

Review article (accepted manuscript): Ionic liquids and deep eutectic solvents for the stabilization of biopharmaceuticals: A review

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Review

Ionic liquids and deep eutectic solvents for the stabilization of biopharmaceuticals: A review

Nathalia Vieira Porphirio Veríssimo ^{1,2*}, Cassamo Usemane Mussagy ³, Heitor Buzetti Simões Bento ¹, Jorge Fernando Brandão Pereira ⁴, and Valéria de Carvalho Santos-Ebinuma ^{1,*}

¹Department of Bioprocess Engineering and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University, CEP: 14801-902, Araraquara SP, Brazil. NVPV: nathalia.v.santos@unesp.br; HBSB: heitor.bento@unesp.br; VCSE: valeria.ebinuma@unesp.br

²Department of Pharmaceutical Sciences, School of Pharmaceutical Sciences, São Paulo University, CEP: 14040-020, Ribeirão Preto, SP, Brazil.

³Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Quillota 2260000, Chile. cassamo.mussagy@pucv.cl

⁴Univ Coimbra, CIEPQPF, Department of Chemical Engineering, Pólo II – Pinhal de Marrocos, 3030-790 Coimbra, Portugal. jfbpereira@eq.uc.pt

*Shared correspondence.

Correspondence and requests for materials should be addressed to Nathalia Vieira Porphirio Veríssimo – E-mail: nathalia.v.santos@unesp.br or Valéria de Carvalho Santos-Ebinuma – E-mail: valeria.ebinuma@unesp.br. Universidade Estadual Paulista (UNESP), School of Pharmaceutical Sciences, Campus (Araraquara), Department of Engineering Bioprocess and Biotechnology, Rodovia Araraquara-Jaú/ Km 01, Campos Ville - Araraquara/SP 14800-903 - Araraquara - SP/Brazil. Phone: Phone: 55-16-3301-4647.

Abstract

Biopharmaceuticals have allowed the control of previously untreatable diseases. However, their low solubility and stability still hinder their application, transport, and storage. Hence, researchers have applied different compounds to preserve and enhance the delivery of biopharmaceuticals, such as ionic liquids (ILs) and deep eutectic solvents (DESs). Although the biopharmaceutical industry can employ various substances for enhancing formulations, their effect will change depending on the properties of the target biomolecule and environmental conditions. Hence, this review organized the current state-of-the-art on the application of ILs and DESs to stabilize biopharmaceuticals, considering the properties of the biomolecules, ILs, and DESs classes, concentration range, types of stability, and effect. We also provided a critical discussion regarding the potential utilization of ILs and DESs in pharmaceutical formulations, considering the restrictions in this field, as well as the advantages and drawbacks of these substances for medical applications. Overall, the most applied IL and DES classes for stabilizing biopharmaceuticals were cholinium-, imidazolium-, and ammonium-based, with cholinium ILs also employed to improve their delivery. Interestingly, dilute and concentrated ILs and DESs solutions presented similar results regarding the stabilization of biopharmaceuticals. With additional investigation, ILs and DESs have the potential to overcome current challenges in biopharmaceutical formulation.

Keywords: proteins, nucleic acid, neoteric solvents, stability of biomolecules, pharmaceutical formulations, preservatives, biopharmaceuticals, ILs, DESs, protein stability

Abbreviations

Ionic liquids (ILs) and Deep Eutectic Solvents (DESS)

Ammonium-based ILs

tetrabutylammonium bromide ($[N_{4,4,4,4}]Br$)
triethylammonium phosphate ($[N_{0,2,2,2}]PO_4$)
triethylammonium sulfate ($[N_{0,2,2,2}]SO_4$)
trimethylammonium acetate ($[N_{0,1,1,1}][CH_3COO]$)
trimethylammonium dihydrogen phosphate ($[N_{0,1,1,1}]H_2PO_4$)
trimethylammonium hydrogen sulfate ($[N_{0,1,1,1}]HSO_4$)

Ammonium IL-Robed siRNA

benzyltrimethyloctylammonium robod-siRNA1 ($[N_{1,1,(Bz),8}]$ -siRNA1)
benzyltrimethylstearylammmonium robod-siRNA1 ($[N_{1,1,(Bz),18}]$ -siRNA1)
benzyltrimethyltetradecylammmonium robod-siRNA1 ($[N_{1,1,(Bz),14}]$ -siRNA1)

Cholinium-based DESSs

choline chloride and ethylene glycol ($[Ch]Cl-[(CH_2OH)_2]$)
choline chloride and glycerol ($[Ch]Cl-[C_3H_5(OH)_3]$)
choline chloride and carbamide ($[Ch]Cl-[CO(NH_2)_2]$)
cholinium geranate 1:2 ($[Ch][C_9H_{15}COO]-[C_9H_{15}COOH]$)
cholinium geranate 1:4 ($[Ch][C_9H_{15}COO]-[C_9H_{15}COOH]_3$)
cholinium hydroxyacetic acid 1:2 ($[Ch][HOCH_2COO]-[HOCH_2COOH]$)
cholinium hydroxyacetic acid 2:1 ($[Ch][HOCH_2COO]-[Ch]$)

Cholinium-based ILs

cholinium acetate ($[Ch][CH_3COO]$)
cholinium butanoate ($[Ch][C_3H_7COO]$)
cholinium citrate ($[Ch][C_5H_7O_5COO]$)
cholinium dihydrogen phosphate ($[Ch]H_2PO_4$)
cholinium geranate ($[Ch][C_9H_{15}COO]$)
cholinium hydroxyacetate ($[Ch][HOCH_2COO]$)
cholinium hexanoate ($[Ch][C_5H_{11}COO]$)
cholinium indole-3-acetate ($[Ch][C_9H_8NCOO]$)
cholinium L-argininate ($[Ch][Arg]$)
cholinium L-asparaginate ($[Ch][Asn]$)
cholinium dodecanoate ($[Ch][C_{12:0}]$)
cholinium L-glutamate ($[Ch][Gln]$)

cholinium L-lysinate ([Ch][Lys])
cholinium cis-9-octadecenoate ([Ch][C_{18:1}])
cholinium phenylpropanoate ([Ch][PhC₂H₅COO])
cholinium propanoate ([Ch][C₂H₅COO])
cholinium 2-oxopropanoate ([Ch][CH₃COCOO])
dicholinium L-asparaginate ([Ch]₂[Asn])
dicholinium L-glutamate ([Ch]₂[Gln])

Phosphonium-based ILs

tetrabutylphosphonium bromide ([P_{4,4,4,4}]Br)

Imidazolium-based ILs

1-butyl-3-methylimidazolium acetate ([C₄MIm][CH₃COO])
1-butyl-3-methylimidazolium bromide ([C₄MIm]Br)
1-butyl-3-methylimidazolium chloride ([C₄MIm]Cl)
1-butyl-3-methylimidazolium dicyanamide ([C₄MIm][N(CN)₂])
1-butyl-3-methylimidazolium hydrogen sulfate ([C₄MIm]HSO₄)
1-butyl-3-methylimidazolium iodine ([C₄MIm]I)
1-butyl-3-methylimidazolium methanesulfonate ([C₄MIm][CH₃SO₃)
1-butyl-3-methylimidazolium nitrate ([C₄MIm]NO₃)
1-butyl-3-methylimidazolium thiocyanate ([C₄MIm][SCN])
1-butyl-3-methylimidazolium tosylate ([C₄MIm][CH₃BzSO₃)
1-butyl-3-methylimidazolium tricyanomethanide ([C₄MIm][C(CN)₃)
1-butyl-3-methylimidazolium trifluoroacetate ([C₄MIm][CF₃COO])
1-decyl-3-methylimidazolium acetate [C₁₀MIm][CH₃COO]
1-dodecyl-3-methylimidazolium acetate ([C₁₂MIm][CH₃COO])
1-ethyl-3-methylimidazolium bromide ([C₂MIm]Br)
1-ethyl-3-methylimidazolium acetate ([C₂MIm][CH₃COO])
1-hexyl-3-methylimidazolium acetate ([C₆MIm][CH₃COO])
1-octyl-3-methylimidazolium acetate ([C₈MIm][CH₃COO])

Lidocainum-based IL

lidocainum etodolac IL, Ionic Liquid Transdermal System from MEDRx (ILTS[®])

Poly(vinyl pyrrolidone)-based DES

poly(vinyl pyrrolidone) and propanedioic acid 1:1 ((C₆H₉NO)_n–[CH₂(COOH)₂)

Other

active pharmaceutical ingredients (APIs)

adenine (A)
analytical ultracentrifugation (AUC)
entropy change (ΔS)
Gibbs Free Energy change (ΔG)
circular dichroism (CD)
compound annual growth rate (CAGR)
cryogenic electron microscopy (Cryo-EM)
cytosine (C)
deoxyribonucleic acid (DNA)
differential scanning calorimetry (DSC)
differential scanning fluorimetry (DSF)
European Union (EU)
Fourier transform infrared (FTIR)
generally recognized as safe (GRAS)
glucagon-like peptide 1 (GLP-1)
grand average hydrophobicity index (GRAVY)
guanine (G)
half-life ($t_{1/2}$)
hydrogen bond acceptor (HBA)
hydrogen bond donor (HBD)
immunoglobulin G1 (IgG1)
instability index (II)
interleukin-2 (IL-2)
ionic liquids (ILs)
isothermal titration calorimetry (ITC)
L-Asparaginase (L-ASNase)
lysozyme (Lys)
nuclear resonance spectroscopy (NMR)
melting enthalpy (ΔH_m)
melting temperature (T_m)
molecular dynamics (MD)
ovalbumin epitope (OVA)
protein-protein interactions (PPIs)
ribonucleic acid (RNA)

SHAPE (Selective 2'-Hydroxyl Acylation analyzed by Primer Extension)

small interfering RNA (siRNA)

molecular weight (MW)

thermal shift assay (TSA)

thymine (T)

ultraviolet (UV)

uracil (U)

α -chymotrypsin (CT)

1. Introduction

Biopharmaceuticals have allowed the successful treatment of illnesses with low recovery rates, such as cancers, autoimmune diseases, and metabolic disorders (Rasmussen et al., 2021). These compounds can include a wide variety of biomolecules based on amino acids and nucleic acids (Walsh, 2013), such as peptides, proteins, DNA, and RNA derivatives. Their effectiveness has led to a growing demand, confirmed by a compound annual growth rate (CAGR) of 9.3% between 2016 and 2024, with an expected value of USD 405 billion by 2024 (Kim et al., 2022). However, due to their high prices and low stability outside of cold chains, most biopharmaceuticals are still inaccessible to low-income countries and communities (Ferrari, 2022). For example, many bioactive macromolecules are degraded or have poor absorption and bioavailability *in vivo* even when presenting pharmacological action *in vitro* (Manning et al., 2010). The inherent instability of biopharmaceuticals when outside their natural environment may cause limitations not only in their medical application, but also in their production, storage, and transportation. Therefore, the development of new formulations to stabilize and enhance the delivery of biopharmaceuticals can potentially expand their applications and improve their access in marginalized communities.

Several strategies have been applied to enhance the stability of biopharmaceuticals, such as the use of neoteric “green” solvents as additives in their formulations. For example, different classes of ionic liquids (ILs) and deep eutectic solvents (DESs) can be employed to improve the stability and delivery of biomolecules such as proteins and nucleic acids (Egorova et al., 2021; Veríssimo et al., 2022). Thus, the development of novel green solvent formulations for biopharmaceuticals may lead to more efficient, sustainable, and environmentally friendly production and application of biologics (Dhiman et al., 2023).

To elucidate the advances in this field, this review will organize the state-of-the-art on the use of ILs and DESs for improving the stability and delivery of biopharmaceuticals. We will demonstrate the trends and knowledge gaps in this area and provide a perspective on the use of biocompatible ILs and DESs as additives in biopharmaceutical formulations. Firstly, we will present the main classes of biopharmaceuticals, their types of stability, and how to assess them. Then, we will discuss the properties of ILs and DESs, their biocompatibility, and their potential for application in biological systems. We will then demonstrate the effects of ILs and DESs on protein and nucleic acid biopharmaceuticals by examining their effects on individual biopharmaceuticals, followed by a debate regarding their overall use to stabilize and enhance the delivery of biotechnological medicines. As concluding remarks, we will provide our expert opinion concerning the trends and opportunities in the field, along with limitations and challenges to overcome.

2. Biopharmaceuticals

Biopharmaceuticals are generally defined as medicines based on amino acid and nucleic acid and produced by biotechnological processes (Walsh, 2013). Therefore, small organic molecules like penicillin-

based antibiotics (*e.g.*, benzylpenicillin) are not considered biopharmaceuticals even if obtained through biotechnological means, being classified as pharmaceuticals. Additionally, biological products such as donated blood and organs are not considered biopharmaceuticals as they are not obtained through biotechnological methods. However, there is still no unified definition for biopharmaceuticals by regulatory agencies such as the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA), which regulates them at times in the broader category of biological products and others with traditional pharmaceuticals (EMA, 2023; FDA, 2017).

Regarding their constitution, amino acid-based biopharmaceuticals include peptides, proteins, and glycosylated peptides and proteins, while nucleic-based are mostly comprised of DNA, RNA (*e.g.*, siRNA, mRNA), viral vectors, antisense oligonucleotides, aptamers, and ribozymes. As for their classes of application, the most relevant include vaccines, anticancer biopharmaceuticals, gene therapies, genome editing biopharmaceuticals, antisense therapy, enzymes, hormones, monoclonal antibodies, blood product derivatives, cytokines and interferons, growth factors, and fusion proteins (Ho, 2013).

Historically, the origins of biopharmaceuticals can be traced back to the therapeutic use of insulin sourced from animal pancreas in the 1920s (Alyas et al., 2021). While this naturally-derived insulin served as a precursor to modern biopharmaceuticals, the true era of biotechnological medicines began with the development of recombinant DNA technology. The first biopharmaceutical, a recombinant human insulin developed using Genentech's techniques and then commercialized as 'Humulin' by Eli Lilly, was approved by the FDA in 1982 (Nielsen, 2013). As biotechnological techniques matured, the late 20th and early 21st centuries saw the rise of monoclonal antibodies, effectively targeting specific disease agents and mechanisms. The evolution of biopharmaceuticals reached a new frontier with the advent of nucleic acid-based therapies, with the first nucleic acid-based drug ("Glybera", from uniQure) approved for use in the European Union (EU) in 2012 (Kesik-Brodacka, 2018; Moran, 2012). However, despite the success of biopharmaceuticals in treating complex diseases, their unstable nature and vulnerability to denaturation still limit their widespread therapeutical application (Lin et al., 2021).

Biopharmaceuticals have significantly greater molecular weight (MW) than traditional pharmaceuticals based on small organic compounds (*e.g.*, MW of 5,734 g/mol for human insulin and 334 g/mol for benzylpenicillin, respectively), with more complex intra and intermolecular interactions required to maintain their structural integrity. Although their intricate nature can improve their therapeutical effectiveness and specificity, this characteristic can also lead to higher sensitivity to adverse environmental conditions and

increased inactivation or degradation during their clinical use. Moreover, these substances can exhibit complex mechanisms of action and are potentially immunogenic, which hinders their study and application (Kesik-Brodacka, 2018; Molowa and Mazanet, 2003).

In this sense, research regarding the use of ILs and DESs as greener and biocompatible stabilizer additives in the composition of biopharmaceuticals has demonstrated that they can be an alternative to the limitations of biomolecules. For example, ILs and DESs can enhance drug delivery, increase stability, and confer protection to bioactive compounds, which are valuable combinations in pharmaceutical formulations (Wang et al., 2022). Before discussing in detail the application of ILs and DESs in biopharmaceuticals, we will explore the relevant parameters and methods associated with the stability of macromolecules and the conventional strategies applied by the pharmaceutical industry to stabilize complex macromolecules.

3. Stability of biopharmaceuticals

To comprehend the stability of biopharmaceuticals, it is necessary to understand their composition, structure, and the different parameters and methods that can be employed to determine their preservation. Proteins, peptides, and nucleic acids are intricate and diverse systems (Nelson and Cox, 2012). The structure of proteins and peptides is comprised of a chain of amino acids while nucleic acids are composed of a nucleotide sequence. Both proteins and nucleic acids will arrange their chains in complex three-dimensional arrangements that can be divided into four organizational levels. It should be noted that not all proteins, peptides, and nucleic acids present all four organizational levels, as their complexity varies depending on their size and function. To demonstrate these different levels, **Figure 1** presents a summary of protein and nucleic acid structures.

