Ionic-Liquid-Based Materials for Protein-related Applications

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Supported ionic liquids (SILs) have been investigated as alternative supports for enzymes in biocatalysis and as new supports in preparative liquid chromatography for the purification of high-value proteins and enzymes. SILs are materials in which ionic liquids are introduced to modify the surface and properties of materials, e.g. as ligands covalently bond or physiosorbed.

Keywords: ionic liquids ; supported ionic liquids ; supports ; purification platforms ; chromatography ; proteins ; enzymes ; biocatalysis

1. Introduction

Proteins are macromolecular polypeptides with application in biochemical, biotechnology, chemical, food and pharmaceutical industries [1]. However, their biological function and activity, which is associated with the three-dimensional structure, can be disrupted through modifications in the medium temperature, pH, or composition [2]. Thus, the preservation of the structure of proteins in their applications is the main challenge, which may be achieved by favoring particular interactions, for instance hydrogen bonding, hydrophobic, and electrostatic forces with a solvent or material [3]. Several approaches such as immobilization, chemical modification, or genetic modification have been established to carry out the stabilization of proteins out of their native environments and improve their biological functions. Ionic liquids (ILs) have been investigated in this field as well, with a large fraction of the respective publications dealing with the interactions of ILs with proteins, while seeking the fundamental knowledge and factors that improve their stability in the different IL environments. Other scientific fields involving proteins and ILs deal with proteins' solubility and separation [4].

The wide structural variety of ILs and proteins/enzymes is responsible for a multiplicity of possible solvent–protein interactions [5], which are summarized in **Figure 1** [4]. Several studies have revealed a higher concentration of cations at the protein surface than the one verified for anions, regardless of the charge presented by the protein [6][7][8]. For proteins in aqueous media, this event can be justified by the preferential hydrogen-bonded environment established between IL anions and water molecules in the bulk phase [4]. However, if properly designed, IL cations have an amphiphilic nature, where long alkyl chains provide apolar regions and can interact favorably with the non-polar protein surface moiety [3][6]. Cations (red arrows in **Figure 1**) compete with the IL anions (dark blue arrows) for solvation positions at the polar protein surface, and in this way, most of the cationic alkyl chains point away from the surface of the protein. Notwithstanding that proteins with negative charge attract more cations in comparison to anions, the latter continue to compete with water molecules (light blue arrows in **Figure 1**) to establish hydrogen bond interactions with the amino acids present on the surface [4].

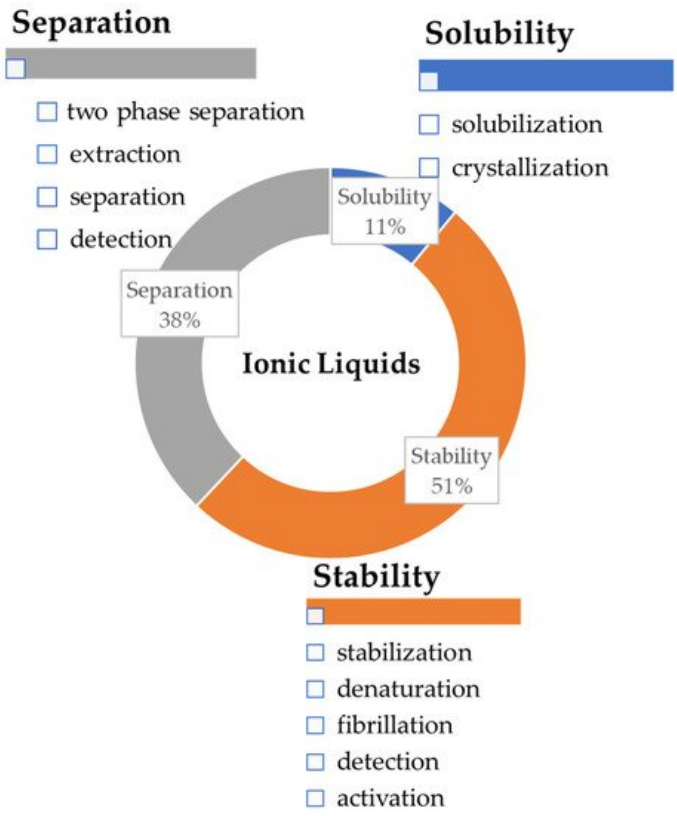

Figure 1. Publications between 2009 and 2020 about the use of ILs in different protein and enzyme applications using the following keywords: “ionic liquids”, “enzyme”, “proteins”, and “applications” (Source of Information: ISI Web of Knowledge, https://www.webofscience.com/wos/woscc/basic-search, accessed on 30 December 2020).

