same GFP variant.

Figure 22 – Average Fluorescence Intensity [FI, $\times 10^3$ unities of fluorescence (UF)] of EGFP ($5.4 \mu\text{g.mL}^{-1}$) over pH. FI was acquired at EGFP peak (F1, λ_{ex} 488 nm, λ_{em} 510 nm) in triplicate with a blank. Results were fitted to a growth/sigmoidal dose-response model to calculate pKa.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

The fit of the sigmoidal curve for EGFP had an $R^2 > 0.99$, and small variations of pH produced discernable variations in the FI, particularly in the range of 5-8. Considering some relevant diseases like tumors and joint diseases can present a decrease in pH around the afflicted areas (with the average pH for tumors around 7.0, and around 7.4 for healthy tissues) (TANNOCK; ROTIN, 1989; TREUHAF; MCCARTY, 1971; WIKE-HOOLEY *et al.*, 1984), there is a strong potential for application of EGFP in the development of biosensors for these conditions.

This study provides an update on previous knowledge regarding EGFP behavior at different pH and is in accord with recent studies on the crystalline structure and fluorescence properties of EGFP. This further confirms the similarities between EGFP and wtGFP, with both having protonated and deprotonated states which cause reversible fluorescence quenching. This knowledge, allied with the strong dependence of EGFP fluorescence on pH also confirms that this protein presents the required properties for the development of novel biocompatible and environmentally friendly neutral-to-acidic pH-biosensors.

4.4 EFFECT OF IONIC LIQUIDS ON EGFP ACTIVITY

Section adapted from two original manuscripts in preparation. (DOS SANTOS et al., 2020b, 2020c)

This section aimed to evaluate the effect of imidazolium and cholinium-based ILs (and salts) on the fluorescence activity of EGFP to gain insights into the interactions between ILs and proteins. The main goal was to find ILs solutions with high potential to maintain EGFP fluorescence activity at room temperature and even protect the EGFP structure under stress conditions.

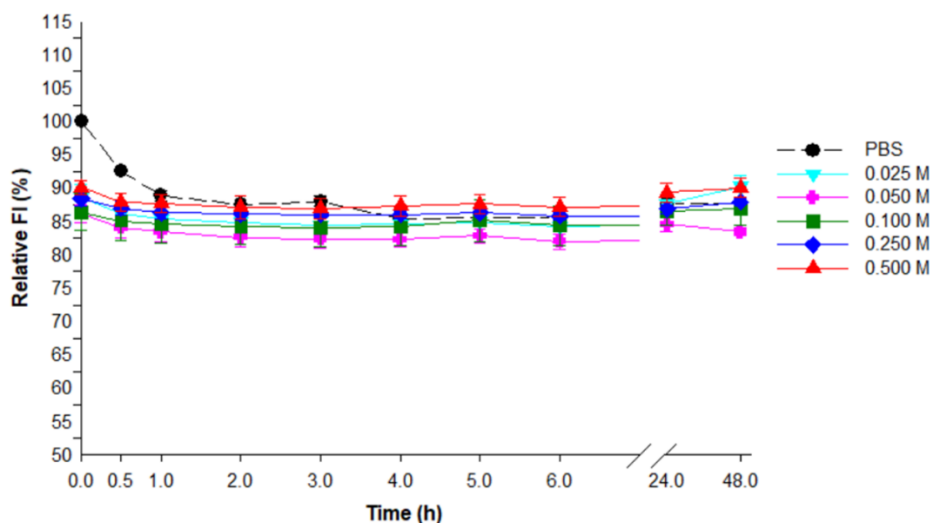
The pH of all buffers, salts, and ILs aqueous solutions was kept at 7.4 ± 0.1 during all experiments to avoid the strong impact of pH on EGFP fluorescence (as demonstrated in the section “**4.3 EGFP activity and structure at different pH**”). The pH and conductivity values of the aqueous solutions with ILs and denaturing agents are detailed in **Table H.1** and **Table H.2** from the **Appendices**, respectively. In this section, all data is presented as Relative FI (%) considering EGFP in PBS (at pH 7.4) at 0 h and 25 °C as the reference for 100 %. The fluorescence intensity values in the first hour of monitoring were not considered for the discussion, as they correspond to the initial conditioning period of proteins to their new environment (*i.e.*, conditioning time). During the conditioning time, different peaks and declines in FI are observed, which do not necessarily represent an increase or decrease in protein stability.

4.4.1 Short-term effect of aqueous solutions of ILs on EGFP fluorescence

The first set of experiments was a 48-h screening to evaluate the effect of different aqueous ILs (and salts) solutions on EGFP fluorescent activity at 25 °C, namely, studying the influence of time and concentration to select the conditions with the highest potential for the long-term and stress studies.

Before the screening using ILs solutions, the influence of ionic strength on EGFP relative FI was determined by evaluating if low concentrations of a conventional salt (namely, NaCl) could change EGFP fluorescence activity. Hence, EGFP FI was monitored for 48 h (at 0, 0.5, 1, 2, 3, 6, 24, and 48 h) in NaCl aqueous solution (at 0.025, 0.050, 0.100, 0.250, and 0.500 mol.L⁻¹), as shown in **Figure 23**.

Figure 23 – Effect of ionic strength (NaCl concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol.L⁻¹ and PBS as control) on EGFP Relative FI (%) over time (48 h). EGFP in PBS at 0 h is the reference for 100 %. Results were evaluated in triplicate with a blank and presented as the average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.



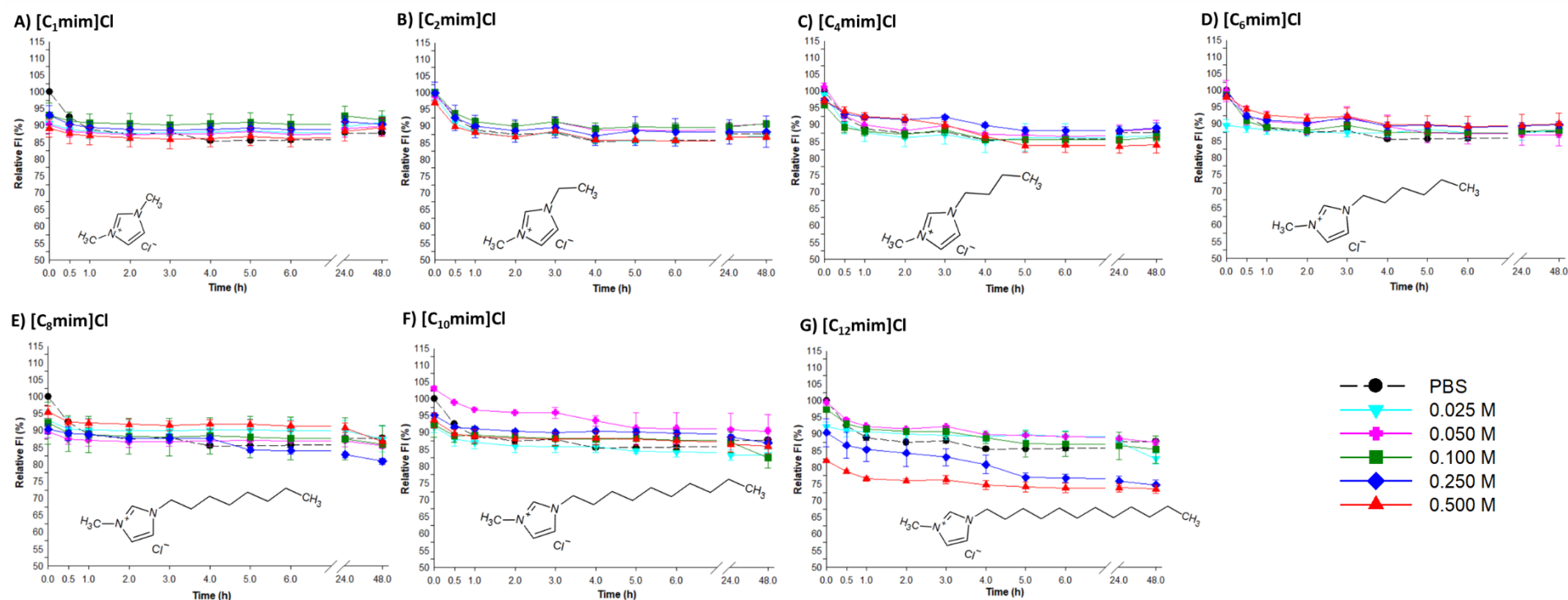
Source: Produced by the author.

As can be seen in **Figure 23**, independent of the time of exposure, all aqueous solutions of NaCl (from 0.025 to 0.500 mol.L⁻¹) had little to no impact on EGFP Relative FI (%) when compared to EGFP fluorescence in PBS at pH 7.4. These salt concentrations were then selected for the experiments using ILs solutions.

A subsequent screening using aqueous solutions of ILs (and salts) from the imidazolium ([C_nmim]Cl) and cholinium families ([Ch]X) on EGFP fluorescence activity was carried out, evaluating the effect of increasing the alkyl chain length of the cation and the anion, respectively.

The results in **Figure 24** show that, similarly to NaCl aqueous solutions and independent of the concentration and time, almost all [C_nmim]Cl had little impact on EGFP relative FI when compared with PBS. The exception was [C₁₂mim]Cl at concentrations of 0.250 and 0.500 mol.L⁻¹, which caused a considerable quenching on EGFP fluorescence. In this particular case, the quenching effect could be due to the surfactant nature of [C_nmim]Cl with longer alkyl chain length, (HEZAVE *et al.*, 2013) but additional experiments are required to confirm these unfavorable self-assembly interactions.

Figure 24 – Effect of imidazolium ILs (concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol.L⁻¹) on EGFP Relative FI (%) over time (48 h). EGFP in PBS at 0 h is the reference for 100 %. PBS is presented as a control. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.

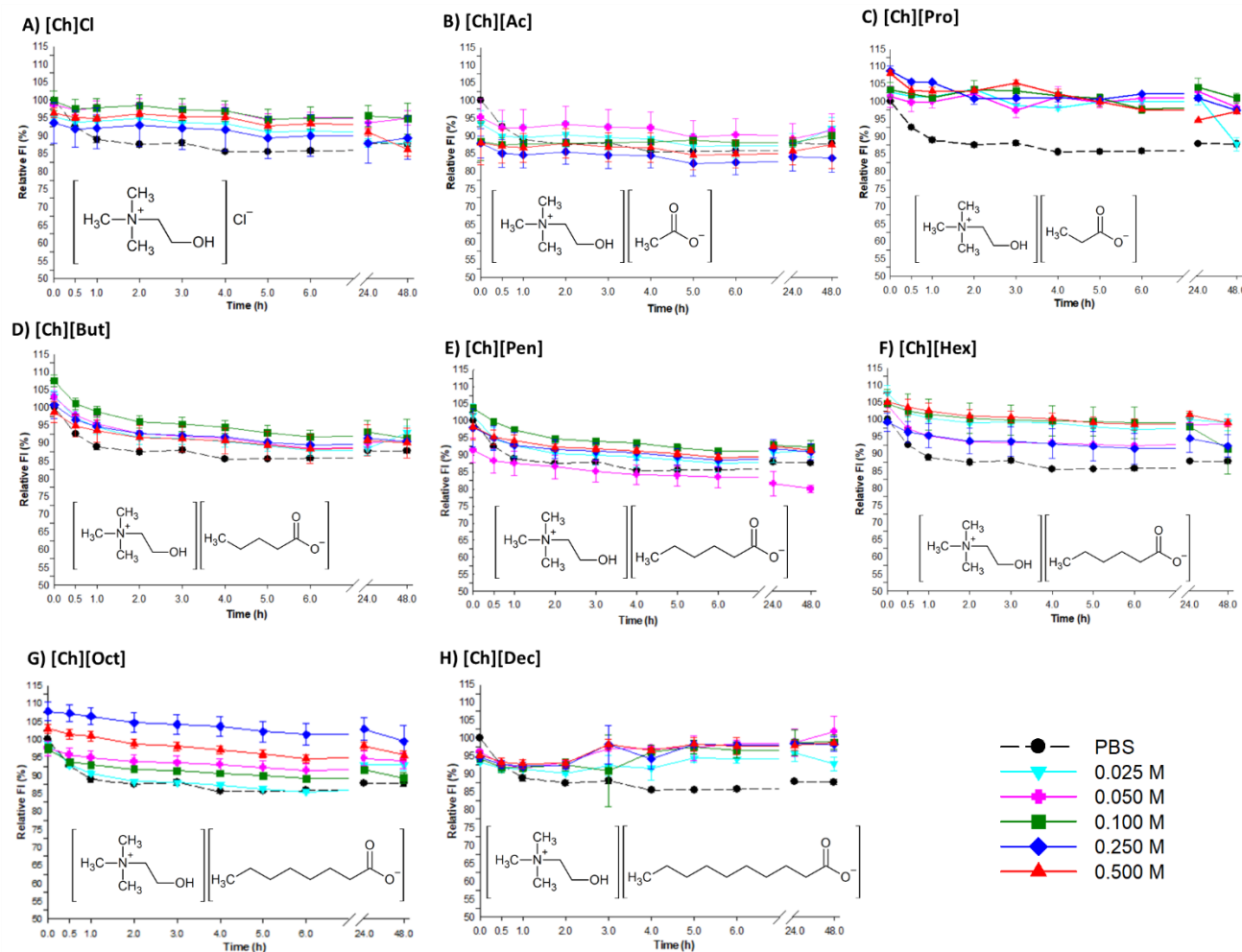


Source: Produced by the author.

As can be seen in **Figure 25**, for most of the conditions under study, the [Ch]X favored EGFP fluorescence. [Ch]Cl solutions at intermediary concentrations of 0.050 and 0.100 mol.L⁻¹ had a small positive effect on EGFP FI, followed by 0.500, 0.025, and 0.250 mol. L⁻¹, which exhibited a more negligible effect on fluorescence activity. Similarly, most of [Ch][Ac] solutions presented negligible impact on EGFP fluorescence (*i.e.*, variations in FI lower than 5 %), except a slight increase in fluorescence observed with the [Ch][Ac] solution at 0.050 mol.L⁻¹ after 2, 3 and 4 h of exposure. [Ch][Pro] increased EGFP relative FI for all concentrations (around 10-15 %) compared to PBS, with little difference among the samples. [Ch][But] and [Ch][Pen] also promoted a small increase in EGFP fluorescence with a concentration of 0.100 mol.L⁻¹, followed by less significant increases (or no increases) for the remaining concentrations. Interestingly, with the increase of the anionic chain length, higher fluorescence activities were obtained, namely, for [Ch][Hex] and [Ch][Oct], which had the highest aptitude to preserve EGFP fluorescence during this 48-h study. For [Ch][Hex], the best concentrations were 0.500 = 0.100 = 0.025 mol.L⁻¹, followed by 0.250 and 0.050 mol.L⁻¹. For [Ch][Oct], the concentration sequence for EGFP relative FI (from highest to lowest) was 0.250 > 0.500 > 0.050 > 0.100 > 0.025 mol.L⁻¹.

Although the [Ch][Dec] solution also presented a positive impact on EGFP fluorescence for all concentrations (with a slightly lower effect for the lowest concentration of 0.025 mol.L⁻¹), this fluorescence enhancement was only observed after 3 h of exposure. This curious phenomenon of an increase of FI after 3 h of incubation with [Ch][Dec] is not fully understood, particularly because the positive effect of [Ch][Dec] on EGFP fluorescence was comparable to the ones for [Ch][Hex] and [Ch][Oct] solution after 24 h. Probably due to the larger anion, the interactions between [Ch][Dec] and EGFP can take longer to stabilize than for the other cholinium ILs. As the [C_nmim]Cl solutions, additional experimental self-assembly characterization and molecular dynamics computational studies are required to fully elucidate the stabilization mechanisms occurring at a molecular level.

Figure 25 – Effect of cholinium ILs and salts (concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol.L⁻¹) on EGFP Relative FI (%) over time (48 h). EGFP in PBS at 0 h is the reference for 100 %. PBS is presented as a control. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.



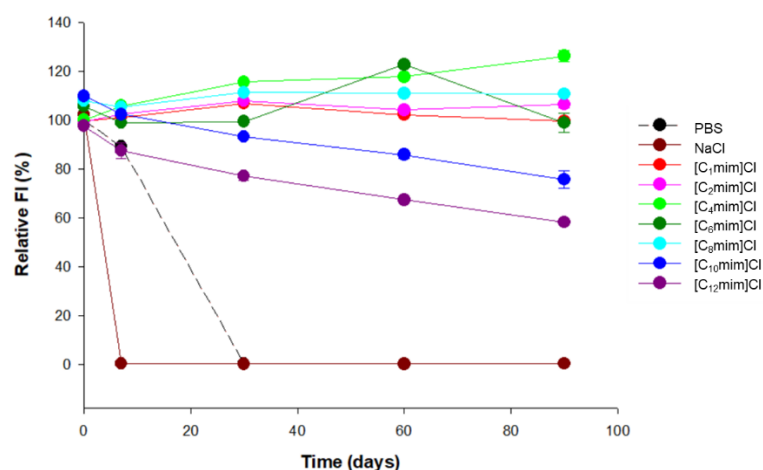
Source: Produced by the author.

As this 48-h screening study had the objective to define the most appropriate concentration for the long-term and under stress EGFP stabilization studies, the concentration of 0.100 mol.L⁻¹ was chosen for presenting similar results for all IL solution under study. Particularly, at 0.100 mol.L⁻¹, equivalent results for PSB and IL solution were obtained with [C_nmim]Cl (*n* from 2 to 10) and [Ch][Ac], while a positive impact on EGFP fluorescence activity was seen for [Ch]Cl, [Ch][But], [Ch][Hex] and [Ch][Dec]. Although the concentration of 0.100 mol.L⁻¹ had a lower fluorescence than in the samples of 0.250 mol.L⁻¹ and 0.500 mol.L⁻¹ for [Ch][Oct], this concentration was not selected because at these other two concentrations quenched EGFP fluorescence for [C₁₂mim]Cl and was not the best condition for most of the ILs.

4.4.2 Long-term effect of aqueous solutions of ILs on EGFP fluorescence

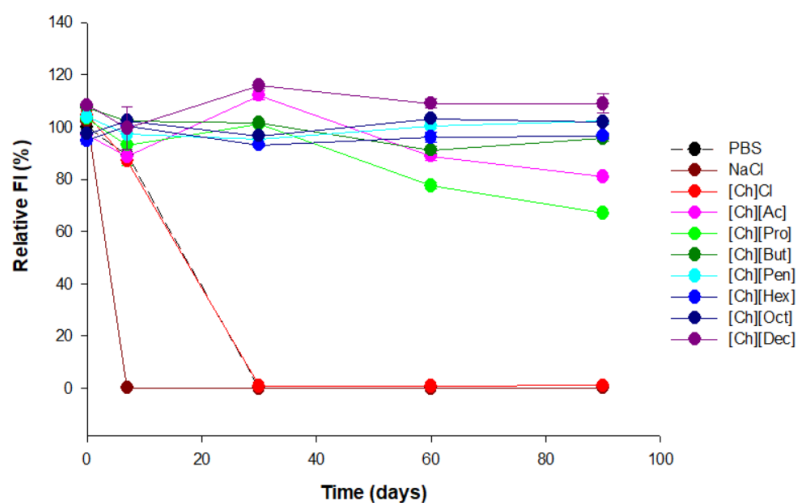
The relative IF of EGFP was determined after 0, 7, 30, 60, and 90 days of exposure of the protein to different [C_nmim]Cl and [Ch]X aqueous solutions (at 0.100 mol.L⁻¹) to assess the long-term effect of ILs on EGFP activity at room temperature (*i.e.* 22-25 °C), as depicted in **Figure 26** and **Figure 27**, respectively. To compare the effect of ILs with buffers and conventional salt, PBS and NaCl acted as control groups.

Figure 26 – EGFP Relative FI (%) for 90 days (3 months), at 25 °C, and absence of light, in the presence of aqueous solutions of imidazolium-based ILs (0.100 mol.L⁻¹) and buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) controls. The results were evaluated in triplicate with a blank sample and presented as average ± standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.



Source: Produced by the author.

Figure 27 – EGFP Relative FI (%) for 90 days (3 months), at 25 °C and absence of light, in the presence of aqueous solutions of cholinium-based ILs (0.100 mol.L⁻¹) and buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) controls. The results were evaluated in triplicate with a blank sample and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.



Source: Produced by the author.

In contrast to the modest differences found in the short-term studies, the long-term evaluation of the effect of aqueous ILs solutions to EGFP fluorescence showed substantial differences between ILs, the buffer (PBS) and salt control (NaCl), and even the cholinium salt [Ch]Cl. **Figure 26** shows that [C_nmim]Cl had a remarkable ability to preserve EGFP fluorescence activity at room temperature when compared to the buffer (PBS) and conventional salt (NaCl) controls. The [C_nmim]Cl with alkyl side cationic chains between 1 and 8 carbons guaranteed the maintenance of the EGFP fluorescence activity during the 90 days of study, while the protein completely loses its fluorescence at day 7 in NaCl aqueous solution and day 30 for the PBS control solution. Interestingly, although a decrease of EGFP relative FI from day 30 to 60 was observed for the [C_nmim]Cl with longer cationic chains (*i.e.*, C10 and C12), these two solutions were better at preserving the EGFP activity at room temperature than the two controls (PBS and NaCl). The rank of the effectivity of the [C_nmim]Cl to preserve EGFP relative FI was the following (highest to lowest): [C₄mim]Cl > [C₈mim]Cl = [C₂mim]Cl > [C₁mim]Cl $\approx$ [C₆mim]Cl > [C₁₀mim]Cl > [C₁₂mim]Cl. This rank is in line with the trend observed in the short-term study as well as in protection experiments (described below in section “**4.4.4 Protective or disruptive effect of ILs on EGFP fluorescence under stress by chemical agents**”), in which the higher is the cationic alkyl chain length of the IL the lower was its ability to preserve EGFP fluorescence.

Concerning the long-term stabilizing effect of [Ch]X on EGFP fluorescence (**Figure 27**), similarly to the [C_nmim]Cl solutions, all [Ch]X maintained EGFP fluorescence activity for

much longer than the buffer (PBS) and conventional salt (NaCl) solutions. Interestingly, the [Ch]X with the highest melting temperature, *i.e.*, [Ch]Cl, which by common definition is considered as a high melting organic salt, had a low aptitude to preserve the fluorescence activity, exhibiting a stabilizing profile similar to the PBS control solution (no fluorescence after 30 days of exposure). However, it is important to note, that contrarily to the [C_nmim]Cl solutions, longer [Ch]X anionic alkyl chain length had a higher capacity to preserve EGFP relative FI, as demonstrated by the following order of the [Ch]X stabilizing ability (from highest to lowest): [Ch][Dec] > [Ch][Oct] $\approx$ [Ch][Pen] > [Ch][Hex] $\approx$ [Ch][But] > [Ch][Ac] > [Ch][Pro]. In this case, only the solutions of cholinium ILs with shortest anionic chain lengths ([Ch][Ac] and [Ch][Pro]) were not able to guarantee the full maintenance of the EGFP relative FI during all 3-months of study, as noted by a decrease in between 20 to 30% of FI from 60 to 90 days of storage time.

The inability of [Ch]Cl to preserve EGFP fluorescence is likely related to its similarity with common “salting-in” inorganic salts. This result is strong evidence that ILs solutions, even at low concentrations, do not act as conventional salts aqueous solutions in the stabilization of proteins, providing a set of other favorable interactions between corresponding ions and proteins, which increase their potential as stabilizing agents for biopharmaceuticals. Additionally, the opposite effect in the maintenance of the EGFP fluorescence observed with the increase of the cationic chain in [C_nmim]Cl versus the increase of anionic chain in [Ch]X indicates that the impact of cations and anions in the protein structure is very distinct, in which the choice of the ion pair is critical and should be taken into consideration according to the target-protein. Most of the previous studies report a stronger anion influence on protein stability, (BUI-LE *et al.*, 2020; OKUR *et al.*, 2013; WEINGÄRTNER *et al.*, 2012; ZHANG; CREMER, 2010), but the cation can also have a strong on protein stabilization in aqueous environments, in line with few previous studies with different proteins. (ATTRI *et al.*, 2011; LANGE *et al.*, 2005; NASCIMENTO *et al.*, 2019a)

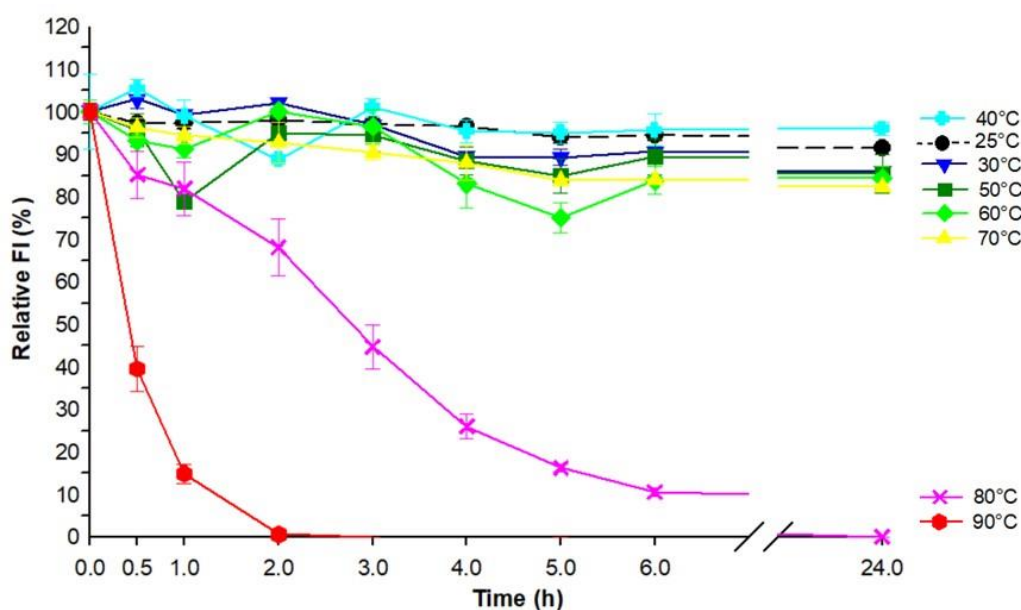
4.4.3 Effect of physical and chemical stress on EGFP fluorescence

In addition to understanding the interactions between ILs and EGFP in an aqueous solution, this work also aimed to evaluate if ILs can be used to protect EGFP structure under unfavorable stress conditions. Therefore, as a first step, the effect of physical and chemical unfavorable conditions to EGFP protein was determined by measuring the fluorescence activity of EGFP solutions at high temperatures and in the presence of distinct chemical denaturing

agents, namely, acidic, alkaline, oxidizing, and surfactant agents. This initial investigation aimed to find moderate stress conditions (*i.e.*, denaturing agents nature and concentration, and time of exposure), in which a moderate fluorescence quenching (in between 40 to 80 %) was found. An intermediate quenching was envisaged to avoid major and irreversible changes in the EGFP structure since a full denaturation of EGFP could overshadow the positive impact of ILs on EGFP stability.

Firstly, the effect of high temperatures on EGFP fluorescence was assessed by measuring the Relative FI (%) over 24 h in a temperature range from 25 to 90 °C (**Figure 28**). Afterward, the effect of chemical denaturing agents was evaluated by exposing EGFP to distinct classes of denaturing agents in different concentrations, for 30 h at 25 °C. The agents selected were GuHCl as acid, urea as an alkaline agent, H₂O₂ as an oxidizing agent, and SDS as a surfactant, as presented in **Figure 29 A, B, C, and D**, respectively.

Figure 28 – Effect of temperature (25 to 90 °C) on EGFP Relative FI (%) over time (48 h). EGFP in PBS 0 h is the reference for 100 %. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.