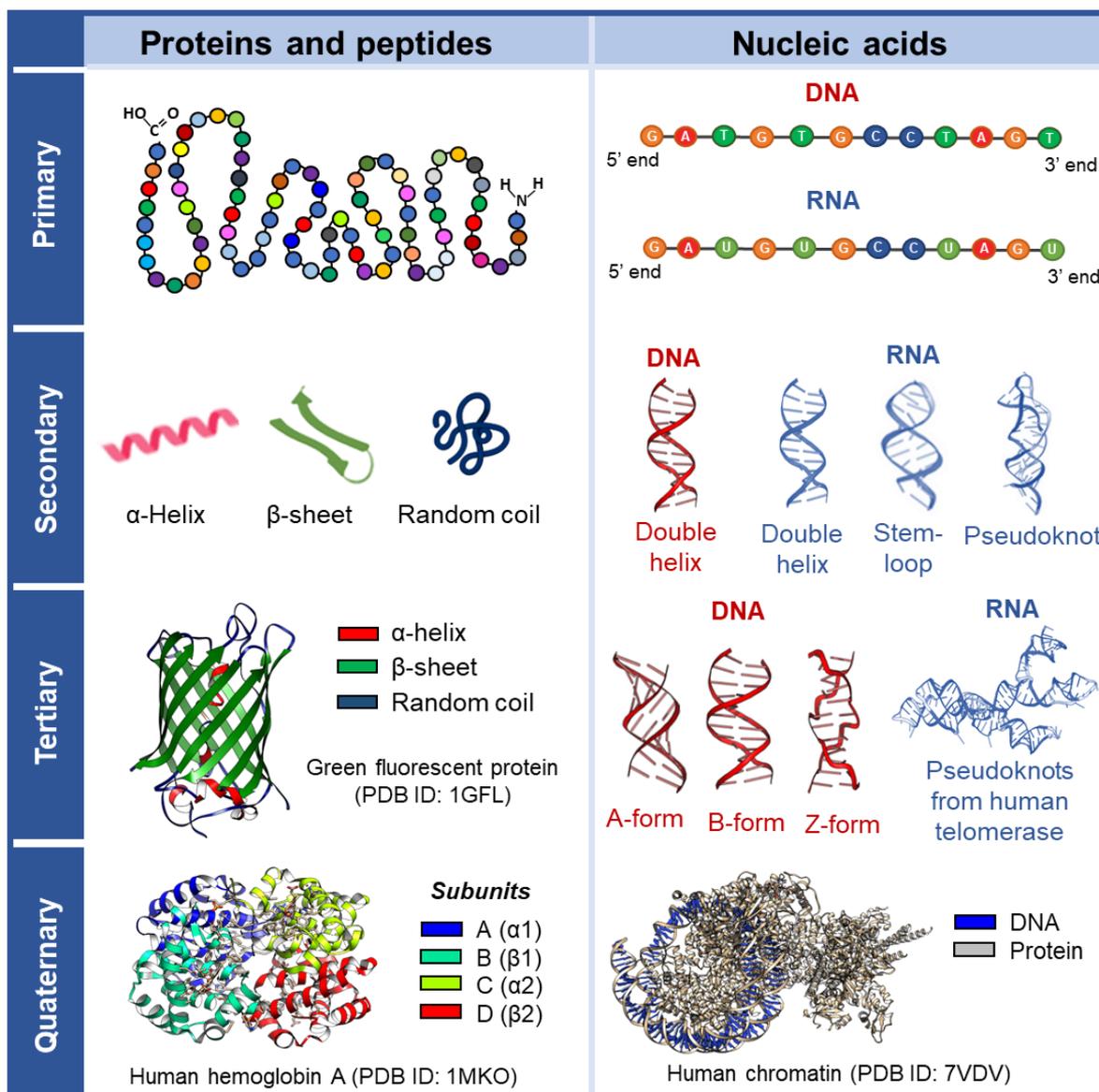


Figure 1. Summary of protein, peptide, and nucleic acid structures (primary, secondary, tertiary, and quaternary). Sources: Protein Data Bank (PDB) IDs 1GFL, 1MKO, and 7VDV. Images of the proteins were produced with the PDB structures using UCSF Chimera 1.14 (Berman et al., 2002; Pettersen et al., 2004). Secondary and tertiary structures of nucleic acids from Thomas Shafee (Creative Commons 4.0) (Shafee, 2017; Thomas Shafee, 2016).

As presented in **Figure 1**, proteins are composed of amino acids, which are organic molecules with amino and carboxylic acid functional groups. The primary structure of proteins comprises a polypeptide chain formed by amino acids linked by peptide bonds (Nelson and Cox, 2012). Its secondary structure includes the interactions of the polypeptides in motifs such as α -helix, β -sheets, and coils. As for the tertiary structure, it comprehends the three-dimensional folding of the protein structure, while the quaternary corresponds to the packing of distinct subunits of the protein (Voet and Voet, 2021).

The structural levels of amino acids follow the same logic as the proteins but with differences in their composition and arrangement. Nucleic acids are composed of nucleotides, a base containing nitrogen [*i.e.*, adenine (A), guanine (G), cytosine (C) in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA); uracil

(U) in RNA; thymine (T) in DNA], a sugar (*i.e.*, ribose in RNA; deoxyribose in DNA), and one phosphate (Neidle and Sanderson, 2021). Their primary structure is formed by the connection of nucleotides by phosphodiester bonds linking the oxygens between different nucleotides in carbons 5' to 3'. The secondary structure of nucleic acids is formed by the specific interaction between the purine (*i.e.*, A, G) and pyrimidine bases (*i.e.*, C, U, T). These base pairings will lead to the formation of structures such as the helices (contiguous base pairs), stem-loops, and pseudoknots (unpaired nucleotides surrounded by helices). While the interactions of bases will form the secondary structure of DNA and RNA, their three-dimensional arrangement will determine its tertiary structure. Especially for DNA, their secondary and tertiary structures are closely related. DNA can take double-helical forms such as A-DNA, B-DNA, and Z-DNA, that differ based on the number of base pairs per turn, right- or left-handed helix, length of the helix, and size between minor and major grooves. As for RNA, it can form double helices, major and minor groove triplexes, quadruplexes, helical stacking, and other arrangements. Finally, their quaternary structure involves the interaction of different nucleic acids (*e.g.*, the RNA enzyme Varkud satellite ribozyme) or between nucleic acid and protein (*e.g.*, DNA and histones to form nucleosomes).

Considering their complex nature, the structure of proteins and nucleic acids is not static, and environmental conditions will alter their conformation and can even denature them (*i.e.*, irreversibly alter their structural arrangements). The conditions include changes in temperature, pH, ionic strength, pressure, enzymes, and chemical and organic substances (Manning et al., 2010; Neidle and Sanderson, 2021). Because the activity and behavior of biopharmaceuticals are directly related to the integrity of their macromolecular arrangements, it is necessary to characterize and monitor their structure and interactions to assure their security and effective application. However, multiple parameters can be evaluated to determine protein stability. **Table 1** compiles the main parameters to evaluate the stability of protein, peptides, and nucleic acids, namely the four levels of structure of proteins, thermodynamic parameters, and activity. It also includes the most common techniques used to determine each type of stability and the metrics evaluated for each parameter.

1 **Table 1.** Level of stability, composition, and recurrent evaluation methods for the different levels of proteins, peptides, and
 2 nucleic acids structure.

Stability type	Overall stability	Composition	Evaluation methods
Proteins and peptides			
Primary structure	Very high	Amino acid chain.	Amino acid sequencing (<i>e.g.</i> , mass spectrometry, Edman degradation), electrophoresis, western blotting, chromatography, protein half-life.
Secondary structure	High to intermediary	Local interactions of the polypeptide backbone (<i>e.g.</i> , α -helix, β -sheet, random coil).	CD, infrared spectroscopy.
Tertiary structure	Intermediary to low	Three-dimensional folding of the protein structure.	<u>Direct</u> : X-ray crystallography, neutron and X-ray scatterings, NMR, dual polarization interferometry, Cryo-EM. <u>Indirect</u> : fluorescence and absorbance (<i>e.g.</i> , intrinsic UV-absorbance and fluorescence of proteins, proteins with chromophores and fluorophores, fluorescence labeling and probes), dynamic and static light scattering and AUC (protein aggregation and oligomeric state), Raman spectroscopy, computational modeling.
Quaternary structure	Low	Packing of different subunits of the protein.	
Thermal	Variable	Thermodynamic parameters (<i>e.g.</i> , T_m , ΔH_m , ΔS , ΔG).	DSF, ITC, TSA, and DSC. CD and infrared spectroscopies associated with controlled heating.
Activity	Variable	Varies depending on its intended application.	<i>E.g.</i> , enzymes: kinetic parameters; biosensors: absorbances and fluorescence; vaccines: potency, immunogenicity, and immunomodulation; monoclonal antibodies: binding.
Nucleic acids			
Primary	High	Nucleotide sequence.	DNA and RNA sequencing, microarrays, southern blots, and <i>in situ</i> hybridization, fluorescence-based assays with probes.
Secondary	Intermediary	Interactions between bases (<i>e.g.</i> , DNA and RNA: double helix; RNA: stem-loops, pseudoknots).	CD, infrared spectroscopy, SHAPE assay.
Tertiary	Intermediary to low	3D folding of the nucleic acid chains. DNA: A-form, B-form, Z-form; RNA: <i>e.g.</i> , helical duplexes, triple-stranded structures.	<u>Direct</u> : X-ray crystallography, neutron and X-ray scatterings, NMR, Cryo-EM. <u>Indirect</u> : electrophoresis and chromatography (size determination), fluorescence (probes and labeling), UV absorbance (usually, λ 260 nm), computational modeling.
Quaternary	Low	Higher level of organization of nucleic acids (<i>e.g.</i> , DNA: chromatin; RNA: interaction of RNA units in the ribosome).	
Thermal	Easily variable	Thermodynamic parameters (<i>e.g.</i> , T_m , ΔH_m , ΔS , ΔG).	ITC, DSF, DSC. CD, UV, and infrared spectroscopies associated with controlled heating.
Activity	Easily variable	Varies depending on its intended application.	<i>E.g.</i> , viral vectors: transduction efficiency; vaccines: potency, immunogenicity, and immunomodulation.

3 For proteins and peptides, their primary structure is highly resistant to stress, with
4 only extreme conditions or enzymes breaking its peptide bonds (Bischof and He, 2006).
5 Its evaluation consists of the direct sequence of their amino acid chain (*e.g.*, mass
6 spectroscopy alone or combined with chromatography (Callahan et al., 2020), Edman
7 degradation (Zhou et al., 2012) or indirectly estimating alterations by assessing its size
8 with electrophoresis, western blotting, and chromatography or verifying its half-life
9 (Deller et al., 2016).

10 The secondary structure of proteins and peptides is still considerably resistant to
11 alterations but to a lesser degree than the primary. Conventional methods to evaluate it
12 include circular dichroism (CD) and infrared spectroscopies, such as Fourier transform
13 infrared (FTIR) and 2D-infrared (Greenfield, 2006; Kong and Yu, 2007), which will
14 estimate the proportions of secondary protein motifs chains (*e.g.*, α -helix, β -sheet,
15 random coil). It should be noted that certain methods to evaluate the tertiary structure of
16 proteins can also provide information on their secondary forms, as we are listing the
17 most prominent methods for each case.

18 The tertiary and quaternary protein structures are more susceptible to alterations
19 due to their environment. The techniques for the tertiary structure need to demonstrate
20 the folding of the protein or suggest alterations to its 3D structure. As for the quaternary
21 structure, they should indicate changes in the protein oligomeric state. Overall, the
22 techniques to determine the tertiary and quaternary structure of proteins overlap. Direct
23 methods include nuclear magnetic resonance spectroscopy (NMR), X-ray
24 crystallography, dual polarisation interferometry, cryogenic electron microscopy (Cryo-
25 EM), and neutron and X-ray scatterings (Alberts et al., 2002; Ilari and Savino, 2008;
26 Kikhney and Svergun, 2015; Milne et al., 2013; Petoukhov and Svergun, 2007; Swann
27 et al., 2004). To indirectly verify the tertiary structure of proteins, it is possible to assess
28 alterations in the absorbances and fluorescence of proteins in the case of proteins with
29 chromophores in their structure, such as fluorescent proteins or proteins with
30 fluorescent amino acid residues, such as tyrosine, tryptophan, and phenylalanine (dos
31 Santos et al., 2020, 2019). Even if the protein has no intrinsic fluorescence, conjugates
32 like fluorescein can be added to recombinant proteins or it is possible to evaluate the
33 interaction of the protein with fluorescence probes (Toseland, 2013). It is also possible
34 to monitor alterations to the oligomeric quaternary state of the protein or verify
35 aggregations by size exclusion chromatography, electrophoresis, dynamic and static
36 light scattering, and analytical ultracentrifugation (AUC) (Ahrer et al., 2003; Deller et

37 al., 2016; Liu et al., 2006). There are also *in silico* models such as molecular dynamics
38 (MD) and molecular docking, homology modeling, and protein-protein interactions
39 (PPIs) targets that allow the prediction of protein structure, stability, and interactions
40 with other molecules (Lee et al., 2017; Watson et al., 2005; Xiang, 2006; Xu et al.,
41 2008).

42 The thermal stability of proteins is a property that can easily vary depending on the
43 environment of the protein. To assess it, the main indicator is the melting temperature
44 (T_m) *i.e.*, the temperature of denaturation; however, there are other thermodynamic and
45 kinetic parameters such as activation energy, melting enthalpy (ΔH_m), change in entropy
46 (ΔS), change in Gibbs free energy (ΔG) and also half-life ($t_{1/2}$). The T_m of proteins can
47 be directly determined by differential scanning calorimetry (DSC), isothermal titration
48 calorimetry (ITC), and differential scanning fluorimetry (DSF, also called thermal shift
49 assay - TSA) (Bischof and He, 2006; Deller et al., 2016). However, by monitoring the
50 alterations of the secondary structure of proteins under controlled heat, it is also
51 possible to discover T_m . From the curves obtained by different techniques and related to
52 the concentration or amount of proteins as a function of the independent variable such
53 as temperature for thermal denaturation curves the other thermodynamic parameters can
54 be determined (Bischof and He, 2006; Schellman, 1987). Moreover, different models
55 can be used to estimate the thermodynamic parameters. As for the determination of the
56 biological activity of proteins, the methods will vary according to their intended
57 applications. For example, for protein vaccines, it is possible to measure their potency,
58 immunogenicity, and immunomodulation, for biosensors their absorbance and
59 fluorescence can be acquired, for monoclonal antibodies their binding can be tested, and
60 the kinetic parameters can be used to verify the activity of enzymes (dos Santos et al.,
61 2020; Iyer and Ananthanarayan, 2008; Schofield, 2009; Veríssimo et al., 2021).

62 For nucleic acids, their primary structure also presents high stability. Their
63 integrity can be determined by gene sequencing or other methods to evaluate specific
64 nucleic acid sequences, such as microarrays, southern blots, and *in situ* hybridization
65 (Brown, 1993; Dorado et al., 2021; Jensen, 2014; Stoughton, 2005). The evaluation of
66 the secondary structure of nucleic acids will quantify their structural motifs, such as
67 double helices for DNA and RNA, and stem-loops and pseudoknots for RNA. Similar to
68 proteins, the methods employed will be CD and infrared spectroscopies (Kypr et al.,
69 2009; Sosnick et al., 2000; Tsuboi, 1970).

70 The tertiary and quaternary structures of nucleic acids have overall lower stability
71 than proteins, and the determination of their higher-level structure was challenging. The
72 traditional methods to directly assess tertiary and quaternary structures of a nucleic acid
73 include X-ray crystallography (Lin et al., 2011), neutron and X-ray scatterings (Oliver
74 et al., 2019), and NMR (Liu et al., 2021). However, cryo-EM is a fairly recent method
75 that has allowed the determination of novel structural forms of nucleic acids (Ma et al.,
76 2022). Hence, even more motifs for nucleic acids may be found soon. There are also
77 indirect methods to estimate changes to the tertiary and quaternary structures of nucleic
78 acids, such as electrophoresis and chromatography (size determination) (Largy and
79 Mergny, 2014; Wei et al., 2022), fluorescence (probes and labeling) (Juskowiak, 2011;
80 Michel et al., 2020), UV absorbance (usually, at a λ of 260 nm) (Barbas et al., 2007),
81 and computational modeling (Feng et al., 2022; Ponce-Salvatierra et al., 2019).

