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Fluorinated ionic liquids for protein drug delivery systems: Investigating their impact on the structure and function of lysozyme

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ABSTRACT

Since the approval of recombinant human insulin by FDA in 1982, more than 200 proteins are currently available for pharmaceutical use to treat a wide range of diseases. However, innovation is still required to develop effective approaches for drug delivery. Our aim is to investigate the potential use of fluorinated ionic liquids (FILs) as drug delivery systems (DDS) for therapeutic proteins. Some initial parameters need to be assessed before further studies can proceed. This work evaluates the impact of FILs on the stability, function, structure and aggregation state of lysozyme. Different techniques were used for this purpose, which included differential scanning fluorimetry (DSF), spectrophotometric assays, circular dichroism (CD), dynamic light scattering (DLS), and scanning and transmission electron microscopy (SEM/TEM). Ionic liquids composed of cholinium-, imidazolium- or pyridinium- derivatives were combined with different anions and analysed at different concentrations in aqueous solutions (below and above the critical aggregation concentration, CAC). The results herein presented show that the addition of ionic liquids had no significant effect on the stability and hydrolytic activity of lysozyme. Moreover, a distinct behaviour was observed in DLS experiments for non-surfactant and surfactant ionic liquids, with the latter encapsulating the protein at concentrations above the CAC. These results encourage us to further study ionic liquids as promising tools for DDS of protein drugs.
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1. Introduction

The pharmaceutical market is undergoing increasing economic pressure due to the reduction of healthcare costs, increased competition from generic medications and high rates of drug development failure in clinical trials, generally around 90% (Dimitrov, 2012). This situation, together with the demand of the society and authorities for safer and more efficient drugs, has contributed to the revived interest in protein drug candidates. Therapeutic proteins, which are natural biological products, have emerged in the early 1980s and promise to become a kind of panacea for all known diseases (Ibraheem et al., 2014). More than 200 natural and modified therapeutic proteins have been approved for clinical use in the European Union and the USA (Dimitrov, 2012). Therapeutic proteins are undergoing clinical trials for the treatment of cancers, Alzheimer's, immune disorders, infectious diseases, Parkinson's, autoimmune diseases and AIDS/HIV, among others (Brasnjevic et al., 2009; Leader et al., 2008; Tan et al., 2010). However, therapeutic proteins have their downsides: i) they are very sensitive to environmental conditions (e.g. they cannot be delivered orally due to the threat of degradation in the acidic environment of the gastrointestinal tract); ii) their three-dimensional structure, spatial occupation and hydrophilic/hydrophobic nature can hinder *in vivo* delivery; iii) they have poor bioavailability due to their short half-lives in the blood stream resulting from their degradation by enzymes such as proteases; iv) high doses are needed in order to obtain therapeutic efficacy, which creates the potential for side effects and toxicity; and v) they are most commonly administered by intravenous injections, which are not always well tolerated by the recipient (Dai et al., 2005). Drug delivery systems (DDS) can be the solution to these various obstacles because they can minimize adverse side effects, improve

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the action of a drug through faster onset, time-controlled or site-specific delivery, or reduce the drug dosage needed (Brasnjevic et al., 2009).

In recent years, ionic liquids (ILs) have attracted considerable attention because of their biological and pharmaceutical potential. Specifically, their ability to solubilize and stabilize proteins *in vitro* for extended times has been studied (Fujita et al., 2007; Ohno et al., 2003). Other previous investigations have emphasized the potential of ILs to dissolve poorly soluble active pharmaceutical ingredients as well as biochemical compounds, such as nucleic acid bases (Araújo et al., 2013, 2014). The first work using ILs as drug delivery devices was reported in 2010 (Moniruzzaman et al., 2010). Since then, these compounds have been studied as additives in several formulations as well as enhancers of drug solubility (Mahkam et al., 2014; Goindi et al., 2015; Wang et al., 2016; Tang et al., 2016).

Although the number of publications dealing with ILs has grown exponentially, there are still numerous quasi-unexplored topics, such as that of the fluorinated ionic liquids (FILs) family, herein defined as ILs with fluorous tags equal to or longer than four carbon atoms. The interest in these novel FILs is that they can combine the best properties of highly fluorinated materials (such as outstanding chemical and biological inertness, low surface tension and unique components and tools for the engineering of stable, self-assembled supramolecular and colloidal systems) with those of many ionic liquids in general (such as vanishing vapor pressure, tuneable solvent quality, high thermal stability, high electrical conductivity, non-flammability and greener potential). Inert fluorinated compounds have been widely used in relevant biomedical applications, e.g. as imaging agents (Rossi et al., 2010; Schutt et al., 2003; Stride and Edirisinghe, 2009), and as fluorocarbon gels, nanocompartmented supramolecular assemblies and colloids (Krafft, 2006; Krafft and Riess, 1994). Similarly, they have been employed in the control and stabilization of emulsions, microbubbles and other colloids (Bertilla et al., 2004; Garnett et al., 1999) as well as in the pulmonary delivery of drugs and genes (Courrier et al., 2004) and oxygen therapeutics (Riess, 2001, 2005, 2006) where these compounds are used as gas carriers in liquid ventilation and intravenous formulations.