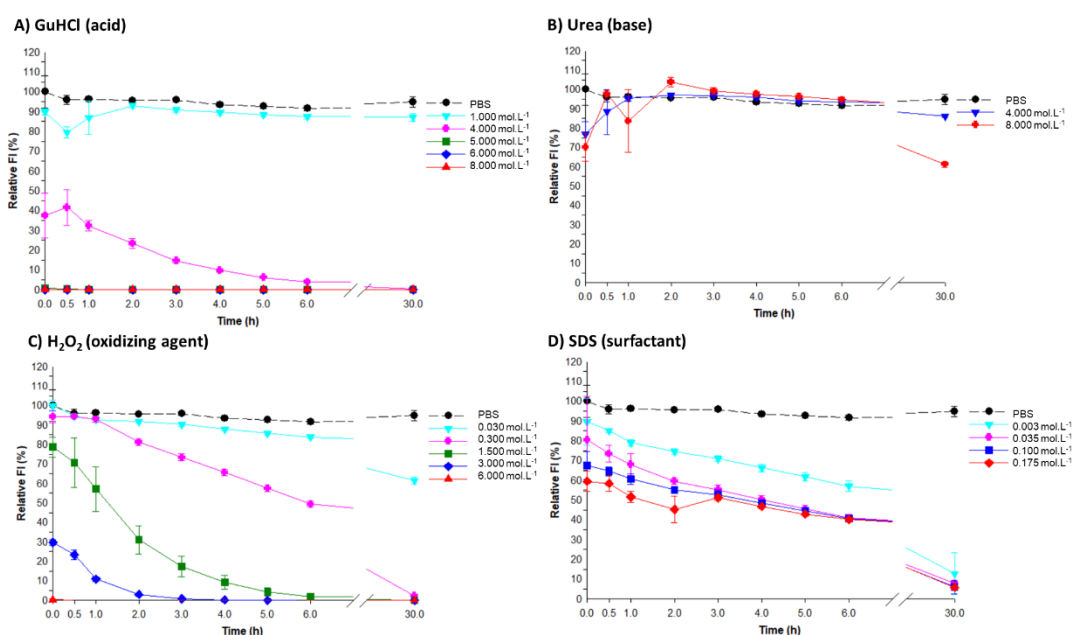


Source: Produced by the author.

As shown in **Figure 28**, EGFP presented no changes when exposed temperatures from 25 to 70 °C, in a period of 24 h. However, at 80 °C, a crescent quenching of EGFP fluorescence with the exposure-time was observed, exhibiting a $\approx$ 50% relative FI after 3 h of exposure, and after 6 h with only 10% of residual fluorescence. The increase of temperature up to 90° C enhanced the thermal denaturation of EGFP, leading to a decrease of 85% of the relative FI after 1 h, and a complete quenching of EGPF fluorescence with 2 h of exposure at that

temperature. This result is consistent with data obtained by Scheyhing *et al.*, which found a thermal unfolding transition temperature for GFP_{mut1} (*i.e.*, EGFP) at 79.5 °C. (SCHEYHING; MEERSMAN; EHRMANN; HEREMANS *et al.*, 2002)

Figure 29 – Effect of chemical denaturing agents on EGFP Relative FI (%) over time (48 h). **A)** GuHCl (acid), **B)** Urea (base), **C)** H₂O₂ (oxidizing agent) and **D)** SDS (surfactant). EGFP in PBS 0 h is the reference for 100 %. PBS is presented as a control. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.



Source: Produced by the author.

For the acid denaturing agent, **Figure 29.A** shows that GuHCl had no impact on EGFP relative FI (%) for the 1.000 mol.L⁻¹ concentration, and above 5.000 mol.L⁻¹, the fluorescence was completely suppressed immediately after the addition of the denaturation agent (*i.e.*, at 0 h). At a concentration of 4.000 mol.L⁻¹, there was a slower fluorescence quenching, with a decline of the relative FI from 37.5 ± 11.2 % at 0 h to 23.4 ± 2.4 % at 2 h, and a complete fluorescence suppression after 30 h. As for the base denaturing agent, the results from **Figure 29.B** show that EGFP is very stable in the presence of urea, even at the highest concentrations (*i.e.* 4.000 and 8.000 mol.L⁻¹). These reports demonstrate EGFP is very resistant to alkaline conditions but sensitive to acids, which is in accord with the literature (SCHEYHING; MEERSMAN; EHRMANN; HEREMANS *et al.*, 2002; WARD; PRENTICE; ROTH; CODY *et al.*, 1982) and the previous results presented in section “4.3. EGFP activity and structure at different pH”.

The oxidizing agent H₂O₂ (**Figure 29.C**) had a concentration-dependent denaturing of EGFP, increasing at higher concentrations until complete suppression of fluorescence at

6.000 mol.L⁻¹. Additionally, there was a gradual decrease in EGFP fluorescence over time for concentrations of 0.300 mol.L⁻¹ and 1.500 mol.L⁻¹ of H₂O₂, which presented an intermediate FI suppression around 1 to 2 h. The free radical species of H₂O₂ can damage the protein and even denature it via oxidative reactions. (FINNEGAN *et al.*, 2010)

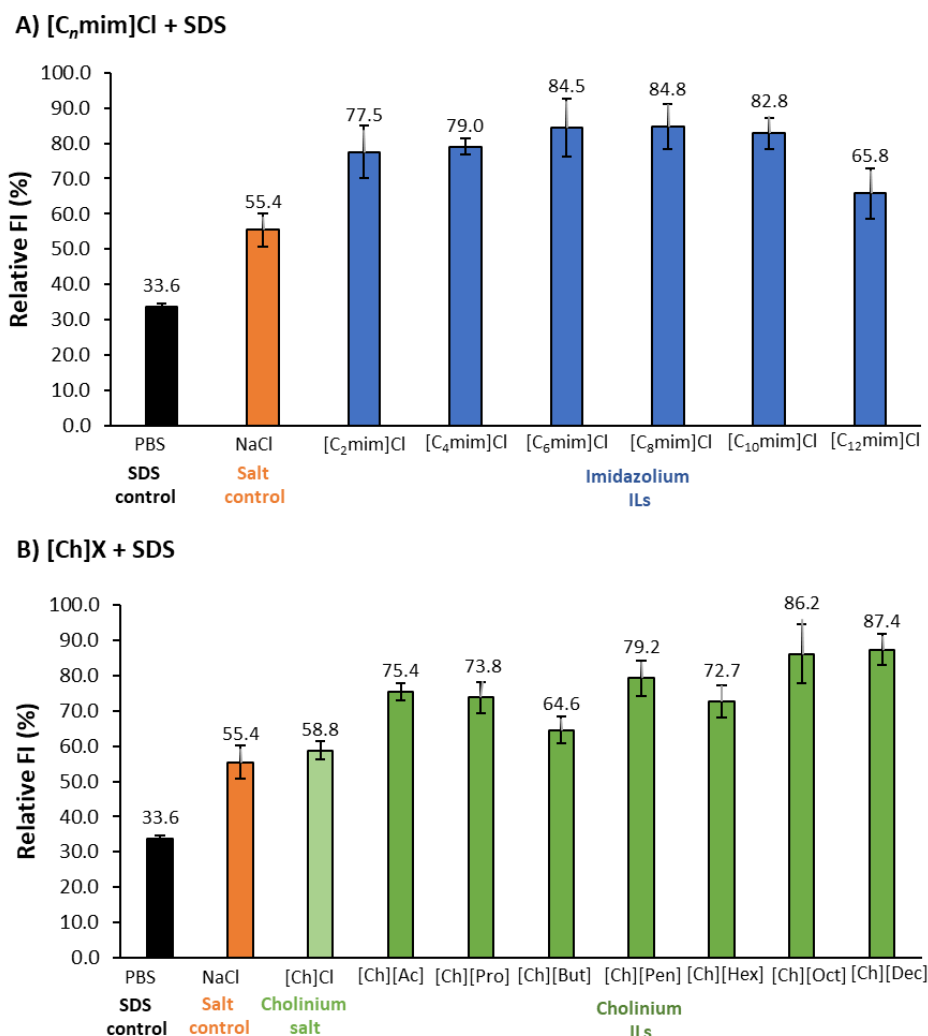
As for the surfactant, **Figure 29.D** showed a reduction of the relative FI of EGFP with the addition of SDS for all concentrations. Although the concentration of 0.175 mol.L⁻¹ allowed a faster fluorescence quenching when compared to the concentrations of 0.035 and 0.100 mol.L⁻¹, in general, the different concentrations of SDS induced a similar fluorescence quenching. In this condition, there was a decrease of 40-50 % of EGFP fluorescence after 3 h, and a complete suppression after 30 h of exposure even for the concentration of 0.003 mol.L⁻¹. The critical micellar concentration of SDS is 0.007-0.010 mol.L⁻¹ (20-25 °C) according to Sigma-Aldrich®; hence, at 0.003 mol.L⁻¹, the loss of EGFP fluorescence is likely caused by a surfactant effect of SDS. (SIGMA-ALDRICH, 2020) The general mechanism of denaturation of proteins with SDS follow a model called “pearl necklace”, because the proteins wrap around the micelles of SDS in a fluid configuration that resembles a pearl necklace. By interacting with the SDS micelles, the protein can drastically change its structure and denature. (WINOGRADOFF *et al.*, 2020)

Considering the results from **Figure 28** and **Figure 29**, the following conditions were selected for the study of protection of EGFP fluorescence using ILs aqueous solutions: SDS at 0.175 mol.L⁻¹; GuHCl at 4.000 mol.L⁻¹; H₂O₂ (1.500 mol.L⁻¹). These conditions were chosen based on their moderate EGFP fluorescence quenching (40-80%) after 2 h of exposure. Urea and high temperature were discarded from the protection study considering EGFP already presents a very high resistance to elevated temperatures and alkaline substances.

4.4.4 Effect of ILs solutions on EGFP fluorescence under chemical stress conditions

The fluorescence of EGFP in the presence of aqueous solutions of ILs (0.100 mol.L⁻¹), control solutions, and each of the denaturing agents was assessed at 0 h and after 2 h of exposure, namely: EGFP in PBS pH 7.4 (as reference for 100%); EGFP in PBS + denaturing agents aqueous solutions (positive control); EGFP in NaCl (0.100 mol.L⁻¹) + denaturing agents aqueous solutions (salt control); EGFP in ILs + denaturing agents aqueous solutions (samples). All solutions were compared in terms of relative FI (%), with the respective results for the denaturing agents SDS, GuHCl, and H₂O₂ in **Figure 30**, **Figure 31**, and **Figure 32**, respectively.

Figure 30 – Effect of **A)** imidazolium-based ILs and **B)** cholinium-based ILs and salts (0.100 mol.L⁻¹) on EGFP fluorescence at 2 h and 25 °C in the presence of the surfactant SDS (0.175 mol.L⁻¹). Data is presented as Relative FI (%) of EGFP. A buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) in the presence of SDS (0.175 mol.L⁻¹) are control groups. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation.



Source: Produced by the author.

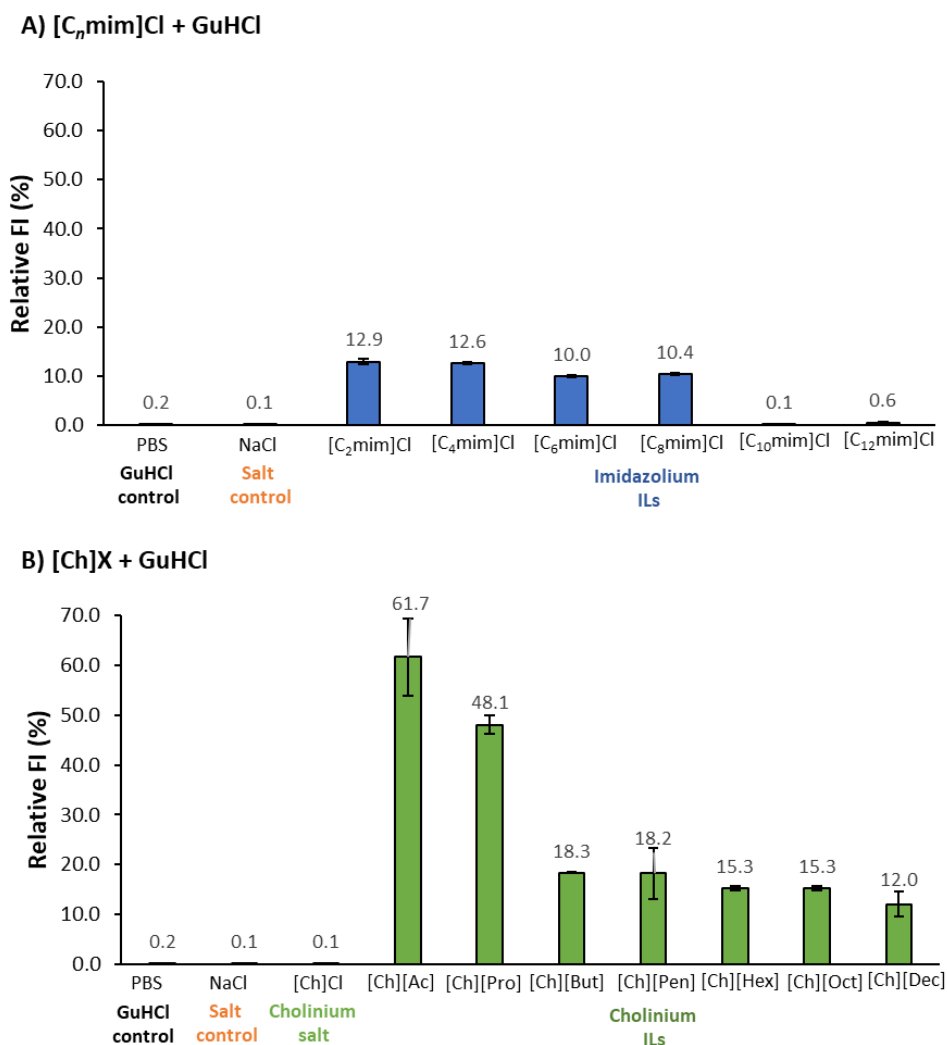
As can be seen in **Figure 30**, even the conventional salt (NaCl) decreased the disruptive effect of SDS on EGFP fluorescence, maintaining 55.5 % of EGFP relative FI in comparison with the PBS + SDS sample (with a residual relative FI of 33.6 %). However, all ILs in the study had an even greater capacity to preserve EGFP fluorescence in the presence of SDS. For example, the [C_nmim]Cl solutions in **Figure 30.A** maintained the EGFP relative FI from 65.8 to 84.8 %, showing they grant additional protection to the protein against the surfactant. The ability of [C_nmim]Cl to preserve EGFP fluorescence from SDS quenching increased from the cationic chain length from C2 to C8, with a minimal decrease for C10 and a considerable decrease for C12. This outcome reinforces the results from the long-term studies in **Figure 26**, with all [C_nmim]Cl protecting EGFP fluorescence in comparison with PBS and salt control, but

with [C₁₂mim]Cl followed by [C₁₀mim]Cl presenting the lowest aptitudes to preserve EGFP relative FI. As for the [Ch]Cl (cholinium salt) in **Figure 30.B**, it also had a protective effect for EGFP when compared to PBS, however, it had the same results as NaCl (salt control). Contrarily, all [Ch]X ILs presented a higher ability to preserve EGFP fluorescence after 90 days, with similar results to the long-term evaluation in **Figure 27**. Additionally, a small trend showed that the increase of the [Ch]X anionic chain length can improve the protector effect of the ILs for EGFP.

For both IL families, it is clear that ILs have a higher potential to preserve EGFP when compared to buffers and salts. The protective effect of [C_{*n*}mim]Cl and [Ch]X on EGFP against SDS can be due to an interaction between the ILs with SDS or an interaction of the ILs with the protein to increase its stability. Although previous reports have already demonstrated the self-assembly of longer imidazolium ILs (*i.e.*, $n > 8$) and SDS in water, (MISKOLCZY *et al.*, 2004) due to the complexity of interactions that can occur between ILs and proteins (*e.g.*, hydrogen bonding, hydrophobic interactions, strong Coulomb interaction, dispersion forces) (SCHRÖDER, 2017) further experimental and computational structural evaluations are required to reveal the mechanisms and corresponding impact on the EGFP fluorescence quenching/dequenching.

In **Figure 31** are presented the results for the protection study with GuHCl.

Figure 31 – Effect of **A)** imidazolium-based ILs and **B)** cholinium-based ILs and salts (0.100 mol.L⁻¹) on EGFP fluorescence at 2 h and 25 °C in the presence of the acid GuHCl (4.000 mol.L⁻¹). Data is presented as Relative FI (%) of EGFP. A buffer (PBS) and conventional salt (NaCl 0.100 mol.L⁻¹) in the presence of GuHCl (4.000 mol.L⁻¹) are control groups. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation.



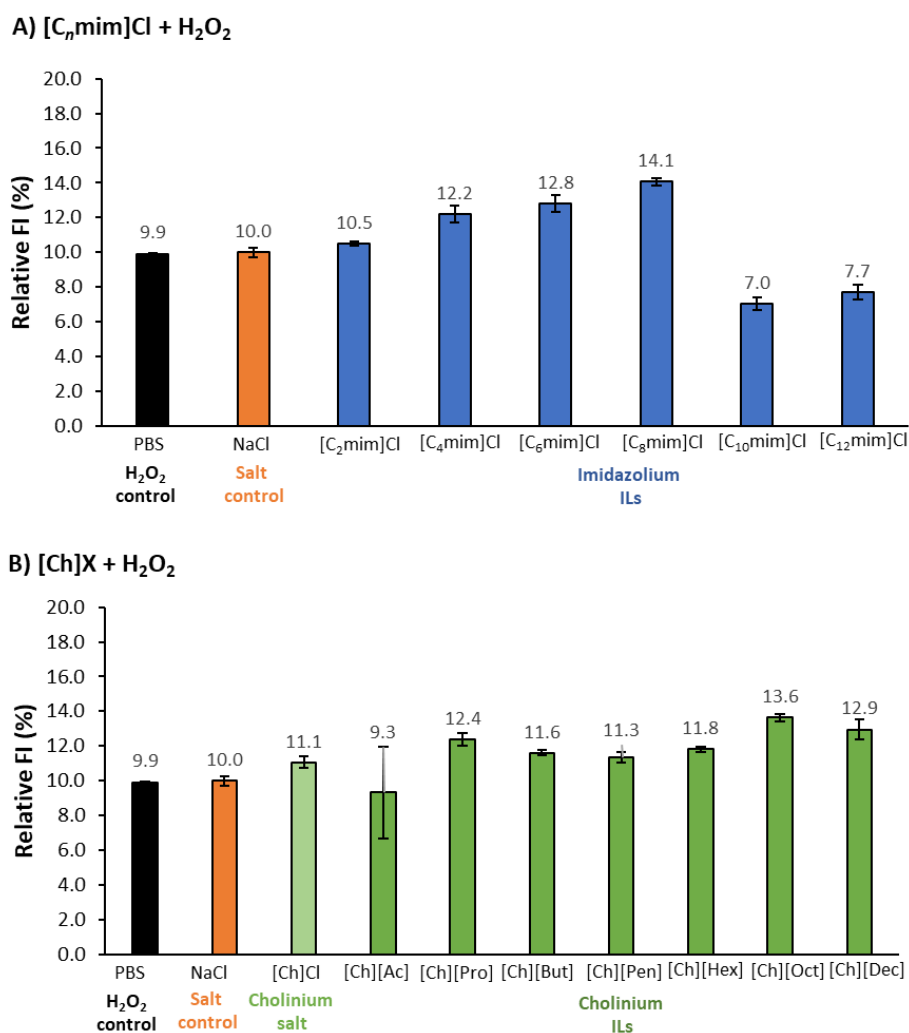
Source: Produced by the author.

For the [C_nmim]Cl, shown in **Figure 31.A**, the [C_nmim]Cl with cation chain length from C2 to C8 provided small protection to EGFP fluorescence against GuHCl, while C10 and C12 had the same response as the PBS and NaCl controls. Regarding the protective effect of [Ch]X against GuHCl, presented in **Figure 31.B**, [Ch][But], [Ch][Pen], [Ch][Hex], [Ch][Oct] and [Ch][Dec] conferred a small protection to EGFP against GuHCl when compared to the PBS and NaCl controls, but this effect was considerably more expressive for [Ch][Ac] and [Ch][Pro]. This effect is in contrast to the previous results with SDS and in the long-term study, where the increase of [Ch]X increase their ability to protect EGFP. This shows that, under certain conditions, trends can be reversed due to the complexity of the interactions

between proteins, ILs, and denaturing agents. The protection of EGFP with the shorter chain [Ch]X could be either due to a direct interaction between these ILs and the protein, but there is also the possibility of a chemical reaction between [Ch]X and the GuHCl. Further characterization would be required to elucidate this mechanism.

Figure 32 presents the results for the EGFP protection study with ILs and the oxidizing agent (H_2O_2).

Figure 32 – Effect of **A)** imidazolium-based ILs and **B)** cholinium-based ILs and salts (0.100 mol.L^{-1}) on EGFP fluorescence at 2 h and 25°C in the presence of the oxidizing agent H_2O_2 (1.500 mol.L^{-1}). Data is presented as Relative FI (%) of EGFP. A buffer (PBS) and conventional salt (NaCl 0.100 mol.L^{-1}) in the presence of H_2O_2 (1.500 mol.L^{-1}) are control groups. Results were evaluated in triplicate with a blank and presented as average $\pm$ standard deviation.



Source: Produced by the author.

For the protection study with H_2O_2 in **Figure 32**, there were almost no differences in EGFP relative FI for all samples. No variations were observed for [Ch]X in **Figure 32.B**, but

there was a small trend for $[C_n\text{mim}]\text{Cl}$ to increase EGFP protection from C2 to C8 and reduce for C10 and C12 in **Figure 32.A**.

Overall, $[C_n\text{mim}]\text{Cl}$ and $[\text{Ch}]\text{X}$ ILs protected EGFP from SDS, with the best results for the shorter chain cations for the $[C_n\text{mim}]\text{Cl}$ and longer chain anions for the $[\text{Ch}]\text{X}$. The $[C_n\text{mim}]\text{Cl}$ from cation chains C2 to C8 and all $[\text{Ch}]\text{X}$ ILs protected EGFP from GuHCl, but C10 and C12 did not. Contrarily to the other studies, the $[\text{Ch}]\text{X}$ with shorter chains ($[\text{Ch}][\text{Ac}]$ and $[\text{Ch}][\text{Pro}]$) had the best aptitudes to preserve EGFP relative FI against GuHCl denaturation. Overall, there was little effect of ILs over the disruption of EGFP fluorescence with H_2O_2 . When comparing these results with the studies of the previous sections, in general, it is possible to notice the persistence of a trend among the experiments. For the imidazolium ILs, the increase of the cation chain length impaired its ability to preserve EGFP fluorescence activity, while longer anionic chain for the cholinium ILs improved their protection of EGFP fluorescence.

Considering the literature on this topic, Nascimento *et al.* (NASCIMENTO *et al.*, 2019b, 2019a) also evaluated the potential of $[C_n\text{mim}]\text{Cl}$ to stabilize or enhance the activity for proteins using lipase as a case study. The tendencies observed by Nascimento *et al.* (NASCIMENTO *et al.*, 2019a) for the effect of imidazolium ILs on lipase were similar to the results in this section, with $[C_n\text{mim}]\text{Cl}$ with short cation chains presenting a positive impact over the protein, while longer chains impaired lipase stability. Additionally, the experiments by Rawat and Bohidar using ILs $[C_n\text{mim}]\text{Cl}$ (C6 and C8) suggested that proteins (namely, BSA, human serum albumin, IgG, β -lactoglobulin, and gelatin-B) preferentially bind to these ILs through electrostatic interactions between charged regions of proteins and the chloride ion of the molecule, or else with the possible formation of hydrogen bonds and van der Waals forces. (RAWAT; BOHIDAR, 2012) Other researchers have also observed similar trends for other proteins, such as Attri *et al.* for chymotrypsin, (ATTRI *et al.*, 2011) Lange *et al.* for HEWL and the ScFvOx antibody fragment, and Guerra and Irabien for BSA. (ALVAREZ-GUERRA; IRABIEN, 2011)

Specifically, for fluorescent proteins and ILs, a recent work of Bui-Le *et al.* provided insights into the complex mechanisms involved in the interactions of imidazolium and pyrrolidinium ILs and the GFP variant sfGFP. (BUI-LE *et al.*, 2020) In an interesting multi-technique approach using CD, fluorescence, UV-vis spectroscopy, NMR, and SAXS to evaluate the interactions between ILs and sfGFP, the group showed that individual techniques could lead to misleading conclusions regarding the effects of ILs and proteins. Only by allying a vast range

of methods, it was possible to obtain the full picture of the intricate (and sometimes, multifactorial) mechanisms involved in the interactions of proteins and ILs. The first difficulty in defining these mechanisms are mainly due to the complexity of proteins by itself, that present a diversity of residues and regions that can propitiate a wide range of interactions with ions, including hydrophobic interactions, strong Coulomb interaction, dispersion forces, and hydrogen bonding. (SCHRÖDER, 2017) In addition to the intrinsic complexity of proteins, ILs can also behave differently depending on their composition and concentration. Besides the traditional “ionic liquid effects” (*e.g.*, a tendency towards ion-pairing, high organization), (NETO; SPENCER, 2012) ILs can also act as conventional salts at low concentrations, or as surfactants in ILs with longer chain lengths and at specific concentrations. (GREAVES; DRUMMOND, 2008) For this reason, even though the present section provides strong indications for the trends and behavior of $[C_n\text{mim}]\text{Cl}$ and EGFP using fluorescence spectroscopy, additional evaluations in a multi-technique approach is still necessary to provide a robust and reliable description of the molecular interactions between these ILs and EGFP.

For the cholinium ILs, Guncheva *et al.* (2015) also showed that $[\text{Ch}]\text{X}$ could improve hemocyanin activity against *in vitro* cell tumors by rearrangements in the secondary structure of the protein. (GUNCHEVA *et al.*, 2015) Likewise, Magri *et al.* (MAGRI *et al.*, 2019) and Nascimento *et al.* (NASCIMENTO *et al.*, 2019b) have studied the effect of dilute aqueous solutions of $[\text{Ch}]\text{X}$ with anion alkyl chain length from C2 to C6 on protein activity, particularly for L-asparaginase and lipase, respectively. However, in contrast with the present study, Magri *et al.* and Nascimento *et al.* found that the increase of the anion chain length reduced the positive impact of $[\text{Ch}]\text{Cl}$ on protein activity. As explained for the $[C_n\text{mim}]\text{Cl}$, the interactions between proteins and ILs are complex and multifactorial, and differences of proteins and conditions of study (*e.g.*, concentration, temperature, ionic strength of the medium, chain length). Therefore, a multi-technique structural evaluation would be required to determine the interactions for each protein-IL system, so it is would be possible to explain (and in the future, predict) the outcome of these associations.

The results of this work show that dilute solutions of different imidazolium and cholinium ILs have great potential for application as solvents or additives for preserving proteins at room temperature or protecting them from denaturing substances. Additionally, this study also demonstrates that the increase of the alkyl chain of both cations and anions can have severe impacts on protein activity. This impact can be positive or negative depending on the nature of the ILs and proteins and the conditions of their medium (*e.g.*, concentration, presence

of disruptive substances) and time of exposure (*e.g.*, long-term storage). For example, the increase of the cation chain length for $[C_n\text{mim}]\text{Cl}$ impaired its ability to maintain EGFP fluorescence, while longer anion chain lengths for $[\text{Ch}]\text{X}$ improved their potential to protect EGFP activity. However, to elucidate the complex mechanisms behind these IL-protein interactions, in-depth structural experiments and molecular dynamics modeling are still required.

