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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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Supervisor: Prof. Dr. Jorge F. B. Pereira
Co-supervisor: Prof. Dr. Fernando L. Primo

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Araraquara, 26 de novembro de 2020

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	*Associação Brasileira de Normas Técnicas* (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	*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (Coordination of Superior Level Staff Improvement)
CAPES-PROEX UNESP	*CAPES-Pró-Reitoria de Extensão da UNESP* (CAPES-Extension board from UNESP)
CD	circular dichroism
CHO	Chinese hamster ovary cells
CNPq	*Conselho Nacional de Desenvolvimento Científico e Tecnológico* (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
E. coli	*Escherichia coli*
EC	extinction coefficient
e.g.	*exempli gratia* (for example)
EGFP	enhanced green fluorescent protein
EOPO	copolymer of ethylene oxide and propylene oxide
et al.	*et alii* (and others)
EU	European Union
Fab'	antigen-binding fragment
FAPESP	*Fundação de Amparo à Pesquisa do Estado de São Paulo* (São Paulo Research Foundation)
FCPC	fast centrifugal partition chromatography
FCT	*Fundação para a Ciência e Tecnologia de Portugal* (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	*Human papillomavirus*
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
P. pastoris	*Pichia pastoris*
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
rhIFNα-1	recombinant human interferon α-1
rHSA	recombinant human serum albumin
RNA	ribonucleic acid
RSC	Royal Society of Chemistry
RSD	relative standard deviation
S. cerevisiae	*Saccharomyces cerevisiae*
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	*Universidade Estadual Paulista "Júlio de Mesquita Filho"* (São Paulo State University)
USA	United States of America
USD	American dollars
UV	ultraviolet light
viz.	*videlicet* (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

× *g*	times gravity
$(NH_4)_2SO_4$	ammonium sulfate
$[C_1mim]Cl$	1,3-dimethylimidazolium chloride
$[C_2mim]Cl$	1-ethyl-3-methylimidazolium chloride
$[C_4mim][N(CN)_2]$	1-butyl-3-methylimidazolium dicyanamide
$[C_4mim]Cl$	1-butyl-3-methylimidazolium chloride
$[C_4mpyr]Cl$	1-butyl-3-methylpyridinium chloride
$[C_6mim]Cl$	1-hexyl-3-methylimidazolium chloride
$[C_8mim]Cl$	1-octyl-3-methylimidazolium chloride
$[C_{10}mim]Cl$	1-decyl-3-methylimidazolium chloride
$[C_{12}mim]Cl$	1-dodecyl-3-methylimidazolium chloride
[Ch][Ac]	cholinium acetate
[Ch][Bit]	cholinium bitartrate
[Ch][But]	cholinium butanoate
[Ch][Dec]	cholinium decanoate
[Ch][Gly]	cholinium glycolate
[Ch][Hex]	cholinium hexanoate
[Ch][Lac]	cholinium lactate
[Ch][Oct]	cholinium octanoate
[Ch][Pen]	cholinium pentanoate
[Ch][Pro]	cholinium propanoate
[Ch][Bic]	cholinium bicarbonate
[Ch]Cl	cholinium chloride
[Ch][OH]	cholinium hydroxide
[Ch]X	cholinium-based ILs
$[C_nmim]Cl$	*n*-3-methyl-imidazolium-based ILs
$[EGFP]_0$	initial concentration of EGFP
$[EGFP]_B$	concentration of EGFP in the bottom phase
$[EGFP]_{EGFP\text{-rich phase}}$	concentration of EGFP in the EGFP-rich phase
$[EGFP]_{rel}$	relative concentration of EGFP by total protein
$[EGFP]_T$	concentration of EGFP in the top phase
$[N_{2,2,2,2}]Br$	tetraethylammonium bromide
$[N_{4,4,4,4}][BES]$	tetrabutylammonium 2-(bis(2-hydroxyethyl)amino)ethanesulfonate
$[N_{4,4,4,4}]Br$	tetrabutylammonium bromide
$[TP]_{EGFP\text{-rich phase}}$	concentration of total proteins in the EGFP-rich phase
$[\theta]$	mean residue ellipticity
°C	Celsius degrees
^1H NMR	proton nuclear magnetic resonance
A	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	*degrees* 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
$FP_{FP\text{-}poor\ phase}$	fluorescence intensity of the FP in the FP-poor phase
$FP_{FP\text{-}rich\ phase}$	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
Hi	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
n	total number of samples/variables
Na_2CO_3	sodium carbonate
Na_2HPO_4	sodium phosphate dibasic
Na_2SO_4	sodium sulfate
NaCl	sodium chloride
NaH_2PO_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
$TP_{FP\text{-rich phase}}$	fluorescence intensity of the TP in the FP-rich phase
U	enzyme unit
V_0	initial volume
V_B	bottom volume
$V_{EGFP\text{-rich phase}}$	EGFP-rich phase volume
V_{inj}	injection velocity
V_T	initial volume
w1	weight fraction of PEG-2000
w2	weight fraction of K_2HPO_4/KH_2PO_4
w3	weight fraction of H_2O
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