Literature shows that DDS for therapeutic proteins have been developed using liposomes, microparticles, nanoparticles, fluorocarbons surfactants and microencapsulation (Dai et al., 2005; Krafft and Riess, 1998; Tan et al., 2010). Additionally, an area referred to as protein engineering has discussed and reviewed the general effects on the essential properties of peptides and proteins arising from the incorporation of fluorinated amino acids (Salwiczek et al., 2012). Nevertheless, more than a decade of research in protein DDS has still failed to produce efficient systems. Studies tackling FILs surfactants as drug delivery systems for therapeutic proteins are not yet available.

Novel surfactants based on FILs might increase the safety and effectiveness of therapeutic proteins, increasing the pre-intake protection until reaching the target receptor, while decreasing their immunogenicity. Furthermore, playing with the van der Waals, coulombic and hydrogen bonding interactions, the size of fluorinated domains (Pereiro et al., 2013a), surfactant behaviour, and the solubility in water and biological fluids (Pereiro et al., 2015) will provide the ingredients needed to use FILs in biological applications where fluorocarbon compounds in general present a handicap (overly low solubility in water and biological fluids).

The main purpose of this work is to take the first steps into the development of a novel and improved strategy for protein drug delivery using biocompatible FILs with reduced immunogenicity that might deliver protein drugs to the body at therapeutics levels. The FILs analysed in this study have been selected based on previous works. They are totally miscible in water with improved surfactant behaviour as compared to traditional surfactants (Pereiro et al., 2015) and present low cytotoxicity in two different human cell lines (Caco-2 and HepG2) (Pereiro et al., 2013b). In these reports, it is shown that only the perfluorooctanesulfonate anion presents some cytotoxicity in one of the human cell cultures at high FIL concentrations (Caco-2 cell with log EC50 = 3.65, 4467 μM; but HepG2 with log EC50 >4, >10000 μM) (Pereiro et al., 2013b). Even so, this value is negligible when compared with the corresponding acid, perfluorooctane sulfonic acid (log EC50 = 1.48, 30 μM in PC12 cells) (Oldham et al., 2012), which belongs to a new class of environmental contaminants, globally distributed and persistent, bio-accumulative and toxic to various species (Lindstrom et al., 2011). It is thus important to become aware of the expansion of production and use of these perfluorinated compounds in the forthcoming years due to their utility, economic value and industrial application (Lindstrom et al., 2011). While the perfluorobutanesulfonate anion, $[C_4F_9SO_3]^-$, shares some of the oleophobic and hydrophobic properties of perfluorooctanesulfonate, $[C_8F_{17}SO_3]^-$, it is not considered to be as bio-accumulative or toxic (Newsted et al., 2008). In fact, the perfluorobutanesulfonate acid has recently been commercialized as an alternative to the perfluorooctanesulfonate acid in a variety of applications (3M, Technical Data Bulletin, 2002). The FILs based on the perfluorobutanesulfonate anion studied herein might be used as neoteric alternative compounds, being more environmentally friendly and presenting tuneable physicochemical properties as compared to other fluorinated compounds. Furthermore, it has been shown that the critical aggregation concentration (CAC) values of fluorinated surfactants are analogous to those of hydrogenated surfactants with a chain length approximately 1.5 times longer (Pereiro et al., 2015; Szajdzinska-Pietek and Wolszczak, 2000; Wadekar et al., 2012). FILs with only four carbon atoms used in this work improve the surfactant power of conventional perfluorosurfactants with eight carbon atoms and the hydrogenated counterparts (see Fig. S1). On the other hand, octanol-water partition coefficients and cytotoxicity in other two human cell cultures (HaCaT and EA.hy926) are currently under study in our lab. Preliminary results show that the FILs studied in this work are non-toxic in these four different human cell culture types (Caco-2, HepG2, HaCaT and EA.hy926) and that their octanol-water partition coefficients are small when compared with traditional solvents. Taking these studies into account, the size of the side fluorinated alkyl chain must be smaller than eight carbon atoms in order to yield non-toxic FILs. Nonetheless, the physiological safety, the biodegradability, and several health studies, among others, will have to be carried out to fully evaluate the actual possible application of these compounds.

Lysozyme (or muramidase, EC 3.2.1.17) is a ubiquitous protein present in a wide range of biological fluids and tissues including bacteria, avian egg, plant and animal secretions. It is well known for its antibacterial properties by breaking the bonds between N-acetylglucosamine and N-acetylmuramic acid, which are peptidoglycan components in the cell wall of Gram-positive bacteria. Lysozyme also exhibits antiviral, antitumor and immune modulatory activities (Abeyrathne et al., 2013; Aminlari et al., 2014; Leśnierowski and Cegielska-Radziejewska, 2012; Tenovuo, 2002; Sava et al., 1989; Sava, 1996; Lee-Huang et al., 1999). Moreover, lysozyme is extensively characterized and often used as a model protein in a variety of studies, some of which involving traditional ionic liquids (Du et al., 2014; Kowacz et al., 2012, 2015; Weaver et al., 2012; Takekiyo et al., 2012; Vrikkis et al., 2009).

The main goal of this work is to investigate the effect of FILs on the stability, structure, and activity of lysozyme using various biophysical and biochemical techniques. Two traditional and non-surfactant ILs have also been included in this work to compare their behaviour with the novel FILs. Finally, the self-aggregation behaviour of this protein with surfactants based on FILs in aqueous

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