4.5 PURIFICATION OF EGFP USING AQUEOUS BIPHASIC SYSTEMS

Section adapted from the published original article in ACS Sustainable Chemistry & Engineering, v. 6, n. 7, 9383-9393, 2018. (DOS SANTOS et al., 2018b)

Cholinium-based ABS are promising platforms for cheaper and more sustainable purification of biomolecules such as GFP. Hence, this work aimed to evaluate the potential of these systems for the recovery and purification of EGFP from cell lysate of recombinant *E. coli* BL21. Thus, this study extensively evaluated EGFP extraction and purification aptitude of different ABS composed of [Ch]Cl and biocompatible polymers like PEG, PPG, or NaPA. The EGFP extraction efficiencies of each ABS were determined and their purification aptitudes were evaluated by 3D fluorescence and SDS-PAGE and compared with more traditional polymer/buffer-based ABS. Additionally, a back-extraction procedure was designed to improve the EGFP purification yields. Envisaging the industrial application of the methodologies developed in this work, an integrated process of purification of EGFP was assembled by using ABS (with the highest purification performance) with ultrafiltration, thus demonstrating the recycling of the phase-forming agents and allowing the EGFP polishing.

4.5.1 EGFP extraction

Firstly, the extraction efficiencies and purity of EGFP extracted from the supernatant of the cell lysate of recombinant *E. coli* BL21 with plasmids pLysS and pET28(a) using various ABS were determined to identify the system allowing the complete recovery and purification of EGFP without losses on its fluorescence structural integrity. The success of the use of ABS as purification platforms is dependent on the properties of coexisting aqueous phases and their ability to separate the target protein from other contaminants. Therefore, the physicochemical characterization of the target bio-product and the ABS coexisting phases are essential for the understanding and design of the best fractionation system.

To evaluate the selective extraction of EGFP from the raw extract (after lysis) the following ABS were studied: PEG-1500/potassium phosphate buffer; PEG-2000/ potassium phosphate buffer; PEG-600/NaPA-8000 + [Ch]Cl as adjuvant; PEG-600/[Ch]Cl; PPG-400/[Ch]Cl. These systems were chosen according to the main mechanisms that control the partitioning of biomolecules, namely, salting-out effect (PEG/phosphate ABS), hydrophobicity/hydrophilicity balance (polymer/[Ch]Cl ABS), and electrostatic repulsion (PEG/NaPA ABS). Different mixtures at the biphasic region were chosen according to previously published phase diagrams for each type of ABS, as listed in **Table 5**. The phase

diagram of the PEG-2000 + K₂HPO₄/KH₂PO₄-based ABS is presented in **Figure I.1.**, as well as the corresponding ternary weight fraction composition (**Table I.1**) and detailed description of phase diagrams determination methodologies (section “**I. Phase diagram determination**”), from the **Annex**. The methods for preparation and characterization of ABS are described in section “**3.3.4.3 Extraction and purification of EGFP**”, and methods for determination of the EGFP 3D fluorescence spectrum and quantification of EGFP and Total Protein (TP) are detailed in section “**3.3.4.2 Quantification of EGFP and total protein**”.

The extraction and purification of EGFP in each series of ABS were assessed in terms of EGFP extraction efficiency (EE_{EGFP}), EGFP mass balance (MB_{EGFP}), and relative concentration of EGFP by total protein ($[EGFP]_{rel}$), according to Equations 5 to 7. The EE_{EGFP} was used to evaluate the capacity of extraction of EGFP in the EGFP-rich phase of each system, the MB_{EGFP} was assessed to verify EGFP fluorescence losses in the extraction process and the $[EGFP]_{rel}$ (%) was calculated to effectively validate the purification capabilities of each system, and thus define which ABS can be used in the purification of EGFP.

Each phase of the ABS studied was also characterized for their physicochemical properties considering the variables pH, conductivity (mS.cm⁻¹ at 25 °C), viscosity (mPa.S), density (g.cm⁻³), and water content (wt%), as presented in **Table 8** and methods described in section “**3.3.4.8 Physicochemical characterization of ABS coexisting phases**”. This characterization was important to determine the overall composition of each phase in association with the FTIR analysis presented in **Figures E.1 to E.4**. In addition to the determination of the ABS phases’ composition, the physicochemical characterization of the phases allows the understanding of the partitioning mechanisms that will be addressed in the next paragraphs.

Table 8 – Detailed weight fraction compositions, physicochemical characteristics [pH, conductivity ($\text{mS}\cdot\text{cm}^{-1}$ at 25 °C), viscosity ($\text{mPa}\cdot\text{S}$), density ($\text{g}\cdot\text{cm}^{-3}$), water content (wt%)] of each ABS. (continue)

ABS	Weight fraction (wt%)			pH		Conductivity ($\text{mS}\cdot\text{cm}^{-1}$ 25 °C)		Viscosity ($\text{mPa}\cdot\text{S}$)		Density ($\text{g}\cdot\text{cm}^{-3}$)		Water content (wt%)	
	Polymer 1	Polymer 2	Buffer or salt	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
Polymer/Polymer with [Ch]Cl as adjuvant ABS	PEG-600 (Top)	NaPA-8000 (Bottom)	[Ch]Cl										
	15.156	15.226	1.024	7.66	7.97	7.34	49.41	5.037	37.326	1.0613	1.1890	67.11	68.21
	15.010	15.026	3.042	7.92	7.99	13.67	47.25	4.907	46.882	1.0637	1.1943	65.12	66.66
PEG-600/NaPA-8000 + [Ch]Cl	15.561	14.984	5.055	7.89	7.94	18.25	45.05	5.415	58.733	1.0694	1.2016	63.25	64.55
	20.035	15.211	1.006	7.70	7.48	4.61	44.70	7.182	113.960	1.0760	1.2324	59.68	63.87
	20.224	15.206	3.085	7.30	7.02	9.74	43.38	6.826	94.529	1.0734	1.2217	59.52	63.36
	20.133	15.360	5.081	7.39	7.44	13.40	42.05	7.807	89.989	1.0787	1.2187	55.76	60.12
Polymer/Buffer ABS	PEG-1500 (Top)	-	$\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (Bottom)										
PEG-1500 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$	15.020	-	14.957	6.82	6.60	6.74	122.30	10.513	2.033	1.0876	1.1984	64.58	71.63
	PEG-2000 (Top)	-	$\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (Bottom)										
PEG-2000 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$	14.963	-	14.949	6.88	6.62	5.35	124.40	14.778	1.875	1.0856	1.1959	75.40	78.85

Table 8 – Detailed weight fraction compositions, physicochemical characteristics [pH, conductivity ($\text{mS}\cdot\text{cm}^{-1}$ at 25 °C), viscosity ($\text{mPa}\cdot\text{S}$), density ($\text{g}\cdot\text{cm}^{-3}$), water content (wt%)] of each ABS.

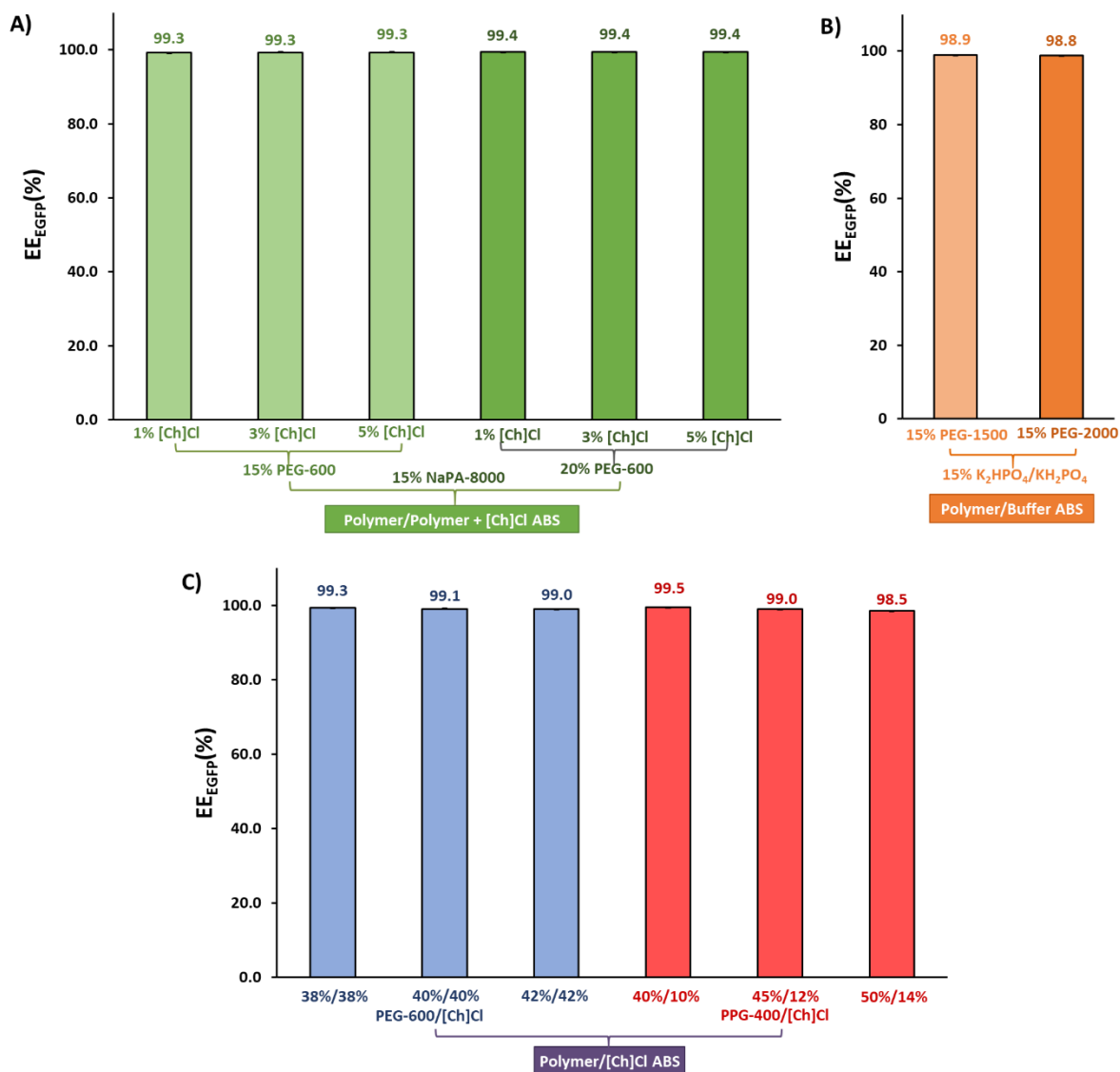
ABS	Weight fraction (wt%)			pH		Conductivity ($\text{mS}\cdot\text{cm}^{-1}$ 25 °C)		Viscosity ($\text{mPa}\cdot\text{S}$)		Density ($\text{g}\cdot\text{cm}^{-3}$)		Water content (wt%)	
	Polymer 1	Polymer 2	Buffer or salt	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
Polymer/[Ch]Cl ABS	PEG-600 (Bottom)	-	[Ch]Cl (Top)										
	38.325	-	37.807	6.12	5.66	31.72	0.53	15.738	100.840	1.0942	1.1202	33.08	14.81
PEG-600/[Ch]Cl	39.919	-	40.081	6.01	6.25	37.31	0.30	14.948	115.210	1.0927	1.1209	31.65	12.41
	41.940	-	41.955	5.79	6.01	31.32	0.13	18.232	118.290	1.0956	1.1215	29.14	12.31
	PPG-400 (Top)	-	[Ch]Cl (Bottom)										
	40.342	-	9.967	5.33	5.53	0.59	26.70	55.770	4.667	1.0273	1.0341	24.70	62.46
PPG-400/[Ch]Cl	45.548	-	11.933	5.62	5.83	0.30	45.60	63.100	3.425	1.0250	1.0362	16.86	63.15
	49.973	-	14.066	5.47	5.87	0.14	64.21	69.200	3.047	1.0222	1.0460	13.06	56.44
Back-extraction ABS	PEG-600 (Top)	NaPA-8000 (Bottom)	[Ch]Cl										
1st extraction	14.923	14.975	3.054	7.75	7.57	12.99	39.50	5.002	54.595	1.0644	1.1997	65.49	66.83
	PEG-600 (Bottom)	Top phase from 1^o extraction	[Ch]Cl (Top)										
2nd extraction	25.269	32.26	34.878	8.18	7.92	28.24	0.57	17.419	74.920	1.1003	1.128	30.11	18.46

Source: Produced by the author and published (adapted) in (DOS SANTOS *et al.*, 2018b).

The ABS mostly presented pH values above 6, an environment in which EGFP is overall stable (TSIEN, 1998) and generally negatively charged, considering EGFP isoelectric point (pI) is 6.2. (GURUNATHAN *et al.*, 2014) The water content in the PEG/NaPA + [Ch]Cl-based ABS was almost identical in the top and bottom phases (with an average difference of only 2.72 ± 1.56 wt%), both rich in water (around 60 wt%). As for the PEG/potassium phosphate buffer ABS, the buffered phase was slightly more hydrophilic (around 5 wt% of difference), but both phases were rich in water (64.58 to 78.85 wt% of water). However, in the polymer/[Ch]Cl systems, there was a greater difference in the water content among the phases, ranging from 29.14–33.08 and 56.44–63.15 wt% for the [Ch]Cl phase in the PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, respectively, and 12.31–14.81 and 13.06–24.70 wt% for the polymer phase of the cited ABS. The average difference of top and bottom phase for the PEG-600 and PPG-400 ABS were 18.11 ± 1.21 wt% and 42.48 ± 4.34 wt%, respectively. As for the conductivity, in the PEG/NaPA + [Ch]Cl ABS, it ranged from 4.61 to 18.25 mS.cm⁻¹ in the PEG rich-phase and from 42.05 to 49.21 mS.cm⁻¹ in the NaPA rich-phase (average difference of 37.3 ± 3.4 mS.cm⁻¹); in the PEG/potassium phosphate buffer ABS, the PEG phase presented a conductivity of 5.35 to 6.74 mS.cm⁻¹ and the buffer phase of 122.30 to 124.40 mS.cm⁻¹; and finally, the polymer/[Ch]Cl ABS had less than 1 mS.cm⁻¹ of conductivity for the polymer phase and the [Ch]Cl phase for the PEG-600 system presented readings from 31.32 to 37.31 mS.cm⁻¹ and the PEG-600 system from 26.70 to 64.21 mS.cm⁻¹, with respective average differences between top and bottom phases of 33.1 ± 3.4 and 45.2 ± 19.0 mS.cm⁻¹.

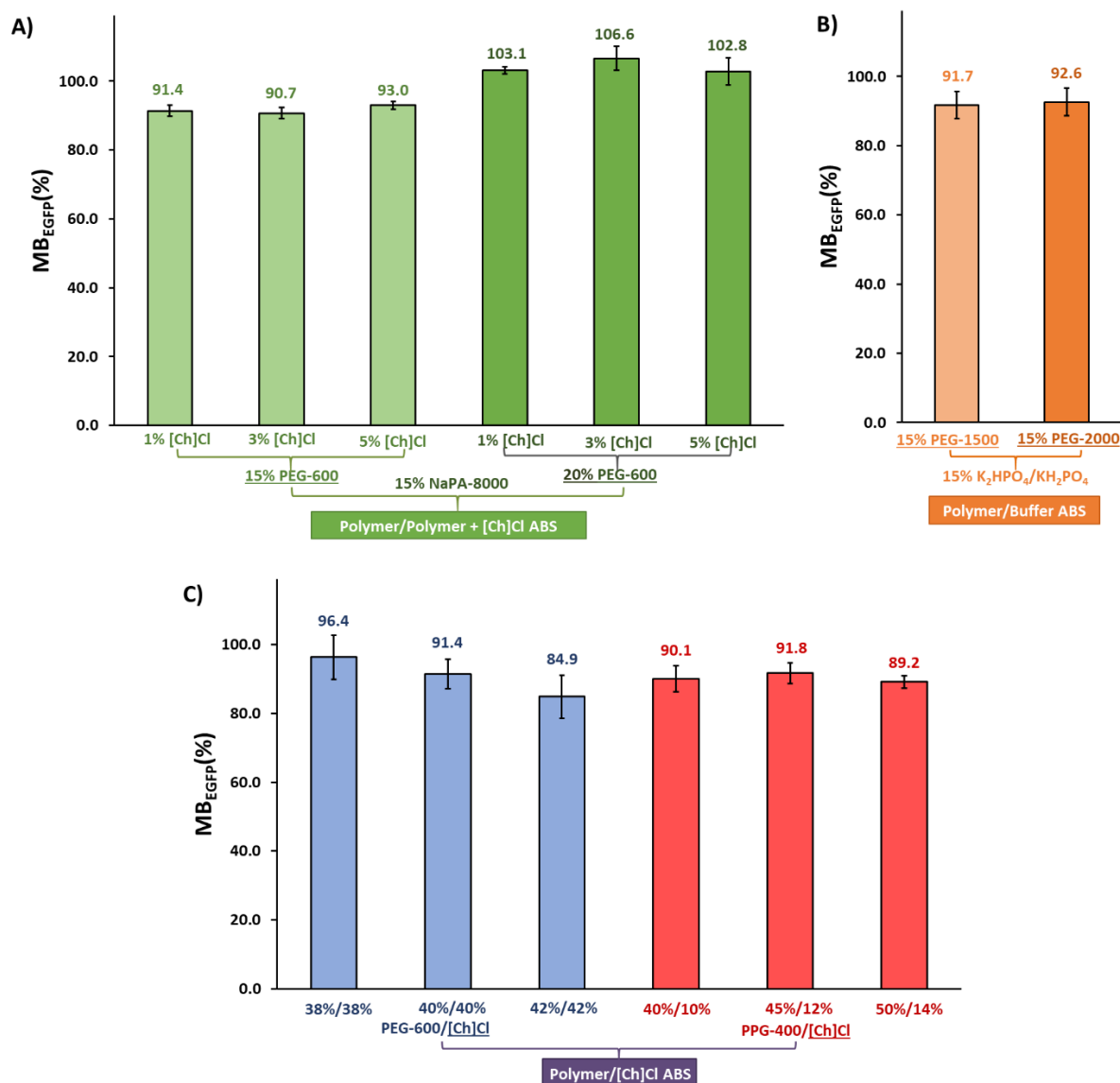
The ABS described in **Table 5** were initially studied for EGFP extraction, where the influence of the type and length of the polymers, type of salts, and the concentration of the phase-forming agents were the parameters evaluated. The comparison of the average EE_{EGFP} (%) MB_{EGFP} (%) and of three independent assays (and respective standard deviations) for all systems are compiled in **Figure 33** and **Figure 34**, and the detailed weight fraction compositions (wt%) and physicochemical characteristics of coexisting phases are fully provided in **Table 8**.

Figure 33 – EGFP extraction efficiency [EE_{EGFP} (%)] for the: **A)** PEG-rich phase using ABS composed of 15 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%) (■); and 20 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%) (■); **B)** PEG-rich phase using ABS composed of 15 wt% PEG-1500 + 15 wt% K_2HPO_4/KH_2PO_4 (■); 15 wt% PEG-2000 + 15 wt% K_2HPO_4/KH_2PO_4 (■); **C)** [Ch]Cl-rich phase using ABS composed of PEG-600 (38, 40 or 42 wt%) + [Ch]Cl (38, 40 or 42 wt%) (■) and PPG-400 (40, 45 or 50 wt%) + [Ch]Cl (10, 12 or 14 wt%) (■) (concentration: polymer wt%/[Ch]Cl wt%). The results represent the average of three independent experiments $\pm$ standard deviation errors.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Figure 34 – EGFP mass balance [MB_{EGFP} (%)] for the: **A)** PEG-rich phase using ABS composed of 15 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%) (■); and 20 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%) (■); **B)** PEG-rich phase using ABS composed of 15 wt% PEG-1500 + 15 wt% K_2HPO_4/KH_2PO_4 (■); 15 wt% PEG-2000 + 15 wt% K_2HPO_4/KH_2PO_4 (■); **C)** [Ch]Cl-rich phase using ABS composed of PEG-600 (38, 40 or 42 wt%) + [Ch]Cl (38, 40 or 42 wt%) (■) and PPG-400 (40, 45 or 50 wt%) + [Ch]Cl (10, 12 or 14 wt%) (■) (concentration: polymer wt%/[Ch]Cl wt%). The results represent the average of three independent experiments $\pm$ standard deviation errors.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

The results, as depicted in **Figure 33.A** and **Figure 33.B**, show that independently of the composition of PEG-600 and [Ch]Cl in the PEG-600/NaPA + [Ch]Cl-based ABS and the MW of the PEG used in the PEG/potassium phosphate buffer systems, EGFP was fully extracted into the PEG-rich phase (EE_{EGFP} (%) > 99 %). Also, the MB_{EGFP} (%) for these systems were higher than 90 % (**Figure 34.A** and **Figure 34.B**), demonstrating that these ABS were quite compatible with EGFP and caused no significant losses on its fluorophore structural

integrity and conformational structure. It is important to note that even with 20 wt% of PEG-600 and 15 wt% of NaPA-8000, the EGFP maintained its fluorescence capability on the PEG-rich phase, as demonstrated by MB_{EGFP} around 100 %. The maintenance of EGFP structure and fluorescence integrity using these ABS is expected, since the concentrations of each polymer, [Ch]Cl, and buffer are low and adequate for protein stability, (JOHANSSON *et al.*, 2008a; PERSSON *et al.*, 1999) avoiding the precipitation of proteins or undesirable structural changes. GFP present many variants and stability ranges, but overall, GFP is a robust protein, (POPPENBORG *et al.*, 1997) showing pH stability between 5.5 to 12 (WARD *et al.*, 1982) and, although in its native form it undergoes denaturation above 37 °C, (WARD, 1981; WARD; BOKMAN, 1982) many recombinant GFP (as EGFP) can retain its fluorescence at 65 °C. Regarding the stability of GFP in the presence of solvents, it can withstand low concentrations of surfactants like 1 % m/v SDS solutions and even some concentrations of chaotropic agents. (BOKMAN; WARD, 1981; CUBITT *et al.*, 1995; ROTH, 1985) Also, GFP extraction studies show that GFP is stable in the presence of phosphate salts, (LI; BEITLE, 2002) PEG and NaPA-8000, (JOHANSSON *et al.*, 2008a) results that are aligned with the outcomes of this study.

In the PEG-600/NaPA-8000 + [Ch]Cl-based ABS, EGFP was completely recovered in the PEG-rich phase at all compositions studied. These results agree with previous works of Johansson *et al.*, (JOHANSSON *et al.*, 2008a) in which more than 90 % of a recombinant GFP was recovered in the PEG-rich phase using systems composed of PEG-400 + NaPA-8000 + Na_2SO_4 as adjuvant. The authors explained the protein preferential partition to the PEG-rich phase as a combination of hydrophobic and entropic effects, suggesting that due to the small size of PEGs (from 400 to 1500 g.mol⁻¹), the proteins are more easily accommodated in the PEG-rich than in the NaPA-rich phase. (JOHANSSON *et al.*, 2008a) This preferential partition to the PEG-rich phase (considered by the authors as the hydrophobic phase) was suggested to be a result of the reduction of entropic (due to the low PEG size) and the increase of enthalpic driving forces (due to the high PEG-concentration). (JOHANSSON *et al.*, 2008a) Herein, it was observed that the GFP fully partitioned to the PEG-600-rich phase, and considering **Table 8**, there was no significant difference in the phases water content (with an average difference of only 2.72 ± 1.56 wt%, the PEG-rich phase cannot be considered as the hydrophobic one), so further understanding of the GFP partition mechanism is needed. As observed in previous studies using PEG/NaPA-based ABS, the partition of proteins, (JOHANSSON *et al.*, 2008a, 2008b) irrespective of its hydrophilic/hydrophobic nature, was always for the PEG-rich phase. Considering that the EGFP pI is 6.2, (GURUNATHAN *et al.*, 2014) the protein is negatively

charged at pH 7 to 8 (pH of the systems). Thus, since at neutral pH the NaPA-8000 is negatively charged due to the presence of carboxylic acid groups in the principal polymeric chain, (JOHANSSON *et al.*, 2008b) electro-repulsive forces of NaPA-8000 over charged solutes govern the protein partitioning towards the PEG-rich phase. Therefore, more than the entropic effects with volume exclusion or hydrophobic effects, the complete extraction for the PEG-600-rich phase seem to be a result of the electro-repulsiveness nature of NaPA-8000 over the negatively charged GFP.

Similarly, in the PEG/potassium phosphate buffer-based ABS, the results from **Figure 33.B** also shown that EGFP completely partitioned to the PEG rich-phase. Thus, despite the EGFP pI 6.2, (GURUNATHAN *et al.*, 2014) and an overall negative surface charge at pH 7, EGFP still had a higher affinity to the low ionic strength (PEG-rich) phase, where the conductivities (mS.cm^{-1}) are 5.35 or 6.75 for both PEG/potassium phosphate buffer studied (for comparison conductivities of the phosphate buffer phases are 122.3 or 124.4 mS.cm^{-1}). Besides, both phases are very rich in water (ranging from 64.58 to 78.85 %), being the difference in water content (wt%) between the two coexisting phases lower than 5 %, **Table 8**. Therefore, this rules out the preferential partition of the hydrophilic EGFP due to the hydrophobic-hydrophilic effects (the PEG-rich phase being the most hydrophobic). Thus, since phosphate salts have a strong salting-out ability, (YI *et al.*, 2014) it seems that the salting-out phenomenon is the mechanism that controls the partition of EGFP on these ABS. Similar behavior was reported by Pereira *et al.* (PEREIRA *et al.*, 2016b) for the purification of ovalbumin from egg white using PEG-salt ABS, also dominated by the salting-out effect.

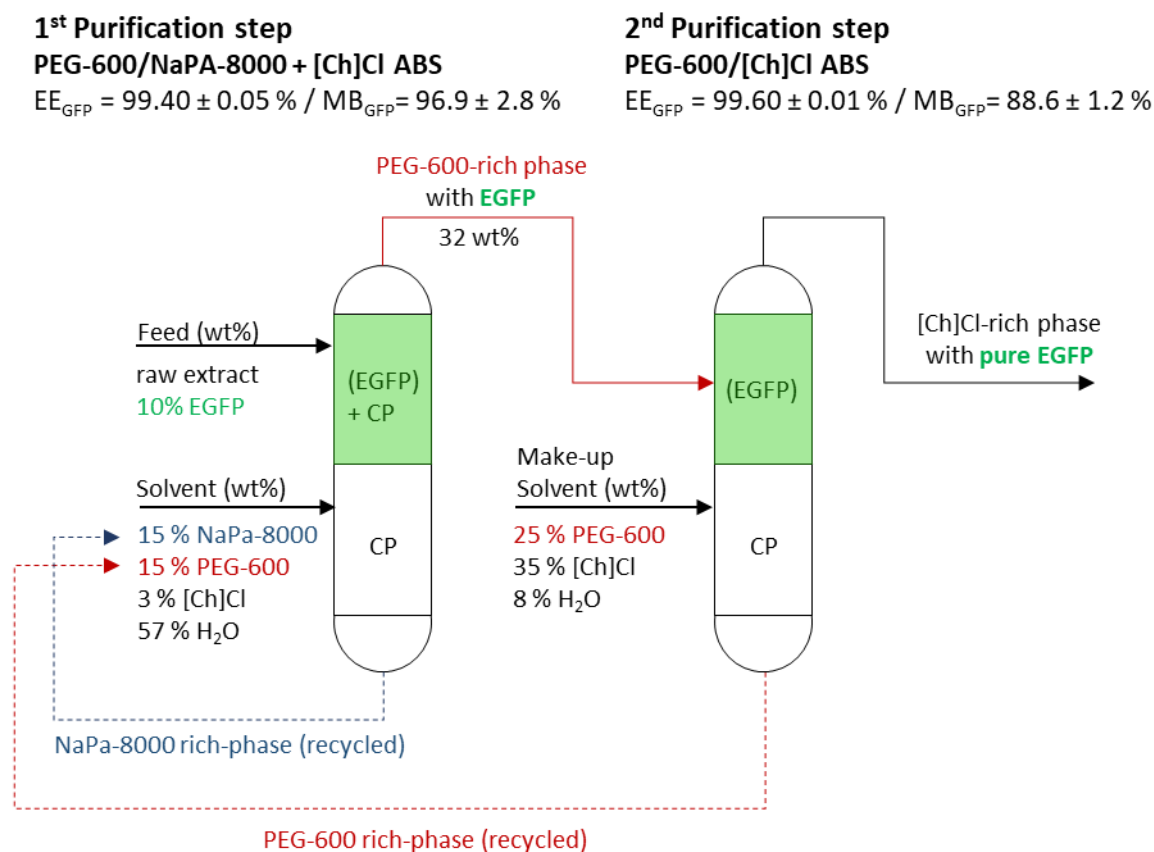
In opposition, on both PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, the EGFP was completely extracted for the [Ch]Cl-rich phase (EE_{EGFP} (%) greater than 99 %), as shown in **Figure 33.C**). These systems are also highly biocompatible with EGFP, exhibiting MB_{EGFP} (%) values closer to or higher than 90 % (**Figure 34.C**). It is important to notice that for both systems there was no apparent difference between the three concentrations of polymer/[Ch]Cl evaluated. Regarding the mechanisms that control the EGFP partition in the PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, these are also completely different from the previous ABS studied. In this system, EGFP was extracted into [Ch]Cl-rich phase (the most hydrophilic phase), considering the water content (wt%) of the phases. Additionally, the water content (wt%) was considerably different for each phase, being around 29-33 and 56-62 wt% for the [Ch]Cl phase in the PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, respectively, and 12-15 and 13-25 wt% for the polymer phase of the ABS, respectively. Thus, due to the hydrophilic

character of EGFP, the hydrophobic-hydrophilic interactions had a major role in the partition mechanism for both ABS, where the EGFP partitioned always for the phase with the highest water content. Previously, Salabat *et al.* (SALABAT *et al.*, 2010) performed some studies on the extraction of the protein using polymer/salt ABS (by combining PEG-600 and PPG-425 polymers and mgSO_4 , Na_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$ inorganic salts), demonstrating a selective extraction of hydrophobic protein for the polymeric phase and the partition of the hydrophilic proteins towards the salt-rich phase. The partition of GFP using other types of polymer/salt ABS was previously reported (PEG with salts mgSO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 or buffer $\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$), (JOHANSSON *et al.*, 2008a; LI; BEITL, 2002) but usually exhibiting low purification capacity (as will be further discussed in the next sections), thus requiring the association of other techniques like chromatography to significantly increase the purification levels.