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SUMMARY

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 UniProti (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.

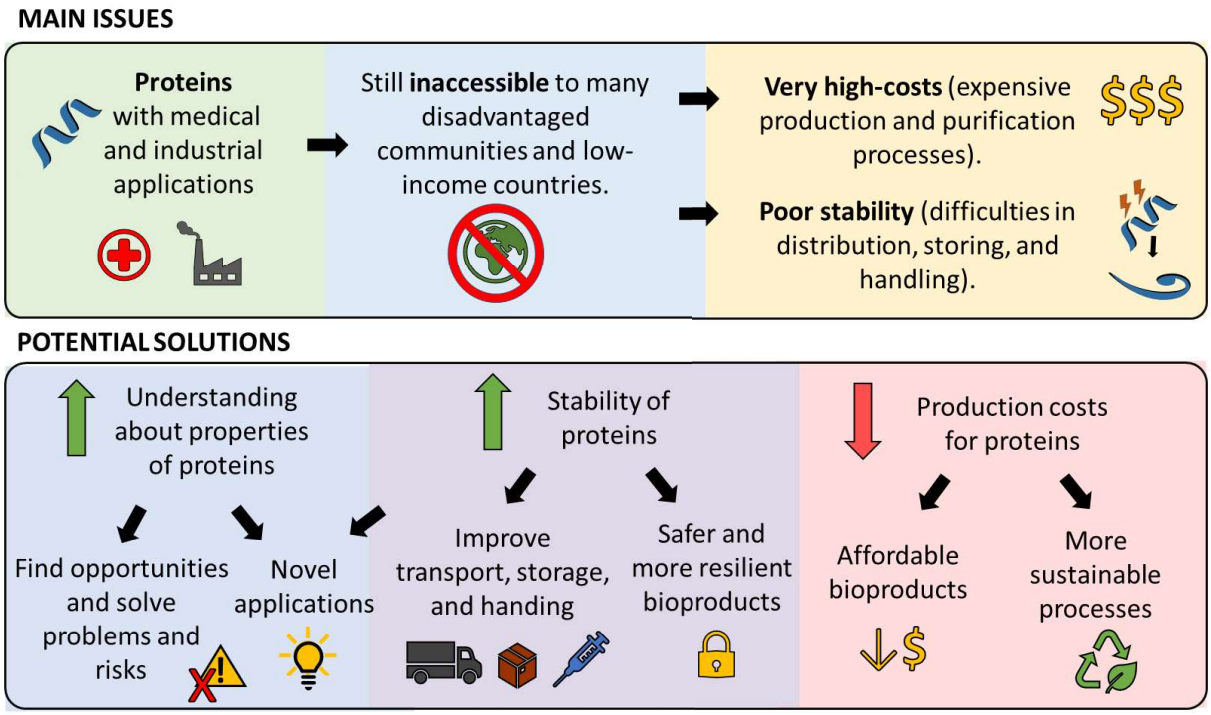
The design of simpler purification methods can help to decrease the costs and complexity of the downstream processing of biomolecules. In this sense, liquid-liquid