Numerous studies have demonstrated that the performance of important biomolecules such as proteins and enzymes can be substantially improved by using ILs. Several studies revealed that the functionality of these biomolecules can be improved in the presence of ILs [9][10][11][12][13][14][15][16][17]. Relevant works have been published demonstrating that proper ILs improve the activity and stability of various proteins or enzymes, comprising lysozyme (lyz), laccase, alcohol dehydrogenases, and cytochrome c (cyt-c), among others [2].

Although the largest fraction of works on enzymes and proteins combined with ILs comprises their use in liquid-liquid extraction processes, relevant evidence has been published on SILs’ potential in similar fields, i.e., as novel enzymatic supports and in proteins/enzymes purification.

2. Supported Ionic Liquids (SILs)

It was in the 1970s–1980s that the supported liquid phase field started [18], being achieved by the physical deposition of a liquid onto a material. However, since then, many studies disclosed the evaporation of the loaded liquid, which was a particular problem, especially when applying water as the liquid phase, making the concept appropriate merely for slurry-phase reactions with hydrophobic reaction mixtures [18]. Thus, new approaches to obtain liquid-comprising solid materials that do not have the ability to evaporate have emerged, particularly by modification of the surface of a porous solid through dispersion of a thin film of IL, which was described as supported ionic liquids (SILs). SILs are a particular group of materials obtained by the immobilization of ILs in a suitable solid support [19]. SILs present several advantages such as adjustable solvent properties with an efficient immobilization on a confined environment [19]. Since IL properties are transferred to solid surfaces, the SIL concept allows creating and customizing the solid material (“designer surfaces”), resulting in a surface that is well-defined and presents new specific properties and regulated chemical reactivity, thus constituting an attractive methodology to traditional material science [18].

Depending on the nature of the interactions settled between the IL and the support material, two principal categories can be distinguished: (i) the physical confinement of the IL in the materials (physisorption); and (ii) covalent grafting (chemisorption), in which covalent bonding between the support and the IL exists [19][20][21]. In the physisorption method, the immobilization of ILs occurs through deposition with non-covalent binding involved. Usually, SILs obtained by this method are present as a monolayer, although multilayers or brushes can also be obtained [22]. In this type of SIL, the properties of neat ILs are almost preserved. These materials have been mainly investigated in the capture and separation of gases [23][24][25]. On the other hand, immobilizing ILs on materials through covalent attachment is an attractive approach to avoid ILs leaching that may occur in the physical deposition method [26]. However, in these materials, the specific

properties noted in the IL bulk phase may no longer be present in the prepared support, since there is not a liquid phase present [26]. Despite this condition, the IL chemical structure diversity and their designer solvents ability are translated into SILs, contributing to enhancing the selectivity as well as the adsorption ability of the material in target applications [26][27].

Due to the recognized advantages obtained with SILs, numerous applications in different areas, varying from chemical to biological sciences, have been proposed [19]. In their use in solid-phase extractions from liquid phases or in biocatalytic applications, SILs with ILs covalently attached are preferred, since they avoid leaching. Solid-liquid systems exhibit numerous advantages in comparison to classical gas-liquid or liquid-liquid systems, such as an elevated high surface area provided by the support material, a thin film composed of liquid that avoids mass transport issues, and their application in fixed-bed or fluidized-bed reactor technologies. However, the preparation of this type of SIL exhibits some limitations and drawbacks: (1) a more challenging preparation since at least one reaction step is necessary, additionally including the pre-treatment requirement of inert materials; and (2) usual low density of IL ions onto the support, while being an IL monolayer [28].

3. SILs Applications in Protein-Related Fields

Up to date, SILs have been broadly employed in almost all fields involving ILs and have led to expansions on the ILs arena [29][26]. Below it is provided an overview on the application of SILs as alternative supports for the purification of proteins and enzymes.

