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Biomedical Applications Using Ionic Liquids

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Ao meu avô, que sempre me apoiou e
que tudo fez para que eu conseguisse chegar aqui

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Keywords

Ionic Liquids, Artificial blood substitutes, active pharmaceutical ingredients, Thermophysical characterization; Toxicity; Octanol-water partition coefficients

Abstract

The first ionic liquid (IL) was synthetized in 1914. Since then the interest in this field is in exponential growth. These compounds are present in diverse areas of science such as electrochemistry or biomedical research.

In this work fluorinated ionic liquids (FILs), a specific family of ionic liquids, where studied in order to evaluate their capacity to replace partial or totally perfluorocarbons (PFCs) in PFCs emulsions, actually used as artificial blood substitutes, also known as oxygen therapeutics. With this aim in mind, the thermophysical characterization was performed as also citotoxicity assays and octanol – water partition coefficients. This last parameter is used to understand the potential of these FILs to penetrate cell membranes.

In last years a new third generation of ionic liquids appeared which is directly linked to pharmaceutical research. A lot of studies were done in order to develop a new formulation that conjugates diverse active pharmaceutical ingredients (APIs) with ILs. This conjugation was made to avoid some disadvantages related to the parent API, particulary poor water solubility. The purpose of this work is to compare the thermical properties, toxicity and partition properties of this new formulation API-ILs with the parent API. These novels ILs are based on cholinium as a benign cation combined with three different APIs like anions.

Palavras - chave

Liquidos Iónicos, Substitutos artificiais do sangue, Príncipios Activos Farmacêuticos, Caracterização Termofísica, Toxicidade, Coeficientes de Partição Octanol-Água

Resumo

Em 1914 foi sintetizado o primeiro líquido iónico (LI),e desde então o interesse nesta área tem vindo a aumentar exponencialmente. São diversos os ramos de utilização destes compostos, desde a electroquímica até às aplicações biomédicas.

Uma família específica de liquidos iónicos, os líquidos iónicos fluorados (LIFs) são estudados neste trabalho de modo a que possam vir a substituir parcial ou totalmente os perfluorocarbonetos (PFCs) das emulsões de PFCs, actualmente utilizadas como subsitutos artificiais do sangue, e também designadas de terapêuticas de oxigénio. Com este objectivo efectuou-se a avaliação termofísica de diversos FILs, a toxicidade, assim como o estudo da sua partição no modelo octanol-água, que ajuda a perceber o potencial dos LIFs em permear as membranas celulares.

Ao londo dos anos o estudo dos LIs tem vindo a passar por diversas fases, sendo a última chamada de 3ª geração, a que estuda a sua aplicação directa na indústria farmacêutica, de modo a contornar determinados problemas, como a pouca solubilidade de muitos príncipios activos farmacêuticos (PAFs) De modo a contornar este e outros problemas, vários têm sido os estudos que formulam novos príncipios activos farmacêuticos em conjunto com liquidos iónicos. O objectivo deste trabalho visa comparar as propriedades térmicas, a toxicidade e a partição destes novos compostos com o príncipio activo farmacêutico original. Sendo estas formulações compostas por um catião de origem benigna, a colina, e por diversos PAFs, como aniões.

Contents

List of Abreviatures

IL	Ionic Liquid
FIL	Fluorinated ionic liquid
HIV	human immunodeficiency virus
PFCs	Perfluorochemicals
APIs	Active pharmaceutical ingredients
API-ILs	Ionic liquids based on active pharmaceutical ingredients
EYP	Egg yolk phospholips
FDA	United States Food and Drug Administration
WHO	World Health Organization
FPP	Finished pharmaceutical product
TGA	Thermogravimetric analyses
DSC	Differential scanning calorimeter
NMR	Nuclear magnetic resonance
RSD	Relative standard deviation
D	Demal (1 g equivalent of solute dissolved in $1dm^3$ solvent)
KCl	Potassium chloride
S.D	Standard deviations
VFT	Vogel-Fulcher-Tammann
EC50	Cytotoxic effective concentration
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
PES	Phenazine ethosulfate
Caco-2	Human colon carcinoma cell line
HepG2	Human hepatocellular carcinoma cells
HaCaT	Spontaneously immortalized human keratinocyte cell line
EA.hy926-	Human , umbilical vein cell line
FBS	Fetal bovine serum
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
K_{ow}	Octanol-water partition coefficient
HPLC	Ultraviolet visivel

UV - VIS	High performance liquid chromatography

List of Symbols

N_A	Avogadro's constant
f	Degrees of freedom
K	Kelvin
T	Temperature
T_{dec}	Decomposition temperature
T_{start}	Start temperature
T_{onset}	Onset temperature
T_m	Melting temperature
T_g	Glass transition
$T_{sol\text{-}sol}$	Solid-solid transition
η	Dinamic viscosity
ρ	Density
ε_0	Electric constant
Ø	Fluidity
α	Non randomness parameter
α_e	Electronic polarizability
M_w	Molecular weight
V_m	Molar volume
R_m	Molar refraction
f_m	Free volume

n_{D}	Refractive index
C_i	Extremely dilute concentration
C_i^o	Concentration of the test compound in octanol phase
C_i^w	Concentration of the test compound in water phase

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1. General Introduction

1.1 General Context

The unique chemical and physical properties of ionic liquids (ILs) allow a great variety of applications, in areas like chemistry, biotechnology and medical industry (Florindo et al. 2013). The focus of this work is the application of ILs in biomedical research, particularly in the development of a new generation of artificial blood substitutes based on fluorinated ionic liquids (FILs). Furthermore, the development of cholinium-based ILs with pharmaceutically active anion was also studied.

Human blood substitutes, also known as oxygen therapeutics had been in development since the 1990s. This interest emerges after human immunodeficiency virus (HIV) appears (Chang 2003).However, the fear of a contaminated blood donor is just one of the reasons to advance the studies in this area. Every day medical community had to replace a huge quantity of blood loss during surgery or following trauma. Furthermore, procedures like angioplasty and chemotherapy in cancer treatment require the presence of blood like adjuncts. A lot of time is wasted for the study of cross-match blood for donor−recipient compatibility, and for some critical situations it could be an enormous disadvantage. In order to avoid some side effects, the evaluation of minimal infectious risks or concerns about immunogenicity is essential. However, this evaluation increases the costs of blood transfusions (Riess 2001). Also ethical issues turn this scientific research very attractive, once that some religions refuse red blood cells transfusion (Lowe 1999; Stollings and Oyen 2006).

Actually, there are two approaches for research in artificial blood substitutes for. The first one is based on haemoglobin, including products of recombinant technology, and the second are emulsions of inert compounds, called perfluorochemicals (PFCs), often described simply as fluorocarbons(Lowe 1999). Emulsions based on PFCs are now the preferred artificial blood candidates.

With this project the thermophysical characterization, toxicity and partition coefficient of FILs were evaluated. This study was carried out in order to select the best FIL to partial or totally replace the PFCs in emulsion used nowadays for oxygen therapeutics. This substitution is linked to several advantages, such as enhancement of respiratory gas solubility, emulsion stability and lowering of vapour pressure.

As mentioned above, the unique characteristics of ILs make them very useful in different areas of interest. Pharmaceutical industry is one of them, where the design of

compounds with ILs and active pharmaceutical ingredients (APIs) allow the balance between the chemical desired effect, the physical properties (required for manufacturing and transport procedures), the chemical stability, the solubility and the bioavailability of the final product (Florindo et al. 2013). Some studies report the capacity of ILs to increase the solubility of poorly soluble APIs and the potential for their application in drug delivery platforms (Mizuuchi et al. 2008; Moniruzzaman, Kamiya, and Goto 2010). More recently some groups develop a new abroad where ILs are themselves the APIs (Ferraz et al. 2011; Hough et al. 2007)

The application of ILs in pharmaceutical industry could overcome some problems during drug development process, related to the poor water solubility and bioavailability of mostly APIs. Elimination of polymorphisms, a big problem in the pharmaceutical industry can also be achieved with this approach (Hough et al. 2007; Rogers and Seddon 2003).

The other important goal of this work is the development of novel API-ILs compounds, with the combination of cholinium cation, classified as Vitamin B4, with different APIs (nalidixic acid, niflumic acid and pyrazinoic acid). Cholinium is an essential nutrient, common in many functions of organism. Their biocompatibility makes them a very attractive cation for biomedical applications.

After measurements of thermal properties, cytotoxicity and partition properties, a complete analysis of the benefits of this novel API-ILs design comparatively to the classical API model were performed.

The results obtained of this work represent a big step in ionic liquid community, due to the great possibilities emerging in medical research using ionic liquids.

1.2 Artificial blood substitutes as oxygen therapeutics

Sometimes the designation of "blood substitute" is miss understood once that these compounds do not replace all of blood functions. Artificial blood substitutes are only responsible for transport oxygen to cells and carbon dioxide from cells to lungs during a limited period of time (Lowe 1999). Functions related to immunity, coagulation and metabolic regulation are not supply for these chemicals (Riess 2001).