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

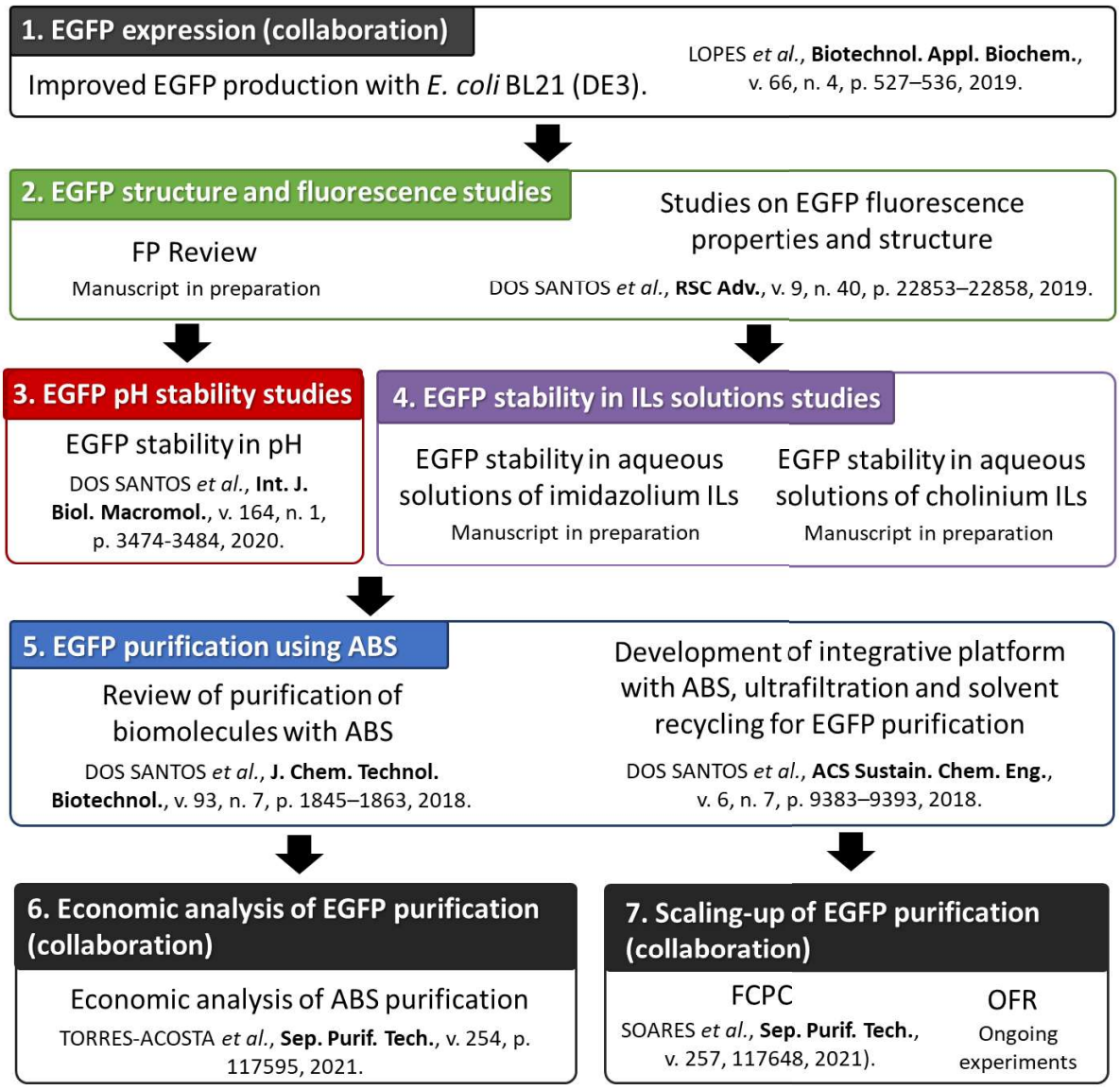
In light of the importance and applications of stabilizing bioformulations and lowering the cost of purification platforms for proteins, this work studied the Enhanced Green Fluorescent Protein (EGFP) as a proof-of-concept for developing novel platforms to stabilize and purify proteins. EGFP is a protein 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) (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). (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) (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). (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). (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). (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). (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.