4.5.2 Back-extraction of EGFP

Although the PEG/NaPA + [Ch]Cl-based ABS were effective to completely extract the EGFP from the supernatant of *E. coli* lysate, a purification using these systems was not completely satisfactory, as further detailed in section “4.5.3 Purification of EGFP”. The low levels of purification using PEG/NaPA and adjuvants were previously reported by Johansson *et al.*, (JOHANSSON *et al.*, 2008a) being highlighted that these yields could not be increased due to the impossibility of to perform a back-extraction of recombinant GFP with small size PEG polymers, which had the highest partition coefficients. However, as demonstrated in this study, EGFP could be completely extracted to opposite phases: (1st) for the PEG-600-rich phase for the systems composed of PEG-600/NaPA-8000 + [Ch]Cl and (2nd) for the [Ch]Cl-rich phase considering the PEG-600/[Ch]Cl-based ABS. Thus, the combination of these PEG/[Ch]Cl-based ABS with the PEG/NaPA + [Ch]Cl system can enable the back-extraction of EGFP, consequently increasing the EGFP purification levels. Considering this, a back-extraction procedure combining two extraction stages using these two ABS platforms was designed, with methods detailed in section “3.3.4.5 Back-extraction of EGFP”. The back-extraction was performed with a first purification step using the PEG-600/NaPA+[Ch]Cl, followed by a second fractionation using an ABS composed of [Ch]Cl and PEG-600, as sketched in **Figure 35**. Moreover, the EE_{EGFP} (%), MB_{EGFP} (%) for the first and second purification steps are also provided.

Figure 35 – EGFP back-extraction diagram representation showing the integration of a 1st purification using PEG-600/NaPA-8000 + [Ch]Cl-based ABS, followed by a 2nd purification step using a PEG-600/[Ch]Cl-based ABS. The EE_{EGFP} (%) and MB_{EGFP} (%) of each system are also presented. MB_{EGFP} (%) considering the overall back-extraction process. CP acronym corresponds to contaminant proteins.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

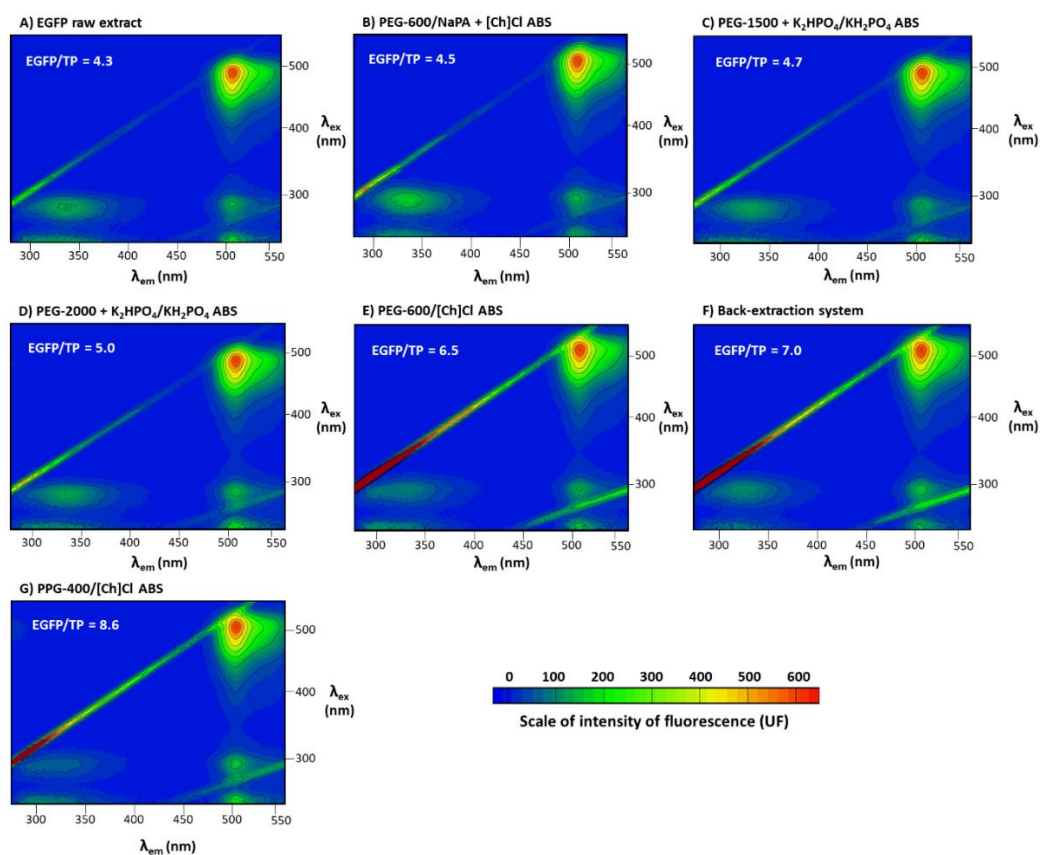
The overall EE_{EGFP} (%) and MB_{EGFP} (%) of the back-extraction procedure were $99.60 \pm 0.01 \%$ and $88.6 \pm 1.2 \%$, respectively, demonstrating that the integration of two purification units was effective on the EGFP recovery, with no significant losses (lower than 12 wt%). The results here obtained are quite interesting since they demonstrated the possibility of creating a back-extraction using PEG with a low molecular weight that completely extracted EGFP while also enhancing the purification yields, as shown in the next section.

4.5.3 Purification of EGFP

Considering the excellent extraction efficiencies obtained with all the ABS under study, as well as with the back-extraction procedure, the purification ability of each system was then assessed. To compare the fraction of EGFP on the total protein, and thus, to obtain an initial evaluation of the EGFP purity, a method using 3D fluorescence spectra was developed (as detailed section “3.3.4.2 Quantification of EGFP and total protein”). For this new method,

purified samples (phases where EGFP was extracted) and non-purified (EGFP raw extract obtained after lysis) were diluted to present the same concentration of EGFP ($12.7 \pm 0.2 \text{ mg.L}^{-1}$). Each solution was evaluated through the analysis of complete 3D fluorescence spectra, allowing the inspection of the EGFP fluorescence region (maximum fluorescence at λ_{ex} 488, λ_{em} 510 nm), and TP fluorescence region (residues of Trp, Tyr, and Phe, with a maximum fluorescence at λ_{ex} 276 nm, λ_{em} 336 nm) in the same figure. The ratio between the fluorescence intensity of EGFP and the fluorescence intensity of TP, EGFP/TP (directly proportional to the EGFP purity relative to other proteins in the sample) was then determined. The complete 3D spectra, and respective EGFP/TP ratios for the EGFP raw extract and the ABS' phases where the EGFP was extracted are shown in **Figure 36**.

Figure 36 – 3D Fluorescence spectra (units of fluorescence, UF) of **A)** EGFP raw extract and the EGFP-rich phase of the following ABS: **B)** PEG-600/NaPA-8000 + [Ch]Cl, **C)** PEG-1500 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, **D)** PEG-2000 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, **E)** PEG-600/[Ch]Cl, **F)** back-extraction and **G)** PPG-400/[Ch]Cl. All 3D fluorescence spectra correspond to a diluted sample with an EGFP concentration of $12.7 \pm 0.2 \text{ mg.L}^{-1}$. The 3D Fluorescence Spectra shows the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the intensity of fluorescence is given by the color scale from blue to red. The EGFP/TP is the ratio between the EGFP and total protein fluorescence intensities (UF).

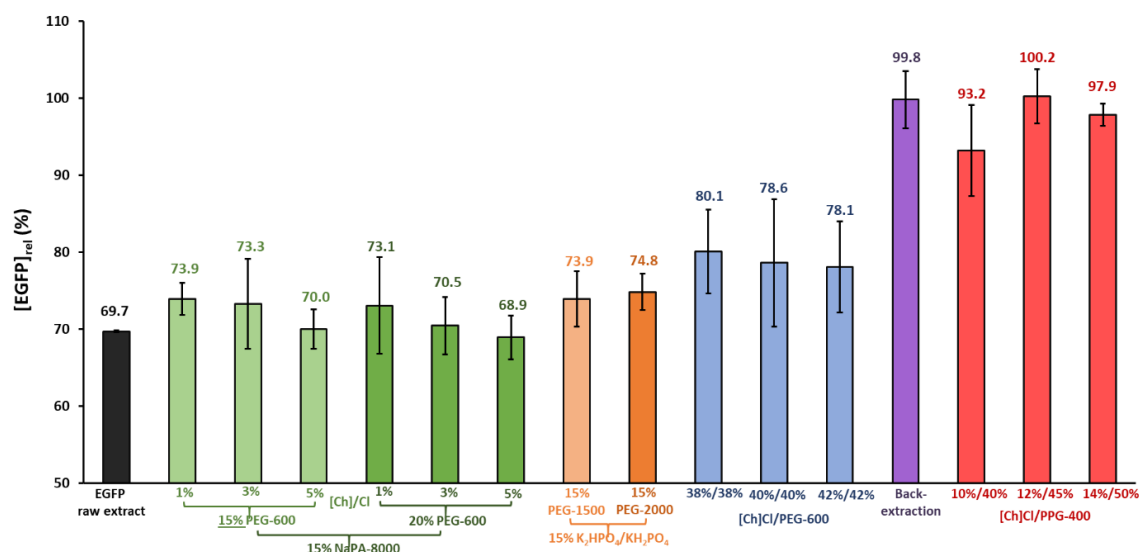


Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Figure 36 has two distinct regions for all the spectra (the first around λ_{ex} 276 nm and λ_{em} 336 nm and the second around λ_{ex} 488 nm and λ_{em} 510 nm), corresponding to the

fluorescence of the proteins containing the fluorescent amino acids and EGFP, respectively. Since the two regions are separated, it was possible to obtain a rough evaluation of the EGFP purity through the analysis of the EGFP/TP fluorescence ratio. Comparing the EGFP/TP ratio from the EGFP raw extract and those from the PEG-1500 + K_2HPO_4/KH_2PO_4 , PEG-2000 + K_2HPO_4/KH_2PO_4 , and PEG-600/NaPA-8000 + [Ch]Cl ABS, only a slight increase was observed, from 4.3 to 4.7, 5.0, and 4.5 respectively. On the other hand, the polymer/[Ch]Cl-based ABS exhibited a more significant increase in EGFP/TP ratio, namely, from 4.3 to 6.5, in the PEG-600/[Ch]Cl ABS, and from 4.3 to 8.6 in the PPG-400/[Ch]Cl ABS. Interestingly, the back-extraction procedure also enhanced the EGFP/TP ratio to 7.0. Although this comparison is based on the total intensity of fluorescence units, without the determination of EGFP and TP concentration through calibration curves, it suggests that the PEG/potassium phosphate buffer and PEG-600/NaPA-8000 + [Ch]Cl-based ABS have a limited purification ability, while the PEG-600/[Ch]Cl, PPG-400/[Ch]Cl ABS and back extraction systems are quite effective in removing the contaminant proteins, consequently improving the purification of EGFP. To quantify the purification capabilities of each system, the $[EGFP]_{rel}$ was calculated and the comparison of the average of $[EGFP]_{rel}$ (%) of three independent assays (and respective standard deviations) for all systems and EGFP raw extract is shown in **Figure 37**.

Figure 37 – Relative concentration of EGFP by TP ($[EGFP]_{rel}$ (%)) for the EGFP raw extract (■) and the EGFP-rich phases for the ABS composed of: 15 % PEG-600, 15 % NaPA-8000 + [Ch]Cl (1, 3 or 5%) (■); 20 % PEG-600, 15 % NaPA-8000 + [Ch]Cl (1, 3 or 5%) (■); 15 wt% PEG-1500 + 15 wt% K_2HPO_4/KH_2PO_4 (■); 15 wt% PEG-1200 + 15 wt% K_2HPO_4/KH_2PO_4 (■); [Ch]Cl/PEG-600 (■), back-extraction (■) and [Ch]Cl/PPG-400 (■). The results are the average of three independent experiments $\pm$ standard deviation.

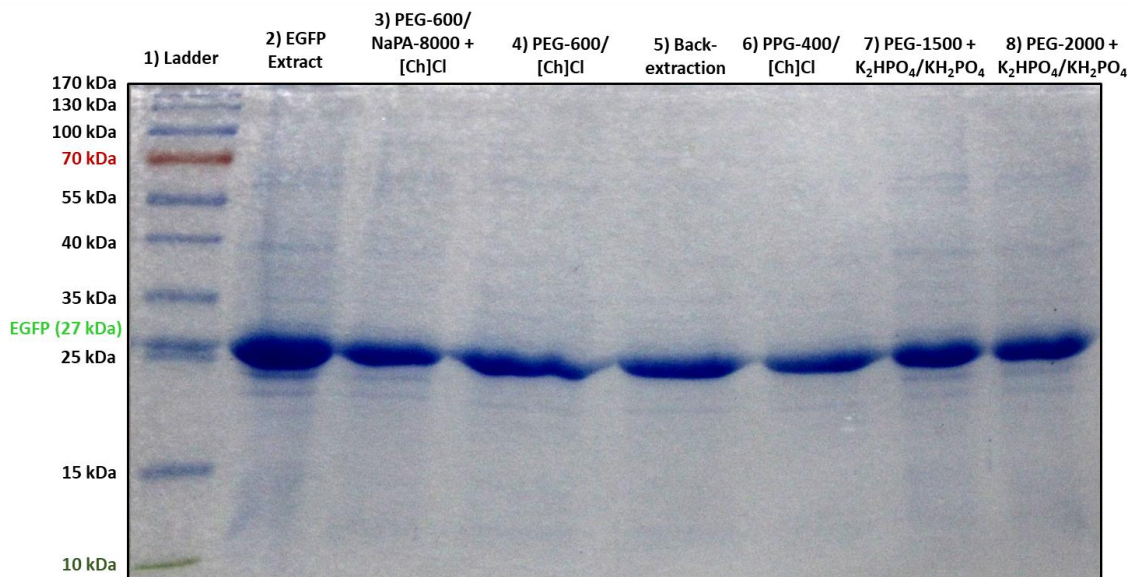


Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

From **Figure 37**, it is observed that the EGFP raw extract (black bar) after lysis has a $[EGFP]_{rel}$ (%) of almost 70 % of the total protein of the cell lysate, which is expected because

EGFP recovered is arising from recombinant *E. coli* BL21(DE3) and other proteins are also produced as a result of microorganism metabolism. As observed in the 3D fluorescence (EGFP/TP ratio) from **Figure 36**, the determination of $[EGFP]_{rel}$ (%) demonstrates that the use of the systems PEG/potassium phosphate buffer (PEG-1500 and PEG-2000, light and dark orange bars respectively) or the PEG-600/NaPA-8000 + [Ch]Cl (light and dark green, respectively) did not promote an increase on the EGFP purity, since there was no significant increase of $[EGFP]_{rel}$ (%) for any of the concentrations of the aforementioned ABS. For the PEG-600/[Ch]Cl-based ABS, the $[EGFP]_{rel}$ (%) showed only a slight increase (around 10%), with no apparent differences between the various concentrations of polymer and [Ch]Cl, which corresponds to limited potential for the purification of the target protein. However, if integrating the prior PEG-600/NaPA-8000 + [Ch]Cl-based system with a further extraction step using a PEG-600/[Ch]Cl ABS (purple bar), the EGFP purification yields are effectively enhanced, being attained $[EGFP]_{rel}$ (%) close to 100 %, revealing that the back-extraction procedure completely purifies the EGFP, removing the other contaminant proteins. This is consistent with the EGFP/TP ratio, increasing from 4.5 from the single-step extraction using PEG-600/NaPA-8000 + [Ch]Cl ABS to 7.0 with the back-extraction associating the aforementioned ABS with a following extraction with the PEG-600/[Ch]Cl-based ABS. Interestingly, the total purification of the EGFP was also achieved in a single purification step with the PPG-400/[Ch]Cl-based ABS, in which $[EGFP]_{rel}$ (%) values from 93 to 100 % were achieved. The high EGFP purification is in close agreement with the EGFP/TP ratio (an increase from 4.3 to 8.6), proving that this PPG-400/[Ch]Cl ABS is the best option to perform a quick and simple EGFP purification. To further confirm the purification, SDS-PAGE analysis was used, a method described in section “**3.3.4.9 Evaluation of EGFP purification by SDS-PAGE**”. To identify the protein present in the extracts after the use of each ABS, samples of each system and the raw extract containing 15 μ g of EGFP were added to the gel, and an SDS-PAGE run was performed, as shown in **Figure 38**.

Figure 38 – SDS-PAGE electrophoresis of the EGPF extract and the ABS containing the same mass of EGFP in each well (15 µg). Ladder (lane 1), EGFP raw extract (lane 2) and extracts after purification using the ABS: PEG-600/NaPA-8000 + [Ch]Cl (lane 3); PEG-600/[Ch]Cl (lane 4); back-extraction (lane 5); PPG-400/[Ch]Cl (lane 6); PEG-1500 + K₂HPO₄/KH₂PO₄ (lane 7); PEG-2000 + K₂HPO₄/KH₂PO₄ (lane 8). EGFP has 27 kDa and is represented by the strongest bands between 25-35 kDa.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

In the SDS-PAGE, a strong band around 27 kDa is indicative of EGFP, while bands in other regions are indicative of the presence of contaminant proteins. All samples present a strong band between 25 and 35 kDa, corresponding to the 27 kDa of EGFP. (HINK *et al.*, 2000) The SDS-PAGE results from **Figure 38** are in close agreement with the EGFP/TP ratio and [EGFP]_{rel} (%) data, suggesting that the 3D fluorescence spectra analysis can be used as a simple and quick method to evaluate the purification of proteins. Moreover, and as shown in the gel electrophoresis, the EGFP raw extract (lane 2) exhibited, in addition to the EGFP band, several bands outside the region between 25-35 kDa, these corresponding to contaminant proteins. While in the PEG-600/NaPA-8000 + [Ch]Cl (lane 3) and PEG/potassium phosphate buffer (lanes 7 and 8) the number and intensity of the impurities were not significantly reduced, these bands diminished in the PEG-600/[Ch]Cl ABS (lane 4), indicating a moderate purification potential. However, the best results were effectively obtained with the back-extraction system (lane 5) and PPG-400/[Ch]Cl (lane 6), in which the impurities bands are practically not visible, showing the purification potential of these ABS.

Considering the partition mechanisms discussed in the section “**4.5.1. EGFP extraction**”, it is possible to suggest that when the EGFP migration is mainly controlled by electro-repulsive forces (for the PEG-600/NaPA-8000 + [Ch]Cl ABS) or by salting-out

mechanisms (for the PEG/phosphate buffer ABS), all the other contaminant proteins are concentrated in the same phase, leading to low EGFP purification yields. On the other hand, when the partitioning mechanisms are based on the hydrophilicity-hydrophobicity balance, in both PEG-600/[Ch]Cl and PPG-400/[Ch]Cl, the EGFP purification is considerably more efficient. It seems that the protein contaminants (other cellular proteins) are less hydrophilic than EGFP, thus preferentially migrating into the most hydrophobic phase (PEG-rich phase). This is also clearly observed by the increase in the EGFP purification yields when the PEG-600 was replaced by the PPG-400 polymer (more hydrophobic), resulting in higher water content (wt%) difference among coexisting phases (as described in **Table 8**).

Previously, Ishihama *et al.* profiled more than 1000 proteins present in the cytosol of *E. coli*, which showed that the most abundant proteins in *E. coli* presented a Grand Average of Hydropathicity (GRAVY, using the mean relative hydrophobicity in Kyte-Doolittle scale) of -0.25, (ISHIHAMA *et al.*, 2008) while EGFP presents a GRAVY of -0.47, (lower GRAVY indicate that the proteins are more hydrophilic). The method for the calculations is described in section “**3.3.4.4 Determination of relative hydrophobicity of proteins**”. Although the most abundant proteins in *E. coli* are still considered hydrophilic (negative GRAVY values), EGFP is even more hydrophilic, and thus, this can explain the difference in the migration pattern in the polymer/[Ch]Cl ABS. This effect was also observed by Salabat *et al.* (SALABAT *et al.*, 2010) in a study with different polymer/salt-based ABS (polymers PPG-425 and PEG-600), where the PPG-425 systems had the best results for the selective separation of hydrophobic and hydrophilic proteins. Additionally, we have demonstrated that the integration of PEG/NaPA + [Ch]Cl and PEG/[Ch]Cl-based ABS, two protein purification platforms with different partitioning mechanisms, can be a feasible approach to increase the protein purification abilities.

To demonstrate the excellent GFP purification results achieved in this work, **Table 9** shows a comparison of the results obtained with the PPG-400/[Ch]Cl-based ABS and back-extraction system with the alternative GFP purification techniques already established in the literature (in general, liquid-liquid extractions in a single step or associated with back-extraction or chromatography techniques). As can be seen in **Table 9**, most alternative techniques for GFP purification either involve a series of liquid-liquid extractions and their association with chromatography techniques, or present low PF or K. Thus, both approaches proposed in this study are effective alternatives, allowing high GFP recovery and purity yields through the use of a simpler extraction platform using ABS. The results confirmed the ability of ABS composed

of biocompatible and low toxicity solvents to purify EGFP in a single or two-phase liquid-liquid extraction unit, however, to further assess their industrial potential, the recycling of the phase formers and EGFP polishing were also investigated.

Table 9 – Comparison of different liquid-liquid extraction approaches (single step liquid-liquid extraction, or association with back-extraction or chromatography techniques) for the purification of GFP. GFP recovery (%), [GFP]_{rel} (%) (indicating GFP purity), purification factor (PF), and partition coefficient (*K*) values are presented when available.

Purification techniques	GFP Recovery (%)	[GFP] _{rel} (%)	PF	<i>K</i>	Reference
PEG-600/NaPA-8000/[Ch]Cl ABS + back extraction with PEG-600/[Ch]Cl ABS	89 ^a	100			This study
PPG-400/[Ch]Cl ABS	92 ^a	100			
Multiple liquid-liquid organic extractions + Phenyl-Sepharose Chromatography	36		14		(YAKHNIN <i>et al.</i> , 1998)
PEG-6000/C ₆ H ₅ Na ₃ O ₇ ABS	91		3.3		(LI; BEITLE, 2002)
PEG-6000/C ₆ H ₅ Na ₃ O ₇ ABS + IMAC ^b	81		89		
PEG-6000/C ₆ H ₅ Na ₃ O ₇ ABS + back extraction with PEG-6000/Dextran T500 ABS	39		4.5		
PEG-6000/C ₆ H ₅ Na ₃ O ₇ ABS + back extraction with PEG-6000/Dextran T500 ABS + IMAC ^b	32		126		
PEG-6000/C ₆ H ₅ Na ₃ O ₇ ABS + back extraction with PEG-6000/NaH ₂ PO ₄ + Na ₂ HPO ₄ ABS	73		4		
PEG-6000/C ₆ H ₅ Na ₃ O ₇ ABS + back extraction with PEG-6000/NaH ₂ PO ₄ + Na ₂ HPO ₄ ABS + IMAC ^b	71		94		
PEG-3000/NaPA-8000/Na ₂ SO ₄ ABS	86			8.1	(JOHANSSON <i>et al.</i> , 2008a)
PEG-3000/NaPA-8000/Na ₂ SO ₄ ABS + back extraction with PEG/Na ₂ SO ₄ ABS	96 ^c			0.2 ^c	

^a For this study, MB_{GFP} (%) represents GFP Recovery (%).

^b IMAC - immobilized metal affinity chromatography.

^c calculated concerning the single extraction with PEG-3000/NaPA-8000/Na₂SO₄ ABS.

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Considering the great potential of the PPG-400/[Ch]Cl ABS to extract and purify EGFP from *E. coli* cell lysate, and to validate if this performance is maintained for other recombinant proteins produced by *E. coli*, the extraction of two other FP from *E. coli* cell lysate, Fezziwig Yellow Fluorescent Protein (YFP) and Red Fluorescent Protein (RFP), was also evaluated (as described in the section “**3.3.4.6 Extraction and purification of other recombinant fluorescent proteins produced by *E. coli* using PPG-400/[Ch]Cl ABS**”). As can be seen in **Table 10**, PPG-400/[Ch]Cl ABS was once more able to completely extract (Relative Extraction for the FP, RE_{FP} (%) > 99%) both proteins in the [Ch]Cl-rich phase. Additionally, to evaluate the purification potential of the system, 3D Fluorescence spectra of YFP and RFP raw extracts

(A and C, respectively) and YFP and RFP after the extraction step using PEG-600/[Ch]Cl ABS (B and D, respectively) were compared (**Figure 39**), and the FP/TP relation determined. As shown in **Figure 39**, there was a small increase in the FP/TP relation for the YFP (from 4.4 to 4.8) and a larger increase for the FP/TP relation of RFP (from 4.0 to 5.5) after extraction, suggesting that the PPG-400/[Ch]Cl ABS can extract efficiently and purify, to a certain extent, other recombinant fluorescent proteins from *E. coli* cell lysate. These relative ratios do not allow to directly compare the purification efficiency of the three fluorescent proteins (EGFP, YFP, and RFP), but indicate qualitatively that all of them were purified to a certain extent using the PPG-400/[Ch]Cl ABS. Although further studies are required to understand the degree of purification, these preliminary studies indicate that this system has a great potential to be used as a high-performance platform for the recovery and purification of different recombinant proteins produced by *E. coli*.