82 The thermal stability of nucleic acids can be evaluated by similar thermodynamic
83 parameters to proteins, such as T_m , ΔH_m , ΔS , and ΔG (Rozners et al., 2015). The T_m can
84 also be determined but some of the same methods, such as ITC, DSF, and DSC, or an
85 association of controlled heating and CD, UV, or infrared spectroscopies (Rozners et
86 al., 2015; Silvers et al., 2015). Their activity will also depend on the intended
87 application of the biopharmaceutical. For example, you can test nucleic acid vaccine
88 stability by verifying its potency, immunogenicity, and immunomodulation with *in vivo*
89 or *in vitro* studies (Chavda et al., 2021; Chen et al., 2022), and viral vectors by their
90 transduction efficiency (Chen et al., 2018).

91 As presented in this section, there are multiple parameters and methods used to
92 evaluate the stability of biopharmaceuticals. When designing an experiment to assess or
93 improve biological drugs, it is necessary to consider its intended application, as certain
94 formulations can increase one type of stability to the detriment of another. For example,
95 2.4 M of cholinium dihydrogen phosphate ($[\text{Ch}]\text{H}_2\text{PO}_4$) improves the thermal stability
96 of lysozyme (Lys) to the detriment of its activity (Weaver et al., 2012), while 0.5 M of
97 N-butylpyridinium chloride has the opposite effect (Yamamoto et al., 2011). Moreover,
98 the same IL can enhance the stability of one specific biopharmaceutical and impair
99 another. For instance, around 0.3 - 0.5 M of 1-butyl-3-methylimidazolium acetate
100 ($[\text{C}_4\text{MIm}][\text{CH}_3\text{COO}]$) improves the structural stability of insulin (Todinova et al., 2016)
101 but will decrease it for α -chymotrypsin (CT) (Kumar et al., 2015). Concentration range
102 is also relevant when developing the stabilizing formulations, as the same IL can have
103 different effects on the same biopharmaceutical depending on the concentration range,

104 such as $[\text{Ch}]\text{H}_2\text{PO}_4$ maintaining the structural integrity of lysozyme (Lys) at 1.2 M but
105 decreasing it at 2.4 M (Weaver et al., 2012). With this in mind, the next section will
106 discuss the most prevalent problems related to the instability of biopharmaceuticals and
107 briefly explore the uses and limitations of conventional additives and solvents for the
108 stabilization of biologics.

109 ***3.1. Stabilization of biopharmaceuticals with additives and solvents***

110 The low stability of biopharmaceuticals in adverse conditions (*e.g.*, low and high
111 pH, extreme temperatures, and the presence of proteases, nucleases, and other
112 denaturing compounds) limits their systematic application, oral and transdermal
113 delivery, production, storage, and transportation (Manning et al., 2010). Therefore, the
114 pharmaceutical industry is constantly searching for additives, formulations, or structural
115 modifications that enhance the resistance of macromolecules to denaturation. However,
116 although engineering recombinant protein and nucleic acids is a highly effective method
117 to improve their stability (Jiang, 2019), this strategy is long and costly, as modified
118 biopharmaceuticals (*e.g.*, biobetters) are treated as new medicines by most regulations
119 and require all safety and clinical trials of novel drugs (Kesik-Brodacka, 2018).
120 Although all additives in pharmaceuticals must be Generally Recognized As Safe
121 (GRAS) in the USA or have an equivalent classification in other countries (Manchanda
122 et al., 2018), after being declared safe for medical use in humans, they can be applied to
123 several pharmaceutical formulations with fewer tests than novel medicines (Use, 2007).
124 Hence, the discovery of additives and solvents to stabilize and improve the delivery of
125 biopharmaceuticals can streamline the development of more effective medicinal
126 formulations.

127 The stabilization of macromolecules relies on obtaining a balance between
128 stabilizing and destabilizing forces that maintain their native folding. In solution, the
129 stabilizing forces are mainly due to the intramolecular interactions of the
130 macromolecule and the interactions between them and the solvents or other solutes in
131 the environment (Veríssimo et al., 2022). When the solute-macromolecules interactions
132 are positive, the additives can either form a hydration layer around the macromolecules
133 or directly bind to them, helping to prevent unfolding and aggregation. However, if the
134 macromolecule is lyophilized, the stabilization process will be due to the direct binding
135 of the additives to the protein (Ohtake et al., 2011). As for the destabilizing forces, they

136 will be caused by the increase of the entropy of unfolding, leading to denaturation, loss
137 of activity, and aggregation (Veríssimo et al., 2022).

138 There are already different classes of molecules used as excipients of
139 biopharmaceuticals, particularly vaccines. For example, sugars, amino acids, proteins,
140 polyols, polymers, surfactants, salts, and organic molecules (Butreddy et al., 2021).
141 These excipients can be applied as preservatives to prevent contamination (*e.g.*,
142 thimerosal), as adjuvants to improve activity (*e.g.*, aluminum salts to stimulate the
143 immune response in vaccines), or as stabilizers to preserve biopharmaceuticals during
144 processing or storage (Centers for Disease Control and Prevention, 2019). However,
145 although lyophilized protein pharmaceuticals and vaccines can be stored for months at
146 room temperature or in the fridge (Remmele et al., 2012), nucleic acids usually require
147 storage at $-70\text{ }^{\circ}\text{C}$ (Uddin and Roni, 2021) and many biopharmaceuticals can lose their
148 potency a few hours after reconstitution (Center for Drug Evaluation and Research,
149 2018).

150 Thus, the development of formulations to improve the stability of
151 biopharmaceuticals is still a field of interest in medicine and public health. In this
152 context, ILs and DESs appear as new classes of compounds with unique properties that
153 have been applied as additives to stabilize biomolecules (Egorova et al., 2021). Thus,
154 the next section will explore the suitability of ILs and DESs for pharmaceutical
155 formulations.

156 **4. ILs and DESs**

157 Green solvents are one of the hot topics of green chemistry (Mussagy et al., 2022b)
158 because they are commonly considered more environmentally friendly and less toxic
159 alternatives than traditional solvents (Mussagy et al., 2020; Veríssimo et al., 2022).
160 Green solvents are considered green because they have a lower environmental impact
161 and a reduced risk of exposure to human health and the environment. ILs and DESs are
162 newer classes of green solvents designed to be even more environmentally friendly and
163 sustainable than traditional solvents, characterized by low toxicity, low flammability,
164 and low volatility (Mussagy et al., 2022a; Quintana et al., 2022).

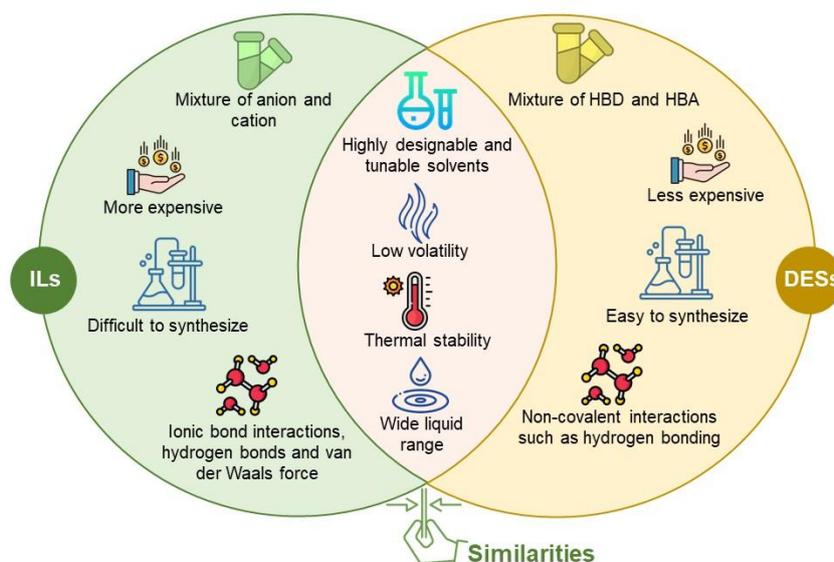
165 ILs are a type of salt that is liquid at room temperature, consisting of a mixture of
166 cations and anions held together by intramolecular interactions such as ionic bonds,
167 hydrogen bonds, and van der Waals forces (Mamusa et al., 2023; Myrdek et al., 2021).
168 ILs are considered an ideal mixture due to the combination of two significant properties:

169 the liquid state of molecular liquids and the ionic character of ionic compounds (Kaur et
170 al., 2022). Unlike traditional salts (*i.e.*, most of them solid at room temperature), ILs are
171 composed of ions that have unique properties such as low volatility, high thermal
172 stability, and high solvating power (Rashid, 2021). ILs are often used as green solvents
173 in a wide range of applications including pharmaceuticals for the formulation of
174 biopharmaceuticals (Kaur et al., 2022; Quintana et al., 2022). DESs are eutectic
175 compounds usually formed by mixing a low molecular weight organic compound
176 (Hydrogen Bond Acceptor - HBA) with a salt (Hydrogen Bond Donor - HBD) *via* non-
177 covalent interactions such as hydrogen bonding (Sun et al., 2022). Since DESs are
178 mostly composed of ionic species, they are now widely acknowledged as a new class of
179 IL analogs because they share the same properties and characteristics as ILs (Afonso et
180 al., 2023). In addition, if the HBD and HBA components of the DES are suitably
181 selected, they can be designed to be highly biodegradable and sustainable for further
182 application in biopharmaceuticals (Hong et al., 2020).

183 ILs and DES are highly designable and tunable solvents that generally share many
184 properties, such as a wide range of low volatility, thermal stability, high solvating
185 power, and ability to dissolve organic or inorganic compounds with significant interest
186 in the biopharmaceutical industry (Sun et al., 2022). However, there are relevant
187 distinctions between ILs and DESs, particularly for industrial applications. ILs are
188 typically formed from a mix of organic heterocyclic cations and anions, whereas DESs
189 are usually based on environmentally benign hydrogen bond donors and acceptors
190 (Płotka-Wasyłka et al., 2020). Moreover, the synthesis of ILs often involves multiple
191 chemical steps, making the process more complex and resource-intensive. In contrast,
192 DESs are synthesized through more sustainable and direct methods, such as mixing
193 hydrogen bond donors with acceptors under mild conditions. In terms of application
194 scope, ILs are versatile, with consolidated applications in specialized fields such as
195 electrochemistry and catalysis (Quintana et al., 2022). On the other hand, DES
196 applications are still limited to research state due to their novelty, but they are gaining
197 traction in fields that prioritize environmental impact and safety, like biotechnology and
198 pharmaceuticals (Shamshina and Rogers, 2023). Thus, while ILs and DESs share
199 specific properties, their distinct synthesis processes, sustainability profiles, and
200 application scopes set them apart, particularly in industrial and environmental
201 applications.

202 In the last years, ILs and DES have been studied for their potential in drug
 203 production and delivery, extraction, and purification of biopharmaceuticals, and as
 204 reaction media for chemical synthesis (Quintana et al., 2022). For example, ILs and
 205 DESs can be employed as alternative mediums to water and volatile organic solvents to
 206 improve chemical and biological reactions, such as amino acid and peptide ligations
 207 (Nolan et al., 2022). Furthermore, due to their high selectivity and multiple interactions
 208 with target compounds and analytes (Makoś et al., 2018; Momotko et al., 2022, 2021),
 209 ILs and DESs are frequently applied for the selective separation of biomolecules
 210 (Castro-Muñoz et al., 2022; Khajavian et al., 2022). Some ILs and DESs even present
 211 pharmaceutical properties, such as antimicrobial and anticancer activity (Ibsen et al.,
 212 2018; Veríssimo et al., 2023).

213 Despite the interesting properties of ILs and DES, it is also important to consider
 214 some potential drawbacks and limitations of these green solvents such as high cost,
 215 some extent of volatility, high viscosity, low moderate instability in the presence of
 216 some acids and bases, and limited availability in the market (Chen and Mu, 2021)
 217 (**Figure 2**). Furthermore, the solubility of some biopharmaceuticals in ILs and DESs
 218 can be limited, leading to difficulties in the development of pharmaceutical formulations
 219 (Veríssimo et al., 2022). The aforementioned drawbacks cannot be ignored, and to
 220 enhance the sustainability of the processes and overcome some of these limitations, the
 221 design of these solvents must be thoroughly evaluated.



222

223 **Figure 2.** Differences and similarities of ILs and DESs.

224 Although ILs and DESs are generally regarded as green solvents, they are also
225 classes of very diverse chemicals due to the countless combinations of cations and
226 anions to form ILs and mixtures of substances capable of resulting in DESs. For
227 example, the most prevalent ions in the first generation of ILs were the anions
228 hexafluorophosphate, tetrafluoroborate, and bis[(trifluoromethyl)sulfonyl]imide and the
229 cations imidazolium, pyridinium, and pyrrolidinium (Veríssimo et al., 2022). These
230 aromatic cations and hydrophobic anions overall presented high terrestrial and aquatic
231 toxicity and low compatibility with biological molecules (Cho et al., 2021; Greer et al.,
232 2020). Depending on the composition and solubility of the ILs and DESs, they can also
233 exhibit microbial toxicity (Marchel et al., 2022a), contribute to atmospheric pollution
234 and cross-contamination (Janjhi et al., 2023), and contaminate water sources (Marchel
235 et al., 2023). Even for ILs and DESs not regarded as toxic, researchers must consider
236 the whole life cycle of the compound when projecting their industrial application. For
237 example, some DESs could promote eutrophication (*i.e.*, excessive nutrient enrichment
238 of water bodies) even though they present no general toxicity, leading to harmful algal
239 blooms and oxygen depletion, adversely affecting aquatic ecosystems (Vieira Sanches
240 et al., 2023). This phenomenon was demonstrated by Vieira Sanches et al. in a study
241 evaluating the effect of choline-, betaine- and proline-based DESs in marine
242 microorganisms and invertebrates (Vieira Sanches et al., 2023). Therefore, researchers
243 should not overlook the environmental footprint and potential toxicity of ILs and DESs.

244 Nonetheless, the extensive array of ion and compound combinations forming ILs
245 and DESs is estimated to surpass one million substances (Bakis et al., 2021), allowing
246 the potential to design chemicals with a wide range of properties. Hence, the tunability
247 of the ILs and DESs properties and a driven approach by researchers to develop solvents
248 with low toxicities, negligible vapor pressure, and non-flammable to favor industrial
249 applications can explain why they are usually labeled as green solvents despite the
250 presence of classes with high toxicity.

251 Recently, some efforts have been made to develop new classes of biocompatible
252 ILs and DES to reduce their potential toxic effects and improve sustainability (Chen and
253 Mu, 2021). These new classes of solvents, such as cholinium-based ILs ([Ch]ILs) and
254 DESs, are designed to be non-toxic and environmentally friendly, making them suitable
255 for use in the stabilization and formulation of several active pharmaceutical ingredients
256 (APIs) (Li et al., 2022). Cholinium-based ILs and DESs are among the most promising

257 alternatives, as they are derived from vitamin B8, a quaternary ammonium cation, and
258 usually present low toxicity and cost, high biodegradability, and improved delivery and
259 solubilization of APIs (Boethling et al., 2007; Kunz and Häckl, 2016; Pereira et al.,
260 2016; Petkovic et al., 2010; Santos et al., 2015; Ventura et al., 2014). Furthermore,
261 researchers can select anions and compounds with low toxicity to pair with choline,
262 such as specific carboxylic acids and amino acids, to develop biocompatible ILs and
263 DESs. Other types of new non-toxic and biodegradable ammonium-based ILs [*e.g.*,
264 triethylammonium phosphate ($[N_{0,2,2,2}]PO_4$), trimethylammonium dihydrogen phosphate
265 ($[N_{0,1,1,1}]H_2PO_4$), trimethylammonium acetate ($[N_{0,1,1,1}][CH_3COO]$)], synthesized using
266 biodegradable and renewable resources have been used as a promising alternative to
267 conventional solvents (*e.g.*, ethanol, acetone, ethers) (Moshikur et al., 2020; Silva et al.,
268 2021).