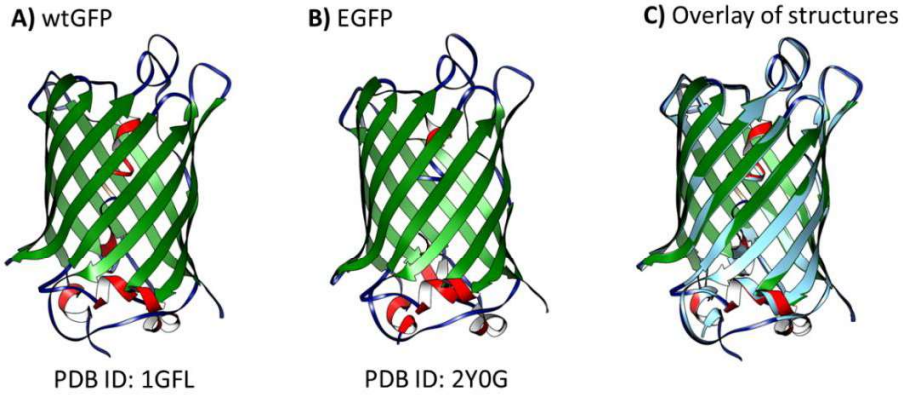
1.3 FLUORESCENT PROTEINS (FP)

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 ^{0}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 helixes 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 ^{0}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-Savoye 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 μmol.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 (55,000-57,000 L.mol^{-1}.cm^{-1}), (TSIEN, 1998) relative brightness (33,000 L.mol^{-1}.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 L.mol^{-1}.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 μmol.L^{-1}) (MYATT *et al.*, 2017) and its environment (*i.e.*, biding 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 (A) 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. (TREUHAFT; 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 (H_2O_2). (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 GPF 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 ≤ 0.5 $mol.L^{-1}$), 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 rβ-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 of 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 periplasmatic 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.

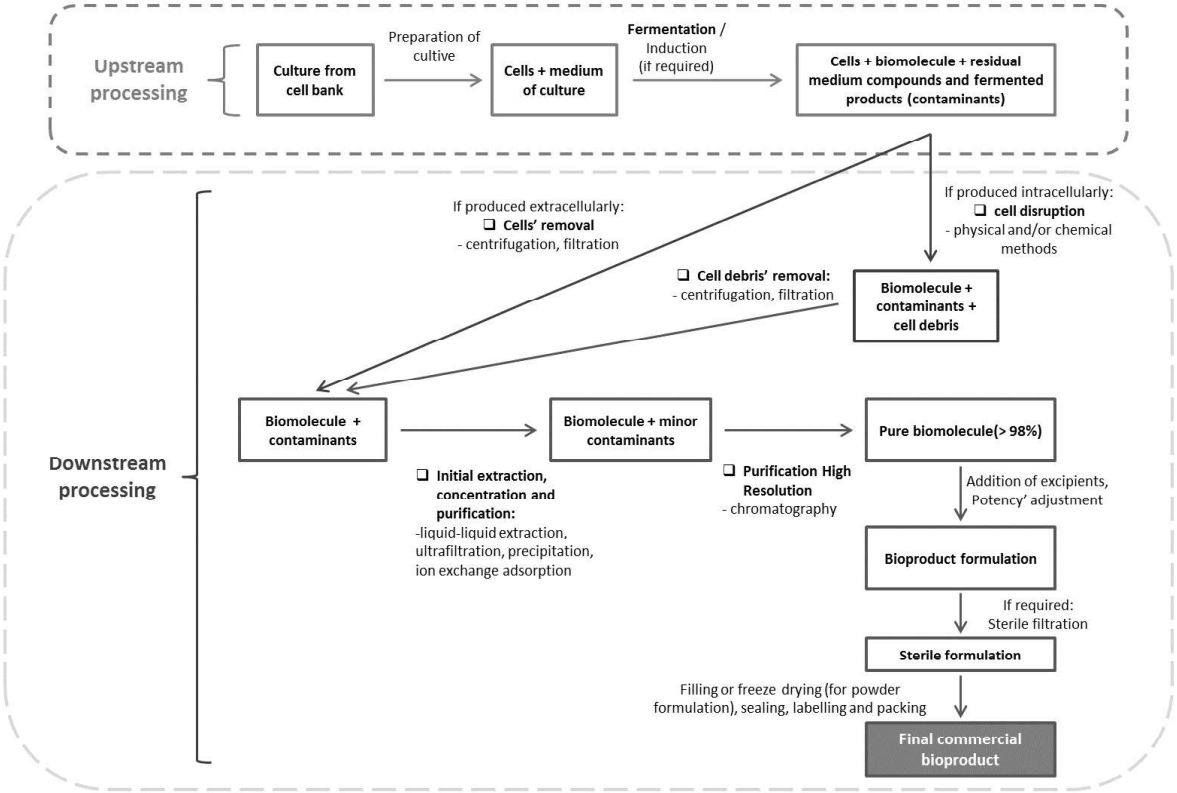
In the case of intracellular biomolecules, it is also necessary to add at least one cell disruption stage to liberate the compounds from the cell interior. (DECHOW, 1989) In addition to the incorporation of an extra step for cell disruption, biomolecules recovered from the intracellular environment present 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.