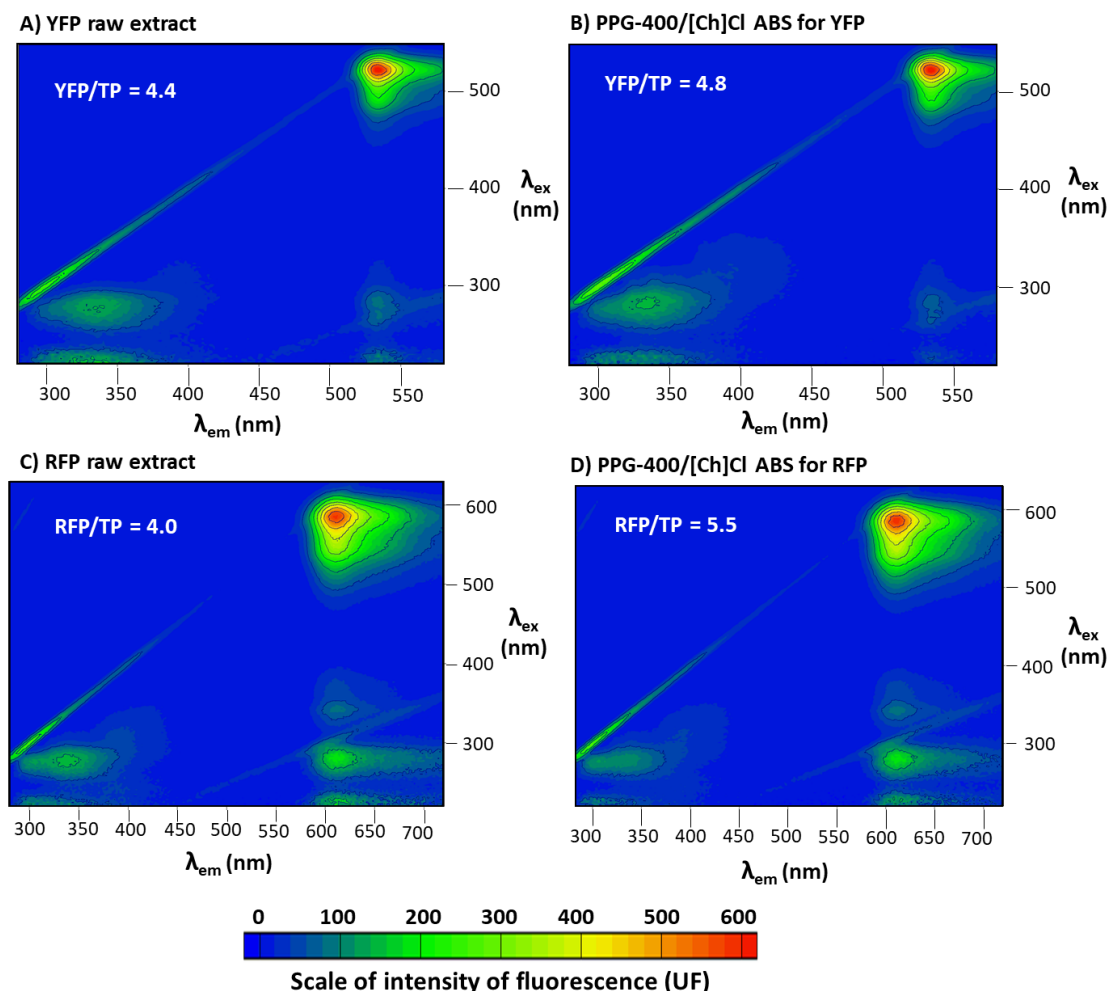
Table 10 – Weight fraction (wt%) and RE_{FP} (%) for extraction of YFP and RFP with PPG-400/[Ch]Cl ABS. Results correspond to the average of three independent assays and respective standard deviations.

PPG-400/[Ch]Cl ABS	Weight fraction (wt%)			RE_{FP} (%) ^a
	PPG-400	[Ch]Cl	FP extract	
YFP	45.03	12.07	9.98	99.83 ± 0.17
RFP	45.08	12.07	10.04	99.9 ± 0.07

^a Extraction in the [Ch]Cl-rich phase.

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Figure 39 – 3D Fluorescence spectra (units of fluorescence, UF) of **A)** YFP raw extract and **C)** RFP raw extract and FP-rich phase of the PEG-600/[Ch]Cl ABS for the purification of **B)** YFP and **D)** RFP. All 3D fluorescence spectra correspond to a diluted sample to present the same intensity of fluorescence in the peak of each FP (600 ± 8 UF). The 3D Fluorescence Spectra shows the excitation wavelengths in the Y-axis (λ_{ex}), the emission wavelengths in the X-axis (λ_{em}), and the intensity of fluorescence is given by the color scale from blue to red. The FP/TP relation (FP being YFP or RFP) is the ratio between the FP and total protein fluorescence intensities (UF).



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

4.5.4 Recycling of the ABS phase-former components and EGFP polishing

To address one of the critical issues regarding the application of ABS for biomolecules' purification, namely the use of high concentrations of relatively expensive phase-forming agents, (DOS SANTOS *et al.*, 2018a) a method for recycling the phase-forming components of the ABS with best EGFP purification performance, PPG-400/[Ch]Cl-based ABS, was attempted (methods described in “3.3.4.7 Recycling of the ABS phase-former components and EGFP polishing”). It is important to note that the successful recycling of the solvents decreases not only the costs but also the environmental impacts of the downstream process. After some initial tests, the ultrafiltration was chosen for the recycling and polishing studies.

Precipitation methods using cold acetone or ammonium sulfate were also attempted, but it failed due to the precipitation of some phase-forming components with the EGFP. Thus, an integrative approach for the extraction/purification with PPG-400/[Ch]Cl ABS and the respective recycling of phases through ultrafiltration was proposed.

After a first purification step using PPG-400/[Ch]Cl ABS, both top (PPG-rich) and bottom ([Ch]Cl-rich phase, containing EGFP) phases were recovered and submitted to ultrafiltration (with a 10 kDa membrane) processes. After filtration of the PPG-rich phase, the permeate containing the PPG-400 aqueous solution free of contaminant proteins, which were removed in the retentate, was then recycled in the liquid-liquid extraction unit. Similarly, the [Ch]Cl-rich phase was also filtered and then, the [Ch]Cl aqueous solution recovered in the permeate (without EGFP) was also recycled in the liquid-liquid extraction unit. It is important to note that extra amounts of ABS phase-forming components can be added in the system, to fulfill some solvent losses occurring in the process. The pure EGFP recovered in the retentate was collected and it can be either used in the liquid for the formulation of some bio-product of interest or go for further processing, for example, freeze-drying or encapsulation procedures. The weight fraction (wt%), EE_{EGFP} (%), MB_{EGFP} (%), and $[EGFP]_{rel}$ (%) for the PPG-400/[Ch]Cl-based ABS before and after solvent recycling using ultrafiltration were determined, as compiled in **Table 11**. Additionally, the purity of the polished GFP (recovered after the ultrafiltration step) was evaluated employing the $[EGFP]_{rel}$ (%). Additional information about the recycling process (mass of top and bottom phases recovered after ultrafiltration, the total mass of the PPG-400/[Ch]Cl ABS before and after ultrafiltration, and recovery percentage (wt%) of the recycled ABS) is presented in **Table 12**.

Table 11 – Weight fraction (wt%), EE_{EGFP} (%), MB_{EGFP} (%), and $[EGFP]_{rel}$ (%) for the PPG-400/[Ch]Cl ABS before and after solvent recycling using ultrafiltration. $[EGFP]_{rel}$ (%) of the polished EGFP, recovered after the ultrafiltration step is also presented. Results correspond to the average of three independent assays and respective standard deviations.

PPG-400/[Ch]Cl ABS	ABS Composition			EE _{EGFP} (%)	MB _{EGFP} (%)	[EGFP] _{rel} (%) ^a
	Weight fraction (wt%)					
Before ultrafiltration	PPG-400	[Ch]Cl	EGFP raw extract			
	44.96	12.04	10.05	99.14 ± 0.06	94.4 ± 3.0	98.2 ± 3.8
After recycling ^b	Recycled PPG-rich phase	Recycled [Ch]Cl-rich phase	EGFP raw extract			
	52.74	37.19	10.07	99.11 ± 0.09	93.0 ± 2.0	98.1 ± 2.9
EGFP Polishing after ultrafiltration						98.5 ± 1.0

^a $[EGFP]_{rel}$ (%) of extract: 69.7 ± 0.1 %. ^b Solvent recovery: 60.7 ± 2.2 wt%.

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Table 12 – Weight (g) of the recovered top and bottom phases after the ultrafiltration of the PPG-400/[Ch]Cl ABS phases; total mass of the PPG-400/[Ch]Cl ABS before and after the solvent recycling process, with the respective recovery percentage (wt%).

ABS	Weight (g)		
	Mass of each phase recovered after ultrafiltration		
	PPG-400-rich phase (top)	[Ch]Cl-rich phase (bottom) ^a	
PPG-400/[Ch]Cl	3.5667 ± 0.0587	6.0282 ± 0.1015	
	Total mass of the ABS before recycling	Total mass of the ABS after recycling	Recovery (wt%)
PPG-400/[Ch]Cl	10.0203 ± 0.0129	6.7685 ± 0.2480	60.7 ± 2.2

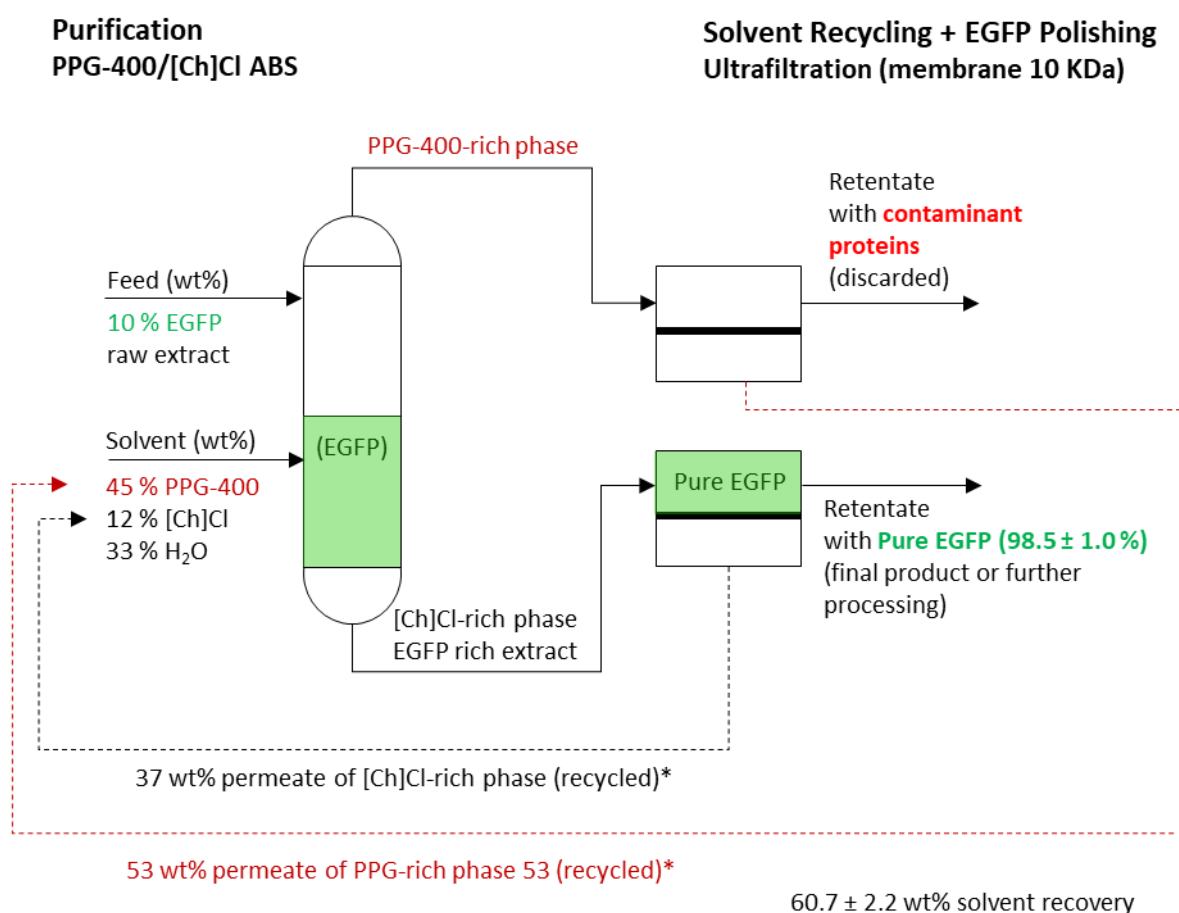
^a Not all the content of the bottom phase was used in the formation of the recycled ABS; 2.5205 ± 0.2346 g of the recovered bottom phase was added in 3.5667 ± 0.0587 g of the recycled top phase until the formation of a new ABS.

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

As can be seen in **Table 11**, the values of EE_{EGFP} (%), MB_{EGFP} (%), and $[EGFP]_{rel}$ (%) for the PPG-400/[Ch]Cl ABS were maintained even after solvent recycling (60.7 ± 2.2 wt% of the solvents were recovered). These results confirm that the reuse of the phase-forming agents after ultrafiltration does not compromise the purification capabilities of the ABS. However, it is important to note that it is also possible to make up the solvents lost in the process, which can potentially increase the solvent recycling capacities of this system. Also, it was possible to obtain a concentrated aqueous solution of pure EGFP, 98.5 ± 1.0 %. It is important to notice that the low molecular residual impurities that could persist after the extraction with ABS are also removed after this polishing step. Therefore, a highly pure EGFP was obtained after

ultrafiltration, with both PPG-400 and [Ch]Cl effectively recycled and reused to form a new PPG-400/[Ch]Cl-based ABS, allowing a closed and sustainable purification process. Summing up, an integrative process for the purification of EGFP with PPG-400/[Ch]Cl ABS and the respective recycling of the phases through ultrafiltration was herein proposed, as schematized in **Figure 40**.

Figure 40 – Diagram of the integrative process developed for the purification of EGFP using PPG-400/[Ch]Cl ABS and the recycling of the phase-forming agents using ultrafiltration. *Composition (wt%) of the new ABS after solvent recycling.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

As can be seen in **Figure 40**, ABS composed of polymers and salts associated with ultrafiltration are an effective, simple, and biocompatible platform for the purification of EGFP. Additionally, considering the low toxicity of the phase-forming agents and the possibility of recovering more than 60 % of the ABS components, the process is a greener and simpler alternative to conventional purification methods, like organic extraction and chromatography.

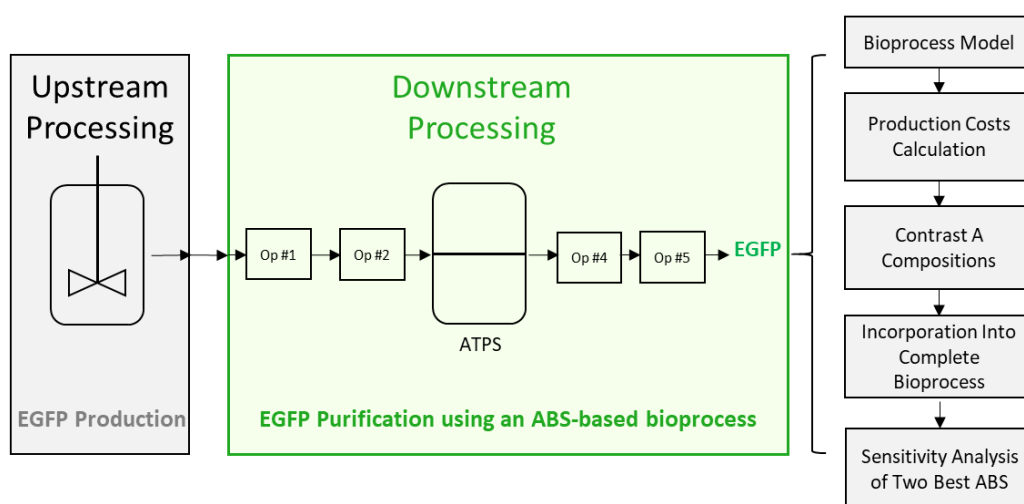
In this section, it was possible to develop a biocompatible and effective platform for the recovery and purification of EGFP from cell lysate of a recombinant *E. coli* BL21, associating

PPG-400/[Ch]Cl ABS and ultrafiltration to recover the protein with > 99 % purity and 60 % recycling of phase-forming agents. Hence, this work demonstrated that [Ch]Cl-based ABS can be an excellent sustainable platform for the purification of recombinant proteins from cell lysates.

4.6 ECONOMIC ANALYSIS OF EGFP PRODUCTION AND PURIFICATION WITH ABS

This work performed bioprocess modeling coupled with economic analysis using the software Biosolve to evaluate the potential and commercial applicability of the ABS for EGFP purification to verify the economic feasibility of the ABS developed in the study of the previous section, “**4.5 Purification of EGFP using aqueous biphasic systems**”. This research analyzed the fourteen ABS to discriminate through production costs while also incorporating the concept of product purity into the calculations. **Figure 41** shows an overview of the stages of this work:

Figure 41 – Overview of the stages of the economic analysis of EGFP production and purification with ABS.



Source: Produced by the author and collaborators and published (adapted) in (TORRES-ACOSTA *et al.*, 2021).

As presented in **Figure 41**, this research evaluated both the upstream and downstream processing for the production and purification of EGFP. In the first step of the bioprocess modeling, the researchers calculate the production costs. Then, the group contrasted different compositions of the ABS to select systems with the best cost-benefit between presenting lower cost and purifying EGFP. The two best systems (the PEG-1500/phosphate buffer and PPG-400/[Ch]Cl ABS) were then placed in a full bioprocess at different scale models (1 to 100 L) to elucidate the viability of applying ABS at a large scale. Although the results showed that the PEG-1500/phosphate buffer ABS exhibited the lowest costs (between USD $3.5 \times 10^3 \cdot \text{g}^{-1}$ at 1 L and USD $0.33 \times 10^3 \cdot \text{g}^{-1}$ at 100 L), for further developments, the inclusion of an ABS granting a higher purity was desirable for the development of simpler bioprocesses. Therefore, as a third approach in this work, a sensitivity analysis was performed to determine the impact of varying different model parameters (recovery yield, material costs discount, and production titer), to elucidate the circumstances under which the PPG-400/[Ch]Cl ABS could overcome the

production costs of the traditional PEG-based ABS. The results indicate that the best cost-effectiveness approach was to improve the production titer (although it can affect all ABS studied), as an increase from 1.33 to 3.8 g/L is enough for the PPG-400/[Ch]Cl ABS to be less expensive than the traditional system at all analyzed scales.

Finally, **Table 13** presents the ABS from the study with the best cost-benefit for applications that require or not high purity levels for EGFP:

Table 13 – Composition and advantage of Aqueous Biphasic System s(ABS) from this study with the highest cost-benefit for applications that require or not high purity levels.

ABS with best cost-benefit for:	Advantage	ABS
Applications which do not demand high EGFP purity (70-80 % purity)	Lower-cost ABS	PEG-1500/Phosphate buffer
Applications that demand high EGFP purity (> 95 % purity)	Highest purity in a single unit	PPG-400/[Ch]Cl

Source: Produced by the author with results from the collaboration article (TORRES-ACOSTA *et al.*, 2021).

As presented in table **Table 13**, the ABS with best for applications that allows EGFP with lower purity levels (70 to 80 % purity) was the PEG-1500/Phosphate buffer ABS because this ABS presented the lowest cost among the systems evaluated. However, for applications that demand very high purity levels (*e.g.*, medical uses), the system with the best cost-benefit was the PPG-400/[Ch]Cl, considering this system had the lowest cost when compared with other systems that presented purification levels above 90 % (*e.g.*, the back-extraction system) and allowed very high purification levels (> 97 %) in a single unit.

This study demonstrates that ABS can greatly reduce EGFP manufacturing costs, which can potentially help to popularize new applications of fluorescent proteins that are currently mostly restricted to research kits due to their high prices.

The complete work, entitled “Economic analysis of the production and recovery of green fluorescent protein using ATPS-based bioprocesses”, was published at **Separation and Purification Technology**, v. 254, p. 117595, 2021, DOI: [10.1016/j.seppur.2020.117595](https://doi.org/10.1016/j.seppur.2020.117595). (TORRES-ACOSTA *et al.*, 2021)

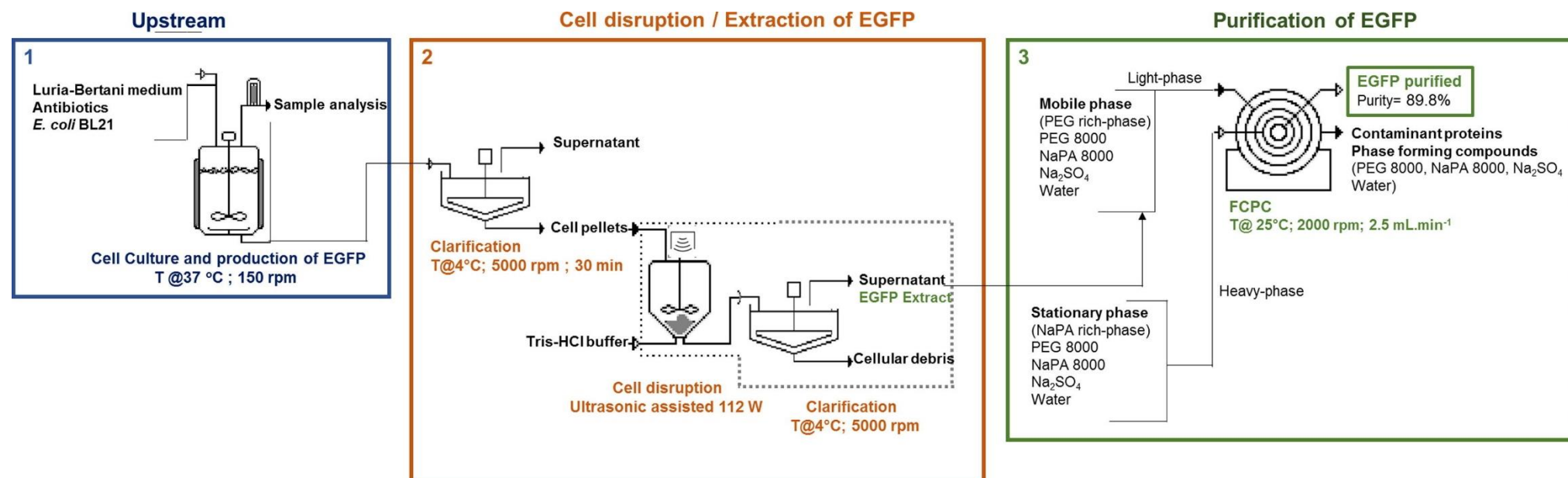
4.7 SCALE-UP OF EGFP PURIFICATION WITH ABS

4.7.1 Fast Centrifugal Partition Chromatography (FCPC) for scale-up of EGFP purification

To prove the feasibility of applying ABS for the purification of EGFP at larger scales, this work aimed to develop automatized industrially-compatible purification platforms to improve EGFP production. This research studied the continuous purification of EGFP by applying ABS composed of PEG-8000, NaPA-8000, and Na_2SO_4) as an electrolyte. An initial screening was carried by increasing the electrolyte content in the ABS. The increase of this condition has demonstrated an increase in the EGFP partition for the PEG-rich phase. The most efficient ABS and, at the same time, with the most appropriate conditions, namely the system composed of 15 wt% PEG 8000 + 4.5 wt% NaPA 8000 + 2.5 wt% Na_2SO_4 was chosen. After a careful selection, it was applied in the FCPC. After optimization, the best operational conditions were identified, *i.e.* a flow rate of $2.5 \text{ mL} \cdot \text{min}^{-1}$ and rotation speed of 2000 rpm at ascending mode, and the best results obtained, resulting in 89.93 % purification and a recovery yield of 82.3 %, confirming the potential of FCPC for the continuous purification of EGFP.

Considering the excellent purification performance, a possible integrated platform for the purification of EGFP was proposed in **Figure 42**, including the units of production and extraction and purification using ABS applied in FCPC.

Figure 42 – Process diagram of the EGFP purification process using ABS in FCPC.



Source: Produced by collaborators and published in the collaboration article (SOARES *et al.*, 2021).

In conclusion, the values of purification in this study support the high potential of ABS for the purification of EGFP using FCPC in a continuous regime from a complex matrix extracted from recombinant *E. coli*, while allowing the technology scale-up.

The complete work, entitled “Purification of green fluorescent protein using fast centrifugal partition chromatography”, was accepted for publication in the **Separation and Purification Technology**, v. 257, p. 117648, 2021, DOI: [10.1016/j.seppur.2020.117648](https://doi.org/10.1016/j.seppur.2020.117648). (SOARES *et al.*, 2021)

4.7.2 Oscillatory Flow Reactor (OFR) for scale-up of EGFP purification

As another alternative for the scale-up of EGFP purification with ABS, the use of an OFR was also attempted in addition to the FCPC. The scale-up of the ABS using OFR was studied in batch and continuous modes, for different amplitudes (A_a) and frequencies of agitation (f_a). The injection velocity (V_{inj}) and times of separation were also evaluated for the continuous system.

The main objective of the study was to increase the scale of the PPG-600/[Ch]Cl ABS using the RFO in batch and continuous modes. Initial tests with water in the RFO allowed the definition of the A_a (2 cm) and f_a (1 and 3 Hz) for the experiments, as well as the V_{inj} (18 and 36 mL.min⁻¹) for the continuous systems. To verify the efficiency of EGFP extraction using the ABS, the fluorescence of the top and bottom phases (after phase separation) was performed and the extraction efficiencies (EE_{EGFP}) were calculated. The purification was suggested by the relation of the FI of EGFP over the FI of TP (FI EGFP/FI TP). For the continuous system, the times required to separate the phases of the systems were also evaluated. **Table 14** presents the main results of the work:

Table 14 – Parameters [amplitudes (A_a), frequencies of agitation (f_a), and injection velocity (V_{inj})] and results [EGFP Extraction Efficiency (EE_{EGFP}), Fluorescence Intensity (FI) of EGFP over the FI of total proteins (FI EGFP/FI TP) and separation times] for the Oscillatory Flow Reactor (RFO) experiments for the scale-up of EGFP purification (from 5 to 50 g) using PPG-400/[Ch]Cl ABS.

	f_a (Hz)	A_a (cm)	V_{inj} (mL.min ⁻¹)	EE_{EGFP} (%)	FI EGFP/FI TP	Separation time (min)
EGFP cell lysate	—	—	—	—	0.98	—
Batch 1	1	2	—	99.9	—	—
Batch 2	3	2	—	99.9	—	—
Continuous 1	1	2	18	99.9	1.75	75
Continuous 2	3	2	18	99.9	1.54	135
Continuous 3	1	2	36	99.9	1.61	100

Source: Produced by the author.

All batch and continuous modes showed complete EGFP extraction (above 99.9 %) for the [Ch]Cl phase (top phase), in accord with the laboratory scale experiments. All three continuous systems increased the FI EGFP/FI TP when compared with the EGFP lysate, indicating EGFP purification. Continuous 1 had the highest FI EGFP/FI TP values, followed by 3 and 2, respectively. The phase separation times of the continuous systems increased at higher f_a , A_a , and V_{inj} , with the shortest separation time (175 min) achieved with the ABS mixed at a f_a of 1 Hz, A_a of 2 cm, and V_{inj} of 18 mL.min⁻¹ (Continuous 1) while the longest separation times (135 min) were obtained in the system with a f_a of 3 Hz, A_a of 2 cm and V_{inj} of 18 mL.min⁻¹ (Continuous 2). The system with f_a of 1 Hz, A_a of 2 cm, and V_{inj} of 36 mL.min⁻¹ (Continuous 3) had an intermediate separation time of 100 min.