269 By applying bio-based ILs and DESs, the concerns with biocompatibility and
270 biodegradability can be addressed. However, even bio-based compounds can present
271 cytotoxicity, particularly substances that can interact with the lipidic membranes of
272 cells. For example, ILs and DESs with long alkyl side chains can present a
273 hydrophobic or surface-active nature, which will impact their biocompatibility and
274 interaction with biological systems. Nonetheless, the surfactant nature and cytotoxicity
275 of certain ILs and DESs can be exploited for therapeutic and delivery purposes, such
276 as antibacterial and antifungal treatments and transdermal delivery of APIs (Gonçalves
277 et al., 2021; Moshikur et al., 2020; Sivapragasam et al., 2019; Tanner et al., 2018; Wu et
278 al., 2021). The use of ILs and DESs to act in synergy with APIs, either by enhancing
279 their activity or improving their administration and distribution, has been the focal point
280 of research for their applications in the medical field recently.

281 In the market, around 50 % of available drugs are administered in their salt form
282 mainly because it is the most preferred method to enhance the physicochemical
283 properties of an active compound. So, the development of biocompatible salts (such as
284 ILs or DESs) of the targeted APIs can be a suitable and effective approach to overcome
285 some current drawbacks (solubility and melting temperature) on drug processing and
286 bioavailability faced by the industry of biopharmaceuticals (Ferraz et al., 2011). ILs and
287 DESs have many potential applications in drug delivery, such as improving thermal
288 stability of biomolecules, controlling drug release, eliminating polymorphism, tailoring
289 their solubility and increasing dissolution, modulating surfactant properties of systems,

290 enhancing permeability of APIs, and modulating cytotoxicity on tumor cells
291 (Shamshina and Rogers, 2023; Veríssimo et al., 2022; Wu et al., 2021). DESs have also
292 been extensively investigated as alternative solvents for the solubilization of APIs,
293 particularly in the context of topical formulations (Smith et al., 2014). For example, the
294 solubility of ibuprofen can be significantly improved, over 5,400 times in DESs
295 compared to its solubility in water (Lu et al., 2016). Additionally, another type of DES
296 composed of cholinium chloride ([Ch]Cl) and glycolic acid has shown substantial
297 solubility enhancements for itraconazole, piroxicam, lidocaine, and posaconazole, with
298 improvements of 6700x, 430x, 28x, and 6400x respectively, compared to their solubility
299 in water (Li and Lee, 2016). Some fatty acids (lauric and palmitic acids) combined with
300 ibuprofen and lidocaine have been successfully used for formulations of API-DESs for
301 transdermal delivery purposes (Benessam et al., 2013; Nazzal et al., 2002).

302 Despite the growing interest in new green solvents for biopharmaceutical
303 applications, the use of ILs or DESs as APIs in formulations is still in the early stages of
304 development, and further research is needed to fully elucidate their properties and
305 applications in these fields. For example, although there are many ILs and DESs with
306 the capacity to improve the stability of biomolecules, there are also reports of these
307 compounds impairing the structural integrity of macromolecules such as proteins
308 (Veríssimo et al., 2022), nucleic acids (Mandal et al., 2020), and polymers (Tan et al.,
309 2018) depending on their structure, interactions, and environmental conditions under
310 study. Furthermore, it is also necessary to assess their toxicity, bioavailability, and
311 dissolution, as well as investigate the irritancy and skin permeation of these
312 formulations.

313 Finally, understanding the stability of the ILs and DESs is another fundamental
314 parameter to allow their application, as degraded compounds in pharmaceutical
315 formulations can lose their function or be detrimental to the safety and efficacy of the
316 medicine. For example, the thermal or long-term degradation of ILs and DESs can
317 cause the formation of toxic byproducts even of initially biocompatible compounds, as
318 demonstrated by Marchel et al. (Marchel et al., 2022b). Moreover, there are other safety
319 concerns related to the overheating of ILs and DESs, such as sample and equipment
320 degradation, increased chemical and biosafety risks, and higher processing costs to add
321 cooling systems to avoid overheating. Hence, in addition to guaranteeing the structural
322 integrity of ILs and DESs at the expected conditions for use of the biopharmaceuticals,

323 long-term stability studies and stress assays [*e.g.*, the thermal stability of ILs and DESs
324 (Cao and Mu, 2014; Chen et al., 2018), chemical stability (Wang et al., 2017)] should
325 also be performed to confirm the potential range of application of these substances.

326 To determine the safety and effectiveness of the clinical use of ILs and DESs, it
327 will be crucial to conduct both *in vitro* and *in vivo* studies, starting with non-clinical
328 trials and progressing to clinical trials. As our understanding of the role of ILs and
329 DESs expands, it is expected that a growing number of cation/anion and HBD/HBA
330 combinations will be utilized to create APIs, resulting in a multitude of options for
331 design and product efficacy. With this in mind, the next section will disclose the state-
332 of-the-art on the use of ILs and DESs to enhance the stability and delivery of
333 biopharmaceuticals.

334 **5. Stability of biopharmaceuticals in ILs and DESs**

335 The effect of ILs and DESs on the stability of protein and nucleic acid
336 biopharmaceuticals will be presented in **Table 2** (biopharmaceuticals in ILs) and **Table**
337 **3** (biopharmaceuticals in DESs). For each biopharmaceutical, we will include its
338 therapeutic use and the effects of the ILs and DESs solutions considering
339 concentration ranges and different stability types (*i.e.*, structural, thermal, aggregation,
340 and activity) and effects (*e.g.*, solubilization, improved delivery, long-term
341 preservation). The symbol (↑) indicates an increase in stability, (=) represents that the
342 solution had a similar effect to the control, and (↓) means a decrease in stability.

343 For proteins and peptides, we will also include their molecular weight (MW),
344 grand average hydrophobicity index (GRAVY), and instability index (II) calculated
345 using their UniProt sequence using the ExPASy ProtParam tool (Artimo et al., 2012;
346 ExPASy, 2018; UniProt, 2020), and their structure in **Figure 3** and **4**. For GRAVY,
347 values below zero represent hydrophilic amino acid chains, while GRAVY above zero
348 means lipophilic chains. For the instability index, values below 40 indicate a stable
349 protein (Veríssimo et al., 2022). The properties of each macromolecule will be
350 discussed in their respective subsection. The type or source of the protein
351 biopharmaceuticals will also be provided in **Tables 2** and **3** when available, considering
352 this can also impact their stability and properties. Finally, we also provide the chemical
353 structures of the cations and anions for the ILs and the components of the DESs from
354 **Tables 2** and **3** in **Figure 4**, so reader can refer to them during the discussions in the
355 next subsections.

356 **Tables 2 and 3** present the properties, use, and stability (according to stability
 357 types) of biopharmaceuticals in the presence of different IL and DES solutions (*i.e.*,
 358 classes and concentrations), respectively.

359

360 **Table 2.** Stability (structural, thermal, activity, aggregation, or simulation) of biopharmaceutical in
 361 different concentrations of ILs and effect of ILs on their delivery. Specific information for each protein
 362 and peptide, namely, UniProt of the most usual variant, molecular weight (MW), instability index (II),
 363 GRAVY‡, and therapeutical use of the biopharmaceuticals are also presented in the table.

364

Biopharmaceutical	Variant or class	ILs	Conc.*	(Stability) [Effect]	Ref.
Peptides					
<u>Glucagon-like peptide 1 (GLP-1)</u> UniProt: P55095, MW: 3.3 kDa II: 17.7 (stable), GRAVY: -0.230 Use: Hormone for treatment of type 2 diabetes.	Native human GLP-1	Cholinium-based ILs			
		[Ch][C ₉ H ₁₅ COO] #	0.7 - 3.7 M (20 - 100 w%)	(= Structural, activity), [Improved pharmacokinetics and sustained release]	(Agatemor et al., 2021)
<u>Interleukin-2 (IL-2)</u> UniProt: P60568; Mw: 15.2 kDa; II: 53.4 (unstable); GRAVY: -0.198 Use: Cytokine for immunotherapy.	DES-ALANYL-1, SER-125 human IL-2	Cholinium-based ILs			
		[Ch]H ₂ PO ₄	0.030 - 0.185 M	(↑ Thermal)	(Weaver et al., 2012)
<u>Insulin</u> UniProt: P01308 (A and B chains) (Human insulin) MW: 5.8 kDa II: 13.61 (stable) GRAVY: 0.218 Use: Hormone for treatment of diabetes.	Zn-free insulin	Ammonium-based ILs			
		[N _{0,2,2,2}]PO ₄ , [N _{0,1,1,1}]HSO ₄ , [N _{0,2,2,2}]SO ₄ , [N _{0,1,1,1}]H ₂ PO ₄ , [N _{0,1,1,1}][CH ₃ COO]	0.5 - 2.0 M	(↑ Thermal, ↓ aggregation)	(Kumar and Venkatesu, 2013)
	Insulin from porcine pancreas	Cholinium-based ILs			
		[Ch][Gln], [Ch] ₂ [Asn]	0.0008 M	(↑ Thermal)	(Guncheva et al., 2019)
		[Ch][Asn]	0.0008 M	(= Thermal)	
		[Ch][Arg], [Ch] ₂ [Gln], [Ch][Lys]	0.0008 M	(↓ Thermal, structural)	
[Ch][Gln], [Ch] ₂ [Asn], [Ch][Asn]	0.0008 M	(↓ Structural)			
FITC-insulin	[Ch][C ₉ H ₁₅ COO]	1.85 - 3.70 M	(= Structural, activity), [↑ Transdermal permeation]	(Banerjee et al., 2017)	
	Human insulin	[Ch][C ₉ H ₁₅ COO]	3.7 M (100%)	(↑ Structural long-term, = activity), [↑ Oral intake, paracellular transportation, sustained]	(Banerjee et al., 2018)

				activity; ↓ enzymatic degradation]	
		[Ch][C ₉ H ₁₅ COO]	0.04 - 0.19 M	[↑ Oral intake, paracellular transportation]	(Peng et al., 2020)
Imidazolium-based ILs					
Porcine insulin		[C ₄ MIm][CH ₃ COO], [C ₄ MIm][CF ₃ COO], [C ₄ MIm][N(CN) ₂] (ILs in KCl/HCl pH 2)	0.3 M	(↑ Structural, thermal)	(Todinova et al., 2016)
		[C ₄ MIm]Cl, [C ₄ MIm][SCN], (ILs in KCl/HCl pH 2)	0.3 M	(= Structural, thermal)	
		[C ₄ MIm][C(CN) ₃] in KCl/HCl pH 2	0.3 M	(↓ Structural, thermal)	
Zn-free insulin		[C ₄ MIm]Cl, [C ₄ MIm]Br	0.01 - 0.04 M	(↑ Structural)	(Kumar and Venkatesu, 2014)
		[C ₄ MIm]Cl, [C ₄ MIm]Br	0.01 - 0.04 M	(↓ Thermal)	
		[C ₄ MIm][SCN], [C ₄ MIm]HSO ₄ , [C ₄ MIm]I, [C ₄ MIm][CH ₃ COO]	0.01 - 0.04 M	(↓ Structural, thermal)	
Porcine insulin (experiment) and human insulin (simulation)		[C ₂ MIm][CH ₃ COO]	3 - 6 M (50 - 90 wt%)	(↑ Simulation, thermal)	
Human insulin		[C ₂ MIm][CH ₃ COO], [C ₄ MIm]Cl, [C ₄ MIm]NO ₃ , [C ₄ MIm][CH ₃ SO ₃], [C ₄ MIm][N(CN) ₂], [C ₄ MIm][CH ₃ COO], [C ₆ MIm][CH ₃ COO], [C ₈ MIm][CH ₃ COO], [C ₁₀ MIm][CH ₃ COO], [C ₁₂ MIm][CH ₃ COO]	~ 1.5 - 7.0 M (75 - 100 wt %)	(↑ Simulation)	(D. Li et al., 2019)
Cholinium-based ILs					
<u>Ovalbumin epitope (OVA)</u> Sequence: SIINFEKL; Mw: 1.0 kDa Use: Cancer antigen for immunostimulation of therapeutic cancer vaccination.					
	OVA257-264 SIINFEKL (H-2 Kb)	[Ch][C _{12:0}], [Ch][C _{18:1}]	~ 0.5 - 0.7 M (20 wt%)	[Skin penetration enhancer]	(Tahara et al., 2020)
		[Ch][C _{18:1}]	0.2 M (8 wt%)	(↑ Activity) [Solubilizer]	
Proteins					
Cholinium-based ILs					
<u>Immunoglobulin G1 (IgG1)</u>					
Unitprot: P01837	IgG from human serum	[Ch][CH ₃ COO]	0.0005 - 0.0025 M	(↑ Structural, thermal)	(Dhiman et al., 2022)
		[Ch][CH ₃ COO], [Ch][C ₅ H ₇ O ₅ COO]	0.0005 - 0.0015 M	(↓ Aggregation)	

MW: 142.6 kDa		[Ch][C ₅ H ₇ O ₅ COO], [Ch]H ₂ PO ₄	0.0005 - 0.0025 M	(= Structural, thermal)	
II: 37.16 (stable)		[Ch][CH ₃ COO], [Ch][C ₅ H ₇ O ₅ COO]	0.0020 - 0.0025 M	(↑ Aggregation)	
GRAVY: -0.442		[Ch]H ₂ PO ₄	0.0005 - 0.0025 M	(↑ Aggregation)	
Use: Immunization, immunotherapy, infection treatments, hematologic and autoimmune diseases treatments, and others.	IgG from rabbit serum	[Ch][HOCH ₂ COO], [Ch][C ₅ H ₇ O ₄ COO]	~ 0.9 - 1.4 M (25 wt%)	(= Structural, aggregation)	
		[Ch][CH ₃ COCOO]	1.3 M (25 wt%)	(= Aggregation)	(Mondal et al., 2016)
		[Ch][C ₉ H ₈ NCOO], [Ch][CH ₃ COCOO]	~ 1.1 - 1.3 M (25 wt%)	(↓ Structural)	
		[Ch][C ₉ H ₈ NCOO]	1.1 M (25 wt%)	(↑ Aggregation)	
		[Ch][HOCH ₂ COO]	~ 1 - 4 M (20 - 70 v%)	(↑ Activity)	
Antihuman TNF- α mouse IgG1	[Ch][HOCH ₂ COO]	~ 3 M (50 v%)	(= Structural)	(Angsantikul et al., 2021)	
	[Ch][HOCH ₂ COO]	~ 4 - 5 M (80 - 90 v%)	(↓ Activity)		
Imidazolium-based ILs					
Human IgG1	[C ₂ MIm]Cl, [C ₄ MIm]Cl, [C ₆ MIm], [C ₈ MIm]Cl		0.04 M	(↑ Structural, ↓ aggregation)	(Rawat and Bohidar, 2015)
	[C ₄ MIm][CH ₃ BzSO ₃], [C ₄ MIm][N(CN) ₂], [C ₄ MIm][CH ₃ COO], [C ₂ MIm]Br, [C ₄ MIm]Br, [C ₄ MIm]Cl		~ 0.1 - 0.3 M (5 wt%)	(= Structural)	(Ferreira et al., 2016)
Ammonium-based ILs					
IgG from rabbit serum	[N _{1,1,1,1}]Br, [N _{2,2,2,2}]Br, [N _{3,3,3,3}]Br		~ 0.1 - 0.2 M (5 wt%)	(= Structural)	(Ferreira et al., 2016)
	[N _{4,4,4,4}]Br		~ 0.2 M (5 wt%)	(↓ Structural)	
Phosphonium-based ILs					
		[P _{4,4,4,4}]Br	~ 0.1 M (5 wt%)	(↓ Structural, ↑ aggregation)	(Ferreira et al., 2016)
Cholinium-based ILs					
<u>L-Asparaginase (L-ASNase)</u>					
Uniprot: P00805 (Using tetrameric form), MW: 137.7	L-ASNase EC 3.5.1.1	[Ch][CH ₃ COO], [Ch][C ₂ H ₅ COO], [Ch][C ₃ H ₇ COO]	0.001–0.050 mol IL/mol total	(↑ Activity)	(Magri et al., 2019)

	(tetramer)					
II: 19.84 (stable)		[Ch][C ₅ H ₁₁ COO]		0.001–0.010 mol IL/mol total	(↑ Activity)	
GRAVY: -0.194						
Treatments of acute lymphoblastic leukemia and lymphoblastic lymphoma.		[Ch][C ₅ H ₁₁ COO]		0.025–0.050 mol IL/mol total	(↓ Activity)	
Nucleic acids						
Cholinium-based ILs						
siRNA against CD45	siRNA				(↑ Structural, activity, thermal)	(Mazid et al., 2014)
Use: Treatment of autoimmune diseases.		[Ch]H ₂ PO ₄		1.0 - 2.5 M (20 - 50 wt%)	[Long-term storage]	
Cholinium-based ILs						
siRNA against NFKBIZ	siRNA	[Ch][PhC ₂ H ₅ COO]		~ 2 M (50 v%)	(↑ Structural)	(Mandal et al., 2020)
		[Ch][C ₉ H ₁₅ COO]		~ 2 M (50 v%)	(↓ Structural)	
		[Ch][C ₉ H ₁₅ COO],		~ 2 M (50 v%)	[Skin penetration enhancer]	
		[Ch][PhC ₂ H ₅ COO]			(↑ Structural, simulation, activity) / [Skin penetration enhancer]	
		[Ch][C ₉ H ₁₅ COO]:[Ch][PhC ₂ H ₅ COO]		~ 1 M each (25 v% each)		
Ammonium IL-Robed siRNA						
siRNA against GAPDH and MMP12	siRNA				[Skin penetration and cell internalization enhancer]	(Zakrewsky and Mitragotri, 2016)
Use: Treatment of skin diseases.		[N _{1,1,(Bz),8}]-siRNA1, [N _{1,1,(Bz),14}]-siRNA1, [N _{1,1,(Bz),18}]-siRNA1		0.00005 M		
Lidocainum-based IL						
STAT6 decoy oligonucleotide	Oligonucleotide				[Skin penetration enhancer]	(Handa et al., 2019; Kubota et al., 2016)
Use: Treatment of skin inflammation.		ILTS®		Not disclosed		

365 ‡ GRAVY - grand average hydrophobicity index, below 0 indicates that the protein sequence is
366 hydrophilic. * Approximate conversions (when possible) to molar (M) using MW and density (when
367 available on the manufacturer's site or literature). # There is still an ongoing discussion about whether
368 choline and geranic acid 1:1 ([Ch][C₉H₁₅COO]) leads to the formation of an IL or a DES (Rogers and
369 Gurau, 2018). Considering the suggestion of Rogers and Gurau (Rogers and Gurau, 2018),
370 [Ch][C₉H₁₅COO] was addressed in this review as an IL.