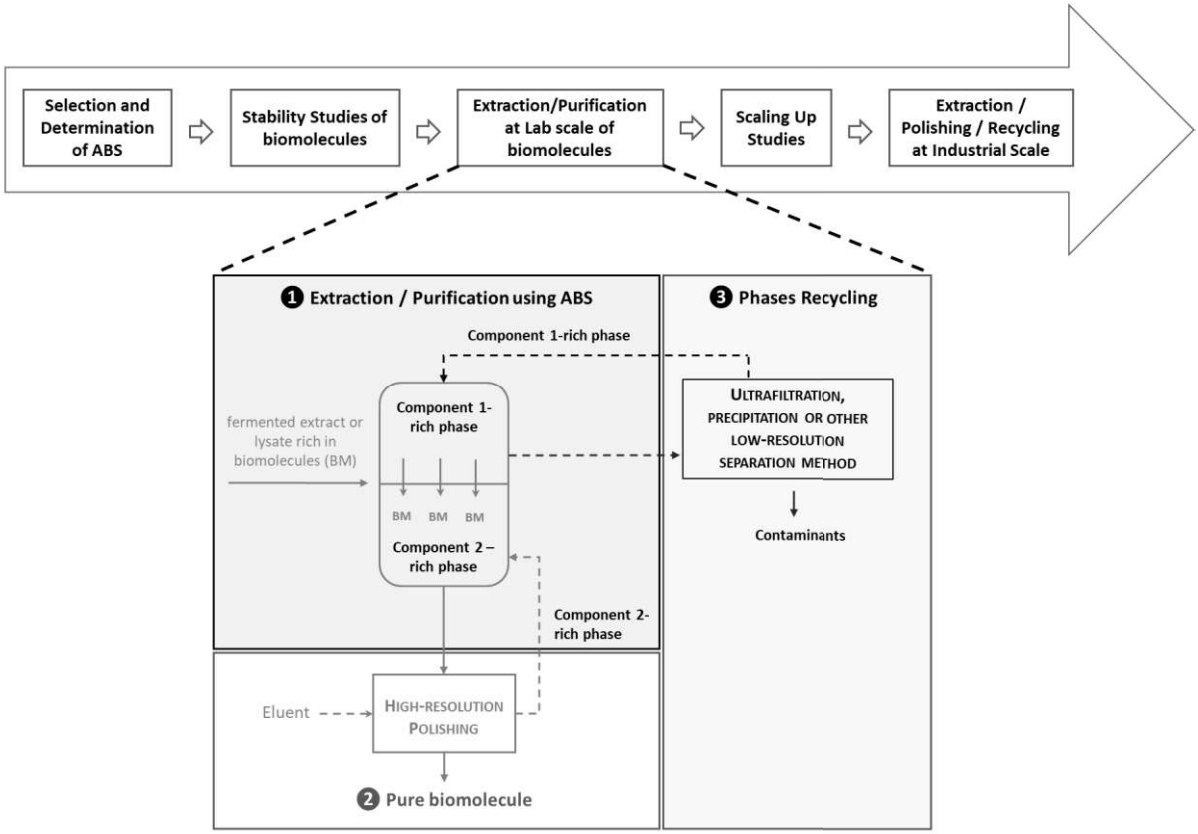
1.5.1.2 ABS for the purification of biomolecules