SILs have been studied in SPE in order to take advantage of the chemical versatility and tunability of ILs. Most studies with SILs in this field used silica or polymers as solid phases. However, this field is less developed than the use of SILs in biocatalysis, with their application in separation processes of proteins being still limited for standard proteins such as lyz, BSA, bovine hemoglobin (BHb), cyt-c, ovotransferrin (OVT), and hemoglobin (Hb). The findings reported in the literature proving the SILs capability in the separation/recovery of some proteins are given in **Table 1**.

Table 1. Summary of the reported studies showing the potential of SILs in the biocatalysis field.

Support(s)	Immobilization Type	Enzyme(s)	Reaction/Application	Reference
Polymeric hybrid monolith	Physisorption	CALB	Vinyl propionate and 2-propanol continuous gas-phase transesterification	[30]
Polymeric monolith	Physisorption	CALB	Continuous flow synthesis of citronellyl propionate in supercritical CO_2 conditions	[31]
Polymers	Physisorption	CALB	Biocatalysts for a stereospecific reaction model	[32]
Single-walled carbon nanotubes (SWNT)	Physisorption	Myoglobin, cyt-c and horseradish peroxidase	Bioelectrocatalysis	[33]
Single-walled carbon nanotubes (SWNTs)	Physisorption	Glucose oxidase (GOx)	Oxidation of glucose	[34]
Multiwalled carbon nanotubes (MWNTs)	Physisorption	CALB	Hydrolysis of triacetin	[35]
Silica	Physisorption	*Burkholderia cepacia* lipase	Supports for lipase immobilization	[36]
Mesoporous silica SBA-15	Physisorption and chemisorption	Porcine pancreatic lipase	Hydrolysis of triacetin	[37]
Magnetic carboxymethyl cellulose (IL-MCMC)	Chemisorption	Lipase and penicillin G acylase (PGA)	Supports for enzyme immobilization	[38]
Magnetic chitosan (MCS) composites	Physisorption	Porcine pancreatic lipase (PPL)	Supports for PLL adsorption	[39]
Polystyrene divinylbenzene porous matrix	Physisorption	CALB	Synthesis of biodiesel	[40]

Support(s)	Immobilization Type	Enzyme(s)	Reaction/Application	Reference
Kynol™ ACC 507-15	Physisorption	*B. cepacia* lipase	1-Phenylethanol acylation with vinyl acetate	[41]
Poly(styrene-co-divinylbenzene)	Physisorption	CALB	Catalysis	[42]

Shu et al. [43] prepared [Nmim]Cl/polyvinyl chloride (PVC) materials by the immobilization of imidazolium cations, namely $[Nmim]^+$ moieties onto the PVC chain. Through this new method, [Nmim]Cl/PVC IL at a molar ratio of 4:1 gives rise to a bulk immobilization ratio of 15.1%. These SILs were able to perform the adsorption of lyz, cyt-c, as well as Hb with high efficiencies, viz. 97%, 98%, and 94%, respectively [43]. It was also pointed out by the authors that the prepared hybrid material exhibited potential properties crucial for its separation performance: for instance, an outstanding selectivity to adsorb basic proteins through an effective suppression of the adsorption of non-specific proteins by the pure PVC material, as well as its enhanced biocompatibility that avoids protein denaturation phenomenon during adsorption. In a different work, the selective isolation of Hb was assessed using imidazolium-modified polystyrene (PS) materials [44]. Imidazolium cations ($[mim]^+$) were attached onto the chloromethyl polystyrene (PS-CH_2-Cl) surface, forming PS-CH_2-[mim]Cl, which is a crosslinked rigid polymer that can behave as a support. The adsorption efficiencies of this material to Hb achieved values up to 91%, with almost no protein denaturation detected [44]. It was also stressed by the authors that the immobilization of the imidazolium groups on the surface of the material showed potential properties crucial for its separation performance, such as the ability to suppress the non-specific protein adsorption, promoting the selective adsorption of Hb. Another two polymer materials were synthesized and applied for the separation of BSA, BHb, lyz, and cyt-c, in which 1-allyl-3-butylimidazolium chloride ([aC $_4$im]Cl) and 1-vinyl-3-octylimidazolium bromide ([VC_8im]Br) were employed as functional monomers, and acrylamide was employed as a co-functional monomer [45]. While the [aC_4im]Cl-based polymer material has a high binding capacity for BHb (828.5 mg g^{-1}), the [VC_8im]Br-based polymer material has it for BSA (804.7 mg g^{-1}) [45]. In a similar work using a macroporous polymer material modified with 1-vinyl-3-butylimidazolium chloride ([ViBuIm]Cl), the selective adsorption of five proteins, namely BSA, BHb, equine myoglobin, cyt-c, and lys, was evaluated [46]. The adsorption tests included the adsorption capacity using individual proteins, binary mixtures composed by lyz and BSA, and lyz and cyt-c, as well as a ternary mixture made of lyz, BSA, and cyt-c. The prepared SIL displayed a strong binding capacity for proteins, in particular for Lys, registering a maximum capacity of 755.1 mg g^{-1}. Regarding the mixture of proteins, BSA was acknowledged as a competitive protein in the referred binary and ternary protein solutions, as the amount of adsorbed BSA was higher than the amount of adsorbed lyz. In addition, the amount of adsorbed BSA from the mixtures was higher in comparison to that of only BSA without other proteins, while the amount of adsorbed lyz of the mixtures was lower compared to that of only lyz without other proteins [46]. On the other hand, when the mixtures exhibit cyt-c, the amount of adsorbed lyz and cyt-c decreased when compared to only one protein in the solution due to the competitive adsorption that exists between the two proteins (with similar molecular weight and pI). Comparing the ternary solution composed of lyz, BSA, and cyt-c and the solution containing only cyt-c, the differences in the adsorption capacities of cyt-c were not obvious, suggesting that the interaction between proteins does not present a significant effect on cyt-c adsorption. The authors concluded that electrostatic interactions, hydrophobic forces, as well as hydrogen bonding were the main interactions established between proteins and the SIL [46].