In conclusion, OFR can be used as an alternative platform for EGFP extraction using ABS, since all systems allowed complete EGFP extraction in one of the equilibrium phases, scaling-up the ABS 10 times (from 5 g to 50 g) while still preserving the extraction efficiencies. Different frequencies, amplitudes, and injection velocities influenced only the phase separation times, but not the extraction capacity. However, long separation times were observed (above 1 h) for the continuous setting, and more studies are still required to optimize the process for industrial applications.

5 CONCLUSIONS

In conclusion, this work successfully achieved the main goals of increasing the understanding regarding EGFP properties, structure, and behavior at different conditions, as well as developing novel sustainable platforms for the stabilization and purification of proteins. The topics below present the specific conclusions and outputs for each stage of the research:

Stage 1: The EGFP expression in recombinant *E. coli* BL21 (DE3) was improved by increasing EGFP production yields while reducing the concentration of the costly inducer used in the process.

Stage 2: The fluorescent properties and structural studies of EGFP allowed to reveal of a new fluorescence peak (attributed to the protonated state) and helped to elucidate the contradiction regarding the two conformations of EGFP for an alleged homogeneous fluorescence spectrum.

Stage 3: The studies of the fluorescence activity and structure of EGFP at different pH unraveled the reversible and irreversible activity of the protein, highlighting attractive features for the development of novel neutral-to-acidic pH-biosensors compatible with biological systems.

Stage 4: The fluorescence and structural assays of EGFP in the presence of ILs aqueous solutions (with or without stress) indicated they had different impacts on EGFP fluorescence activity considering their nature and chain-length. Particularly, cholinium ILs with longer anion chain-lengths and imidazolium ILs with shorter cation chain-lengths promoted greater stabilization of EGFP (especially [Ch][Oct] and [Ch][Dec]). Besides, the concentration of solutions also played a role in determining the potential of ILs to protect EGFP. For example, only the two highest concentrations (0.250 and 0.500 mol.L⁻¹) of [C₁₂mim]Cl decreased EGFP fluorescence activity and had a more pronounced impact on the increase of [Ch][Oct] enhancement of EGFP FI. However, for most of the ILs studied (imidazolium with exception of [C₁₂mim]Cl and [Ch][Ac], [Ch][But] and [Ch][Dec]), there was no difference in the effect from concentrations 0.025 to 0.500 mol.L⁻¹. The long-term studies demonstrated some IL solutions could triple the preservation of the activity of EGFP at room temperature, showing great potential for the future development of stabilizing formulations for biomolecules of commercial application.

Stage 5: In the purification studies, it was possible to develop a sustainable and effective process for the recovery and purification of EGFP from cell lysate of a recombinant *E. coli*

BL21. In general, all ABS allowed the full partition of the target protein (above 99 % of extraction efficiency) in the PEG-rich or [Ch]Cl-rich-phase, depending on ABS. The systems composed of PPG-400 and [Ch]Cl, in addition to the excellent extraction performance, also had a great capacity to purify EGFP from contaminant proteins in a single step process (EGFP purity yields close to 100 %). The ABS PEG-600/NaPA-8000 + [Ch]Cl and PEG-600/[Ch]Cl did not exhibit high EGFP purification aptitudes when used as a single step, but when integrated into a two-step purification (as a back-extraction procedure), also allowed to obtain an almost 100 % pure EGFP. Additionally, it was possible to improve the economic and environmental sustainability of the PPG-400/[Ch]Cl-based ABS by recycling 60 % of the phase-forming components through the integration of an ultrafiltration unit after the extraction phase. Besides, the PPG-400/[Ch]Cl-based ABS could even recover and purify other fluorescent recombinant proteins (YFP and RFP) from *E. coli* cell lysate. Therefore, [Ch]Cl-based ABS is a feasible and promising sustainable platform for the purification of proteins from complex medium (such as fermented broth or cell lysates).

Stage 6: The bioprocess and economic model of the best ABS for the purification of GFP allowed to find two systems with the highest potential for commercial applications, namely, PEG-1500/Phosphate buffer (lower-cost ABS, best for applications which do not demand high purity) and PPG-400/[Ch]Cl (the highest purification ABS in a single step, best for applications which require high purity).

Stage 7: The purification of EGFP with ABS was successfully scaled-up, namely, by using FCPC and the ABS composed of PEG 8000/NaPA 8000 + Na₂SO₄ ABS to purify EGFP in a continuous mode. After optimizing the operational conditions (flow rate of 2.5 mL.min⁻¹ and rotation speed of 2000 rpm at ascending mode), it possible to obtain an EGFP with 89.93 % purity and a recovery yield of 82.3 %. The high purification and recovery of EGFP in FCPC demonstrate the ability of this system to increase the scale of purification of proteins using ABS. On the other hand, the scale-up of EGFP purification using PPG-400/[Ch]Cl ABS and OFR was able to maintain the extraction efficiencies and purification of the protein from a 5 g scale to a 50 g scale, showing the potential of this system for the recovery of macromolecules; however, it is still necessary to improve the separation times of the system to allow its industrial application.

As a general conclusion, with this Thesis project, it was possible to demonstrate that the study of EGFP properties and stability, allied with the development of sustainable platforms for the stabilization and purification of EGFP, can broaden its application and large-scale use and

potentially be applied to other protein-based products. The use of EGFP in this Thesis was a great proof-of-concept to demonstrate the potential of novel and more sustainable technologies, in particular using ILs, to stabilize and purify proteins of commercial interest, especially for future use in the manufacturing and formulation of biopharmaceuticals. These innovative solutions could help to solve two of the main drawbacks of biopharmaceuticals, namely their instability at room temperature (that hinders their transport, storage, and application) and their egregious prices (mainly caused by a complex and costly downstream processing). Therefore, these new simple and more environmentally-friendly platforms can potentially support the democratization of access to biotechnological products and guide the path towards a more circular and inclusive economy.

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CHAPTER 2

LIST OF PUBLICATIONS

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DOS SANTOS, N. V.; DE CARVALHO SANTOS-EBINUMA, V.; PESSOA JUNIOR, A.; PEREIRA, J. F. B. Liquid-liquid extraction of biopharmaceuticals from fermented broth: Trends and future prospects. **Journal of Chemical Technology & Biotechnology**, v. 93, n. 7, p. 1845–1863, 2018.

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Awards and mentions

“Mention of Congratulations and Praise” from the City Hall of Rio Claro for her research, City Hall of Rio Claro, Brazil, 2019.

Best poster for Carolina Falaschi Saponi (undergraduate student co-supervised by Nathalia Vieira dos Santos), awarded by ACS Sustainable Chemistry & Engineering for presentation at the “8th International Congress on Ionic Liquids” (COIL-8), Beijing, China, 2019 (co-author).

Best undergraduate student panel at the IX Scientific Congress of UNESP by student Carolina Falaschi Saponi, Pharmaceutical Journey of UNESP, 2019 (co-author).

Liquid–liquid extraction of biopharmaceuticals from fermented broth: trends and future prospects

Nathalia Vieira dos Santos,^a Valéria de Carvalho Santos-Ebinuma,^a Adalberto Pessoa Junior^{b*} and Jorge Fernando Brandão Pereira^{a*} 

Abstract

Biopharmaceuticals are one of the most important groups of biotechnological products, representing one-quarter of all pharmaceutical sales and providing suitable and efficient medical care for many previously untreatable diseases. However, their production and purification usually require complex processes, resulting in extremely expensive final products. Thus, the development of newer, simpler and cheaper methods for biosynthesis and purification of these biocompounds, as well as proper integration between these stages, are crucial to reduce their commercial costs and to increase the scale of biopharmaceuticals' medical use. One solution for this concern relies on the proper integration between upstream and downstream processing applying liquid–liquid extraction systems as an intermediate stage. Recently, several works reported that a proper choice of liquid–liquid systems such as aqueous biphasic systems (ABS) can be a suitable platform to simplify, reduce the number of the downstream stages, improve the recovery and purification yields and, consequently, decrease biopharmaceuticals' manufacturing costs. This review will explain the general concept of biopharmaceuticals, their main production and purification methods, particularly, exploring the use of ABS as effective and integrative platforms for their recovery and purification, and providing further insights into the future trends and prospects in the field.

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Keywords: biopharmaceuticals; fermentation; liquid–liquid extraction; aqueous biphasic systems; downstream process

NOTATION

(NH ₄) ₂ SO ₄	ammonium sulfate
[C ₂ mim]Cl	1-ethyl-3-methylimidazolium chloride
[C ₄ mim]Cl	1-butyl-3-methylimidazolium chloride
[C ₄ mim][CH ₃ SO ₃]	1-butyl-3-methylimidazolium methanesulfonate
[C ₄ mim][N(CN) ₂]	1-butyl-3-methylimidazolium dicyanamide
[C ₆ mim]Cl	1-hexyl-3-methylimidazolium chloride
[C ₈ mim]Cl	1-octyl-3-methylimidazolium chloride
[C ₄ mpyr]Cl	1-butyl-3-methylpyridinium chloride
[Ch]Cl	cholinium chloride
[Ch][Bic]	cholinium bicarbonate
[Ch][Bit]	cholinium bitartrate
[N _{2,2,2,2}]Br	tetraethylammonium bromide
[N _{4,4,4,4}]Br	tetrabutylammonium bromide
[N _{4,4,4,4}][BES]	tetrabutylammonium 2-(bis(2-hydroxyethyl)amino)ethanesulfonate
ABS	aqueous biphasic systems
AMTPS	aqueous micellar two-phase systems
ATPS	aqueous two-phase systems
BHK	baby hamster kidney cells
C ₆ H ₇ NaO	monosodium citrate
CHO	chinese hamster ovary cells
COSMO-RS	conductor like screening model for real solvents
DES	deep eutectic solvents
DOE	design of experiments

<i>E. coli</i>	<i>Escherichia coli</i>
EOPO	copolymer of ethylene oxide and propylene oxide
EU	European Union
Fab'	antigen-binding fragment
FDA	Food and Drug Administration
GFP	green fluorescent protein
HEK293	human cells HEK293
hGH	human growth hormone
hGHA	human growth hormone antagonist
HIC	hydrophobic interaction chromatography
IGF-I	human insulin-like growth factor-I
IgG	immunoglobulin G
IL	ionic liquids

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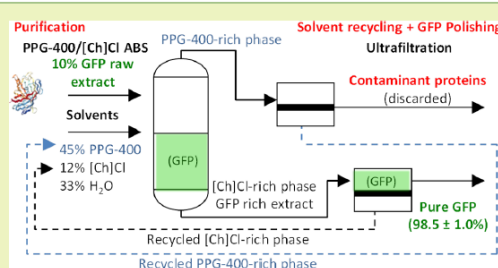
Aqueous Biphasic Systems Composed of Cholinium Chloride and Polymers as Effective Platforms for the Purification of Recombinant Green Fluorescent Protein

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Supporting Information

ABSTRACT: Green fluorescent protein (GFP) has excellent properties as a biosensor and biomarker; however, its widespread use is limited by its purification costs. Alternative low-cost purification techniques can overcome this issue. The aim of this work was to evaluate aqueous biphasic systems (ABS) composed of cholinium chloride ([Ch]Cl) and different polymers as effective platforms to recover GFP from cell lysate of recombinant *Escherichia coli* BL21. All systems completely extracted GFP from cell lysate (>99%) into the polymeric- or [Ch]Cl-rich phases. In general, [Ch]Cl-based ABS allowed a good purification capacity (GFP 80–100% pure), with the best results (approximately 100% pure GFP) achieved with a polypropylene glycol (PPG)-400/[Ch]Cl ABS in a single-step extraction or in a two-step extraction (back-extraction) by the integration of a polyethylene glycol (PEG)/sodium polyacrylate+[Ch]Cl ABS with a following stage using a PEG/[Ch]Cl-based ABS. Additionally, to demonstrate the potential of the PPG-400/[Ch]Cl ABS in downstream processing, solvent recycling and GFP polishing were carried out using ultrafiltration. Finally, the capacity of the PPG-400/[Ch]Cl ABS to extract other fluorescent proteins was also confirmed. The results clearly demonstrated that the PPG-400/[Ch]Cl ABS can be applied in downstream processing for the purification of proteins, not only enhancing purification yields but also providing simpler, quicker, cost-effective, and biocompatible processes.

KEYWORDS: Aqueous biphasic systems, Cholinium chloride, Green fluorescent protein, Purification, Integrated downstream process



INTRODUCTION

Recent advances in biotechnology have pushed the discovery of many bioproducts for diagnostic and therapeutic use, allowing medical care for previously untreatable diseases. However, medical applications require high levels of purity and demand complex processes of production and purification, resulting in expensive end-products, inaccessible to the general public.^{1,2} The optimization and development of sustainable industrial purification techniques is essential to reduce the production costs and allow the use of these novel bioproducts at large scale. Among the biomolecules discovered in recent decades, the green fluorescent protein (GFP) stands as one with a high biotechnological potential, mainly because of its potential applications as a biosensor and biomarker,³ limited by the aforementioned purification and production issues.

GFP is a globular protein originally isolated from jellyfish *Aequorea victoria* species that has an intense and natural

fluorescence,^{4,5} exhibits a large pH and temperature stability range, and through the proper manipulation of its structure, its fluorescence intensity and spectra can be altered for different applications,⁶ including in biological systems as a biomarker and biosensor.³ Through the isolation and cloning of the gene responsible for the production of GFP, the protein was successfully produced by recombinant organisms such as *Escherichia coli* (*E. coli*) and *Caenorhabditis elegans*.³ However, even with the advances in GFP production through innovative molecular biology approaches, the development of downstream processes remains incomplete, and thus, GFP purification costs are still very high, restricting its application to research and small scale procedures.

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Revealing a new fluorescence peak of the enhanced green fluorescent protein using three-dimensional fluorescence spectroscopy†

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Fluorescent proteins have many applications as biomarkers and biosensors in the medical and biological fields. Their success was largely supported by modifications of the first isolated fluorescent protein, the wild-type Green Fluorescent Protein (wtGFP), which allowed the development of improved variants such as the Enhanced GFP (EGFP). The first reports on EGFP indicated that the protein presented a single form and fluorescence peak, in contrast to the two conformations observed in wtGFP. However, after experimental determination of the crystalline structure of EGFP, two conformations were found, generating questions regarding the relationship between EGFP structure and its spectral characteristics. To resolve the controversy, this study evaluated EGFP 3D fluorescence spectra at lower wavelengths and under distinct conditions (different concentrations, pH and temperatures), revealing the existence of a second fluorescence peak for this protein. It was possible to confirm that the new peak was not a reflection of the intrinsic fluorescence of proteins or an artefact from the 3D fluorescence spectroscopy. It was also shown that the second peak is pH dependent, sensitive to high temperatures and linearly related to EGFP concentration, confirming a direct relationship between the new fluorescence peak and EGFP protein structure. In addition to the revelation of the new EGFP fluorescence peak, this study demonstrated that 3D fluorescence can be used as powerful technique in the discovery of other elusive fluorophores.

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1 Introduction

Fluorescent proteins play a major role in modern biological and medical sciences, with extensive use as biomarkers and biosensors^{1–3} and giving around 70 000 results in a PubMed search (January 2019).⁴ Their success is largely due to the modifications in the first isolated fluorescent protein,² wild-type Green Fluorescent Protein (wtGFP),⁵ which created variants with increased fluorescence intensity at longer wavelengths and improved folding above 20 °C, such as the Enhanced GFP (EGFP, mutations F64L/S65T).⁶ Due to its properties, EGFP was successfully used as a fusion protein in the quantification of cells, reactions and cellular components,⁷ monitoring intracellular pH,⁸ whole-body imaging,⁹ and many other applications.^{7,10} The first fluorescence studies on EGFP suggested the protein had a single form and fluorescence peak, in contrast to

what was seen for wtGFP, which presented protonated and unprotonated states with distinct conformations and fluorescence peaks.¹¹ However, after the experimental determination of the crystalline structure of EGFP, multiple groups reported two different conformations for the protein,^{12,13} generating questions regarding the relationship between EGFP structure and spectral characteristics.

Considering fluorescence is usually extremely sensitive to changes in the fluorophore conformation or microenvironment,¹⁴ the first report determining EGFP structure stated that the presence of two crystalline forms was an “apparent contradiction with the single fluorescence lifetime of the protein”.¹² This phenomenon, also reported by Arpino *et al.* 2012,¹³ was explained as a stabilizing effect of the Glu22 residue over the chromophore, which supposedly maintained its structure despite the conformational changes of the protein. However, the multiple fluorescence studies that reported the existence of only a single fluorescence peak for EGFP^{6,12,13,15} only evaluated longer excitation wavelengths (above 350 nm), using two-dimensional (2D) fluorescence spectra and spectrofluorophotometers with resolutions far inferior to what is currently available. Additionally, modern spectrofluorophotometers allow the evaluation of three-dimensional (3D) fluorescence spectra, which analyses both excitation and emission spectra in a single assay across a larger spectral range.

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† Electronic supplementary information (ESI) available: SDS-PAGE electrophoresis of EGFP and details about 3D fluorescence spectra. See DOI: 10.1039/c9ra02567g





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Reversible and irreversible fluorescence activity of the Enhanced Green Fluorescent Protein in pH: Insights for the development of pH-biosensors

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ABSTRACT

Enhanced Green Fluorescent Protein (EGFP) is a biomolecule with intense and natural fluorescence, with biological and medical applications. Although widely used as a biomarker in research, its application as a biosensor is limited by the lack of in-depth knowledge regarding its structure and behavior in adverse conditions. This study is focused on addressing this need by evaluating EGFP activity and structure at different pH using three-dimensional fluorescence, circular dichroism and small-angle X-ray scattering. The focus was on the reversibility of the process to gain insights for the development of biocompatible pH-biosensors. EGFP was highly stable at alkaline pH and quenched from neutral-to-acidic pH. Above pH 6.0, the fluorescence loss was almost completely reversible on return to neutral pH, but only partially reversible from pH 5.0 to 2.0. This work updates the knowledge regarding EGFP behavior in pH by accounting for the recent data on its structure. Hence, it is evident that EGFP presents the required properties for use as natural, biocompatible and environmentally friendly neutral to acidic pH-biosensors.

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1. Introduction

Green Fluorescent Protein (GFP) is a biomolecule with intense and natural fluorescence, which can be easily quantified and evaluated, allowing its application as a biomarker and biosensor [1,2]. However, although GFP and other fluorescent proteins are widely used in research as biomarkers [3], there is a lack of in-depth knowledge regarding the structure and behavior in different environments of many of its variants, such as temperature and pH, which is the basis for a wider development of biosensors [4,5]. For example, only recently the crystalline structure of the most popular recombinant GFP (variant Enhanced GFP, EGFP) was elucidated [6,7], and none of the previous pH studies with EGFP accounted this data for the variant [8–10]. Therefore, crucial information for understanding and predicting EGFP behavior at different pH is still missing, and studies of reversible modifications of this protein are essential to improve its current use as a biosensor and for the development of novel applications for EGFP.

The first fluorescent protein, known as wild-type GFP (wtGFP), was originally isolated from the jellyfish *Aequorea victoria* [2]. Today, wtGFP can be expressed by a series of organisms, like *Escherichia coli* (*E. coli*) and *Caenorhabditis elegans* [1], and is extensively used as a biomarker [11]. In its native form, the fluorophore of wtGFP is located inside a β -barrel scaffold, formed by 11 β -strands and one central α -helix [12], as can be seen in Fig. 1A. The fluorophore of wtGFP is formed by the autocatalytic cyclization of the tripeptide –Ser65-Tyr66-Gly67, which confers two peaks of fluorescence with maximum intensity at the excitation wavelengths of 395 nm for the protonated states of the protein (higher intensity peak) and 475 nm for its deprotonated state (lower intensity peak) [11,13]. Despite its intense and natural fluorescence, wtGFP presents two main disadvantages for biological applications, of 1) its inefficient folding above 20 °C [14] (below the optimal temperature for most microorganisms and cells used in research, like *E. coli*, yeast and mammalian cell cultures [15,16]), and 2) its low intensity of fluorescence when excited at higher wavelengths (which are preferred over lower wavelengths that can damage biological samples) [11,14]. To overcome these limitations, recombinant variants of wtGFP were developed, including EGFP (mutations F64L and S65T) [17], which presented improved folding above 20 °C and high fluorescence intensity when excited at higher wavelengths [2,11].

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Improving the cost effectiveness of enhanced green fluorescent protein production using recombinant *Escherichia coli* BL21 (DE3): Decreasing the expression inducer concentration

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Abstract

Green fluorescent protein (GFP) is a globular protein used as biosensor and biomarker in medical and industrial fields. However, due to the expensive production costs of expressing proteins using high-cost inducers like isopropyl- β -D-1-thiogalactopyranoside (IPTG), the number of GFP applications are still scarce. This work studied the production of enhanced GFP (EGFP) using *Escherichia coli* BL21 (DE3) [pLysS; pET28(a)], aiming to increase its yield and reduce costs. First, the influence of agitation rate, induction time, and concentration of IPTG in the production of EGFP was

evaluated, but only the first two parameters were significant. Subsequently, aiming to reduce costs related to the use of inducer, the IPTG concentration (0.005, 0.010, and 0.025 mM) was decreased and, interestingly, the production levels were maintained or increased. These results show that a proper choice of production conditions, particularly through the decrease of inducer concentration, is effective to reduce the upstream production costs and guarantee high EGFP expression. © 2019 International Union of Biochemistry and Molecular Biology, Inc. Volume 00, Number 0, Pages 1–10, 2019

Keywords: biomarker, *Escherichia coli*, green fluorescent protein, inducer protein expression, IPTG, production

1. Introduction

Biotechnology and recombinant DNA technology allowed the production of several biomolecules with industrial and medical relevance [1, 2]. Among these, the green fluorescent protein

(GFP), originally isolated from jellyfish *Aequorea victoria*, stood out for its great use as biosensor and biomarker, with many applications in the pharmaceutical and industrial fields [3, 4]. GFP presents a chromophore in its structure that confers fluorescence at an excitation wavelength from 395 to 489 nm and emission from 504 to 511 nm, depending on the GFP variant [3, 4], which allows this protein to be easily detectable and quantifiable [5]. GFP has a relatively low toxicity, present intense and natural fluorescence, and a large pH and temperature stability range [5]. Furthermore, by a proper manipulation of its structure, its fluorescence intensity and spectra can be changed, adjusting its use for a wide array of purposes [6], from biological applications as a fusion protein (to quantify cells, cellular components, and reactions), for whole-organism visualization and even as a transcriptional probe to monitor environmental variables such as pH, oxygen, temperature, and nutrient availability [7]. Even considering the advantages of GFP when used as biosensor and biomarker, due

Abbreviations: AU, absorbance unities; *E. coli*, *Escherichia coli*; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; IPTG, isopropyl- β -D-1-thiogalactopyranoside; LB, Luria Bertani broth; OD_{600nm}, optical density at 600 nm; rpm, revolutions per minute.

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Economic analysis of the production and recovery of green fluorescent protein using ATPS-based bioprocesses

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ABSTRACT

Green fluorescent protein (GFP) is a useful biomolecule in biotechnology; however, its price makes its widespread application prohibitive. To overcome this issue, recently, the use of aqueous two-phase systems (ATPS) for GFP purification was proposed as an alternative platform to reduce processing costs. Aligned with this goal, this study performed bioprocess modelling coupled with economic analysis using the software Biosolve to evaluate the potential and commercial applicability of ATPS for GFP purification. This work analysed a collection of fourteen ATPS to discriminate through production costs while also incorporating the concept of product purity into the calculations. The two best systems (a PEG-based and an ionic liquid (IL)-based ATPS) were placed in a full bioprocess at different scale models (1 to 100 L) to elucidate the viability of applying ATPS at large scale. Although the results showed that the PEG-based ATPS exhibit the lowest costs (between USD $3.5 \times 10^3 \cdot \text{g}^{-1}$ at 1 L and USD $0.33 \times 10^3 \cdot \text{g}^{-1}$ at 100 L), for further developments, the inclusion of an ATPS granting a higher purity is desired for the development of simpler bioprocesses. Therefore, as a third approach in this work, a sensitivity analysis was performed to determine the impact of varying different model parameters (recovery yield, material costs discount and production titre), to elucidate the circumstances under which the IL-based system can overcome the production costs of the traditional PEG-based ATPS. The results indicate that the best cost-effectiveness approach is to improve the production titre (although it can affect all ATPS studied), as an increase from 1.33 to 3.8 g/L is enough for the IL-based ATPS to be less expensive than the traditional system at all analysed scales. This study demonstrates that ATPS can greatly reduce GFP manufacturing costs, which can potentially help to popularize new applications of fluorescent proteins that are currently mostly restricted to research kits due to their high prices.

1. Introduction

Green fluorescent protein (GFP) is a biomolecule that has a widespread use in biotechnology [1,2]. Most of its applications are as a biomarker and biosensor [1], which demand high-purity levels. Therefore, and despite of its wide acceptance in biotechnology-related fields, the major drawback for a widespread use of GFP is its high cost, for example, its selling price of around USD 2,000 per mg by Biovision Incorporate company [3].

Currently, there is already a profitable field for GFP in the Reporter Gene Assay Kits market (which includes fluorescent proteins and enzymes like luciferase), which achieved a market value of USD 1.6 billion per year in 2019 [4]. However, considering the exorbitant current prices of GFP (ranging from around 350 to 600 dollars per 0.1 mg) [3,5–7], this application is only commercially viable because it uses very small concentrations of the protein (only around 0.1 mg of GFP per kit for 100 assays) [3,8,9]. Moreover, other fields of application are emerging, enclosing innovative products in the medical [10,11], energy

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Purification of green fluorescent protein using fast centrifugal partition chromatography



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ABSTRACT

The green fluorescent protein (GFP) is a biomolecule used in many biological applications such as biomarkers and biosensors, which require high purity levels. It is usually obtained from recombinant *Escherichia coli* strains, which also produces other endogenous proteins, demanding multiple purification steps, and consequently, increasing the overall costs to obtain pure GFP. Simpler and cheaper purification methods like Aqueous Biphasic Systems (ABS) were already successfully applied to purify GFP at lab scale. Therefore, the development of automatized industrially compatible purification platforms, such as countercurrent chromatography using ABS, can potentially improve the GFP production. This work studied the continuous purification of the variant enhanced GFP (EGFP) by applying ABS composed of polyethylene glycol (PEG 8000), sodium polyacrylate (NaPA 8000) and sodium sulfate (Na_2SO_4) as electrolyte. An initial screening was carried by changing the electrolyte content in the ABS. The increase of this condition has demonstrated an increase on the EGFP partition for the PEG-rich phase. The most efficient ABS and, at the same time, with the most appropriate conditions, namely the system composed of 15 wt% PEG 8000 + 4.5 wt% NaPA 8000 + 2.5 wt% Na_2SO_4 was chosen and applied on the fast centrifugal partition chromatography (FCPC). After optimization, the best operational conditions were identified, i.e. a flow rate of $2.5 \text{ mL} \cdot \text{min}^{-1}$ and rotation speed of 2000 rpm at ascending mode, and the best results obtained, meaning a purification of 89.93% and a recovery yield of 82.3%, confirming the potential of FCPC to the continuous purification of EGFP.

1. Introduction

The green fluorescent protein (GFP) emits a natural and intense visible fluorescence without needing cofactors. It can be genetically encoded by a series of organisms, an advantage that enables its application as biomarker and biosensor [1,2]. It is a relatively small protein (27 kDa) consisting of 238 amino acid residues [3], firstly isolated by Shimomura *et al.* [4] from the jellyfish *Aequorea victoria* in 1960s. Almost 30 years later, some studies revealed GFP as an important tool for mapping cells by connecting its gene with those encoding for other proteins, thus functioning as a tracking lamp in living organisms, forming a fused protein [5–8]. Through the isolation and cloning of the gene coding sequence to obtain GFP, the protein was successfully produced by recombinant organisms such as *Escherichia coli* (*E. coli*) and

Caenorhabditis elegans (*C. elegans*) [5]. Moreover, it has been successfully expressed in many other organisms, such as bacteria, fungi, plant, and animal cells [9]. In addition to that, the development of modified GFP with improved fluorescence properties and folding for biological applications, such as its most popular variant, the Enhanced Green Fluorescent Protein (EGFP) [10], broadened its use as a fluorescent genetic marker and tracking agent of analyzes within living cells or organelles [11].