371

372 **Table 3.** Stability (structural, thermal, activity, aggregation, or simulation) of biopharmaceuticals in
373 different concentrations of DESs and the effect of DESs on their delivery. Specific information for each
374 protein, namely, UniProt of the most usual variant, molecular weight (MW), instability index (II),
375 GRAVY‡, and therapeutical use of the biopharmaceuticals are also presented in the table.

376

Biopharmaceutical	Variant or class	DESs	Conc.*	(Stability) [Effect]	Ref.
Peptides					
<u>Insulin</u>		Cholinium-based DESs			

UniProt: P01308 (A and B chains) (Human insulin) MW: 5.8 kDa II: 13.61 (stable) GRAVY: 0.218 Use: Hormone for treatment of diabetes.	FITC-insulin	[Ch][C ₉ H ₁₅ COO]- [C ₉ H ₁₅ COOH], [Ch][C ₉ H ₁₅ COO]- [C ₉ H ₁₅ COOH] ₃	~ 1.7 - 2.7 M (100 %)	[↑ Transdermal permeation]	(Tanner et al., 2018)
	Human insulin	[Ch][C ₉ H ₁₅ COO]- [C ₉ H ₁₅ COOH]	~ 2.7 M (100%)	[↑ Transdermal permeation]	(Jorge et al., 2020)

Proteins

<u>Immunoglobulin G1 (IgG1)</u>	Cholinium-based DESs				
Unitprot: P01837	IgG from human serum	[Ch]Cl-[CO(NH ₂) ₂], [Ch]Cl-[C ₃ H ₅ (OH) ₃], [Ch]Cl-[(CH ₂ OH) ₂]	~ 0.2 - 1.5 M (5 - 30 wt%)	(↑ Thermal)	(Dhiman et al., 2023)
MW: 142.6 kDa		[Ch]Cl-[CO(NH ₂) ₂]	1.5 M (30 wt%)	[↑ Long-term stability]	
II: 37.16 (stable)		[Ch]Cl-[CO(NH ₂) ₂], [Ch]Cl-[C ₃ H ₅ (OH) ₃]	~ 0.2 - 0.8 M (5 - 15 wt%)	(↓ Aggregation)	
GRAVY: -0.442		[Ch]Cl-[(CH ₂ OH) ₂]	~ 0.3 - 0.5 M (5 - 10 wt%)	(↓ Aggregation)	
Use: Immunization, immunotherapy, infection treatments, hematologic and autoimmune diseases treatments, and others.		[Ch]Cl-[CO(NH ₂) ₂], [Ch]Cl-[C ₃ H ₅ (OH) ₃], [Ch]Cl-[(CH ₂ OH) ₂]	~ 0.2 - 0.8 M (5 - 15 wt%)	(= Structural)	
		[Ch]Cl-[C ₃ H ₅ (OH) ₃], [Ch]Cl-[(CH ₂ OH) ₂]	~ 1.3 - 1.5 M (30 wt%)	(= Long-term stability)	
		[Ch]Cl-[(CH ₂ OH) ₂]	~ 0.8 - 2.5 M (15 - 50 wt%)	(↑ Aggregation)	
		[Ch]Cl-[CO(NH ₂) ₂], [Ch]Cl-[C ₃ H ₅ (OH) ₃]	(~ 1.3 - 2.5 M) 30 - 50 wt%	(↑ Aggregation)	
		[Ch]Cl-[CO(NH ₂) ₂], [Ch]Cl-[C ₃ H ₅ (OH) ₃], [Ch]Cl-[(CH ₂ OH) ₂]	(~ 1.3 - 2.5 M) 30 - 50 wt%	(↓ Structural)	
		FITC-IgG	[Ch][HOCH ₂ COO]-[Ch]	0.03 - 0.08 M	
Antihuman TNF-α mouse IgG1	[Ch][HOCH ₂ COO]-[Ch]	~ 1.4 M (50 v%)	[↑ Oral intake]	(Angsantikul et al., 2021)	
	[Ch][HOCH ₂ COO]-[Ch]	~ 0.7 - 2.5 M (20 - 70 v%)	(↑ Activity)		
	[Ch][HOCH ₂ COO]-[Ch], [Ch][HOCH ₂ COO]-[HOCH ₂ COOH]	~ 2 M (50 v%)	(= Structural)		
	[Ch][HOCH ₂ COO]-[Ch]	~ 3.0 - 3.5 M (80 - 90 v%)	(↓ Activity)		
	[Ch][HOCH ₂ COO]-[HOCH ₂ COOH]	~ 0.8 - 3.5 M (20 - 90 v%)	(↓ Activity)		

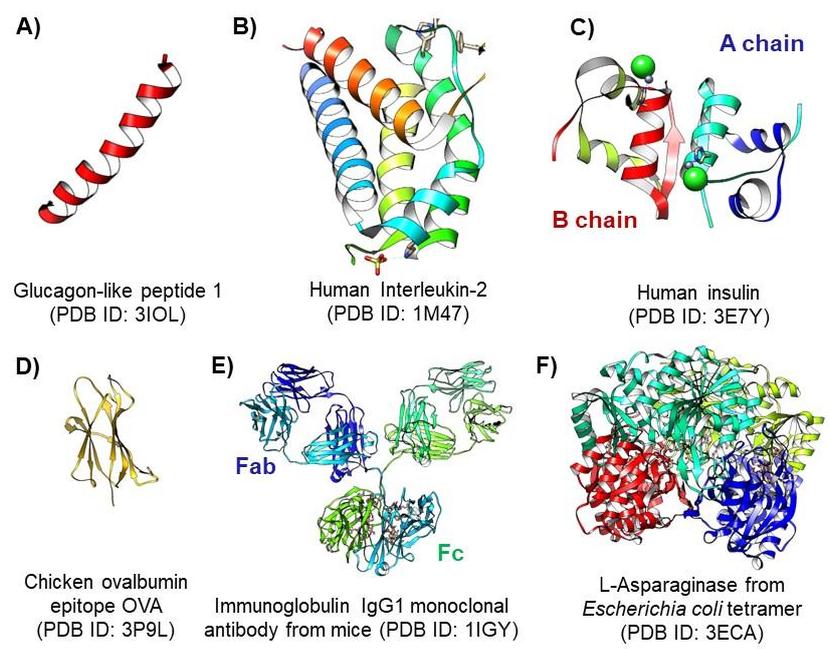
L-Asparaginase (L-ASNase)		Poly(vinyl pyrrolidone)-based DESs		
Uniprot: P00805 (Using tetrameric form), MW: 137.7 (tetramer), II: 19.84 (stable), GRAVY: -0.194 Use: Treatments of acute lymphoblastic leukemia and lymphoblastic lymphoma.	Pure L-ASNase and L-ASNase from <i>E. coli</i> extract	$[(C_6H_9NO)_n]-[CH_2(COOH)_2]$	0.1 M (0.02 g/mL)	(↓ Structural, = activity) [Adsorption of L-ASNase from <i>E. coli</i> extracts]

(Li et al., 2019)

377 ‡ GRAVY - grand average hydrophobicity index, below 0 indicates that the protein sequence is
378 hydrophilic. * Approximate conversions (when possible) to molar (M) using MW and density (when
379 available on the manufacturer's site or literature).

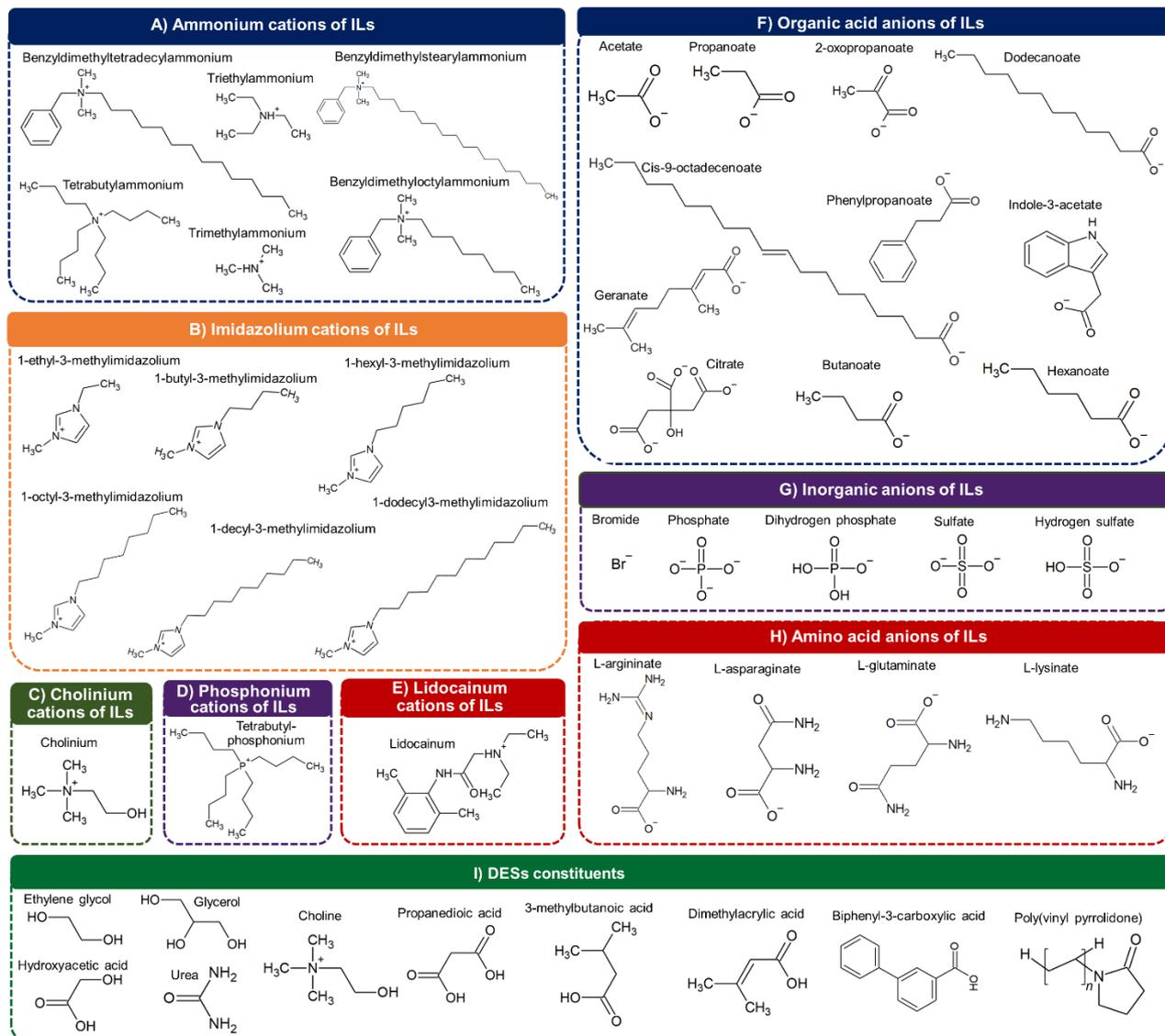
380
381

382 As observed in **Tables 2** and **3**, the effect of ILs and DESs on biopharmaceuticals
383 has been demonstrated in several studies. The interactions between ILs and proteins will
384 not only rely on the IL class, but the properties of the biomolecule, concentration range,
385 and type of stability evaluated. Furthermore, specific ILs and DESs can also enhance
386 the delivery or solubility of biopharmaceuticals. Considering the impacts of each IL and
387 DES on the macromolecules are associated with their structure and specific activity, we
388 will discuss each biopharmaceutical individually. **Figure 3** shows the structure of each
389 of the proteins and **Figure 4** from the ILs and DESs constituents from **Tables 2** and **3**.
390 The next subsections present a detailed examination of the structure, use, drawbacks,
391 and interaction of ILs and DESs with biopharmaceuticals.



392

393 **Figure 3.** Structure of protein biopharmaceuticals. **A)** Glucagon-like peptide 1 (GLP-1), **B)** Insulin, **C)**
 394 Interleukin-2 (IL-2), **D)** Ovalbumin epitope (OVA), **E)** Immunoglobulin G1 (IgG1), and **F)** L-
 395 asparaginase (L-ASNase). Images of the proteins were produced with the PDB structures using UCSF
 396 Chimera 1.14 (Berman et al., 2002; Pettersen et al., 2004).



397 **Figure 4.** Chemical structures of cations and anions of ionic liquids (ILs) and constituents of deep
 398 eutectic solvents (DESS). **A)** Ammonium, **B)** imidazolium, **C)** cholinium, **D)** phosphonium, and **E)**
 399 lidocainium cations of ILs. **F)** Organic acid, **G)** inorganic, and **H)** amino acid anions of ILs. **I)** DESS
 400 constituents.
 401
 402

403 5.1 Biopharmaceuticals in ILs and DESSs

404 5.1.1. Glucagon-like peptide 1 (GLP-1)

405 GLP-1 is an intestinal hormone produced as a response to meal ingestion (Müller
 406 et al., 2019). Its release enhances insulin secretion and helps to normalize the
 407 postprandial glycemic response and achieve blood glucose homeostasis (Müller et al.,
 408 2019). As presented in **Figure 3.A** and **Table 2**, GLP-1 is a small (3.3 kDa) and
 409 hydrophilic (GRAVY: -0.230) peptide formed by a continuous α -helix. Furthermore,

410 GLP-1 pI is 4.6 and is found in a negatively charged monomeric state at physiological
411 pH (Kaasalainen et al., 2015). As a biopharmaceutical, GLP-1 and GLP-1 receptor
412 agonists are applied to lower glucose levels in patients with type 2 diabetes (Walsh,
413 2010). However, GLP-1 is quickly degraded by the circulatory enzyme dipeptidyl
414 peptidase-4 (DPP-4) within minutes of its intravenous administration and around 1 h
415 after subcutaneous administration, which hinders its therapeutical use (Agatemor et al.,
416 2021).