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
E. coli	Apolipoprotein A (Apo A)**	EOPO/Reppal	82	-	2.7	(PERSSON *et al.*, 1998)
	Green Fluorescent Protein (GFP)*	PEG/$C_6H_5Na_3O_7$	91	-	3.3	(LI; BEITLE, 2002)
		PEG/$C_6H_5Na_3O_7$, back extraction with PEG/ Na_2SO_4 + IMAC	71	-	94.0	
	Human growth hormone (hGH)*	EOPO/waxy starch	70	-	5.0	(PERSSON *et al.*, 2005)
	Human growth hormone antagonist (hGHA)*	PEG/$(NH_4)_2SO_4$	83	-	-	(HAYENGA; VALEX, 2002)
	Human recombinant interferon α-1 (rhIFNα-1)*	PEG-phosphate ester/K_2HPO_4/NaCl	99	-	25.0	(GUAN *et al.*, 1996)
	Human recombinant interferon-α2b (IFN-α2b)*	2-propanol/$(NH_4)_2SO_4$/NaCl	75	-	16.2	
		1-Propanol/K_2HPO_4	97	-	2.6	
		2-Propanol/K_2HPO_4	92	-	1.4	
		Ethanol/K_2HPO_4	60	-	0.6	
		1-Propanol/$(NH_4)_2SO_4$	82	-	7.0	(LIN *et al.*, 2013)
		2-Propanol/$(NH_4)_2SO_4$	78	-	12.8	
		Ethanol/$(NH_4)_2SO_4$	35	-	3.2	
		1-Propanol/$C_6H_7NaO_7$	83	-	3.3	
		2-Propanol/$C_6H_7NaO_7$	79	-	5.0	
		Ethanol/$C_6H_7NaO_7$	39	-	1.2	
		PEG/KH_2PO_4/NaCl	41	-	26.3	
	Human insulin-like growth factor-I (IGF-I)*	PEG/Na_2SO_4	70	97	-	(HART *et al.*, 1994)
	L1 protein*	PEG/KH_2PO_4	65	-	-	(RITO-PALOMARES; MIDDELBERG, 2002)
*P. pastoris***	Recombinant human serum albumin (rHSA)	ethanol/K_2HPO_4	100	-	-	(DONG *et al.*, 2012)
	GFP*	PEG/$C_6H_5Na_3O_7$	91		3.3	(LI; BEITLE, 2002)
		ethanol/Na_2CO_3	91	-	-	(DONG *et al.*, 2012)
		ethanol/Na_2SO_4	74	-	-	
		ethanol/$(NH_4)_2SO_4$	75	-	-	
		ethanol/K_2HPO_4 associate with HIC	75	99	-	

[a]PF – 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 (rhIFNα-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 rhIFNα-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 rhIFNα-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 rhIFNα-1 in the bottom phase at pH 6.0. (GUAN *et al.*, 1996)