Despite the described promising results, more studies must be carried out to extend the range of proteins and complexity of the matrices used to fully appraise their potential. However, the described outcomes, although still scarce in the field of proteins separation, indicate that different materials can be designed to adsorb and separate specific proteins and therefore can be seen as potential approaches to separate target proteins from complex real matrices. So far, studies addressing the purification of target proteins and enzymes from real and complex matrices only addressed bovine serum samples. There are also simulation studies on SILs; most of them focused on the absorption and separation of gases [47] [48]. These studies could open the door for the scientific community to start carrying out simulation studies of IL-based materials applied to the protein/enzyme separation and purification fields and thus better understand the molecular-level phenomenon of solid-phase extraction and favorable interactions to improve the material selectivity. The use of IL-based materials for protein separation is yet at an early phase, but this is a novel area where significant progress is estimated in the coming years, confirming the high potential of these materials in proteins downstream processing.

4. Conclusions

The development of novel IL-based materials has shown to be a field of high interest over the last few years. In the field of proteins and enzymes, SILs have been used as physisorbed or chemisorbed ILs in materials, while they have also been applied as enzyme supports in biocatalysis and in the separation of proteins and enzymes. IL-modified materials such as polymeric (hybrid) monoliths, single-walled carbon nanotubes, multiwalled carbon nanotubes, silica gel and mesoporous

silica SBA-15, magnetic carboxymethyl cellulose, magnetic chitosan composites, polystyrene divinylbenzene porous matrix, Kynol™ ACC 507-15, poly(styrene-co-divinylbenzene), polyvinyl chloride, polystyrene, and magnetic graphene oxide, among others, have been used. On the other hand, the proteins and enzymes studied in these fields correspond to lysozyme, hemoglobulin, bovine serum albumin, bovine hemoglobulin, equine myoglobulin, *Candida antarctica* lipase B, myoglobin, horseradish peroxidase, glucose oxidase, *Burkholderia cepacia* lipase, porcine pancreatic lipase, lipase, and penicillin G acylase.

Although the field of biocatalysis and SILs is a well-known and evolving field, the field of proteins separation/purification is still an underexplored area given the plethora of available material types, ILs, and proteins/enzymes. Furthermore, there is a critical need to start working with real and complex biological matrices in the field of proteins separation, as well as to apply spectroscopic and computational tools to better understand the main factors ruling selectivity and adsorption efficiency. On the other hand, these separation techniques based on SILs should be applied as well to separate high-value proteins, as is the case of biopharmaceuticals. Accordingly, it is expected that in the next few years, more investigations will be accomplished, and promising new results in this specific area will be disclosed.

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