417 To improve the delivery and pharmacokinetics of native human GLP-1, Agatemor
418 *et al.* (Agatemor et al., 2021) used cholinium geranate (*i.e.*, geranate also called 3,7-
419 dimethyl-2,6-octadienoate) 1:1 ([Ch][C₉H₁₅COO]) as a vehicle for the subcutaneous
420 administration of this biopharmaceutical in rats. [Ch][C₉H₁₅COO] preserved the
421 structural integrity and activity of the peptide from low to high concentrations (0.7 to
422 3.7 M). Furthermore, it improved GLP-1 pharmacokinetics by reducing its degradation
423 by DPP-4 and possibly by a sustained release due to entrapment of the
424 biopharmaceutical. We must note that there is still an ongoing discussion about whether
425 choline and geranic acid systems lead to the formation of DESs or IL with a complex
426 anion (*e.g.*, CAGE as [choline][geranate2(H)]), as pointed out by Rogers and Gurau
427 (Rogers and Gurau, 2018). Moreover, this discussion is not limited to choline and
428 geranic acid systems, as the distinction between certain DESs and ILs can be complex
429 to discern for specific classes, with some authors even considering DESs an IL subclass
430 (Płotka-Wasyłka et al., 2020). As ILs or DESs, choline and geranic acid systems
431 showed remarkable potential as vehicles to boost the delivery of pharmaceuticals such
432 as GLP-1.

433 5.1.2. Interleukin-2 (IL-2)

434 IL-2 is a cytokine that immunomodulates regulatory T cells and effector
435 lymphocytes (Arenas-Ramirez et al., 2015). As presented in **Figure 3.B** and **Table 2**,
436 the IL-2 is a small (15.2 kDa) and hydrophilic (GRAVY: -0.198) peptide comprised of
437 four α -helices in a bundle. In chemotherapy, IL-2 is used as an immunostimulant to
438 activate T cells against cancer cells, already approved to treat unresectable metastatic
439 renal cell carcinoma and stage IV melanoma (Weaver et al., 2012). As with other
440 protein-based biopharmaceuticals, its stability can be an issue for application, storage,
441 and transportation. For example, according to the supplier R&D systems, IL-2 protein
442 should be used within 24 hours after reconstitution in water (R&D Systems, 2023).

443 Hence, the discovery of additives to stabilize IL-2 can enhance and expand its use in
444 medicine.

445 To improve the thermal stability of human IL-2, Eckstein *et al.* (Weaver et al.,
446 2012) applied the IL $[\text{Ch}]\text{H}_2\text{PO}_4$ at low concentrations. From 0.030 to 0.185 M,
447 $[\text{Ch}]\text{H}_2\text{PO}_4$ preserved the structural integrity of IL-2 and increased its thermal stability.
448 According to the authors, the complementary electrostatic interactions between the IL
449 and the surface of the peptide likely offer protection against IL-2 thermal denaturation
450 without altering its secondary or tertiary protein structure. Therefore, cholinium-based
451 ILs can be applied as additives in very low concentrations to improve the thermal
452 stability of therapeutic peptides without altering their conformation.

453 5.1.3. Insulin

454 Insulin is an endogenous hormone peptide produced in the pancreas and is
455 responsible for blood glucose homeostasis (Mayer et al., 2007). Insulin was the first
456 recombinant biopharmaceutical, and it is still used for the treatment of diabetes (Walsh,
457 2013). As presented in **Figure 3.C**, insulin has two polypeptide chains (A and B) linked
458 by disulfide bonds (Brange and Langkjær, 1993), with A chain formed by two
459 antiparallel α -helices and B chain comprised of an α -helix with a turn and β -strand
460 (Brange and Langkjær, 1993). Insulin presents low solubility in water at neutral pH, but
461 it is possible to solubilize it up to 0.17 mM at pH 2 (Sigma-Aldrich, 2014).
462 Furthermore, this peptide is a weak dimer and can dimerize above 10^{-6} M and form
463 hexamers at 2 mM (Brange and Langkjær, 1993). Regarding its stability, insulin is
464 considered stable and can be stored for one month at room temperature before
465 reconstitution (Center for Drug Evaluation and Research, 2018). However, insulin can
466 lose its potency when diluted or maintained at extreme temperatures and should be
467 discarded if exposed to these conditions (Center for Drug Evaluation and Research,
468 2018). Additionally, there are multiple efforts to develop oral and transdermal delivery
469 systems for insulin, due to the discomfort and distress caused by its main administration
470 via subcutaneous injection (Xiao et al., 2020; Zhang et al., 2019). In this sense, ILs and
471 DESs can help to improve insulin medical use by improving its solubility and stability
472 and in the development of novel oral and transdermal systems for drug delivery.

473 For the thermal stability of insulin, researchers found that different IL classes (*i.e.*,
474 ammonium, cholinium, and imidazolium) and concentration ranges (*i.e.*, 0.0008 to 6 M)
475 can improve its thermodynamic parameters. For example, $[\text{N}_{0,2,2,2}]\text{PO}_4$,

476 trimethylammonium hydrogen sulfate ($[N_{0,1,1,1}]HSO_4$), triethylammonium sulfate
477 ($[N_{0,2,2,2}]SO_4$), $[N_{0,1,1,1}]H_2PO_4$, and $[N_{0,1,1,1}][CH_3COO]$ from 0.5 to 2.0 M increased the
478 T_m of insulin and decreased its aggregation (Kumar and Venkatesu, 2013). Moreover,
479 dilute solutions of cholinium L-glutamate ($[Ch][Gln]$) and dicholinium L-asparaginate
480 ($[Ch]_2[Asn]$) (at 0.0008 M) (Guncheva et al., 2019), and $[C_4MIm][CH_3COO]$, 1-butyl-3-
481 methylimidazolium trifluoroacetate ($[C_4MIm][CF_3COO]$), and 1-butyl-3-
482 methylimidazolium dicyanamide ($[C_4MIm][N(CN)_2]$) (0.3 M at pH 2) (Todanova et al.,
483 2016) also enhanced the thermal stability of insulin. Additionally, high concentrations
484 of 1-ethyl-3-methylimidazolium acetate ($[C_2MIm][CH_3COO]$) (3 to 6 M) had a similar
485 positive effect on the peptide (Li et al., 2019). There were also ILs at low concentrations
486 that did not affect the thermal stability of insulin, such as 0.0008 M of cholinium L-
487 asparaginate ($[Ch][Asn]$) (Guncheva et al., 2019), and 0.3 M of 1-butyl-3-
488 methylimidazolium chloride ($[C_4MIm]Cl$) and 1-butyl-3-methylimidazolium
489 thiocyanate ($[C_4MIm][SCN]$) at acidic pH (Todanova et al., 2016). However, even
490 concentrations below 0.04 M of specific cholinium and imidazolium ILs decreased the
491 T_m of insulin, including cholinium L-argininate ($[Ch][Arg]$), dicholinium L-glutamate
492 ($[Ch]_2[Gln]$), cholinium L-lysinate ($[Ch][Lys]$), (Guncheva et al., 2019) $[C_4MIm][SCN]$,
493 1-butyl-3-methylimidazolium hydrogen sulfate ($[C_4MIm]HSO_4$), 1-butyl-3-
494 methylimidazolium iodide ($[C_4MIm]I$), $[C_4MIm][CH_3COO]$ (Kumar and Venkatesu,
495 2014), and 1-butyl-3-methylimidazolium tricyanomethanide ($[C_4MIm][C(CN)_3]$) in
496 acidic pH (Todanova et al., 2016). Noteworthy, $[C_4MIm][SCN]$ at low concentrations
497 (below 0.3 M) had different impacts on the thermal stability of insulin at different pH.
498 For example, this IL had no impact on insulin T_m at acidic pH (Todanova et al., 2016)
499 but decreased it at neutral conditions (Kumar and Venkatesu, 2014). Therefore, the
500 composition of the IL, its concentration range, and the environment will impact the
501 effect of ILs on protein thermal stability.

502 As for the effect of ILs on the structural stability of insulin in ILs, it varied
503 according to the IL class and concentration range of the solutions. Low concentrations
504 of different imidazolium ILs were detrimental to insulin native structure, such as 0.3 M
505 of $[C_4MIm][C(CN)_3]$, $[C_4MIm]Cl$, $[C_4MIm][SCN]$ at pH 2 (Todanova et al., 2016), and
506 $[C_4MIm][SCN]$, $[C_4MIm]HSO_4$, $[C_4MIm]I$, and $[C_4MIm][CH_3COO]$ from 0.01 to 0.04
507 M (Kumar and Venkatesu, 2014). The exceptions to this trend were
508 $[C_4MIm][CH_3COO]$, $[C_4MIm][CF_3COO]$, $[C_4MIm][N(CN)_2]$ at 0.3 M and pH 2
509 (Todanova et al., 2016) and $[C_4MIm]Cl$ and $[C_4MIm]Br$ from 0.01 to 0.04 M (Kumar

510 and Venkatesu, 2014) that preserved the structure of insulin. There were also dilute
511 imidazolium ILs that did not alter insulin structure, such as [C₄MIm]Cl and
512 [C₄MIm][SCN] at 0.3 M and pH 2 (Todinova et al., 2016). As for cholinium ILs, high
513 concentrations (1.8 to 3.7 M) of [Ch][C₉H₁₅COO] maintained the short-term and
514 improved the long-term structural integrity and activity of insulin (Banerjee et al., 2018,
515 2017). However, dilute cholinium IL solutions (0.0008 M of [Ch][Arg], [Ch]₂[Gln],
516 [Ch][Lys], [Ch][Gln], [Ch]₂[Asn], [Ch][Asn] also impaired the structural stability of the
517 peptide (Guncheva et al., 2019). Interestingly, although [Ch][Gln], [Ch]₂[Asn], and
518 [Ch][Asn] caused a partial unfolding of insulin, they still increased its thermal stability,
519 confirming a certain environment can be positive to a protein stability parameter in
520 detriment of another (Guncheva et al., 2019).

521 Simulation tools such as MD were also used to predict the structural stability of
522 insulin in ILs. For example, Li *et al.* (Li et al., 2019) estimated with MD that high
523 concentrations of imidazolium ILs (75 to 100 wt%) stabilize insulin native state,
524 particularly the ILs with shorter alkyl chains and weak hydrogen bonding. The ILs
525 included [C₂MIm][CH₃COO], [C₄MIm]Cl, 1-butyl-3-methylimidazolium nitrate
526 ([C₄MIm]NO₃), 1-butyl-3-methylimidazolium methanesulfonate ([C₄MIm][CH₃SO₃]),
527 [C₄MIm][N(CN)₂], [C₄MIm][CH₃COO], 1-hexyl-3-methylimidazolium acetate
528 ([C₆MIm][CH₃COO]), 1-methyl-3-octylimidazolium acetate ([C₈MIm][CH₃COO]), 1-
529 decyl-3-methylimidazolium acetate [C₁₀MIm][CH₃COO], and 1-dodecyl-3-
530 methylimidazolium acetate ([C₁₂MIm][CH₃COO]). Moreover, the MD demonstrated
531 that electrostatic interactions are the main forces responsible for the ability of the ILs to
532 stabilize the peptide.

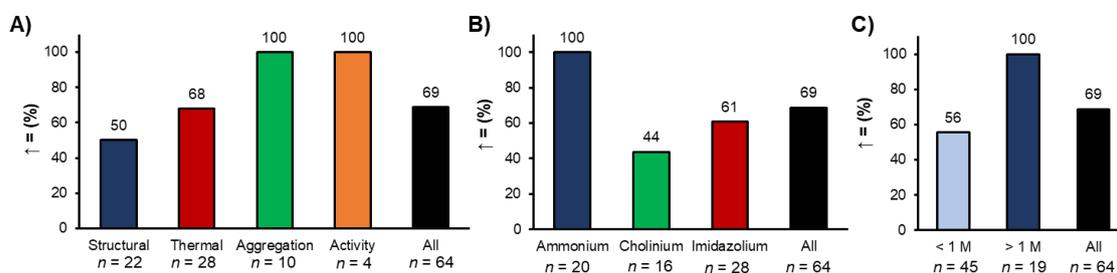
533 Regarding the application of ILs and DESs in pharmaceutical formulations for
534 improved delivery, cholinium-based ILs and DESs have been successfully applied by
535 multiple groups for transdermal and oral administration of insulin. For instance, high
536 concentrations (above 1 M to neat) of cholinium geranate ILs and DESs [*i.e.*, IL
537 [Ch][C₉H₁₅COO]; DESs cholinium geranate 1:2 ([Ch][C₉H₁₅COO]–[C₉H₁₅COOH]) and
538 cholinium geranate 1:4 ([Ch][C₉H₁₅COO]–[C₉H₁₅COOH]₃)] improved the transdermal
539 permeation of insulin in three different studies (Banerjee et al., 2018; Jorge et al., 2020;
540 Tanner et al., 2018). Moreover, [Ch][C₉H₁₅COO] also increased the oral intake and
541 paracellular transportation from 0.04 to 3.7 M (neat IL) in two other works (Banerjee et
542 al., 2018; Peng et al., 2020). Another advantage of cholinium geranate ILs and DESs is

543 their overall biocompatibility, which makes them remarkable candidates for the
544 development of biopharmaceutical delivery systems (Riaz et al., 2022).

545 For insulin, both ILs and DESs presented remarkable applications to improve its
546 delivery by enhancing its transdermal permeation. However, the data is still limited
547 regarding the impact of DESs on the stability of this biopharmaceutical. Hence, we
548 suggest following studies on the topic also account for the effect of DESs on the
549 stability of insulin, considering specific ILs improved while others impaired the stability
550 of this peptide.

551 Considering the extensive number of studies on the effect of ILs on insulin, we
552 evaluated the entries in **Tables 2** and **3** to determine the amount of IL solutions that
553 increased or maintained the stability of insulin according to the stability type, IL class,
554 and concentration range. The results as percentages of the total for each variable are
555 presented in **Figure 5**. This method was chosen to try to establish trends regarding the
556 effect of different conditions on insulin stability. It should be noted that this analysis
557 does not try to be a definitive answer regarding the effects of ILs and DESs on insulin,
558 but it aims to find tendencies and knowledge gaps in this topic and be a guide for future
559 research.

560



561

562 **Figure 5. A)** Percentage of IL and DES solutions that maintained or increased the different types of
563 stability of insulin (structural, thermal, activity, and aggregation). For aggregation, improvement of
564 stability represents ILs that decrease protein aggregation. **B)** Percentage of IL and DES solutions that
565 maintained or increased the stability of insulin according to their class. **C)** Percentage of IL and DES
566 solutions that maintained or increased the stability of insulin according to their concentration (< 1 or ≥ 1
567 M). *n* = total number of samples for each condition.

568

569 As can be seen in **Figure 5**, most IL and DES solutions had a positive effect on
570 insulin stability (69 %, *n* = 64, with *n* representing the number of samples for each
571 condition evaluated). Specifically, half the IL and DES solutions improved the
572 structural stability of insulin (*n* = 22), 68 % increased or maintained its thermal stability
573 (*n* = 28), and 100 % decreased its aggregation (*n* = 10). Although 100 % also
574 maintained or improved insulin activity, the number of samples is still too low for a

575 conclusion ($n = 4$). Hence, we suggest that future studies also include the effect of ILs
576 on the biological activity of insulin. Regarding the effect of different classes,
577 ammonium IL had the most positive effect on insulin stability (100 %, $n = 28$), followed
578 by imidazolium (61 %, $n = 20$), and cholinium-based ILs and DESs (44 %, $n = 16$).
579 However, it should be noted that cholinium ILs and DESs also had a functional effect
580 on insulin oral and transdermal delivery, sustained release, activity, and long-term
581 stabilization, demonstrating its potential for application. As for the concentration range,
582 higher concentrations of ILs and DESs were more compatible with insulin than lower
583 (100 % and $n = 19$, 56 % and $n = 45$, respectively).

584 Therefore, many biocompatible IL and DES solutions can be applied to enhance
585 the stability, activity, and delivery of insulin. The development of more stable insulin
586 formulations with the possibility of oral and transdermal delivery can improve
587 prognosis and patient adherence to diabetes treatments.

588 5.1.4. Ovalbumin epitope (OVA)

589 OVA is a glycopeptide and the major protein constituent from chicken egg whites.
590 Furthermore, OVA is a small (1 kDa) peptide formed by β -sheets and β -turns (**Figure**
591 **3.D**) (Tahara et al., 2020). Because it is mildly immunogenic, OVA is used as an
592 antigen in vaccines to improve immunogenic response. However, OVA and most other
593 antigens used as adjuvants in vaccines have low solubility in oil-based formulations for
594 transdermal delivery (Tahara et al., 2020). Considering it is possible to design
595 amphipathic ILs, they can be a suitable media to solubilize and improve the skin
596 permeation of OVA.