The development of these artificial blood substitutes follow two different approaches take into account the transport mechanism. As was mentioned above, there

are oxygen carriers based on haemoglobin (including products of recombinant technology) and in this case, the oxygen is chemically linked to carrier. Otherwise, for PFCs-emulsions, the oxygen is physically dissolved in carrier (Riess 2001). Both haemoglobin and PFCs-emulsion for artificial blood substitutes are illustrated in *Figure 1.1*

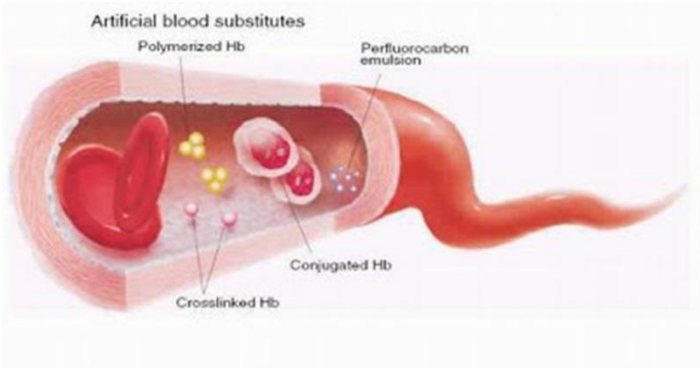


Figure 1.1– Representation of haemoglobin and PFCs emulsion blood substitutes (available in: http://muslimlifemalaysia.blogspot.pt)

Haemoglobin-based oxygen therapeutics had a structure identical to haemoglobin and could derive from human, animal or genetic modified microorganisms and plants (Lowe 1999; Stollings and Oyen 2006). This last class presents some advantages related to the absence of virus and residual red cells contaminants. Through recombinant technology a lot of alterations can be done in order to modulate the desire characteristics (Lowe 1999) and the final product could be available in form of solutions and liposomes (Riess 2001).However, there are a lot of clinical implications in the use of these substitutes, like hypertension, esophageal dysmotility and abdominal discomfort.

Perfluorinated compounds, fluorine-substituted hydrocarbons, are defined as inert organic materials that can dissolve a large volume of non-polar gases. The solubility of respiratory gases in PFCs tends to increase with the increment of fluorine atoms. In PFCs-emulsions, oxygen had solubility greater than in water or blood plasma, once that the dissolution process of oxygen in this systems is a simple passive process where gas molecules occupy the free cavities of PFCs-emulsions (Lowe 1999; Pereiro et al. 2013). Fluorocarbons fill the gaps in ischemic microcirculation, supplying oxygenation and reinstituting aerobic metabolism(Faithfull 1992). PFCs are intravascular administrated in the form of emulsions, due to their immiscibility in aqueous systems, and are coated

with a surfactant that serves to emulsify and stabilize the system. The most common surfactant used in this case is egg yolk phospholips (EYP) (Chang 2003). Recently, it had been discovered that some perfluorocarbons are excreted by lungs and skin in a few days, with no problems to organism(Clark 1973).

Between 1989 and 1990, the first oxygen carrier based on PFCs-emulsion had approval for commercialization by United States Food and Drug Administration (FDA). This first oxygen carrier, *Fluosol*, was used to treat percutaneous transluminal coronary artery ballon angioplasty(Lowe 1994, 1997). However, these first generations of oxygen carriers were not very useful for medical community once that the storage and efficacy of these products were redundant in comparison to the new developed technical in angioplasty. Also some side effects were reported and the product was removed from market (Lowe 1999).

With regard to develop PFCs-emulsion with highly biocompatibility, excretion properties, more stability (through the use of surfactants and perfluorinated stabilizers) and more oxygen carrying capacity, a second generation of PFCs was developed. In this second phase of studies, the physico-chemical properties of emulsions in general, and surfactants and stabilizers in particular, were better evaluated. One of the major problems in increasing the oxygen carrying potential was the high viscosity of the emulsions which is a handicap for their use in vasculature (Chang 2003). Some PFCs-emulsions from second generation, mainly constituted of perflurobron or perfluorodecalin (Oxigent and Oxyfluor, as example) shown to be much better than the first generation. These products offer a lot of advantages comparatively whit the first ones. There were less sensitivity to manufacturing process and storage and showed a viscosity closed to the blood).

Nowadays, a lot of these PFCs-emulsions are in clinical trials, and other biomedical applications for PFCs-emulsion are also in study such as anti-cancer agents, diagnostic imaging agents, ophthalmologic agents, among others (Faithfull 1992).

1.3 Active Pharmaceutical Ingredients

Accordingly to World Health Organization (WHO), active pharmaceutical ingredient (API) is a substance used in a finished pharmaceutical product (FPP) with chemical structure known, intended to furnish pharmacological activity or to otherwise have direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease,

or to have direct effect in restoring, correcting or modifying physiological functions in human beings (World Health Organization 2011). The pharmaceutical products could be administrated in form of tablets, capsules, emulsion, creams and other ones.

In the past years, the growing of pharmaceutical industry is dependent of several issues. There are more demanding regulatory requirements and more time is needed for research and development. With all of these barriers, the development costs of a new medical product are increasing(Paul et al. 2010).

Pharmaceutical industry identify that 40% of the major problems in drug development are connected to poor water solubility of APIs. This factor could shelve or invalidate the development of several new pharmaceutical candidates. Poor aqueous solubility of orally administrated drugs leads to low dissolution rate and low bioavailability. This last property refers to the extent and rate at APIs entry in the systemic circulation and thus access the site of action. Bioavailability depends of the compound solubility in water and their capacity to cross cellular membranes(Araújo et al. 2014a; Florindo et al. 2013). This property could be determined by the properties of the dosage form which depends partly on its design and manufacture. A large amount of compound is required for poor aqueous soluble APIs to reach the therapeutic range concentrations. The consequences of this dose escalation are the topical toxicity in the gastrointestinal tract after oral administration, as also the increasing of manufacturing costs(Araújo et al. 2014b).

Almost all pharmaceutical products are marketed in salts form. The salt formation is a very important procedure in drug development once that could improve some properties of the original compound like solubility, dissolution rate, hygroscopicity, stability, impurity profiles and particle characteristics. The most common cation present in this process is sodium ion, while the most usual anion is chloride (Kumar and Malhotra 2010)

Actually mostly administration of APIs is through solid, primarily crystalline forms. This formulation is the elected by reason of purity, thermal stability, manufacturability and ease of administration. However, there are some disadvantages related to these solid forms which show low solubility and low bioavailability, especially for crystalline solids, polymorphic conversion and tendency of amorphous forms to spontaneously crystallize. All of these structural alterations could affect the solubility, chemical and physical stability, dissolution rate and bioavailability of the

compound. These differences between crystal forms of the same compound create changes in biopharmaceutical properties, like bioavailability, leading to divergences in pharmaceutical efficacy (Hough et al. 2007). In order to avoid these disadvantages, some liquid formulations had been in development, like API-ILs and eutectic mixtures.

1.4 Ionic Liquids

The concept of Ionic Liquid is used to define salts entirely constituted of ions, an organic cation and an organic or inorganic anion (most commonly families of cations and anions are shown in *Figure 1.2 and Figure 1.3, respectively*) (Plechkova and Seddon 2008). These compounds have a melting temperature bellow 100ºC, due to their asymmetric structure (MacFarlane and Seddon 2007) that is a result of weak intermolecular interactions of ions (bonhote, hidrofobic and welton hydrogen bonding).

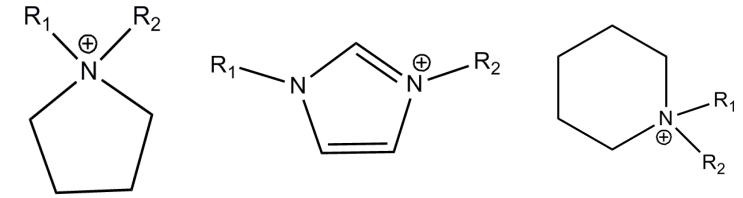


Figure 1.2 – Most common families of cation in ILs

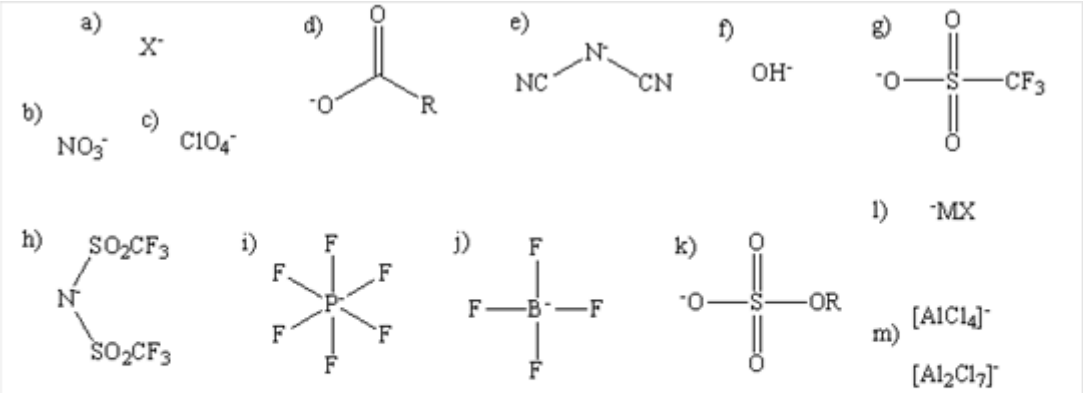


Figure 1.3 – Most common anion structures of ILs. a) halide (Cl^- F^-,Br^-,I^-; b) nitrate; c) chlorate; d) alkanoate; e) dicyanamide; f) hydroxide; g) trifluoromethanesulfonate; h) [bis(trifluoromethyl)sulfonyl]imide; i) hexafluorophosphate; j) tetrafluoroborate; k) alkyl sulfate; l) metal halide; m) cloroaluminates.