Endowed by its remarkable fluorescence, recombinant variants, such as EGFP, exhibit high thermal stabilities and high resistances to alkaline pH, detergents, organic salts and proteases, being thus capable to retain fluorescence up to a pressure of 600 MPa [9,12]. With these properties, recombinant fluorescent proteins became a powerful tool for applications such as fusion tags, reporter gene, data storage, diagnosis,

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Sterilization in pharmaceutical biotechnology

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Scope: Sterilization is essential in most industrial bioprocesses; however, it has a higher relevance for the biotechnological-pharmaceutical industries. This chapter covers the various sterilization methods according to each processing stage, *i.e.*, upstream and downstream, including an overview of the restrictions and procedures for applying sterilization and asepsis procedures in the manufacture of pharmaceutical biotechnology products.

Sterilization in pharmaceutical biotechnology. *In: Pharmaceutical Biotechnology: Focus on Industrial Application*. 2. ed. Taylor & Francis, 2021. (*Accepted*)

APPENDIX

APPENDIX A – EGFP INFORMATION

Fluorescent Protein: Enhanced Green Fluorescent Protein (EGFP) (CORMACK *et al.*, 1996)

Alias: GFPmut1 (CORMACK *et al.*, 1996)

Similar: m-EGFP (monomeric EGFP) (MYATT *et al.*, 2017)

Derived from: wild-type Green Fluorescent Protein (wtGFP) with mutations F64L/S65T (CORMACK *et al.*, 1996)

GenBank: AAB02572.1 (GENBANK, 1996)

Dimerization: Weak dimer (binding affinity of 100 $\mu\text{mol.L}^{-1}$) (MYATT *et al.*, 2017)

Structure: PDB ID 2Y0G (ROYANT; NOIRCLERC-SAVOYE, 2011) and 4EUL (ARPINO *et al.*, 2012)

Amino acid sequence: (GENBANK, 1996)

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVP
WPTLVTTLTLYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEV
KFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRH
NIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLLEFVTA
AGITLGMDELYK

Maximum excitation wavelength: 488 nm (Figure 10)

Stokes shift: 22 nm

Molar extinction coefficient estimate at 488 nm: 55,000-57,000 $\text{L.mol}^{-1}.\text{cm}^{-1}$ (TSIEN, 1998)

Quantum Yield: 0.6 (TSIEN, 1998)

Relative brightness: 33,000 $\text{L.mol}^{-1}.\text{cm}^{-1}$ (TOSELAND, 2013)

Lifetime: 2.5 ns (SCHLEIFENBAUM *et al.*, 2010)

Maturation half-time: 25 min (CORMACK *et al.*, 1996)

Bleaching half-life: 174 s (light: arc-lamp; mode: widefield; temperature: 23 °C) (SHANER *et al.*, 2005)

pKa: 5.7 (BOMATI *et al.*, 2014)

pI: 6.2 (GURUNATHAN *et al.*, 2014)

ProtParam calculations using amino acid sequence (GASTEIGER *et al.*, 2005)

Molar extinction coefficient estimated at 280 nm in water: 21,890 L.mol⁻¹.cm⁻¹

Average molecular weight: 26,941.4811 Da

Monisotopic molecular weight: 26,924.4846 Da

Number of atoms: 3,764

Table A-1 – Amino acid composition of EGFP.

Amino acid composition of EGFP		
Amino acid	Count	Total (%)
Alanine (A)	8	3.4
Arginine (R)	6	2.5
Asparagine (N)	13	5.4
Aspartic acid (D)	18	7.5
Cysteine (C)	2	0.8
Glutamine (Q)	8	3.4
Glutamic acid (E)	16	6.7
Glycine (G)	22	9.2
Histidine (H)	9	3.8
Isoleucine (I)	12	5.0
Leucine (L)	21	8.8
Lysine (K)	20	8.4
Methionine (M)	6	2.5
Phenylalanine (F)	12	5.0
Proline (P)	10	4.2
Serine (S)	10	4.2
Threonine (T)	16	6.7
Tryptophan (W)	1	0.4
Tyrosine (Y)	11	4.6
Valine (V)	18	7.5
Total	239	100.0

Source: Produced by the author with ProtParam. (GASTEIGER *et al.*, 2005)

Charge and hydrophobicity

Negatively charged residues (D, E) = 34 (14.23%)

Positively charged residues (K, R, H) = 35 (14.64%)

Polar residues (C, S, Q, N, T, Y) = 60 (25.10%)

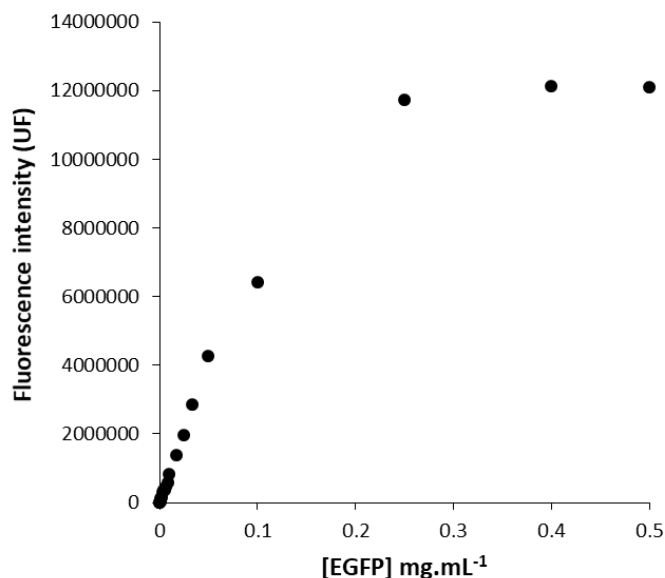
Hydrophobic residues (A, G, I, L, M, P, F, W, V) = 110 (46.03%)

Aliphatic index: 79.04 **Grand Average of Hydropathicity (GRAVY):** -0.467

Instability index: 29.06 (below 40 is considered stable)

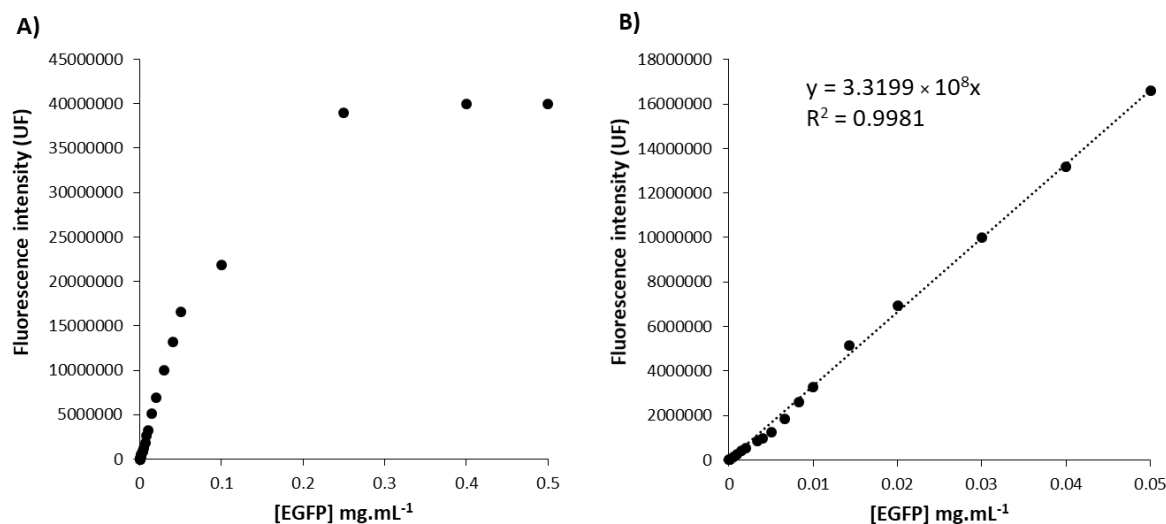
APPENDIX B – CALIBRATION CURVES

Figure B-1 – Complete calibration curve for EGFP F2 peak (λ_{ex} 278 nm, λ_{em} 510 nm), concentration of EGFP [EGFP] (mg.mL^{-1}) in the X axis and fluorescence intensity (UF) in the Y axis.



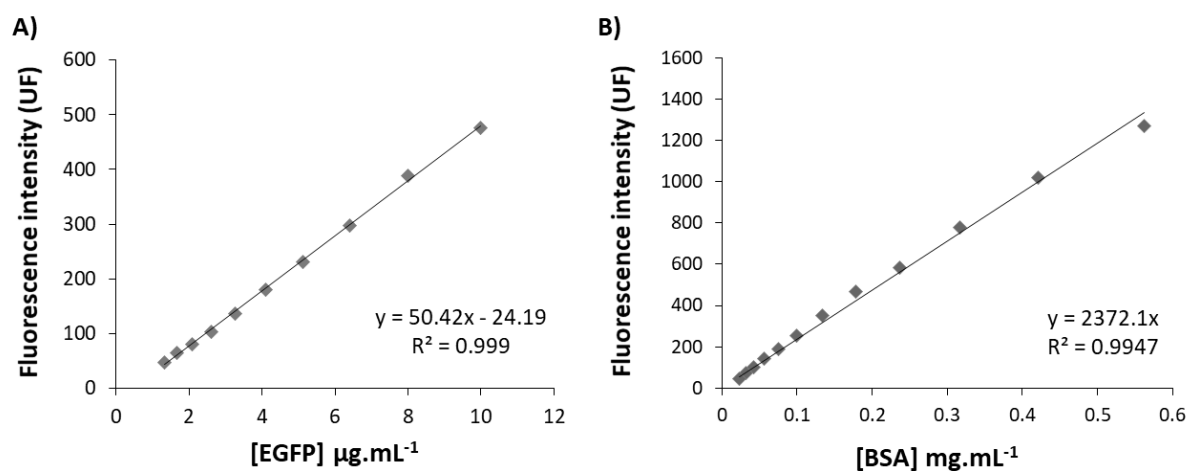
Source: Produced by the author and published in (DOS SANTOS *et al.*, 2019).

Figure B-2 – Calibration curves for EGFP F1 peak (λ_{ex} 488 nm, λ_{em} 510 nm), concentration of EGFP [EGFP] (mg.mL^{-1}) in the X axis and fluorescence intensity (UF) in the Y axis at two different ranges. **(A)** [EGFP] from 0 to 0.5 mg.mL^{-1} and fluorescence intensity from 0 to 45,000,000 UF. **(B)** [EGFP] from 0 to 0.05 mg.mL^{-1} and fluorescence intensity from 0 to 18,000,000 UF).



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2019).

Figure B-3 – Calibration curves for: **A)** EGFP quantification – with EGFP standard (fluorescence analysis at λ_{ex} 488 nm, λ_{em} 510 nm); **B)** Total protein quantification - with BSA as standard (fluorescence analysis at λ_{ex} 276 nm, λ_{em} 336 nm).



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

APPENDIX C – SAMPLES AND PARAMETERS DETAILS FOR EGFP AT DIFFERENT PH STUDIES

Table C-1 – Details of SAXS samples, parameters, and analysis.

(continue)

I) Sample	
Protein	Enhanced Green Fluorescent Protein (EGFP)
Organism	<i>Escherichia coli</i> BL21 (DE3) [pLysS] pET28(a)
Purification	Methods from (DOS SANTOS <i>et al.</i> , 2018b)
Quantification and confirmation of purification	Calibration curves and electrophoresis at (DOS SANTOS <i>et al.</i> , 2019)
Mw (kDa)	26.9 (ARPINO <i>et al.</i> , 2012)
PDB ID	2Y0G (ROYANT; NOIRCLERC-SAVOYE, 2011)
GenBank	AAB02572.1 (GENBANK, 1996)
Extinction coefficient estimate at 280 nm in water ($\text{L.mol}^{-1}.\text{cm}^{-1}$)	21,890 [calculated using ProtParam, (GASTEIGER <i>et al.</i> , 2005)]
Pi	6.2 (GURUNATHAN <i>et al.</i> , 2014)

Table C-1 – Details of SAXS samples, parameters, and analysis.

(continue)

B) Data collection parameters ^a		
	SEC-SAXS	Well-plate SAXS
Instrument	Australian Synchrotron SAXS/WAXS beamline (RYAN <i>et al.</i> , 2018)	Australian Synchrotron SAXS/WAXS beamline (ANSTO, 2020)
Beam Geometry	120 µm point source	120 µm point source
Wavelength (Å)	1.033	1.033
q range (Å⁻¹)	0.006 – 0.53	0.006 – 0.53
Temperature (°C)	25	25
Solvent	Potassium phosphate buffer 10 mmol.L ⁻¹ (pH 5.5, 6.5, and 7.4)	Potassium phosphate buffer 10 mmol.L ⁻¹ (pH 4.0, 5.0, 6.0, and 7.4)
Loading protein concentration (mg.mL⁻¹)	5.0	3.0
Collection Mode	Coflow SEC-SAXS	Well-plate SAXS
Column size and media	5 x 150 superdex S200	–
Volume injection (µL)	50	–
Cell pathlength for UV (cm)	0.3	–
Flow rate of injection (mL.min⁻¹)	0.4	–

Table C-1 – Details of SAXS samples, parameters, and analysis.

(conclusion)

D) SEC-SAXS Structural parameters			
	pH 5.5	pH 6.5	pH 7.4
Concentration at peak during SEC run (mg.mL⁻¹)^d	0.9	1.1	1.2
Guiner I(0) (cm⁻¹) [std.err]	0.015 ± 0.00028	0.014 ± 0.00026	0.011 ± 0.00003
Guinier Rg (Å) [std.err]	23.33 ± 0.69	21.46 ± 0.61	20.36 ± 0.38
qRg (max)	1.29	1.3	1.3
P(r) I(0) (cm⁻¹)	0.01	0.01	0.01
P(r) Rg (Å)	24.17	21.88	20.73
Dmax (Å)	93	74	76

^a Further detail can be found on the ANSTO site. (ANSTO, 2020a)

^b Solvent blanks taken from SEC flow-through before elution of protein for SEC-SAXS and from the blank sample for well-plate SAXS.

^c Due to the high heterogeneity of the well-plate SAXS samples, only qualitative analysis was performed.

^d Calculated using UV 280 nm absorbance from the frames selected for EGFP monomer analysis and ProtParam, (GASTEIGER *et al.*, 2005) with correction for UV cell pathlength from 1.0 cm to 0.3 cm.

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

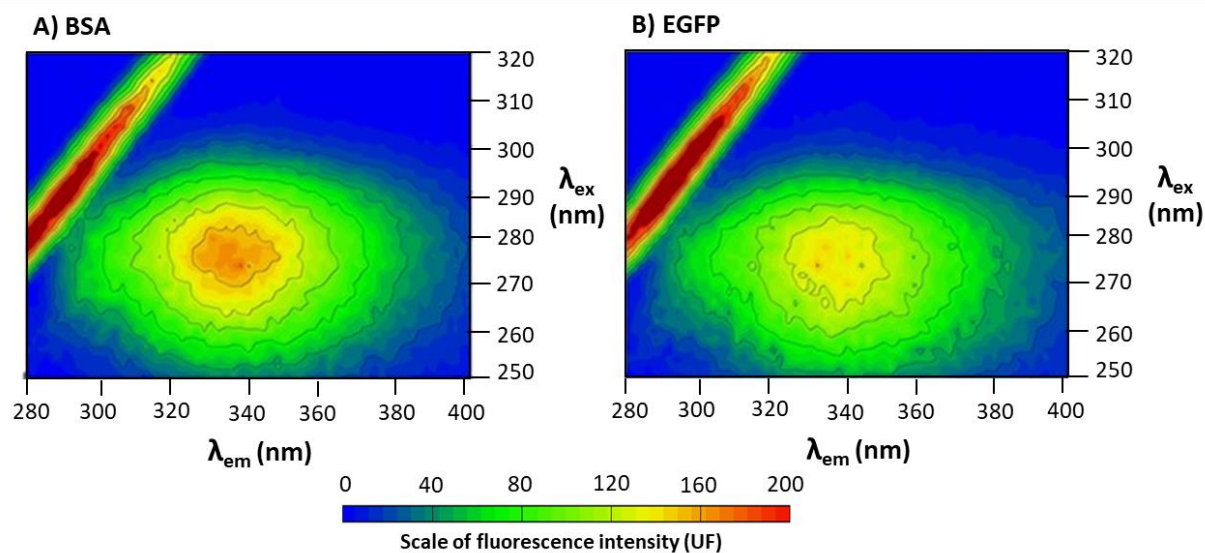
Table C-2 – Real pH and conductivity (mS.cm^{-1} at 25 °C) of samples from **Figure 14**.

pH	real pH	Conductivity (mS.cm^{-1} at 25 °C)
2.0	2.0	19.3
3.0	3.0	17.2
4.0	4.0	16.8
5.0	5.1	18.9
6.0	5.9	16.0
7.0	7.1	16.0
7.4	7.4	16.0
8.0	8.0	15.9
9.0	9.0	16.5
10.0	10.0	16.3
11.0	10.9	16.5
12.0	11.9	18.3
13.0	12.9	19.0

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020).

APPENDIX D – 3D FLUORESCENCE SPECTRA OF BSA AND EGFP

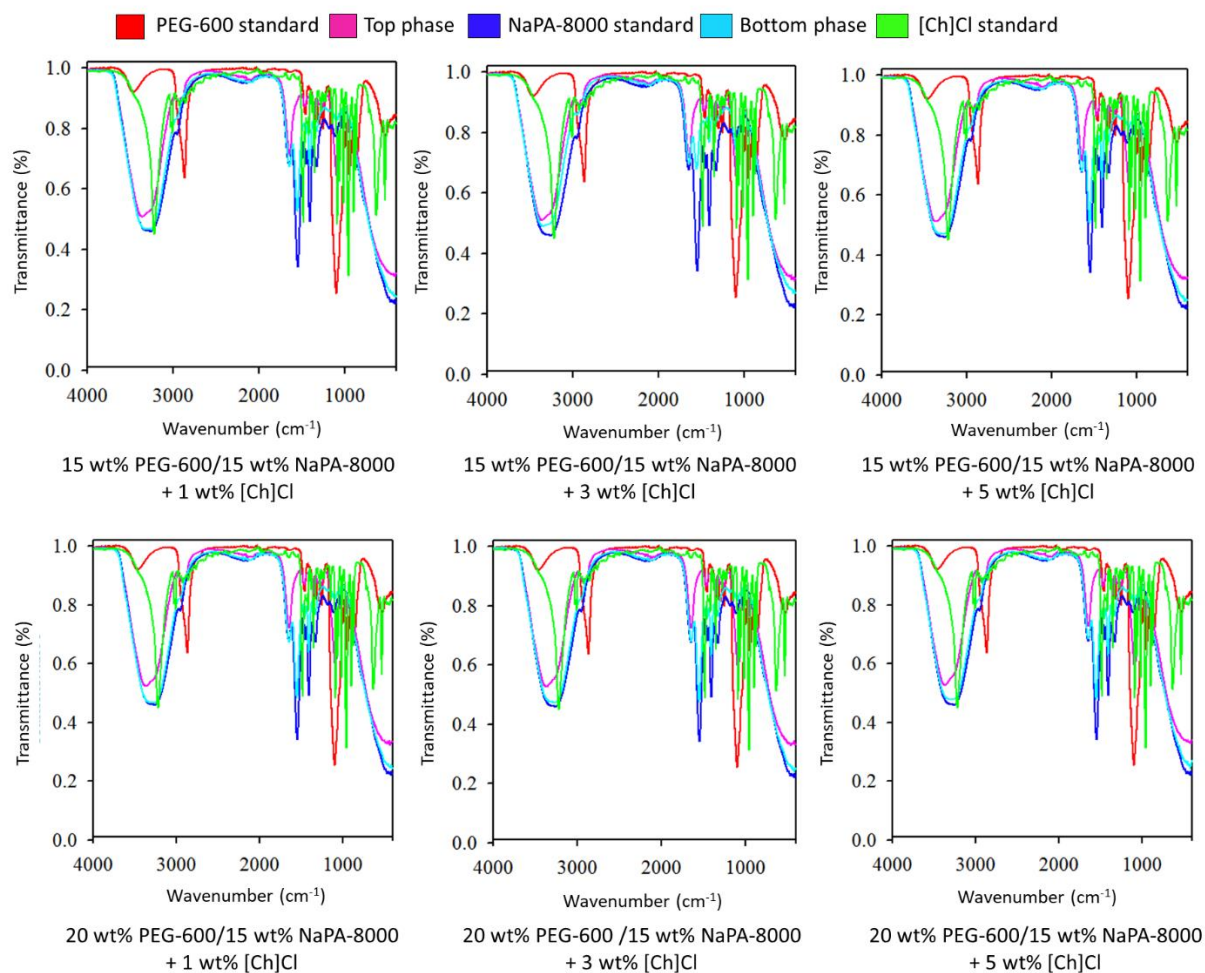
Figure D-1 – 3D Fluorescence spectra (units of fluorescence, UF) of **A)** Bovine Serum Albumin (BSA) and **B)** Enhanced Green Fluorescent Protein (EGFP) raw extract. The 3D Fluorescence Spectra shows the excitation wavelengths in the Y axis (λ_{ex}), the emission wavelengths in the X axis (λ_{em}) and the intensity of fluorescence is given by the color scale from blue to red (0 to 200 UF). 3D fluorescence spectra ranged excitation wavelength from 250 to 320 nm and emission wavelength from 280 to 400 nm.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

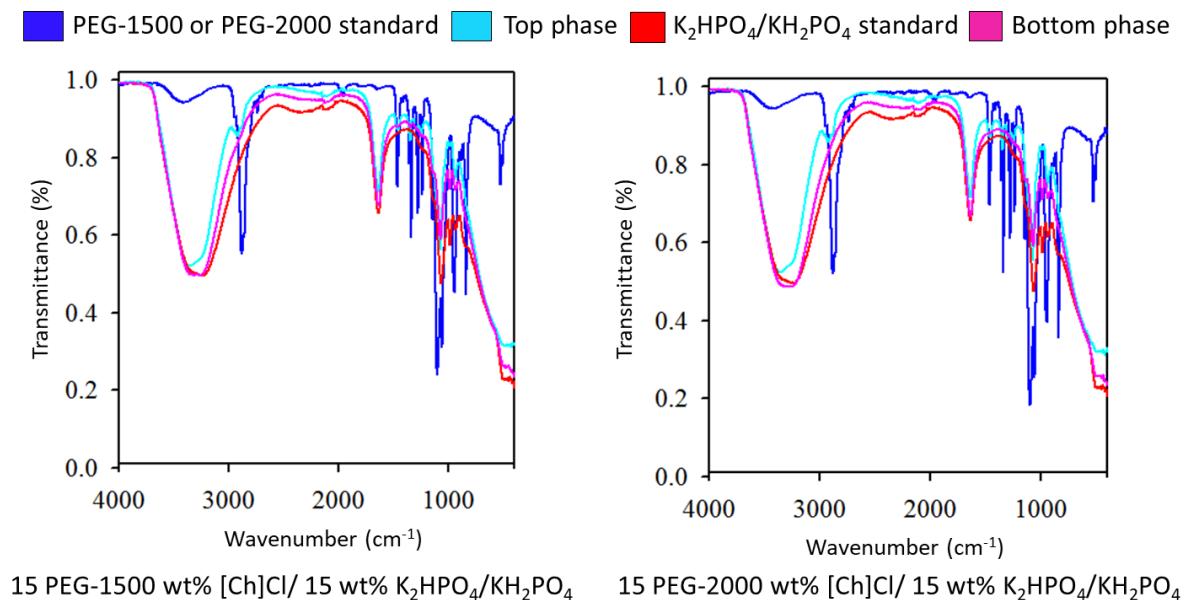
APPENDIX E – FTIR-ATR ANALYSIS OF ABS PHASES

Figure E-1 – Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) of top (pink) and bottom (light blue) phases from ABS composed of PEG-600 (15 or 20 wt%) + NaPA-8000 (15 wt%) + [Ch]Cl (1, 3 or 5 wt%), and standards for PEG-600 (red), NaPA-8000 (blue) and [Ch]Cl (green). Wavenumber (cm^{-1}) in the x axis and transmittance (%) in the y axis.



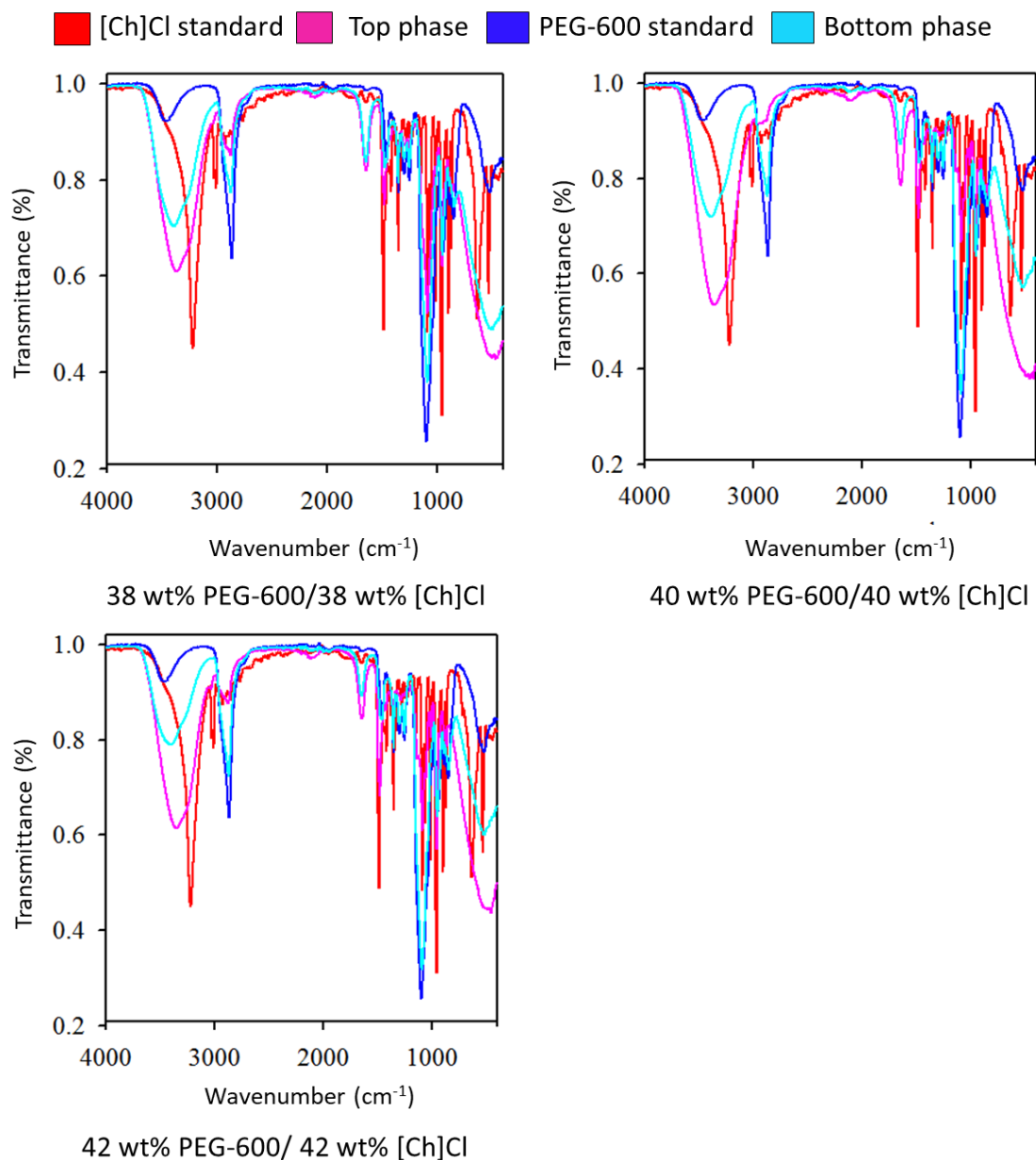
Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Figure E-2 – Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) of top (pink) and bottom (light blue) phases from ABS composed of PEG-1500 (15 wt%) + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (15 wt%) and PEG-2500 (15 wt%) + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, and standards for PEG-1500 or PEG-2000 (red) and $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (blue). Wavenumber (cm^{-1}) in the x axis and transmittance (%) in the y axis.