597 To increase the solubility and skin penetration of OVA, Tahara *et al.* (Tahara et
598 al., 2020) applied cholinium fatty acid-based ILs as additives from 0.2 to 0.7 M. The IL
599 cholinium cis-9-octadecenoate ([Ch][C_{18:1}]) and cholinium dodecanoate ([Ch][C_{12:0}]) at
600 20 wt% (0.5 and 0.7 M, respectively) enhanced the skin penetration of OVA, while
601 [Ch][C_{18:1}] also improved the solubility and activity (suppression of tumor growth *in*
602 *vivo*) of OVA at 0.2 M. [Ch][C_{18:1}] increased 28-fold the delivery of OVA when
603 compared with the control using an aqueous vehicle. Furthermore, [Ch][C_{18:1}] was
604 found to be biocompatible with dendritic cells and to not cause skin irritation. Thus,
605 cholinium fatty-acid ILs can be used for the development of novel transdermal drug
606 delivery systems due to their biocompatibility with cells and skin and their ability to
607 help in the dissolution and skin penetration of hydrophilic biopharmaceuticals.

608 5.1.5. Immunoglobulin G1 (IgG1)

609 IgG1 is the most abundant immunoglobulin in humans and is one of the main
610 antibodies for mediating the humoral response against infections. As presented in
611 **Figure 3.E**, IgG1 is a large globular tetrameric protein (around 140 kDa) with two
612 heavy chains and two light chains (Harris et al., 1998). The identical heavy chains
613 connect at the base of the protein structure forming a Y shape (Fc region, conserved
614 portion). The top portion of the Y is the Fab region, which binds to antigens and is
615 highly variable. In the medical field, IgG1 is the main representative of monoclonal
616 antibodies, the largest class of biopharmaceuticals (Angsantikul et al., 2021). They are
617 used to treat several illnesses, such as cancers, infections, inflammations, and
618 autoimmune diseases. However, because IgG1 is a large and complex protein, it is
619 prone to unfolding and aggregation, losing its affinity towards its specific antigens and
620 in consequence, its function (Manning et al., 2010). Moreover, antibodies are not
621 resistant or have poor absorption in oral administration (Angsantikul et al., 2021). Thus,
622 the development of formulations to enhance the stability and delivery of
623 immunoglobulins can improve and expand their application in medicine.

624 Researchers have applied cholinium ILs and DESs to enhance the thermal stability
625 of IgG1. For example, Dhiman *et al.* used low concentrations of cholinium acetate
626 ([Ch][CH₃COO]) to increase the T_m of IgG1 from human serum (Dhiman et al., 2022).
627 In a follow-up study, the group saw that low to high concentrations (around 0.2 to 1.5
628 M) of cholinium DESs [*i.e.*, choline chloride and carbamide ([Ch]Cl-[CO(NH₂)₂]),
629 choline chloride and glycerol ([Ch]Cl-[C₃H₅(OH)₃]), choline chloride and ethylene
630 glycol ([Ch]Cl-[(CH₂OH)₂])] also have a positive effect on the thermal stability of this
631 protein (Dhiman et al., 2023). Therefore, cholinium ILs and DESs are suitable
632 compounds to decrease the thermal unfolding of antibodies.

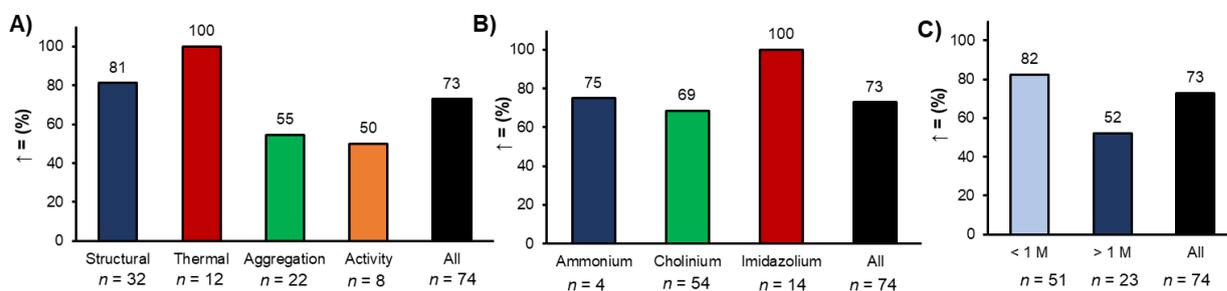
633 There are also several studies on the effects of ILs and DESs on the structural
634 stability of IgG1. In addition to evaluating the effect of cholinium ILs on IgG1 T_m,
635 Dhiman *et al.* showed that 0.0005 to 0.0025 M of [Ch][CH₃COO], cholinium citrate
636 (*i.e.*, citrate also known as 2-hydroxypropane-1,2,3-tricarboxylate)
637 ([Ch][C₃H₇O₅COO]), and [Ch]H₂PO₄ and around 0.2 to 0.8 M [Ch]Cl-[CO(NH₂)₂],
638 [Ch]Cl-[C₃H₅(OH)₃], and [Ch]Cl-[(CH₂OH)₂] preserved the native structure of this
639 antibody (Dhiman et al., 2023, 2022). In a similar approach, Mondal *et al.* (Mondal et
640 al., 2016) and Angsantikul *et al.* demonstrated that high concentrations of cholinium

641 ILs and DESs can also maintain the structural integrity of IgG1 (Angsantikul et al.,
642 2021). Specifically, Mondal *et al.* used around 0.9 to 1.4 M of cholinium
643 hydroxyacetate ([Ch][HOCH₂COO]) (*i.e.*, also called cholinium glycolate) and
644 [Ch][C₅H₇O₄COO] with IgG from rabbit serum, (Mondal et al., 2016) while
645 Angsantikul *et al.* applied around 1.8 to 2.8 M of [Ch][HOCH₂COO], cholinium
646 hydroxyacetic acid 2:1 ([Ch][HOCH₂COO]–[Ch]), and cholinium hydroxyacetic acid
647 1:2 ([Ch][HOCH₂COO]–[HOCH₂COOH]) to maintain the structure of antihuman TNF-
648 α mouse IgG1 (Angsantikul et al., 2021). With similar results, Rawat and Bohidar
649 (Rawat and Bohidar, 2015) showed that dilution solutions (0.04 M) of the imidazolium
650 ILs 1-ethyl-3-methylimidazolium chloride ([C₂MIm]Cl), [C₄MIm]Cl, 1-hexyl-3-
651 methylimidazolium chloride ([C₆MIm]Cl), and 1-octyl-3-methylimidazolium chloride
652 ([C₈MIm]Cl) can increase the structural stability of human IgG1. For Ferreira *et al.*
653 (Ferreira et al., 2016), low concentrations (around 0.1 to 0.3 M) of imidazolium and
654 ammonium ILs also maintain the structure of IgG1 from rabbit serum. The tested ILs
655 included 1-butyl-3-methylimidazolium tosylate ([C₄MIm][CH₃BzSO₃]),
656 [C₄MIm][N(CN)₂], [C₄MIm][CH₃COO], 1-ethyl-3-methylimidazolium bromide
657 ([C₂MIm]Br), 1-butyl-3-methylimidazolium bromide ([C₄MIm]Br), [C₄MIm]Cl,
658 [N_{1,1,1,1}]Br, [N_{2,2,2,2}]Br, and [N_{3,3,3,3}]Br. However, high concentrations (around 1.1 to
659 2.5 M) of certain cholinium ILs and even dilute solutions (0.1 to 0.2 M) of bulkier
660 phosphonium and ammonium ILs caused partial unfolding of the antibodies. The dilute
661 IL solutions that decrease the structural stability of IgG1 were [Ch]Cl–[CO(NH₂)₂],
662 [Ch]Cl–[C₃H₅(OH)₃], [Ch]Cl–[(CH₂OH)₂] (Dhiman et al., 2023), cholinium indole-3-
663 acetate ([Ch][C₉H₈NCOO]), cholinium 2-oxopropanoate ([Ch][CH₃COCOO]) (Mondal
664 et al., 2016), tetrabutylphosphonium bromide ([P_{4,4,4,4}]Br), and tetrabutylammonium
665 bromide ([N_{4,4,4,4}]Br) (Ferreira et al., 2016). Overall, dilute solutions of ILs and DESs
666 are better at improving or maintaining the native structure of IgG1 than concentrated
667 solutions or low concentrations of bulkier ILs and DESs.

668 Regarding the effect of ILs and DESs on the activity of antibodies, there is only
669 one study on this topic. Angsantikul *et al.* (Angsantikul et al., 2021) observed that
670 around 0.7 to 3.9 M of [Ch][HOCH₂COO] and [Ch][HOCH₂COO]–[Ch] improved the
671 antigen binding capacity of antihuman TNF-α mouse IgG1, while around 0.8 to 3.5 M
672 of [Ch][HOCH₂COO]–[HOCH₂COOH] impaired it. As there is still only one study on
673 the impact of ILs and DESs on IgG1 activity, we recommend other studies to address
674 this variable.

675 The ILs and DESs can also affect the long-term storage and delivery of
 676 antibodies. For example, Dhiman *et al.* (Dhiman et al., 2023) observed that 1.5 M of
 677 [Ch]Cl-[CO(NH₂)₂] can preserve IgG1 for 20 days in solution, while there is
 678 aggregation and partial unfolding in phosphate buffer for the same time. In another
 679 approach, Angsantikul *et al.* (Angsantikul et al., 2021) were able to improve the
 680 delivery of antihuman TNF- α mouse IgG1, a monoclonal antibody for the treatment of
 681 gastrointestinal infections and inflammatory bowel disease, using cholinium DESs. The
 682 researchers enhanced the paracellular transportation of the antibody with solutions from
 683 0.03 to 0.08 M of [Ch][HOCH₂COO]-[Ch] and enhanced IgG1 delivery into the
 684 intestinal mucosa and systemic circulation with 1.4 M [Ch][HOCH₂COO]-[Ch]
 685 solution. Furthermore, [Ch][HOCH₂COO]-[Ch] is also biocompatible with Caco-2
 686 Cells and rats, causing no damage to their gastrointestinal tissue and normal liver and
 687 kidney functions. Thus, cholinium DES solutions are suitable and biocompatible
 688 vehicles for the long-term storage and improved delivery of IgG1.

689 As previously presented for insulin, **Figure 6** shows the percentage of IL and
 690 DES solutions that increased or maintained the stability of IgG1 insulin according to the
 691 stability type, IL class, and concentration range.



692

693 **Figure 6. A)** Percentage of IL and DES solutions that maintained or increased the different types of
 694 stability (structural, thermal, activity, and aggregation) of IgG1. For aggregation, improvement of stability
 695 represents ILs that decrease protein aggregation. **B)** Percentage of IL and DES solutions that maintained
 696 or increased the stability of IgG1 according to their class. **C)** Percentage of IL and DEs solutions that
 697 maintained or increased the stability of IgG1 according to their concentration (<1 or \geq 1 M). *n* = total
 698 number of samples for each condition.

699

700 In **Figure 6.A**, most IL and DES solutions improved the stability of IgG1 (73 %, *n*
 701 = 74), with 81 % improving or preserving IgG1 structural (*n* = 32) and 100 % its
 702 thermal stability (*n* = 12), 55 % reducing its aggregation (*n* = 22), and 50 % enhancing
 703 or maintaining its activity (*n* = 8). As for insulin, only one study with 8 solutions
 704 included the effect of ILs and DESs on the biological activity of the protein. Thus, we
 705 suggest more studies include this parameter in their research design, considering the

706 maintenance of the activity of the biopharmaceutical is vital to allow its medical
707 application.

708 Regarding the different classes in **Figure 6.B**, ammonium-, cholinium- and
709 imidazolium-based IL and DESs solutions had a positive effect on IgG1, with 75 % ($n =$
710 4), 69 % ($n = 54$), and 100 % ($n = 14$) preserving its stability, respectively. Furthermore,
711 it should be noted that again, cholinium ILs and DESs also could improve the long-term
712 preservation and oral delivery of the biopharmaceutical, showing potential outside of
713 only enhancing protein stability.

714 As for the concentration range in **Figure 6.C**, dilute solutions (82 %, $n = 51$) were
715 better at preserving IgG1 than concentrated ILs (52 %, $n = 23$). Interestingly, this trend
716 is the opposite of what was observed for insulin, showing the nature of the protein will
717 change the tendencies and types of interactions between proteins and ILs and DESs.

718 As discussed, cholinium-based ILs and DESs have been investigated for their
719 potential to enhance the stability and delivery of IgG1. Both classes were able to
720 increase the thermal stability of IgG1. However, their effect on the structural integrity of
721 the protein differed based on concentration and the specific solvent. Generally, dilute
722 solutions of both ILs and DESs are more successful at maintaining the native structure
723 of IgG1 than concentrated solutions. Nonetheless, certain concentrated cholinium ILs
724 and even some dilute solutions of bulkier ILs have caused partial unfolding of the
725 antibodies. On the delivery front, cholinium DESs have showcased a distinct advantage,
726 demonstrating their ability to not only improve the long-term storage of IgG1 but also
727 enhance its delivery into the intestinal mucosa and systemic circulation. This suggests
728 that while both ILs and DESs can aid in the stabilization of IgG1, cholinium-based
729 DESs might offer superior advantages for antibody delivery applications.

730 In conclusion, ILs and DESs can be applied to improve the stability, activity, long-
731 term preservation, and oral delivery of antibodies. These novel formulations can
732 potentially help in expanding and increasing access to antibodies for the treatment of
733 life-threatening diseases such as cancers, infections, and auto-immune disorders.

734 *5.1.6. L-Asparaginase (L-ASNase)*

735 L-ASNase is an enzyme used for the treatment of acute lymphoblastic leukemia
736 and other types of cancer (Stecher et al., 1999) and in the food industry as an acrylamide
737 mitigation agent (Bento et al., 2022). There are multiple L-ASNase types, with types I
738 and II being more applied in biotechnology. Type II is a bacterial periplasmic or

739 membrane-associated enzyme with a high affinity for L-asparagine and low activity
740 towards L-glutamine, which is ideal for applications as a pharmaceutical (Castro et al.,
741 2021). Type I still has an affinity towards L-asparagine, but its hydrolysis of L-
742 glutamine limits its application to the food industry. The L-ASNase structure is
743 tetrameric with two connected α/β domains (*i.e.*, N-terminal and C-terminal) for each of
744 its four subunits, as shown in **Figure 3.C**. The N-terminal domain presents eight-
745 stranded mixed β -sheets with four α -helices, and the C-terminal of four-stranded
746 parallel β -sheets with four α -helices (Swain et al., 1993). The anti-leukemia property of
747 L-ASNase is based on the hydrolysis of L-asparagine and the depletion of this amino
748 acid necessary for the survival of cancer cells (Van Trimont et al., 2022). However,
749 different sources and environments can alter the catalytic activity of L-ANSase,
750 improve or impair its pharmaceutical use, and reduce or increase its adverse effects.

751 Regarding its stability, Elspar[®] (L-ASNase biopharmaceutical from *Escherichia*
752 *coli*) can be maintained for up to 7 days in solution in the fridge (Stecher et al., 1999),
753 but its activity decreases substantially after a few hours at 50 °C (Magri et al., 2019). L-
754 ASNase from other sources can vary in properties and stability, hence, it is necessary to
755 take this information into account when comparing the enzyme variants (Bento et al.,
756 2022). Furthermore, L-ASNase is usually obtained from microorganisms, which
757 generate many contaminants that can cause unwanted clinical effects, such as
758 hyperglycemia and hepatotoxicity (Li et al., 2019). The purification of L-ASNase to
759 medical grade levels is complex and costly, raising the price of this biopharmaceutical
760 and limiting its access (Dos Santos et al., 2018). Thus, the development of technologies
761 to enhance L-ASNase stability, activity, and extraction can improve its clinical use in
762 cancer treatment.

763 ILs can be used as additives to improve the hydrolysis of L-asparagine by L-
764 ASNase, as observed in a study by Magri *et al.* that worked with L-ASNase EC 3.5.1.1
765 produced by *E. coli* (Magri et al., 2019). The ILs [Ch][CH₃COO], cholinium propanoate
766 ([Ch][C₂H₅COO]), cholinium butanoate ([Ch][C₃H₇COO]) improved asparagine
767 hydrolysis from 0.001 to 0.050 mol IL/mol total and cholinium hexanoate
768 ([Ch][C₅H₁₁COO]) from 0.001 to 0.010 mol IL/mol total at room temperature. However,
769 higher concentrations of [Ch][C₅H₁₁COO] (0.025 to 0.050 mol IL/mol total) or the
770 increase of temperature to 50 °C impaired L-ASNase catalysis for all ILs when
771 compared to the buffer.