The first IL, ethylammonium nitrate, was synthetize by Paul Walden in 1914 in order to find a substitute to nitroglycerin. However, just in 1934 with an industrial application in preparation of cellulose solutions the first patent was registred by Charles Graenacher (Charles 1934)

During the past decades the interest in ionic liquids had increased a lot, with applications in diverse areas of study. In *Figure 1.4* are represented the number of publications of the last 20 years to clarify the "boom" of this area.

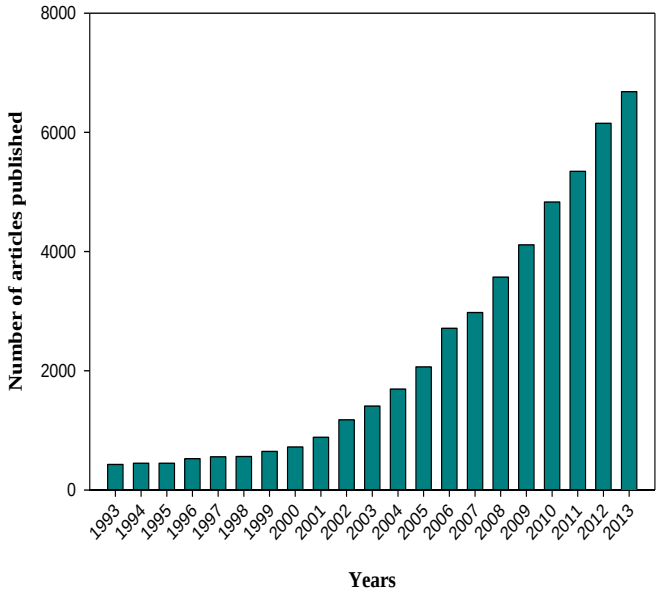


Figure 1.4 – Number of published article in the past 20 year. Values taken from IDI Web of Knowledge.

The first IL application was in electrolytes to batteries, and until now ILs are in the basis of importants electrochemical developments (Sato, Masuda, and Takagi 2004). ILs are included in reaction media for many organic transformations, separations and extractions, nanotechnology, biotechnology, and in engineering processes, among others (see *Figure 1.5*). Due to these diverse applications, ILs were used at an industrial scale by different companies (e. g. BASF, Petronas, Eastman Chemical Company, Institut Français du Pétrole, Degussa, SASOL) to reactions like hydrogenations, polymerizations, alkylation as an illustrative example the numerous possible applications (Olivier-bourbigou and Magna 2002; Plechkova and Seddon 2008).

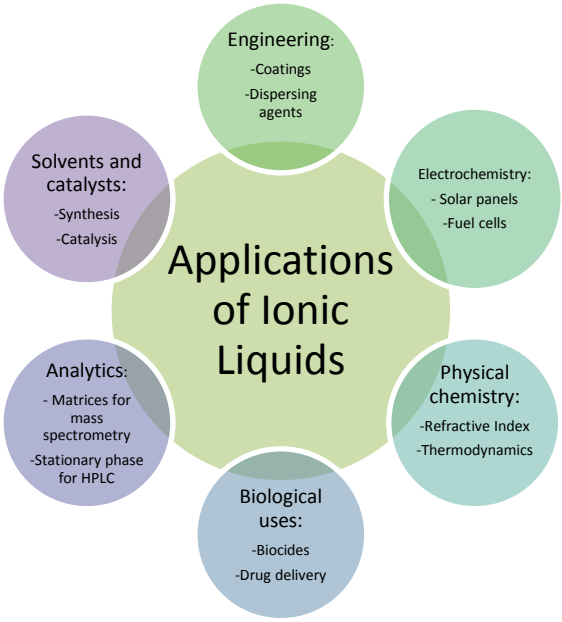


Figure 1.5 – Applications of ionic liquids (adapted fromPlechkova & Seddon, 2008)

A more ambitious research area for ILs application is the pharmaceutical industry. Nowadays several groups try to take advantage of the unique properties of ILs to improve or develop new biomedical strategies (Ferraz et al. 2011; Florindo et al. 2013; Stoimenovski et al. 2010). The Central Glass Company was the first company that produce pharmaceutical intermediates using ionic liquids, and also Eli Lilly adopt ILs for some industrial processes (Plechkova and Seddon 2008).

This large campus of applications (represented in *Figure 1.5*) is the consequence of ILs properties that make that compounds very useful. Besides, ILs have low melting point, a negligible vapour pressure at room temperature, low flammability, high thermal and chemical stability, high ionic conductivity, high solvation ability for organic, inorganic and organometallic compounds, improved selectivity and ease recycling (Earle et al. 2006; Rogers and Seddon 2003).

Another advantage of ILs is the possibility to manage the final characteristics of the compounds, through the diverse possible combination of cations and anions. This tuneability allows the manipulation of some characteristics like hydrophobicity, thermophysical properties, bioavailability and toxicity (Ranke et al. 2007; Rogers and Seddon 2003)

1.4.1 Fluorinated Ionic Liquids (FILs)

Despite the increasing amount of research in the ionic liquids field, there are still quite unexplored themes. That is the case of the fluorinated ionic liquids (FILs) family, here defined as ionic liquids with fluorine tags longer than four carbon atoms. These FILs are distinct from conventional fluorinated ionic liquids based on bis(trifluoromethylsulfonyl)imide, hexafluorophosphate or tetrafluoroborate anions which are ionic liquids studied extensively. It has been shown that fluorous phases present quite distinct behaviour from polar/non polar solvents (Tindale et al. 2007). The introduction of these unusual but potentially useful fluorous groups into the cation or anion pair can impart to ionic liquids attractive properties not observed in more conventional systems. The solvation in fully or partially fluorinated media is of full importance in areas where PFCs are used. This importance is related to the ability of fluorocarbon domains dissolving gases, to their low surface tension and outstanding chemical and biological inertness (Riess 2001). Fluorinated ionic liquids are of particular interest in relevant applications of perfluorocarbons such as imaging agents (Schutt et al. 2003; Stride and Edirisinghe 2009), fluorocarbon gels (Krafft and Riess 1994), pulmonary delivery of drug and genes (Courrier, Vandamme, and Krafft 2004) and oxygen therapeutics (Riess 2005), where these compounds are used as gas carriers in liquid ventilation and intravenous formulations.

The unique properties of fluorinated ionic liquids lead to high gas solubility and low forces required for expelling the gas molecules (regenerating the FIL) due to the repulsive tendency of fluorine atoms. The high solubility of gases, especially carbon dioxide in FILs has already been demonstrated (Almantariotis et al. 2010; Muldoon et al. 2007). A comparison between values for the CO_2 solubility in a fluorinated ionic liquid (Muldoon et al. 2007)and in perfluoro-n-octane (Dias, Daridon, and Pa 2006) at similar conditions indicates that the new FILs have greater solubilisation capacities for CO_2 than the traditional PFCs. On the other hand, FILs have also shown a good behaviour as surfactants, remarkably facilitating the formation and stabilization of dispersions of perfluorocarbons in a conventional IL (Merrigan et al. 2000). All the above mentioned properties of the neat FILs are essential to apply in the (FIL+PFC)-water emulsions as oxygen carriers where FILs could enhance the emulsion stability and increase the solubility of respiratory gas.

The FILs research with longer fluorinated chains are very scarce and are specially focused on synthesis and thermophysical characterization of compounds (Bara et al. 2009; Li et al. 2008; Linder and Sundermeyer 2009).

In this work, the thermodynamic and thermophysical properties, toxicity and bioavailability of FILs were executed in order to evaluate the substitution partial or totally the PFCs in PFCs-emulsions used nowadays as oxygen therapeutics. In literature, there is a scarce of information about toxicity of these compounds (Pereiro et al. 2013) and no information about permeability in cell membranes was found.