More recently, also using PEG/potassium phosphate monobasic (KH_2PO_4)/NaCl-based ABS, 41 % of a human recombinant interferon-α2b (IFN-α2b) 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-α2b, applying alcohol/salts-based ABS. The most promising results demonstrate that the combination of 1-propanol, 2-propanol, or ethanol, with aqueous solutions of K_2HPO_4, ammonium sulfate [$(NH_4)_2SO_4$] or monosodium citrate ($C_6H_7NaO_7$) 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-α2b 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 $(NH_4)_2SO_4$, pH 8.0–8.3 for $C_6H_7NaO_7$ and pH 9.0–9.5 for K_2HPO_4], 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-α2b 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 $(NH_4)_2SO_4$ 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/$(NH_4)_2SO_4$] and added other salts as electrolytes, increasing the PF to 16.2 for a recovery of 75 % of IFN-α2b when used 2-propanol/$(NH_4)_2SO_4$/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-α2b'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/$C_6H_5Na_3O_7$-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; BEITLE, 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; BEITLE, 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 $(NH_4)_2SO_4$] 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/$(NH_4)_2SO_4$-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	*Penicillium* sp.	[C_4mim]Cl	NaH_2PO_4	91.7	-	(LIU *et al.*, 2006b)
	Tetracycline	*Streptomyces aureofaciens*	[Ch][Bic]	PEG-600	81.6		
			[Ch]Cl	PEG-600	82.5	-	(PEREIRA *et al.*, 2013b)
				K_3PO_4	92.4		
	Clavulanic Acid	*Streptomyces clavuligerus*	[Ch]Cl	PEG-600	44.4	22.7	
				PEG-550-OMe	45.7	4.7	(PANAS *et al.*, 2017)
				PEG-600/NaPA-8000	55.9	2.4	
Colorants or Pigments	Natural red colorant	*Penicillium purpurogenum*	[C_4mim]Cl	potassium citrate buffer	62.4	-	
			[$N_{2,2,2,2}$]Br		51.5	-	(VENTURA *et al.*, 2013b)
			[$N_{4,4,4,4}$]Br		80.2	-	
		Talaromyces amestolkiae	[C_8mim]Cl	Triton X-114 [a]	(only reported *K*)	-	(TORRES *et al.*, 2018)
			[C_{10}mim]Cl				
			[C_{12}mim]Cl				
	Red *Monascus* pigment	*Monascus anka*	[C_4mim][PF_6]	Triton X-100 [a]	86	-	(SHEN *et al.*, 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	*Bacillus* sp. ITP-001	$[C_2mim]Cl$	PEG-1500 + K_2HPO_4/KH_2PO_4	77.2	175.6	(SOUZA *et al.*, 2015b)
			$[C_4mim]Cl$		77.5	169.3	
			$[C_6mim]Cl$		76.6	220	
			$[C_8mim]Cl$		90.2	245	
			$[C_2mim]Cl$	PEG-4000 + K_2HPO_4/KH_2PO_4	60.2	181.3	
			$[C_6mim]Cl$		79.3	254	
			$[C_2mim]Cl$	PEG-8000 + K_2HPO_4/KH_2PO_4	89.7	160.6	
			$[C_6mim]Cl$		80.9	222.8	
			[Ch][Bit]	Tetrahydrofuran	90	136.8	(SOUZA *et al.*, 2015a)
			$[C_4mim][N(CN)_2]$	Potassium phosphate buffer	96.1	26	(VENTURA *et al.*, 2012)
			$[C_4mpyr]Cl$		94.2	36.7	
			$[C_4mim]Cl$		90.6	40.8	
			$[C_8mim]Cl$		92.2	51	
		Burkholderia cepacia ST8	$[N_{4,4,4,4}][BES]$	$(NH_4)_2SO_4$	from 93.7 to 98.7	from 8.9 to 22.4	(LEE *et al.*, 2017)
Polysaccharides	*Cordyceps sinensis* polysaccharides	*Cordyceps sinensis*	$[C_4mim]Cl$	K_3PO_4	89.4	-	(YAN *et al.*, 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 $(NH_4)_2SO_4$), buffers (potassium citrate buffer and phosphate buffer K_2HPO_4/KH_2PO_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 ($[C_2mim]Cl$), 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$), 1-hexyl-3-methylimidazolium chloride ($[C_6mim]Cl$), 1-octyl-3-methylimidazolium chloride ($[C_8mim]Cl$)} to benign ammonium-based ILs {such as, tetraethylammonium bromide ($[N_{2,2,2,2}]Br$), tetrabutylammonium bromide ($[N_{4,4,4,4}]Br$), cholinium chloride ([Ch]Cl), cholinium bicarbonate ([Ch][Bic]), cholinium bitartrate ([Ch][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.