772 The recovery and preservation of biopharmaceuticals during their upstream
773 processing is also a topic of relevance for the pharmaceutical industry. With this in
774 mind, Li *et al.* (Li *et al.*, 2019) applied the DES poly(vinyl pyrrolidone) and
775 propanedioic acid 1:1 ($[(C_6H_9NO)_n]-[CH_2(COOH)_2]$) to adsorb L-ASNase from *E. coli*
776 extract. The addition of 0.1 M of $[(C_6H_9NO)_n]-[CH_2(COOH)_2]$ to the bacterial extract
777 facilitates the separation of the enzyme from their complex medium. Even though there
778 was a slight alteration in the protein structure, the catalytic activity of the protein was
779 preserved.

780 Considering the goals of application of the ILs and DESs with L-ASNase were
781 very distinct (*i.e.*, ILs for its stabilization and activity enhancement and DES for its
782 recovery), it is not possible to do a direct comparison of the impacts of each class on
783 this enzyme.

784 As seen in this subsection, ILs and DESs can be applied to enhance the catalysis
785 and promote the recovery of L-ASNase from a complex medium. The improvement of
786 the activity and extraction of this enzyme can allow the reduction of costs and side
787 effects of leukemia treatments.

788 **5.2. Nucleic acid biopharmaceuticals**

789 Nucleic acids such as small interfering RNA (siRNA) and oligonucleotides can be
790 applied as biopharmaceuticals to treat different conditions, such as autoimmune and
791 skin diseases. The siRNA are double-stranded RNA used to silence genes by interfering
792 with their expression (Chakraborty *et al.*, 2017). They have been recently applied as
793 biopharmaceuticals to treat several illnesses, particularly cancer, viral infections, and
794 immune diseases. However, nucleic acids have very poor stability and problems with
795 their delivery, which hinders their applications in medicine (Mandal *et al.*, 2020). In
796 addition to siRNA, oligonucleotides can also be applied as biopharmaceuticals (Handa
797 *et al.*, 2019). However, they present the same drawbacks as siRNA due to their low
798 stability and difficulty in drug delivery. Considering the relevance of nucleic acid
799 biopharmaceuticals for advanced therapies and the current limitations to their
800 application, this section will explore the use and potential of ILs and DESs to stabilize
801 and improve the delivery of this class of biotechnological medicines, as previously
802 displayed in **Tables 2** and **3**.

803 To improve the stability and administration of siRNA, different groups have
804 applied ILs and DESs in siRNA formulations. For example, Mazid *et al.* (Mazid *et al.*,

2014) used high concentrations (1.0 to 2.5 M) of [Ch]H₂PO₄ to stabilize the siRNA against CD45, employed in the treatment of autoimmune diseases. This IL enhanced the structural and thermal stability of the siRNA. Furthermore, it improved the long-term storage of the siRNA, increasing its preservation from 30 min to 3 months while maintaining its ability to induce gene knockdown. Also studying the effect of cholinium ILs and DESs in siRNA, Mandal *et al.* (Mandal *et al.*, 2020) employed high concentrations (1.0 to 2.5 M) of the ILs [Ch][C₉H₁₅COO] and cholinium phenylpropanoate ([Ch][PhC₂H₅COO]), and other cholinium derivatives (*i.e.*, 3-methylbutanoic acid, choline and dimethylacrylic acid, and choline and biphenyl-3-carboxylic acid) to enhance the skin penetration of the psoriasis biopharmaceutical siRNA against NFKBIZ. [Ch][PhC₂H₅COO] preserved the structural integrity and enhanced the skin penetration of the siRNA. On the other hand, [Ch][C₉H₁₅COO] improved skin penetration of the siRNA but slightly decreased its structural stability. With this in mind, the researchers mixed [Ch][C₉H₁₅COO] and [Ch][PhC₂H₅COO] 1:1 (v/v) and studied the effect of the solution on siRNA. The solution of [Ch][C₉H₁₅COO] and [Ch][PhC₂H₅COO] (1 M each) improved the structural stability, activity (*i.e.*, down-regulation of psoriasis-related signals), and skin penetration of the siRNA. Moreover, [Ch][C₉H₁₅COO] and [Ch][PhC₂H₅COO] 1:1 solution was also biocompatible with mice. Thus, ILs, particularly [Ch][PhC₂H₅COO], showed specific capabilities in preserving the structural integrity and enhancing skin penetration of siRNA.

With another approach to improve the topical delivery of siRNA with ILs, Zakrewsky and Mitragotri (Zakrewsky and Mitragotri, 2016) synthesized siRNA against GAPDH and MMP12 robed with ILs moieties for the treatment of skin diseases. The siRNA robed with ammonium-ILs [*i.e.*, benzyldimethyloctylammonium robed-siRNA1 ([N_{1,1,(Bz),8}]-siRNA1), benzyldimethyltetradecylammonium robed-siRNA1 ([N_{1,1,(Bz),14}]-siRNA1), benzyldimethylstearyl ammonium robed-siRNA1 ([N_{1,1,(Bz),18}]-siRNA1)] were able to increase skin penetration and cell internalization of the biopharmaceutical in concentrations as low as 0.00005 M. The efficacy and safety of the siRNA-robed ILs were also confirmed with tests in skin tissue.

To counter this issue related to the low stability and difficulty in drug delivery of oligonucleotides, MEDRx co. developed the Ionic Liquid Transdermal System (ILTS[®]), a lidocainum etodolac IL designed to solubilize and enhance the delivery of pharmaceuticals (Kubota *et al.*, 2016). This system was successfully applied to increase the skin penetration of STAT6 decoy oligonucleotide used for the treatment of skin

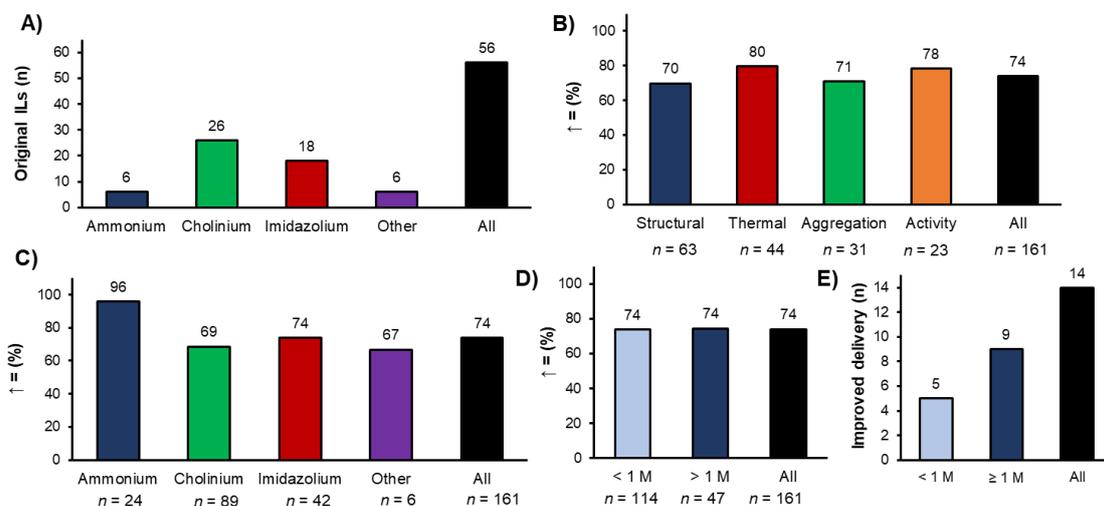
839 inflammation (Handa et al., 2019). As a result, the formulation with ILTS and the
 840 oligonucleotide led to a better therapeutical outcome in the treatment of skin
 841 inflammation in mice when compared to the conventional protocol using Vaseline
 842 (Handa et al., 2019).

843 As can be seen, ILs and DESs are promising alternatives to improve the stability
 844 and drug delivery of nucleic acid biopharmaceuticals. Considering the current
 845 expansion of nucleic acid therapeutics, the demand for formulations to stabilize and
 846 functionalize this class of biopharmaceuticals will escalate in the next years.

847 6. Perspectives for the use of ILs and DESs for the stabilization of 848 biopharmaceuticals

849 In this section, we will present the tendencies of the effects of ILs and DESs on the
 850 stability of biopharmaceuticals in a pragmatic manner, as previously shown for insulin
 851 and IgG1. As stated for insulin, this discussion aims to find patterns and knowledge
 852 gaps in the topic to guide further research and not to be a definitive conclusion on the
 853 effect of ILs and DESs on nucleic acids and proteins. Furthermore, we will give our
 854 insights regarding the opportunities in the area and the perspectives regarding the
 855 expansion of applications of ILs and DESs in the medical field.

856 With this goal, **Figure 7** shows the percentage of IL and DES solutions that
 857 increased or maintained the stability of biopharmaceuticals according to the stability
 858 type (**Figure 7.B**), class (**Figure 7.C**), and concentration range (**Figure 7.E**). Moreover,
 859 **Figure 7.A** has the number of original ILs and DESs per class and **Figure 7.E** has the
 860 number of cholinium ILs and DESs that improved the delivery of biopharmaceuticals
 861 considering their concentration ranges.



862

863 **Figure 7. A)** Number of original ILs and DESs for each class. **B)** Percentage of IL and DES solutions that
864 maintained or increased the different types of stability of biopharmaceuticals (structural, thermal, activity,
865 and aggregation). For aggregation, improvement of stability represents ILs and DESs that decrease
866 protein aggregation. **C)** Percentage of solutions that maintained or increased the stability of
867 biopharmaceuticals according to their IL and DES class. **D)** Percentage of IL and DES solutions that
868 maintained or increased the stability of biopharmaceuticals according to their concentration (<1 or ≥ 1
869 M). **E)** Number of cholinium-based ILs and DESs that improved the delivery of biopharmaceuticals at
870 different concentrations. n = total number of samples for each condition.
871

872 **Figure 7.A** show there were 56 original ILs or DESs in studies of their effect on
873 biopharmaceuticals. The most prevalent class was of cholinium ILs and DESs (26
874 entries), followed by imidazolium- (18) and ammonium-based (6) ILs. This information
875 contrasts with a previous study of the group, where the most used class for non-
876 enzymatic protein stabilization was imidazolium ILs (55 % of entries) (Veríssimo et al.,
877 2022). However, considering that cholinium ILs and DESs are regarded as more
878 biocompatible than imidazolium ILs, it is not surprising that research designed for
879 pharmaceutical applications opt for a class with lower toxicity (Kumar et al., 2017; Riaz
880 et al., 2022). Furthermore, as presented in **Figure 7.E**, cholinium ILs and DESs are also
881 suitable vehicles to improve the delivery of biopharmaceuticals ($n = 14$), adding another
882 reason to justify their selection.

883 Regarding the overall effect of ILs and DESs on biopharmaceuticals stability,
884 around 70 % of the solutions increased or maintained the structural ($n = 63$) and thermal
885 ($n = 44$) stability and aggregation rate ($n = 31$) and activity ($n = 23$) of proteins,
886 peptides, and nucleic acid biologics (**Figure 7.B**), with only small differences between
887 each type. Furthermore, there were also only small differences between the use of low
888 (< 1 M, $n = 114$) or high (≥ 1 M, $n = 47$) concentrations of ILs and DESs (**Figure 7.D**),
889 with both preserving around 70 % of the stability of biopharmaceuticals. This is an
890 interesting result, considering the percentages fluctuate when considering individual
891 macromolecules, as observed in **Tables 2** and **3** and the previous work of the group
892 (Veríssimo et al., 2022). Therefore, larger amounts of data comprising different classes
893 of macromolecules and ILs and DESs must be evaluated when searching for trends in
894 their interactions.

895 As for the IL and DES classes in **Figure 7.C**, ammonium ILs were the most
896 compatible with biopharmaceuticals, with 96 % ($n = 24$) preserving their stability. They
897 were followed by imidazolium ILs with 74 % ($n = 42$) e cholinium ILs and DESs with
898 69 % ($n = 89$).

899 The results are still scarce to allow a proper comparison between ILs and DESs on
900 their particular effect on the stability of biopharmaceuticals, given that DESs were only
901 applied in studies with insulin, IgG1, and L-ASNase. Furthermore, the research with
902 DESs involving insulin and L-ASNase primarily aimed at enhancing the delivery and
903 recovery of the biomolecules, respectively. Therefore, there are only reports on the
904 effect of DESs on the stability of IgG1. For insulin, both ILs and DESs have
905 demonstrated the potential to improve its delivery through enhanced transdermal
906 permeation. Cholinium-based ILs and DESs, when applied to IgG1, showed aptitude in
907 boosting both its stability and delivery. Both ILs and DESs increased the thermal
908 stability of IgG1, but their impact on the structural integrity of the protein varied based
909 on concentration range and specific solvent. Interestingly, while both ILs and DESs are
910 useful for stabilizing IgG1, cholinium-based DESs seem particularly promising for
911 antibody delivery applications, especially for enhancing the long-term storage of IgG1
912 and its delivery into the intestinal mucosa and systemic circulation. In conclusion, while
913 both ILs and DESs have showcased potential in the stabilization and delivery of
914 biopharmaceuticals, their specific impacts vary considerably based on the biomolecule
915 in question and the specific application.

916 The evolving landscape of ILs and DESs in biopharmaceutical applications
917 highlights their significant potential for enhancing the stability and delivery of various
918 biomolecules. While the current data suggest a generally positive impact on the
919 structure and activity of biopharmaceuticals, the variability in effects based on the type
920 of IL or DES, concentration, and specific biomolecule underscores the need for further
921 research. The cholinium-based ILs and DESs are particularly promising for future
922 pharmaceutical applications, considering their biocompatibility and enhanced delivery
923 capabilities.

924 **7. Conclusions**

925 Medical professionals are expanding the use of biopharmaceuticals due to their
926 targeted therapeutic action, reducing adverse effects and improving positive clinical
927 outcomes of complex treatments such as cancers and auto-immune diseases. However,
928 stabilizing biopharmaceuticals and enhancing their delivery are essential steps to allow
929 their sustainable production and application, which are required to fulfill their market
930 demand and clinical use. This issue demands continuous R&D efforts to find disruptive
931 and sustainable solutions for current drawbacks in formulating biopharmaceuticals.

932 In this sense, ILs and DESs offer a diversity of physical-chemical properties and
933 effects that researchers could exploit to improve the stability and delivery of
934 biopharmaceuticals, such as their tunable and "designer" (Freire, 2016; Kunz and Häckl,
935 2016). As presented in this review, most ILs and DESs conditions evaluated (around 70
936 %) preserved or increased the stability or activity of the biopharmaceuticals, enhanced
937 their solubility, or improved their delivery. However, although there are classes with
938 low toxicity, such as ammonium and cholinium ILs and DESs, the complete
939 environmental footprint of these compounds still needs further investigation to
940 guarantee their safe and sustainable use. Moreover, altering the biomolecule,
941 concentration range, and environmental conditions can impact their effect on the
942 biopharmaceuticals and their safety, which could complicate their commercial use.
943 There is also the issue regarding their current low availability, high prices, and lack of
944 approved ILs and DESs for human use by regulatory agencies, which can limit their
945 large-scale applications (dos Santos et al., 2018; Hough and Rogers, 2007; Wakayama
946 et al., 2019).

947 Hence, we believe researchers should further explore biocompatible classes such
948 as cholinium and ammonium-based DESs, which present high preservation of the
949 structure and activity of the biomolecules while also allowing formulations with
950 improved solubilization and delivery of the biopharmaceuticals. Moreover, scientists
951 should work towards increasing the numbers and availability of ILs and DESs and
952 investigate the interactions between biopharmaceuticals and these green solvents to
953 provide insights regarding the transposition of applications for different biomolecules.
954 These advances can allow the development of predictive models and facilitate the
955 design of novel biopharmaceutical formulations.

956

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967 **Author contributions**

968 Conceptualization, methodology: N. V. P. Veríssimo, V. C. Santos-Ebinuma. Writing -
969 original draft preparation, investigation, data curation: N. V. Veríssimo, H. B. S. Bento,
970 C. U. Mussagy. Formal analysis, writing - review and editing, visualization: N. V.
971 Veríssimo, H. B. S. Bento, C. U. Mussagy, J. F. B. Pereira, V. C. Santos-Ebinuma.
972 Supervision, project administration, funding acquisition: V. C. Santos-Ebinuma.

973 **Competing interest declaration**

974 The authors declare no competing interests.

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