1.4.2 Ionic Liquids with Pharmaceutically active compounds

The tuneable characteristic of ILs mentioned above display an important role for their application in different areas such as chemistry, biotechnology and pharmaceutical industry. In general, ionic liquids were grouped in three different families or generations. The first generation of ILs was focused only on their unique intrinsic physical and chemical properties (density, viscosity, conductivity, solubility and thermal and chemical stability). In the second generation of ILs, cations and anions properties could be designed to develop new materials kept the main properties of ILs. Recently, a new generation with pharmaceuticals applications in mind start to grow. This third generation use active pharmaceutical ingredients to produce ILs biological actives. The safety character of ILs in comparision with some high volatile and flammable organic solvents is also an advantage for these developments. Also the utilization of ILs as delivery platforms is nowadays in study. Besides, some APIs were designed as themselves ILs (Araújo et al. 2014b; Ferraz et al. 2011; Florindo et al. 2013; Hough et al. 2007).With these approach physicochemical and biopharmaceutical characteristics of a drug could be managed in conjugation with different counter ions. The addition of ion pair could change the pharmacokinetics of a drug and reduce the undesired effects (*Figure 1.6*). Consequently, the final efficacy and security of the pharmaceutical product will be related to the salt structure (Araújo et al. 2014b; Ferraz et al. 2011).

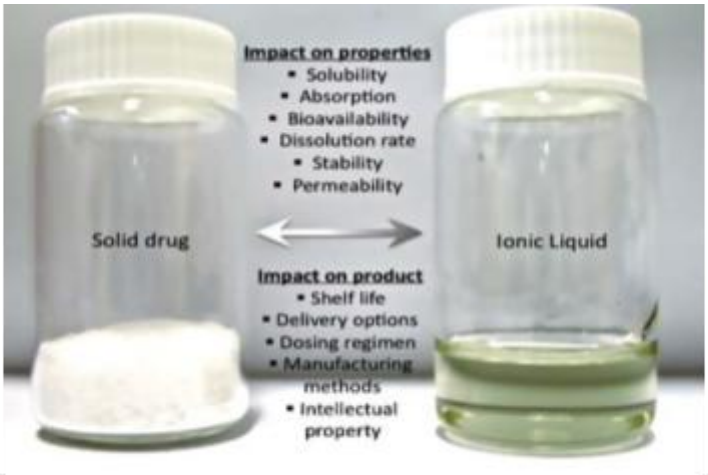


Figure 1.6. – Advantages of API-ILs formulation (Hough et al. 2007)

Finally, this new generation of ILs represents a new opportunity to pharmaceutical industry once that there are an urgent need for innovative and efficient new drugs and therapies. In this work, the development of new cholinium based APIs were made in order to improve the biopharmaceutical characteristics of nalidixic acid, niflumic acid and pyrazinoic acid. The chemical structure of these APIs is represented in *Figure 1.7*. Nalidixic acid is part of a class of anti-microbial drugs called quinolones and is administrated in the treatment of urinary tract infections. However, the solubility in water of this API is less than 0.1 mg/ml and its bioavailability is limited. Niflumic acid is a nonsteroidal anti-inflammatory drug, part of fenamates family and is widely used in rheumatic disorders. Nevertheless, the poorly solubility in water is also a disadvantage for pharmaceutical industry. Pyrazinoic acid is the active metabolite of the anti-tuberculosis drug pyrazinamide. The active form of this drug is not directly used due to poor absorption through the gastrointestinal tract (Araújo et al. 2014b; Takács-Novák et al. 2013).

The aim of this work is evaluate the contribution of formulations with active pharmaceutical ingredients and ionic liquids. Properties such as high solubility were previously demonstrated, then another studies, like thermophysical behavior, cytotoxicity and partition properties were done. This new drug design is of full importance to pharmaceutical industry once that could avoid some disadvantages related to the classic formulations, including the formation of different solid forms, the straight liquid range of application, low solubility and low bioavailability of the original APIs.

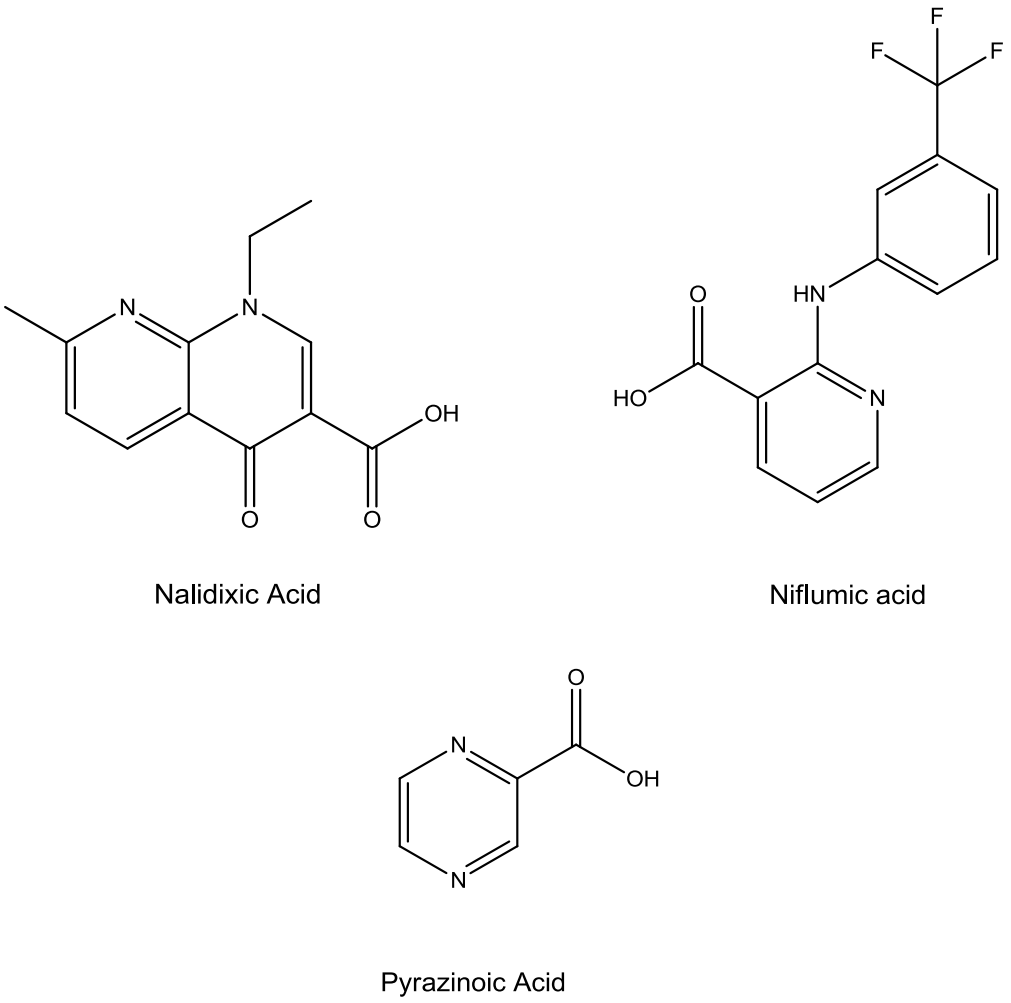


Figure 1.7 - Chemical structure of active pharmaceutical

2.Fluorinated Ionic Liquids as Artificial Blood Substitutes

2.1.**Thermophysical Characterization of Fluorinated Ionic Liquids**

2.1.1.Introduction

Thermophysical and transport properties of ILs like density, viscosity, ionic conductivity and refractive index, are crucial for their application in industry. This information is a vital for process scale up, since it decisively affects technological operations like mixing, pumping, and stirring, and plays a crucial role in other properties like diffusion (Deive, Rivas, and Rodríguez 2011). Some physical properties and thermal characterisation are reported for fluorinated ionic liquids, but measurements of these properties have been performed for specific temperatures only (Almantariotis et al. 2010; Bara et al. 2009; Li et al. 2008; Tindale, Mouland, and Ragogna 2010; Tsukada et al. 2006; Xue and Shreeve 2005). Therefore, there is a clear need for reliable systematic thermodynamic and thermophysical properties for these ionic liquids to point out their availability for use at the industrial processes level.

The physicochemical properties of ILs in general and thus these FILs in particular can be fine-tuned through an adequate combination of the cation and the anion leading to the optimal physicochemical properties for each application. Nevertheless, this process would require wide collection of accurate physicochemical properties data for different families of FILs and the understanding of the relationships between each property and the ionic liquid intermolecular forces. This work provides a critical analysis of the thermodynamic and thermophysical properties of novel, non-volatile, recyclable fluorinated ionic liquids to establishment of guidelines for selecting the most suitable FILs for development of new and improved generation of artificial blood substitute. This characterization involves analysis of decomposition temperature, melting point, density, dynamic viscosity, refractive index, and ionic conductivity at atmospheric pressure in a large temperature interval.

Melting and decomposition temperatures, are important properties of ionic liquids, especially for their application as alternative solvents, because they determine the liquid range of the fluids and thus their range of application. And, the upper operating temperature of an ionic liquid is usually determined by the temperature at which it decomposes. Viscosity and density are the most relevant properties for any fluid phase; viscosity is a measure of the resistance of a fluid which is being deformed by either shear stress or tensile stress. The less viscous the fluid is, the greater its ease of movement (fluidity). All fluorinated ionic liquids

have a viscosity higher than water (Li et al. 2008; Tsukada et al. 2006) or PFCs (Grunert 1994).