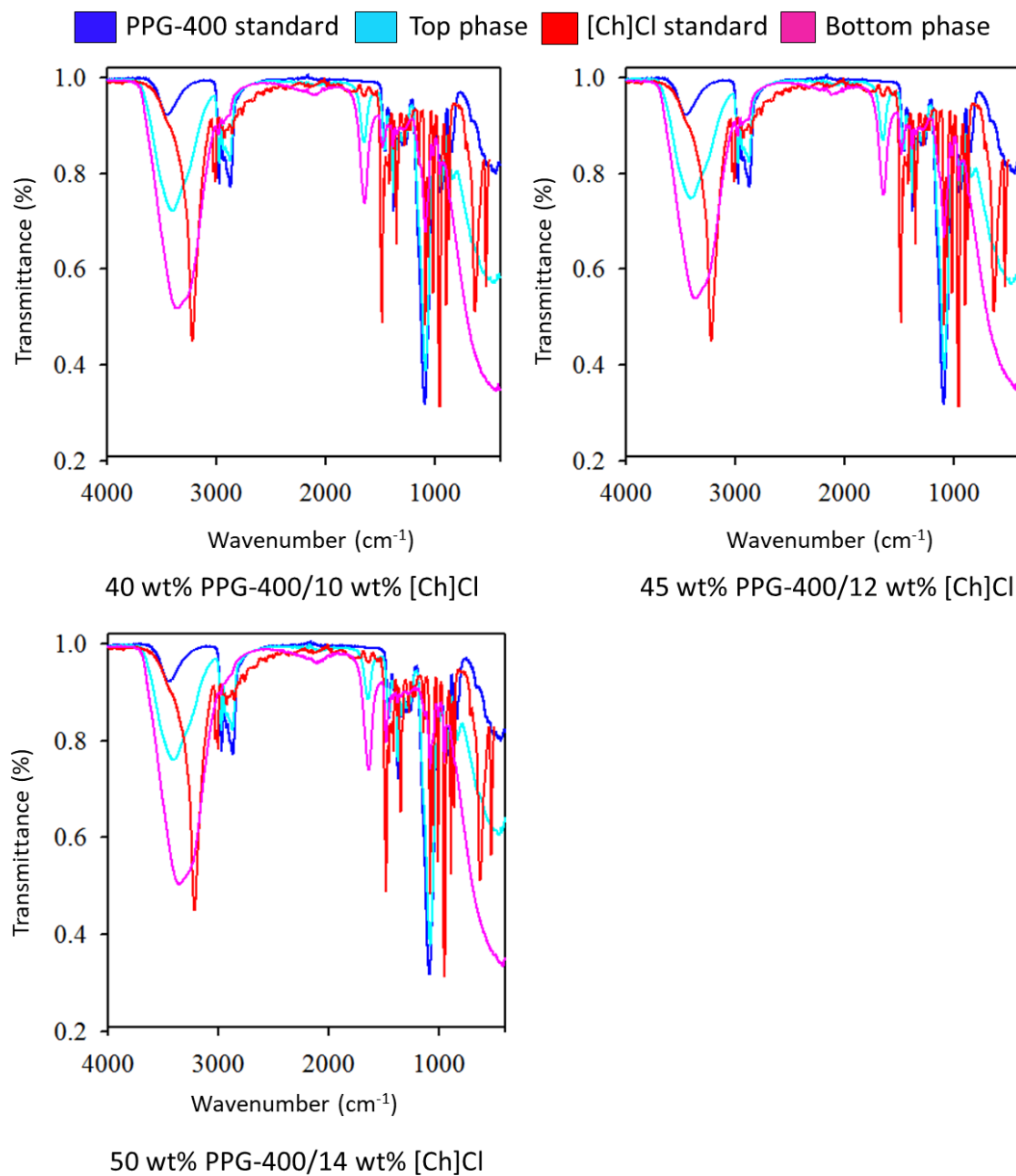
Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

Figure E-3 – Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) of top (pink) and bottom (light blue) phases from ABS composed of PEG-600 (38, 40 or 42 wt%) and [Ch]Cl (38, 40 or 42 wt%), and standards for [Ch]Cl (red) and PEG-600 (blue). Wavenumber (cm^{-1}) in the x axis and transmittance (%) in the y axis.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

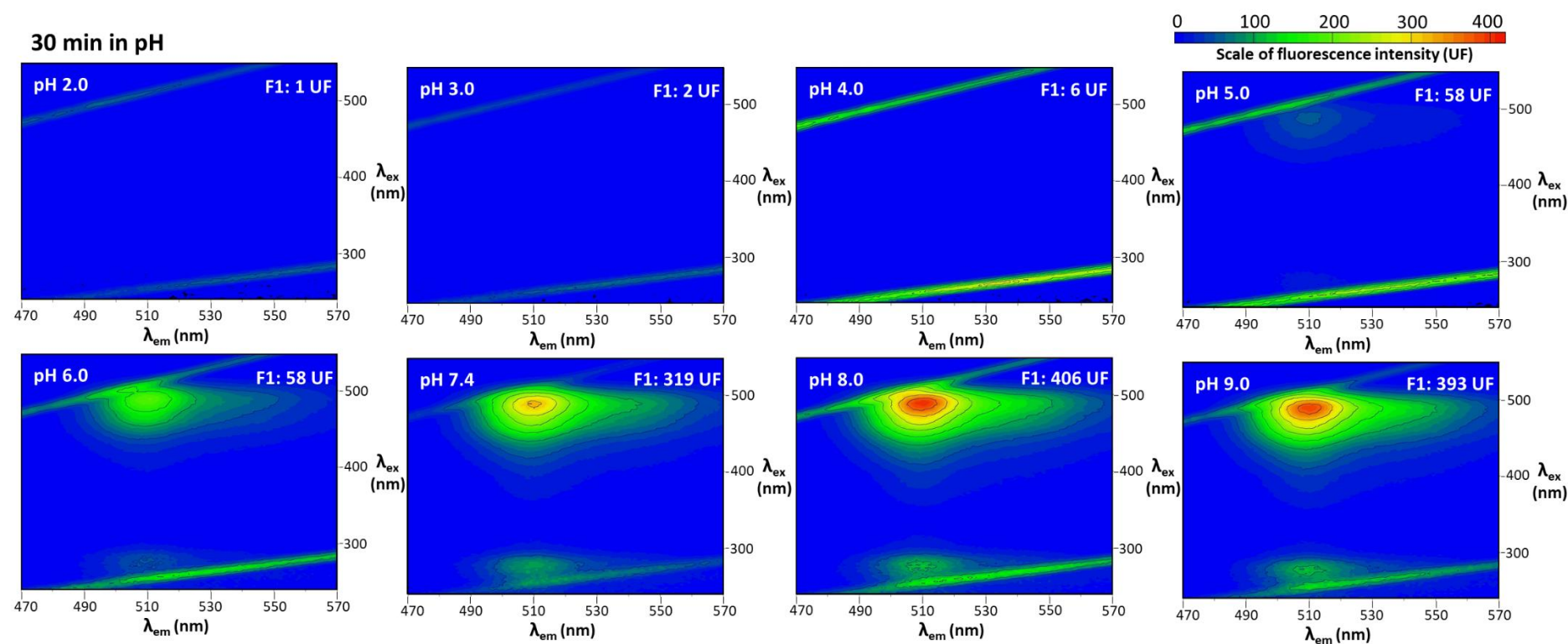
Figure E-4 – Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) of top (light blue) and bottom (pink) phases from ABS composed of PPG-400 (40, 45 or 50 wt%) and [Ch]Cl (10, 12 and 14 wt%), and standards for [Ch]Cl (red) and PPG-400 (blue). Wavenumber (cm^{-1}) in the x axis and transmittance (%) in the y axis.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2018b).

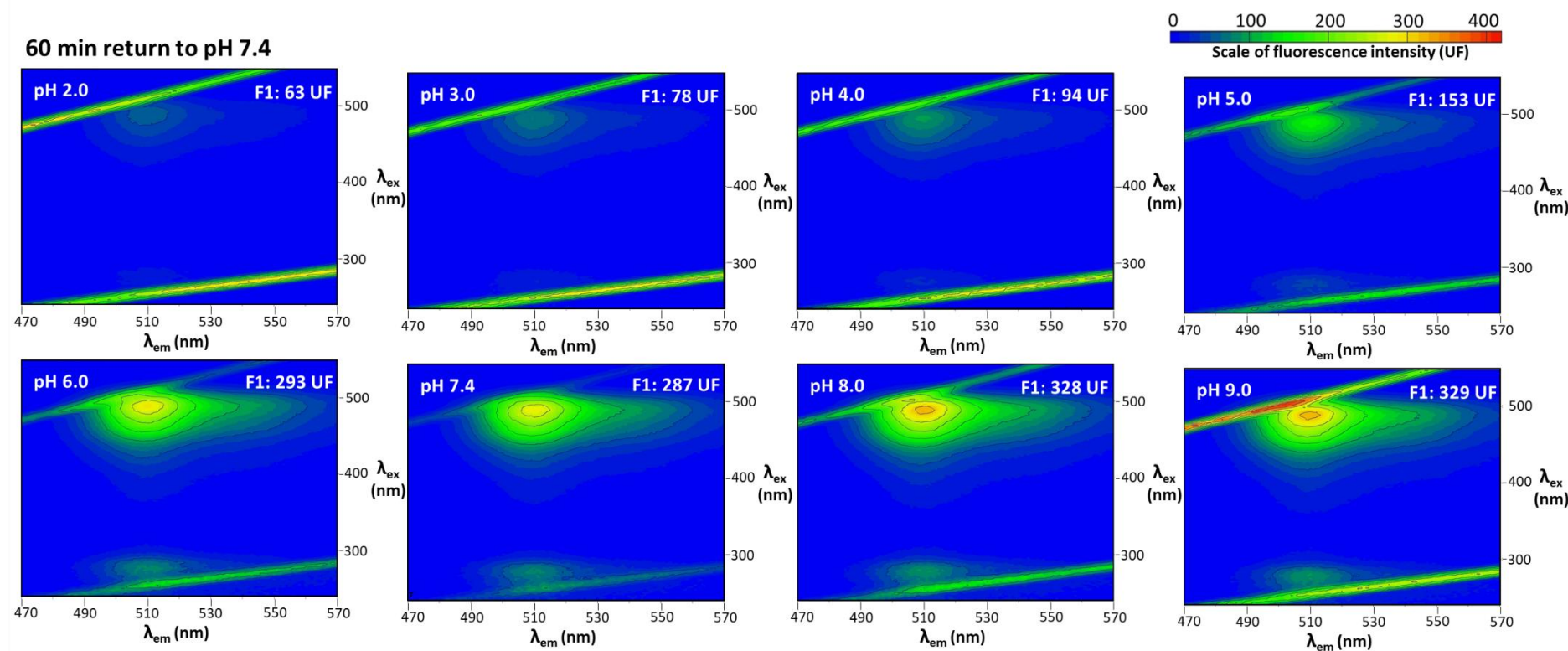
APPENDIX F – 3D FLUORESCENCE SPECTRA ANALYSIS OF EGFP STABILITY IN PH

Figure F-1 – 3D fluorescence spectra of EGFP in PB after 30 min exposure to different pH. The 3D fluorescence spectra show the excitation wavelengths in the Y axis (λ_{ex}), the emission wavelengths in the X axis (λ_{em}) and the fluorescence intensity (units of fluorescence, UF) is given by the color scale from blue to red (0 to 420 UF). 3D fluorescence spectra ranged excitation wavelength from 240 to 550 nm and emission wavelength from 470 to 570 nm. F1 is the fluorescence intensity of EGFP (in UF) at λ_{ex} 488 nm, λ_{em} 510 nm.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

Figure F-2 – 3D fluorescence spectra of EGFP in PB after 30 min exposure to different pH + 60 min return to pH 7.4. The 3D fluorescence spectra show the excitation wavelengths in the Y axis (λ_{ex}), the emission wavelengths in the X axis (λ_{em}) and the fluorescence intensity (units of fluorescence, UF) is given by the color scale from blue to red (0 to 420 UF). 3D fluorescence spectra ranged excitation wavelength from 240 to 550 nm and emission wavelength from 470 to 570 nm. F1 is the fluorescence intensity (in UF) λ_{ex} 488 nm, λ_{em} 510 nm.



Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

APPENDIX G – CIRCULAR DICHROISM ANALYSIS

Table G-1 – Values of EGFP (0.3 mg.mL⁻¹ in PB) secondary structures obtained from circular dichroism analysis in Dichroweb CONTIN-LL^a after 30 min exposure to different pH and 60 min return to pH 7.4. Results are presented in percentage (%) as the average of three samples and the respective standard deviations.

	α -helix (%)		β -strands (%)		Turns (%)	Unordered (%)
	Regular	Distorted	Regular	Distorted		
30 min in pH						
pH 9.0	9 $\pm$ 2	2 $\pm$ 1	35 $\pm$ 0	13 $\pm$ 0	10 $\pm$ 0	31 $\pm$ 2
pH 7.4	10 $\pm$ 2	3 $\pm$ 1	35 $\pm$ 1	13 $\pm$ 0	11 $\pm$ 1	29 $\pm$ 1
pH 6.0	9 $\pm$ 1	1 $\pm$ 0	36 $\pm$ 0	13 $\pm$ 0	10 $\pm$ 0	30 $\pm$ 1
pH 5.0	6 $\pm$ 0	5 $\pm$ 1	31 $\pm$ 1	13 $\pm$ 0	11 $\pm$ 0	34 $\pm$ 0
pH 4.0	5 $\pm$ 1	6 $\pm$ 0	28 $\pm$ 0	13 $\pm$ 0	12 $\pm$ 0	36 $\pm$ 1
pH 3.0	3 $\pm$ 0	7 $\pm$ 1	25 $\pm$ 1	13 $\pm$ 0	12 $\pm$ 0	39 $\pm$ 0
pH 2.0	2 $\pm$ 1	8 $\pm$ 0	25 $\pm$ 0	13 $\pm$ 0	13 $\pm$ 0	40 $\pm$ 1
60 min return to pH 7.4						
pH 9.0	8 $\pm$ 2	2 $\pm$ 1	35 $\pm$ 1	13 $\pm$ 0	11 $\pm$ 0	30 $\pm$ 1
pH 7.4	9 $\pm$ 1	2 $\pm$ 1	35 $\pm$ 1	13 $\pm$ 0	10 $\pm$ 0	30 $\pm$ 1
pH 6.0	9 $\pm$ 1	3 $\pm$ 0	35 $\pm$ 0	13 $\pm$ 0	10 $\pm$ 0	30 $\pm$ 1
pH 5.0	7 $\pm$ 1	3 $\pm$ 0	34 $\pm$ 0	13 $\pm$ 0	11 $\pm$ 0	32 $\pm$ 1
pH 4.0	0 $\pm$ 0	4 $\pm$ 2	31 $\pm$ 1	14 $\pm$ 0	11 $\pm$ 0	40 $\pm$ 1
pH 3.0	0 $\pm$ 0	5 $\pm$ 1	30 $\pm$ 1	14 $\pm$ 0	11 $\pm$ 0	40 $\pm$ 1
pH 2.0	5 $\pm$ 2	5 $\pm$ 1	30 $\pm$ 0	13 $\pm$ 0	11 $\pm$ 0	35 $\pm$ 2

^a Reference set SP175 optimized for 190-240 nm range and results for the closest matching solution with all proteins.

Source: Produced by the author and published in (DOS SANTOS *et al.*, 2020d).

APPENDIX H – PHYSICAL-CHEMICAL PROPERTIES OF SOLUTIONS OF SALTS, ILS, AND DENATURING AGENTS

Table H-1 – Physical-chemical properties (pH and conductivity in mS.cm^{-1} at 25 °C) of solutions of salts and ILS used for the EGFP stability studies.

Concentration (mol.L ⁻¹)	pH					Conductivity (mS.cm^{-1} at 25 °C)				
	0.500	0.250	0.100	0.050	0.025	0.500	0.250	0.100	0.050	0.025
NaCl	7.46	7.42	7.30	7.31	7.40	53.01	35.31	22.92	18.80	16.17
[C _{1min}]Cl	7.30	7.34	7.35	7.33	7.36	46.60	32.00	21.05	17.53	15.29
[C _{2min}]Cl	7.33	7.32	7.33	7.43	7.36	44.00	31.69	23.33	20.02	18.06
[C _{4min}]Cl	7.30	7.42	7.31	7.51	7.30	40.29	30.12	22.64	19.62	18.00
[C _{6min}]Cl	7.31	7.31	7.26	7.33	7.40	26.91	21.31	16.96	15.27	14.50
[C _{8min}]Cl	7.47	7.30	7.32	7.40	7.40	37.78	29.16	22.14	19.30	18.01
[C _{10min}]Cl	7.30	7.30	7.30	7.34	7.32	24.14	20.84	18.83	17.74	16.96
[C _{12min}]Cl	7.32	7.31	7.33	7.38	7.39	30.06	23.62	19.64	17.86	17.10
[Ch][Cl]	7.33	7.38	7.44	7.45	7.33	43.52	29.10	19.41	15.73	15.72
[Ch][Bic]	7.49	7.42	7.30	7.31	7.43	42.10	29.09	19.77	16.80	14.78
[Ch][Lac]	7.46	7.33	7.40	7.34	7.34	29.36	23.09	18.41	16.53	15.59
[Ch][Gly]	7.46	7.44	7.50	7.35	7.37	33.16	24.71	19.83	17.05	15.50
[Ch][Ac]	7.31	7.36	7.30	7.42	7.30	33.16	23.40	17.69	15.30	14.07
[Ch][Pro]	7.37	7.39	7.32	7.39	7.32	28.44	22.72	18.07	16.13	15.27
[Ch][But]	7.33	7.36	7.37	7.44	7.38	30.08	23.43	18.44	16.56	15.01
[Ch][Pen]	7.40	7.39	7.45	7.36	7.37	26.04	21.90	18.36	16.46	15.77
[Ch][Hex]	7.45	7.36	7.44	7.36	7.40	26.05	21.71	17.50	16.45	15.10
[Ch][Oct]	7.48	7.42	7.36	7.46	7.36	23.50	19.94	16.97	16.10	15.16
[Ch][Dec]	7.45	7.48	7.44	7.45	7.36	22.86	18.86	16.84	16.28	15.52

Source: Produced by the author.

Table H-2 – Physical-chemical properties (pH and conductivity in mS.cm^{-1} at $25\text{ }^{\circ}\text{C}$) of solutions of denaturing agents used for the EGFP stability studies.

Denaturing agent	Concentration (mol.L^{-1})	pH	Conductivity (mS.cm^{-1} at $25\text{ }^{\circ}\text{C}$)
SDS	0.003	7.30	15.41
	0.035	7.28	16.02
	0.100	7.14	17.80
	0.175	7.04	19.43
H_2O_2	0.030	7.29	15.13
	0.300	7.32	14.03
	1.500	6.70	13.06
	3.000	6.21	10.93
	6.000	4.87	7.03
Urea	4.000	7.81	7.27
	8.000	8.72	1.01
GuHCl	1.000	6.63	90.29
	4.000	6.09	230. ⁰⁹
	5.000	5.97	257.60
	6.000	5.84	270.60
	8.000	5.65	253.60

Source: Produced by the author.

ANNEX

ANNEX I – PHASE DIAGRAM DETERMINATION

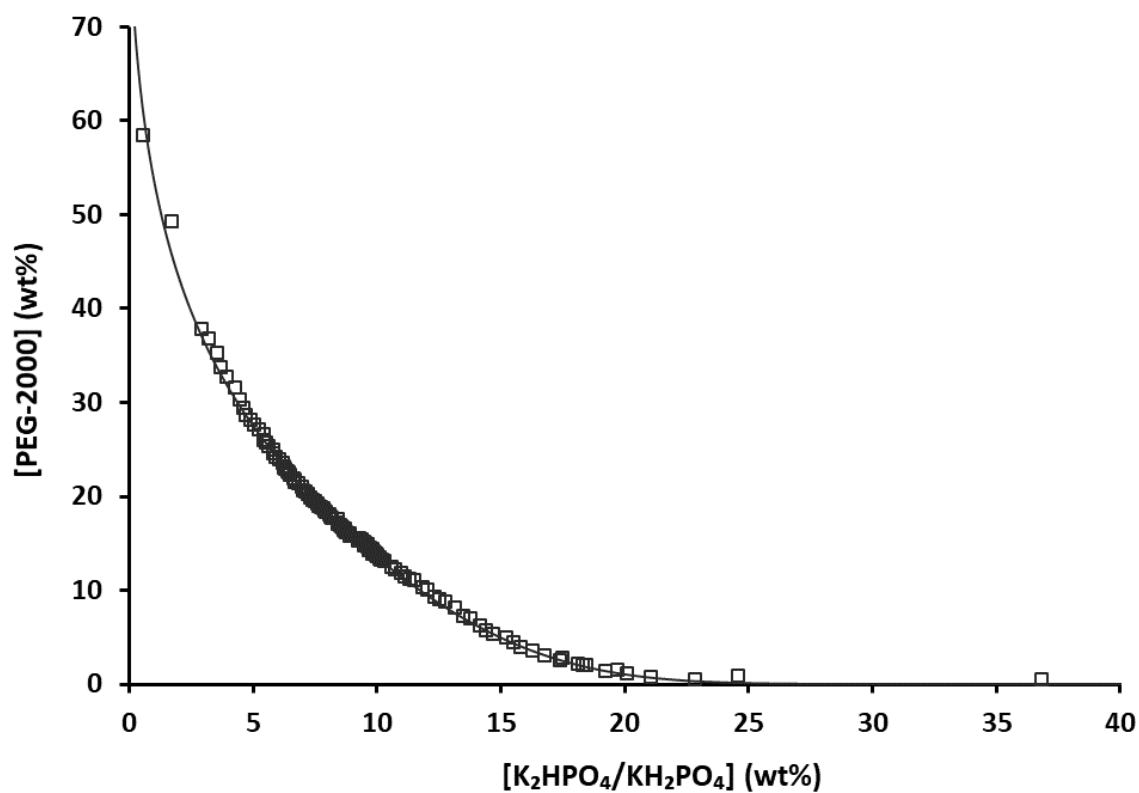
This experiment was organized/executed by a collaborator from the University of Aveiro (M. Martins, S. P. M. Ventura, and J. A. P. Coutinho) and it was also published by Dos Santos *et al.*, 2018. (DOS SANTOS *et al.*, 2018b) The ternary phase diagram for the PEG-2000 + K₂HPO₄/KH₂PO₄ ABS was determined at 298 (± 1) K and atmospheric pressure (**Table I.1**), by the cloud point titration method. (PEREIRA *et al.*, 2010) Aqueous solutions of PEG-2000 at ≈ 60 wt% and aqueous solutions of the buffer K₂HPO₄/KH₂PO₄ (pH 7; ≈ 40 wt%) were prepared and used for the determination of the binodal curves of the polymeric systems. The system compositions were determined by the weight quantification of all components added within ± 10⁻⁴ g. The experimental binodal curves (in weight fraction percentage) were correlated using the Merchuk equation, (MERCHUK *et al.*, 1998) described as equation (I.1):

$$Y = A \times \exp[(BX^{0.5}) - (CX^3)] \quad (\text{I.1})$$

where Y and X, represent PEG and inorganic salt weight percentages, respectively. A, B, and C are constants obtained by the regression of the experimental binodal data.

In the studied ABS, the top phase corresponded to the PEG-rich phase, while the bottom phase was mainly composed of the phosphate-buffer (considering the FTIR from **Figure E.2** and conductivity and viscosity from **Table 7** – PEG phase have lower values for conductivity and higher values for viscosity, and also have a more similar pattern to the PEG standard in the FTIR spectra). The experimental binodal data was fitted by the empirical relationship described by Equation (I.1). The regression parameters were estimated by the least-squares regression, and their values and corresponding standard deviations were 90.36 ± 2.22 , -0.511 ± 0.012 , and $2.684 \times 10^{-4} \pm 1.96 \times 10^{-5}$ for A, B, and C, respectively. In general, a good correlation between the experimental data and the parameters was obtained ($R^2 = 0.9967$ and mean squared deviation = 0.35935), indicating that these fittings can be used to predict data in a specific region of the phase diagram where no experimental data are available, as described in **Figure I.1**.

Figure I-1 – Binodal curve for the ternary systems composed of PEG-2000 + $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7) + H_2O , at $298 (\pm 1)$ K.



Source: Produced by the author with results from collaborators (M. Martins, S. P. M Ventura, and J. A. P. Coutinho) and published in (DOS SANTOS *et al.*, 2018b).

Table I-1 – Weight fraction (w, wt%) data for the ternary systems composed of PEG-2000 (w1) + K₂HPO₄/KH₂PO₄ (w2) + H₂O (w3) at 25 °C and pH 7.

w1	w2	w1	w2	w1	w2
58.42437	0.562859	21.06194	6.972367	13.27194	10.18537
49.26235	1.742253	20.68231	7.017107	13.09396	10.31974
37.89252	2.901231	20.46833	7.123536	12.54766	10.57263
36.81171	3.238451	20.24508	7.234466	12.29095	10.71429
35.26683	3.567477	19.90789	7.301978	11.78791	10.97369
33.75769	3.712223	19.66289	7.415254	11.51793	11.13575
32.69948	3.956477	19.46818	7.515810	11.25842	11.33941
31.57079	4.298149	19.26964	7.596715	11.02314	11.52046
30.27585	4.478772	19.04345	7.658419	10.26256	11.83541
29.41505	4.627360	18.87769	7.744907	10.00918	12.04028
28.72553	4.706418	18.70314	7.834514	9.360700	12.32877
28.10760	4.879526	18.46185	7.875029	9.052108	12.54371
27.58184	5.046982	18.29381	7.951765	8.780667	12.78578
27.16746	5.244942	18.10748	8.069320	8.108906	13.13353
26.57263	5.423158	17.89441	8.121810	7.300127	13.49782
26.01723	5.438430	17.75196	8.194425	6.992313	13.77098
25.72244	5.538211	17.52374	8.420059	6.188999	14.14568
25.40149	5.634937	17.22858	8.414731	5.797979	14.40041
24.92791	5.800055	17.11272	8.436237	5.405427	14.67220
24.52302	5.819312	16.98194	8.500928	4.997708	15.20160
24.21626	5.938375	16.85450	8.567461	4.474634	15.52588
23.90893	6.038794	16.72364	8.614357	3.981884	15.81832
23.61018	6.214740	16.57257	8.723510	3.533446	16.29567
23.21415	6.253219	16.39647	8.655755	3.034510	16.74112
23.03537	6.275140	16.28267	8.730875	2.556691	17.38679
22.90162	6.302573	16.15719	8.742448	2.048639	18.30308
22.71288	6.401785	16.01330	8.887874	1.467205	19.22625
22.49670	6.436388	15.82061	8.927327	1.125973	20.07612
22.23906	6.471210	15.26308	9.266025	0.747296	21.07214
21.95894	6.641614	14.71845	9.480480	0.538774	22.85190
21.75007	6.692859	14.29278	9.655835	0.957874	24.57909
21.55985	6.695819	13.92618	9.842041	0.459675	36.83897
21.34619	6.828344	13.76840	9.976558	–	–

Source: Produced by the author with results from collaborators (M. Martins, S. P. M Ventura, and J. A. P. Coutinho) and published in (DOS SANTOS *et al.*, 2018b).