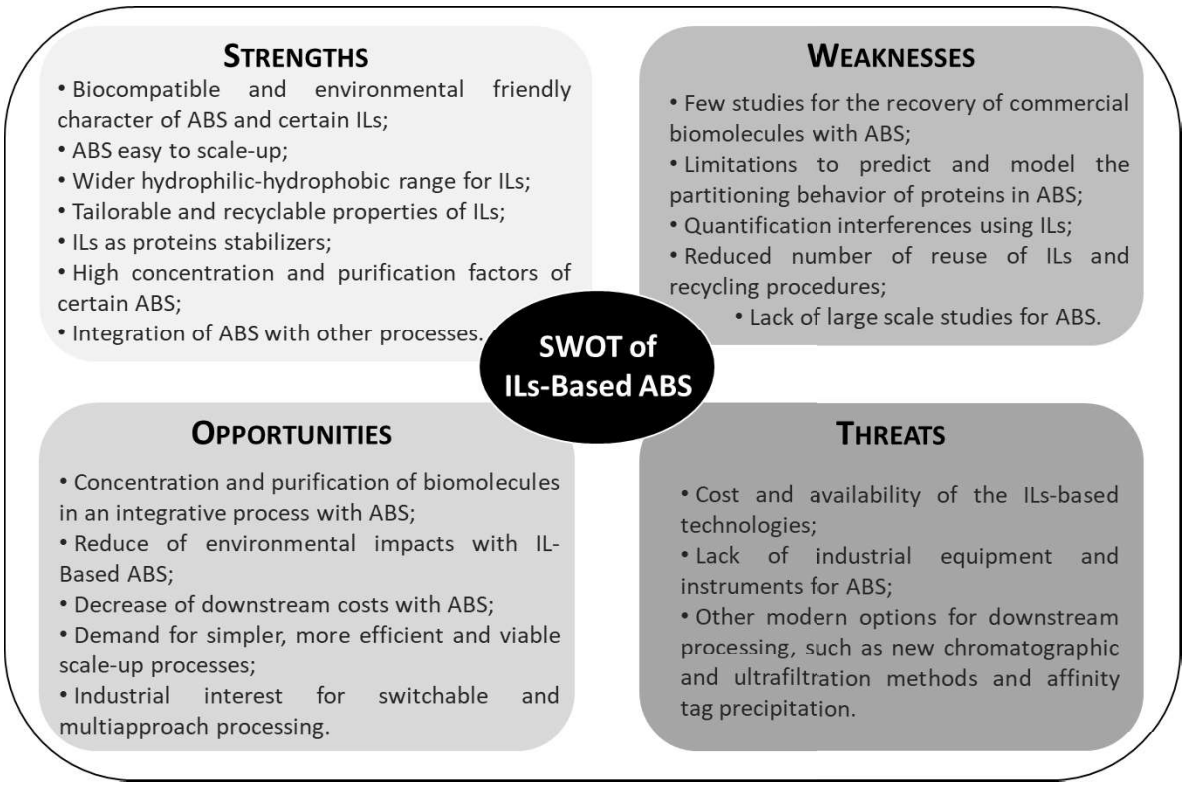
There are also works with applications of IL-based ABS for the recovery of biomolecules of medical use, for example, the purification of the anti-leukemic biopharmaceutical asparaginase produced by *E. coli* cells. (SANTOS *et al.*, 2018) Considering L-asparaginase is used to treat acute lymphoblastic 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 ($[C_4mim][CH_3SO_3]$) 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; DEMAIN; 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
E. coli	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)	Krystexxal™
	Chronic hepatitis C and B, certain cancers	interferon-α2a	Roferon®-A
		interferon-α2b	Intron A®
		interferon-α2b 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®
	Diabetes Mellitus	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
E. coli	Infectious diseases	opebacan (bactericidal/permeability-increasing protein)	Neuprex®
	Lyme disease vaccine	OspA (Outer surface protein A fragment from *Borrelia burgdorferi*)	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®
		filgrastim pegylated	Neulasta®
	Oral mucositis	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®
Neisseria meningitidis	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 meningitides*. (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
S. cerevisiae	Anticoagulant	Hirudine	Refludan®, Revasc®
	Diabetes mellitus	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-*Haemophilus influenza* type B vaccine	various antigens	Tritanrix® HepB
	Diphtheria-Tetanus-Pertussis-Hepatitis B-Poliomyelitis vaccine	various antigens	Infanrix® Penta
	Diphtheria-Tetanus-Pertussis-Hepatitis B-Poliomyelitis-*Haemophilus influenza* 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®
	Human papillomavirus (HPV) vaccine	HPV vaccine	Gardasil®
		HPV surface antigens	Silgard®
	Hyperuricaemia	Rasburicase	Fasturtec®
	Neuropathic, chronic and diabetic ulcer	platelet-derived growth factor-BB	Regranex®
	Neutropenia	Sargramostim	Leukine®
	Production of human therapeutics	Albumin	Recombumin®
P. pastoris	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.

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 $[C_{12}mim]Cl$ 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 $[C_{12}mim]Cl$ 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 + Na_2SO_4 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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