Therefore, the viscosity will increase with the addition of FILs. However, FILs with high viscosity should not be selected because might present problems in promoting the dispersion of fluorinated compound in water. On the other hand, PFCs used in artificial blood substitute have a density around 1.5 (Rizzo et al. 2012) and FILs with similar densities are good candidates to increase the fluorinated domains of the emulsion. Additional transport properties, such as ionic conductivity and its relation with viscosity, known as ionicity, are also important for the characterization of pure fluorinated ionic liquids.

Densities and related properties such as the molar free volumes can be related with the solubility of different species in mixtures containing ionic liquid, especially low molecular weight solutes that are gaseous at normal conditions (Tariq et al. 2009). Refractive index can also provide important insights on molar refractivity, which can be related to the solubility of low molecular weight solutes that are gaseous at normal conditions. These data will allow in the evaluation of these fluorinated ionic liquids to solubilise respiratory gases. The attained results will clearly impact applications using polyfluorinated compounds.

2.1.2.Materials

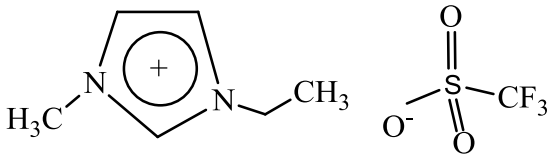
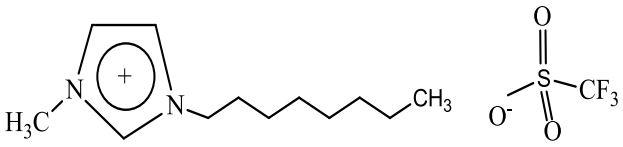
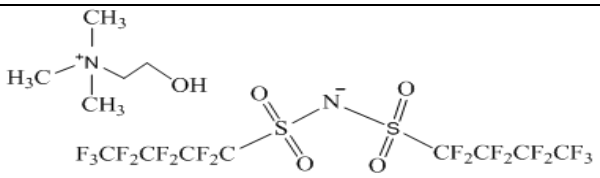
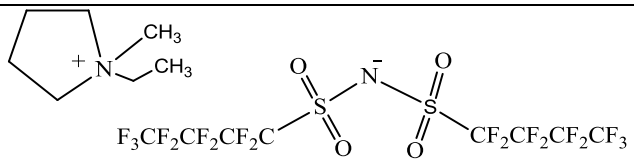
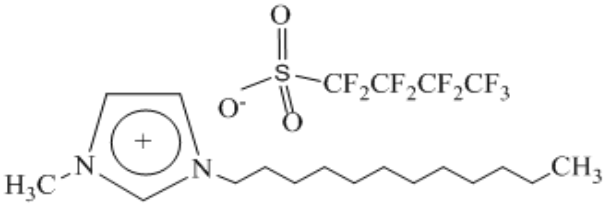
1-Ethyl-3-methylimidazolium trifluoromethanesulfonate, >99% mass fraction purity, 1-methyl -3-octylimidazolium trifluoromethanesulfonate, >99% mass fraction purity (2-hydroxyethyl)trimethylammonium bis(nonafluorobutylsulfonyl)imide, >97% mass fraction purity 1-ethyl–N–methylpyrrolidinium bis(nonafluorobutylsulfonyl)imide; 1-ethyl-3-methylimidazolium perfluorooctanesulfonate; 1-dodecyl-3-methylimidazolium perfluorobutanesulfonate, >98% mass fraction purity; (2-hydroxyethyl)trimethylammonium perfluorobutanesulfonate >97% mass fraction purity, were acquired at Iolitec. The purity of the commercial ionic liquids was checked by ^{1}H-NMR.

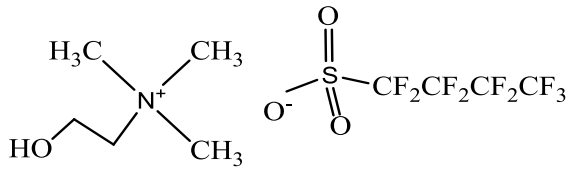
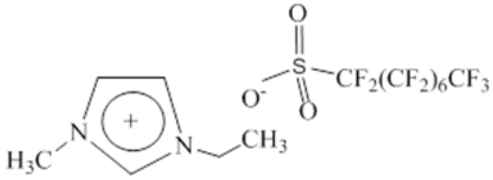
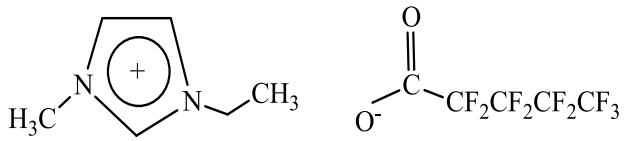
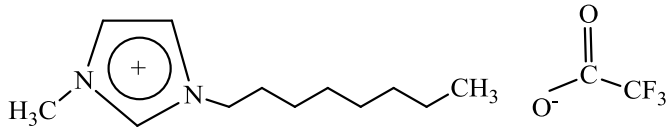
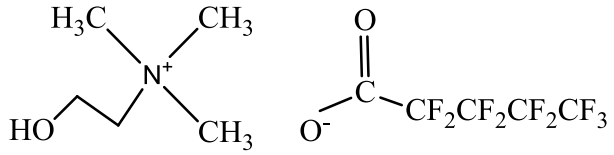
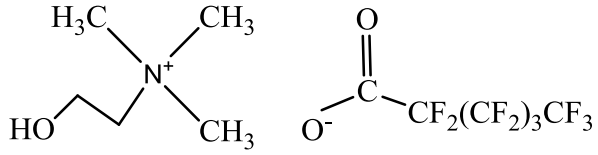
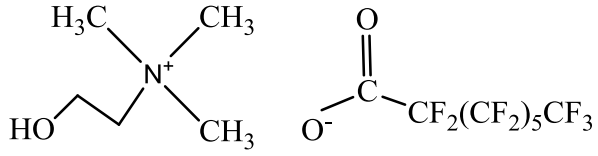
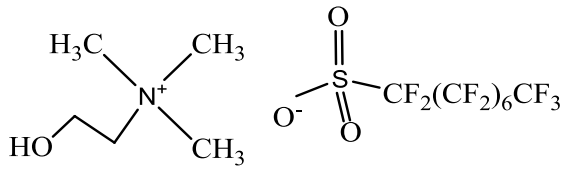
1-Ethyl-3-methylimidazolium perfluoropentanoate, 1-methyl-3-octylimidazolium trifluoroethanoate, (2-hydroxyethyl)trimethylammonium perfluoropentanoate, (2-hydroxyethyl)trimethylammonium perfluorohexanoate, (2-hydroxyethyl)trimethylammonium perfluorooctanoate and (2-hydroxyethyl)trimethylammonium perfluorooctanesulfonate were

synthesized in Molecular Thermodynamic Lab by ion exchange resin methods, as develop by Ohno *et al* (Fukumoto, Yoshizawa, and Ohno 2005).The purity of the final products was checked by ^{1}H, ^{13}C and ^{19}F NMR and elemental analysis.

Structures and acronyms of all fluorinated ionic liquids are shown in *Table 2.1*. All fluorinated ionic liquids were dried under vacuum (3.10^{-2} Torr) and vigorous stirring at 323.15K for at least 2 days, immediately prior to their use. This step is crucial in order to reduce volatile impurities, as well, as water, which can influence the ionic liquids properties. The water content, determined by Karl Fisher titration, was less than 100 ppm for all the studied ionic liquids. No further purification was carried out.

Table 2.1 – Chemical structure and respective abbreviation of fluorinated ionic liquids in study

FIL designation	Chemical Structure
1-Ethyl-3-methylimidazolium trifluoromethanesulfonate **[EtMeIm][(CF_3)SO_3]**	
1-Methyl -3-octylimidazolium trifluoromethanesulfonate **[OcMeIm][(CF_3)SO_3]**	
(2-Hydroxyethyl)trimethylammonium bis(nonafluorobutylsulfonyl)imide **[NM_3(EtOH)][NNf_2]**	
1-Ethyl–N–methylpyrrolidinium bis(nonafluorobutylsulfonyl)imide **[EMpyr][NNf_2]**	
1-Dodecyl-3-methylimidazolium perfluorobutanesulfonate **[DodMeIm][(PFBu)SO_3]**	

FIL designation	Chemical Structure
2-Hydroxyethyl)trimethylammonium perfluorobutanesulfonate **[NM_3(EtOH)][(PFBu)SO_3]**	
1-Ethyl-3-methylimidazolium perfluorooctanesulfonate **[EtMeIm][(PFOc)SO_3]**	
1-Ethyl-3methylimidazolium perfluoropentanoate **[EtMeIm][(PFBu)CO_2]**	
1-Methyl -3-octylimidazolium trifluoroethanoate **[OcMeIm][(CF_3)CO_2]**	
(2-Hydroxyethyl)trimethylammonium perfluoropentanoate **[NM_3(EtOH)][(PFBu)CO_2]**	
(2-Hydroxyethyl)trimethylammonium perfluorohexanoate **[NM_3(EtOH)][(PFPe)CO_2]**	
(2-Hydroxyethyl)trimethylammonium perfluorooctanoate **[NM_3(EtOH)][(PFHep)CO_2]**	
(2-Hydroxyethyl)trimethylammonium perfluorooctanesulfonate **[NM_3(EtOH)][(PFOc)SO_3]**	

2.1.3.Experimental Procedure

Each fluorinated ionic liquid was taken from the respective schlenk flask with a syringe under a nitrogen flow to prevent humidity and was immediately placed in the apparatus.

2.1.3.1.Thermal properties

Thermogravimetric analyses (TGA) were carried out with a TA instrument Model TGA Q50, shown in *Figure 2.1*, where the thermal stabilities and decomposition temperatures of the fluorinated ionic liquids were measured. Nitrogen was used for the TGA measurements at a flow rate of 60 $mL \cdot min^{-1}$. Samples were placed inside of aluminium pans and heated to 873 K at a rate of 1 $K \cdot min^{-1}$ until complete thermal degradation was achieved. Universal Analysis, version 4.4 software, was used to determine the onset (T_{onset}), the starting (T_{start}) and the decomposition (T_{dec}) temperatures corresponding to the temperature at which the baseline slope changed during heating, the weight loss was 1 % and the weight loss was 50 %, respectively. A Differential Scanning Calorimeter (DSC) TA Instrument Model DSC Q200, shown in *Figure 2.2*, was used to measure the thermal properties of the fluorinated ionic liquids. The sample was continuously purged with 50 $mL \cdot min^{-1}$ of dinitrogen. About 5 to 10 mg of fluorinated IL is crimped in an aluminum standard sample pan. Indium (melting point T = 429.76 K) was used as standard compound for the calibration of the DSC.

Figure 2.1 - Thermogravimetric analyses model TGA Q50 (TGA).

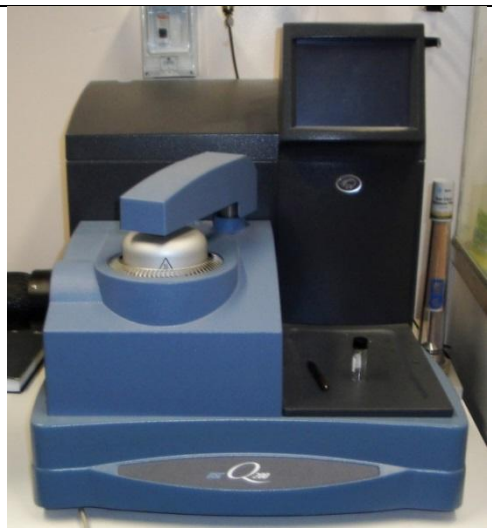


Figure 2.2 - Differential scanning calorimeter Model DSC Q200 (DSC)

2.1.3.2.Viscosity and density measurements

Measurements of viscosity and density were performed in the temperature range between 283.15 and 353.15 K at atmospheric pressure using an automated SVM 3000 Anton Paar rotational Stabinger viscometer-densimeter, *Figure 2.3*. The SVM 3000 uses Peltier elements for fast and efficient thermostability. The temperature uncertainty is ± 0.02 K. The uncertainty of the dynamic viscosity measurements is ± 0.5% and the absolute uncertainty of the density is ± 0.0005 $g \cdot cm^{-3}$. For each fluorinated ionic liquid triplicates were measured and the reported result is the average value with a maximum relative standard deviation (RSD) of 0.51%.

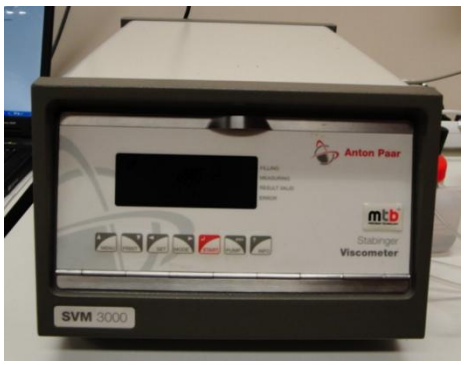


Figure 2.3 - SVM 3000 Anton Paar

2.1.3.3.Refractive index measurements

The refractive index of the pure ionic liquids were determined using the automatic refractometer ABBEMAT 500 Anton Paar, *Figure 2.4*, with a resolution of ± 10^{-6} and the uncertainty in the experimental measurements of ± $4 \cdot 10^{-5}$. The apparatus was calibrated by measuring the refractive index of Millipore quality water and tetrachloroethylene (provided by the supplier) before each series of measurements. For each fluorinated ionic liquid triplicates were measured.

Figure 2.4 - Refractometer ABBEMAT 500 Anton Paar.

2.1.3.4.Ionic conductivity measurements

A CDM210 Radiometer Analytical conductimeter, *Figure 2.5*, was used to measure the ionic conductivities in a jacketed glass cell containing a magnetic stirrer. A water bath controlled to ± 0.01 K was used to thermostatize the cell. Cell temperature was measured by means of a platinum resistance thermometer coupled to a Keithly 199 System DMM/Scanner. The thermometer was calibrated against high accuracy mercury thermometers (0.01 K). For the electrical conductivity measurements, 1.5 mL of the sample was added to the thermostatic cell and vigorously stirred. The cell was closed with screw caps to ensure a secure seal and flushed with dry nitrogen to prevent humidity. Each measurement was performed as quickly as possible to minimize undesired effects, such as self-heating of the samples or ionization in the electrodes (Hamelin, Bose, and Thoen 1990) that might modify the measured conductivity values. This conductimeter uses an alter current of 12 V and a frequency of 2.93 kHz (4.00

mS range) in the range of conductivities measured. The use of high frequency alter current (greater than 600 Hz) and the fact that the electrodes are platinized avoids polarization phenomena at the surface of the cell electrodes The conductimeter was previously calibrated at each temperature with certified 0.01 D and 0.1 D KCl standard solutions supplied by Radiometer Analytical. This technique was validated using the pure ionic liquids 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide and 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide. The obtained results were compared with the published data using the impedance method showing maximum relative deviations of 2%. Every conductivity value was determined at least twice and the uncertainty of the measurements is estimated to be 1% in absolute value.

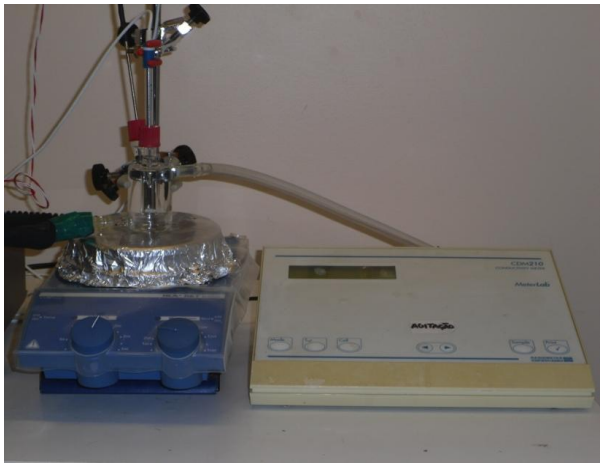


Figure 2.5 - CDM210 Radiometer analytical

2.1.4.Results and Discussion

2.1.4.1.Thermal properties

Melting and decomposition temperatures, are one of the most remarkable properties for ionic liquids, especially for their application as alternative solvents, because they determine the liquid range of the fluids and their range of application, which make them the most studied properties. The thermal properties (thermal stabilities, decomposition temperatures, melting points and glass transition temperatures) of the fluorinated ionic liquids studied in this work are summarized in *Table 2.2* and *Table 2*.3.The melting points and decomposition

temperatures for the fluorinated ionic liquids studied in this work as well as those others found in the literature (Cortes 2013; Pereiro et al. 2013)are illustrated in *Figure 2.6.*.

Table 2.2 - Thermal properties of fluorinated ionic liquids: start temperature, T_{start}, onset temperature, T_{onset}, decomposition temperature, T_{dec}.

FIL	T_{start}[a] / K	T_{onset}[a] / K	T_{dec}[a] / K
[EtMeIm][$(CF_3)SO_3$]	577.95	609.56	633.26
[OcMeIm][$(CF_3)SO_3$]	575.45	606.06	628.95
[NM_3(EtOH)][NNf_2]	576.60	621.71	633.27
[EMpyr][NNf_2]	566.80	619.23	637.59
[EtMeIm][$(PFOc)SO_3$]	567.44	616.21	642.93
[DodMeIm][$(PFBu)SO_3$]	567.55	616.75	636.38
[EtMeIm][$(PFBu)CO_2$]	374.85	391.58	408.58
[OcMeIm][$(CF_3)CO_2$]	415.99	423.26	438.74
[NM_3(EtOH)][$(PFBu)SO_3$]	576.92	608.58	631.32
[NM_3(EtOH)][$(PFBu)CO_2$]	405.66	414.15	420.53
[NM_3(EtOH)][$(PFPen)CO_2$]	405.30	412.30	420.72
[NM_3(EtOH)] [$(PFHep)CO_2$]	412.95	433.61	435.63
[NM_3(EtOH)] [$(PFOc)SO_3$]	512.56	627.38	650.41

a.Note that these values are from scanning TGA, and do not represent isothermal stability

Table 2.3.- Melting point, T_m, solid-solid transition temperature, $T_{sol-sol}$, and glass transition temperature, T_g for fluorinated ionic liquids.

FIL	T_m / K	$T_{sol-sol}$ / K	T_g / K
[EtMeIm] [$(CF_3)SO_3$]	260.41	246.52 255.92	
[OcMeIm] [$(CF_3)SO_3$]	284.14	264.24	
[NM_3(EtOH)][NNf_2]	309.16	287.44	229.08
[EMpyr][NNf_2]		261.41 273.08	
[EtMeIm][$(PFOc)SO_3$]		323.91 368.33	
[DodMeIm][$(PFBu)SO_3$]	310.66	310.66 297.47	
[NM_3(EtOH)][$(PFBu)SO_3$]		236.46 258.68 337.84 376.53	
[EtMeIm][$(PFBu)CO_2$]	277.63	236.74 249.88	
[OcMeIm][$(CF_3)CO_2$]	279.45	254.34	189.12
[NM_3(EtOH)][$(PFBu)CO_2$]		273.93 289.55	
[NM_3(EtOH)][$(PFPe)CO_2$]		314.23	
[NM_3(EtOH)][$(PFHep)CO_2$]		339.67	
[NM_3(EtOH)] [$(PFOc)SO_3$]		221.96 364.46	

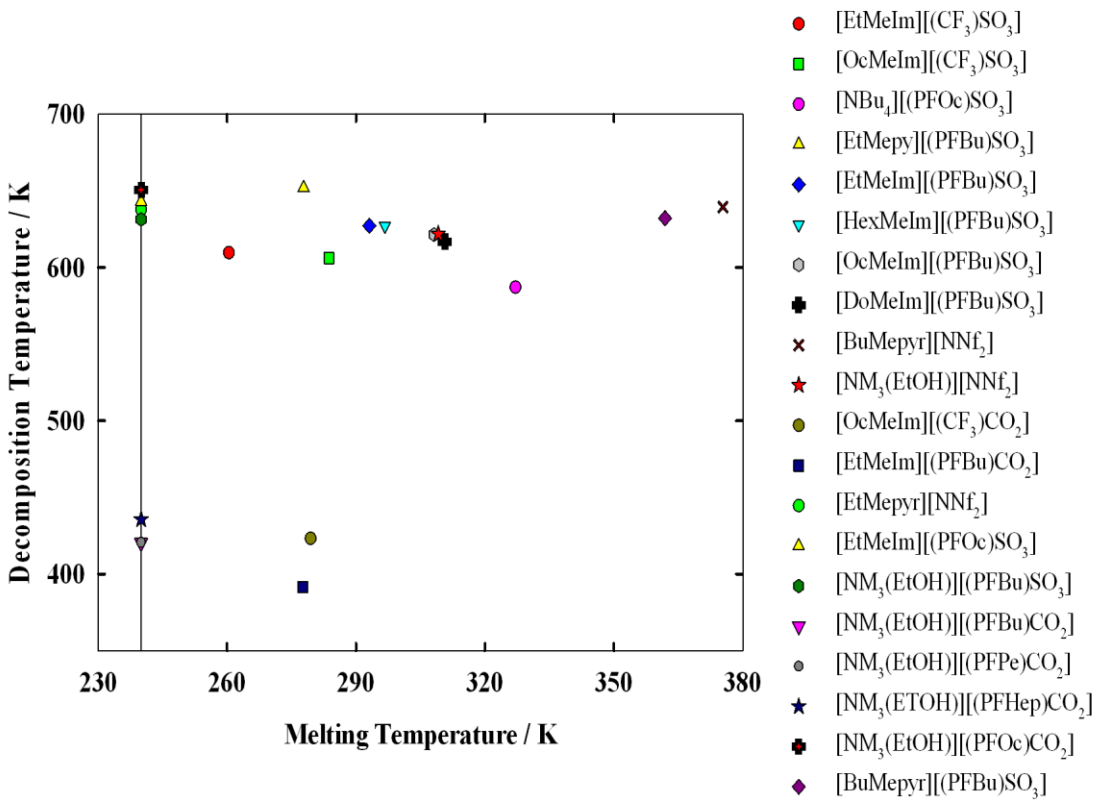


Figure 2.6 - Melting points and decomposition temperatures of the fluorinated ionic liquids in study and the available s in literature (Cortes 2013; Pereiro et al. 2013)

In order to explore the application of fluorinated ionic liquids for artificial gas carriers and blood substitutes the most relevant temperature is 310.15 K since is accepted as the average temperature of human body.

The analysis of experimental data studied in this work and those data available in literature allows us to draw some conclusions.

Almost fluorinated ionic liquids shows one or more solid-solid transition, reported in *Table 2.3*, that behaviour is connected with the presence of different crystalline structures which are formed during cooling ramps.

Once that [EMpyr][NNf_2], [EtMeIm][(PFOc)SO_3], [NM_3(EtOH)][(PFBu)SO_3], [NM_3(EtOH)][(PFBu)CO_2, [NM_3(EtOH)][(PFPe)CO_2], [NM_3(EtOH)][(PFHep)CO_2] and [NM_3(EtOH)] [(PFOc)SO_3] begin the process of decomposition before melting it is not possible to determine that last temperature.

In the presence of cholinium cation, $[NM_3(EtOH)]$, the decomposition temperature are lower for carboxylate than for sulfonate anion. However, an increasing of the fluorinated chain length in carboxylate anions is attached with an increase in decomposition temperature, *Figure 2.6*.

A comparison between two families of anions, sulfonate and carboxylate, was carried out. With this aim in mind, two different imidazolium cations, $[EtMeIm]^+$ and $[OcMeIm]^+$, were considered. In terms of melting temperature ionic liquids based on carboxylate anion have a lower melting point:

- [EtMeIm]+ - $[(PFBu)SO_3]^-$ > $[(PFBu)CO_2]^-$
- [OcMeIm]+ - $[(PFBu)SO_3]^-$ > $[(CF_3)CO_2]^-$

For decomposition temperatures, ionic liquids based on sulfonate anion are more stable because have higher decomposition temperatures.

- [EtMeIm]+ - $[(PFBu)SO_3]^-$ > $[(PFBu)CO_2]^-$
- [OcMeIm]+ - $[(PFBu)SO_3]^-$ > $[(CF_3)CO_2]^-$

The effect of increasing the hydrogenated alkyl chain length of the cation in thermal properties can be studied in imidazoliums and pyrrolidiniums ionic liquids, with the anions triflate, $[(CF_3)SO_3]^-$and perfluorobutanesulfonate, $[(PFBu)SO_3]^-$.

For $[(CF_3)SO_3]^-$ and $[(PFBu)SO_3]^-$, the melting temperature increases with the increment of hidrogenated alkyl chain:

- $[(CF_3)SO_3]^-$ - [EtMeIm]+ < [OcMeIm]+
- $[(PFBu)SO_3]^-$ - $[EtMeIm]^+$ < $[HexMeIm]^+$ < $[OcMeIm]^+$ < $[DoMeIm]^+$

The decomposition temperature of these compounds decreases with the increment of the hydrogenated alkyl chain.

- $[(CF_3)SO_3]^-$ - $[EtMeIm]^+$ > $[OcMeIm]^+$

$[(PFBu)SO_3]^-$ - $[EtMeIm]^+$ > $[HexMeIm]^+$ > $[OcMeIm]^+$ > $[DoMeIm]^+$

The effect of the increment on the fluorinated chain using cholinium, and imidazolium ionic liquids can be also evaluated. In the case of cholinium ionic liquids based on carboxilate anion, melting point cannot be measured in this range of temperatures because these compounds decompose before reaching

the melting point, like mencioned above. On the other hand, the decomposition temperature increases slightly with the increment of fluorinated chain:

- Cholinium - $[(PFBu)CO_2]^- > [(PFPe)CO_2]^- > [(PFHep)CO_2]^-$

For imidazolium ionic liquids based on sulfonate anion, the melting temperature increases in ionic liquids with higher number of fluor atoms in alkyl chain:

- Imidazolium - $[(CF_3)SO_3]^- < [(PFBu)SO_3]^-$

In the case of ionic liquids [EtMeIm][(PFOc)SO3], it was not possible determine the melting temperature because this FIL decomposes before reaching the melting point.

Taking into account the effect of cation in decomposition temperature for perfluorooctanesulfonate anion, $[(PFOc)SO3]^-$, decreases according to the following order:

- Cholinium > Imidazolium > Ammonium

From the exclusive analysis of thermal properties results and taking into account the data available in literature, the better ionic liquids for biomedical applications are $[EtMeIm][(CF_3)CO_2]$ and $[EtMepy][(PFBu)SO_3]$, with lower melting temperatures and more stability in a wide temperature range (high decomposition temperatures).Melting point of ionic liquids based on cholinium cation cannot be determined because they decompose before reaching the melting point. Furthermore, [BuMepyr][NNf2] and [NBu4][(PFBu)SO3] ionic liquids are not good candidates for biomedical applications, because they stay in solid state at temperatures higher than human body. To conclude, no specific overall rule can be extracted for melting and decomposition temperatures. However, if the fluorinated ionic liquids are analysed as separate families, some consistent effects are observed.

2.1.4.2.Thermophysical and thermodynamic properties

The experimental density, dynamic viscosity, refractive index and ionic conductivities of fluorinated ionic liquids as a function of temperature are listed in *Table 2.4.*

Table 2.4 – Density, ρ, dynamic viscosity, η, refractive index, n_D, and ionic conductivity, k, of the pure fluorinated ionic liquids as a function of temperature.

T / K	ρ /g·cm^{-3}	η /mPa·s	n_D	k/mS· cm^{-1}
		[EtMeIm][(CF$_3$)SO$_3$]		
293.15	1.3884	51.40	1.43451	0.767
298.15	1.3842	42.60	1.43294	0.902
303.15	1.3800	35.72	1.43151	1.07
308.15	1.3758	30.29	1.43012	1.23
313.15	1.3717	25.86	1.42870	1.42
318.15	1.3677	22.40	1.42730	1.61
323.15	1.3636	19.52	1.42593	1.82
328.15	1.3595	17.13	1.42459	
333.15	1.3552	15.10	1.42325	
338.15	1.3512	13.45	1.42132	
343.15	1.3471	12.04	1.42007	
348.15	1.3429	10.82	1.41878	
353.15	1.3386	9.75	1.41751	
		[OcMeIm][(CF$_3$)SO$_3$]		
293.15	1.1911	305.1	1.44460	0.436
298.15	1.1873	226.1	1.44319	0.576
303.15	1.1836	170.6	1.44178	0.743
308.15	1.1799	131.1	1.44037	0.937
313.15	1.1763	102.8	1.43890	1.18
318.15	1.1726	81.42	1.43735	1.46
323.15	1.1690	65.59	1.43582	1.78
328.15	1.1654	53.52	1.43425	
333.15	1.1618	44.38	1.43273	

T / K	*ρ* /g·cm^{-3}	*η* /mPa·s	*nD*	*k*/mS· cm^{-1}
		[OcMeIm][(CF$_3$)SO$_3$]		
338.15	1.1583	36.94	1.43128	
343.15	1.1548	31.16	1.42981	
348.15	1.1512	26.53	1.42853	
353.15	1.1476	22.90	1.42714	
		[NM$_3$(EtOH)][NNf$_2$]		
303.15	1908	1908		
308.15	1324	1324	1.37365	0.096
310.15				0.109
313.15	937.4	937.4	1.37234	0.126
315.15				0.151
318.15	677.7	677.7	1.37103	0.171
320.15				0.196
323.15	500.5	500.5	1.36975	0.226
328.15	1.6559	375.7	1.36844	
333.15	1.6501	287.1	1.36714	
338.15	1.6444	222.3	1.36590	
343.15	1.6388	174.7	1.36463	
348.15	1.6311	139.1	1.36340	
353.15	1.6272	112.0	1.36211	
		[DodMeIm][(PFBu)S		
308.15	1.2495	386.1		0.155
310.15				0.176
313.15	1.2450	277.9	1.41616	0.206
315.15				0.239
318.15	1.2406	205.1	1.41467	0.265
320.15				0.292
323.15	1.2364	155.0	1.41317	0.336
328.15	1.2322	155.0	1.41317	
333.15	1.2279	119.6	1.41171	
338.15	1.2237	94.10	1.41026	
343.15	1.2195	74.78	1.40880	
348.15	1.2153	60.48	1.40734	
353.15	1.2110	49.54	1.40590	

T / K	*ρ /g•cm^{-3}*	*η /mPa•s*	*nD*	*k/mS•cm^{-1}*
		[EtMeIm][(PFBu)CO_2]		
283.15	1.4783	262.8	1.40161	0.825
288.15	1.4976	190.1	1.40019	1.11
293.15	1.4921	141.3	1.39877	1.46
298.15	1.4868	107.5	1.39736	1.87
303.15	1.4815	83.32	1.39595	2.35
308.15	1.4762	65.87	1.39454	2.88
313.15	1.4710	52.80	1.39315	3.50
318.15	1.4659	43.09	1.39173	4.19
323.15	1.4607	35.63	1.39036	4.96
328.15	1.4556	29.80	1.38899	
333.15	1.4505	25.27	1.38760	
338.15	1.4454	21.51	1.38626	
343.15	1.4403	18.51	1.38493	
283.15	1.4783	262.8	1.40161	
		[OtMeIm][(CF_3)CO_2]		
283.15	1.1260	421.2	1.45306	0.272
288.15	1.1223	297.2	1.45156	0.385
293.15	1.1186	214.3	1.45010	0.525
298.15	1.1149	159.6	1.44863	0.696
303.15	1.1113	120.9	1.44716	0.901
308.15	1.1076	93.43	1.44572	1.14
313.15	1.1040	73.72	1.44425	1.43
318.15	1.1005	58.71	1.44279	1.76
323.15	1.0969	47.60	1.44132	2.14
283.15	1.1260	421.2	1.45306	0.272
328.15	1.0934	39.11	1.43993	
333.15	1.0899	32.64	1.43850	
338.15	1.0864	27.37	1.43709	
343.15	1.0829	23.24	1.43573	
348.15	1.0794	19.91	1.43432	
353.15	1.0760	17.29	1.43295	

The densities, fluidities (1 / dynamic viscosity) and ionic conductivities of the fluorinated ionic liquids measured in this work, as some of available in literature(Cortes 2013; Pereiro et al. 2013)are presented in *Figure 2.7*, *Figure 2.8* and *Figure 2.9*, respectively

Density and fluidity (inverse of dynamic viscosity) are relevant characteristics when it

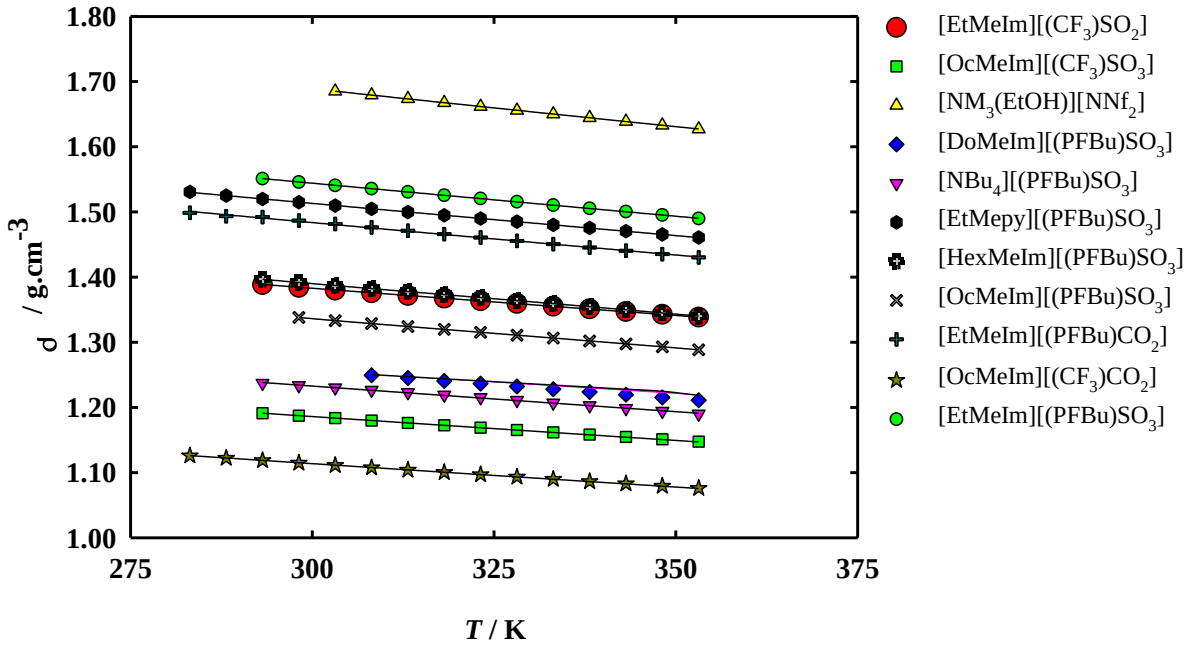


Figure 2.7 - Density and fitted curves as a function of temperature for the fluorinated ionic liquids.

comes to biomedical applications, such as blood substitutes, because this properties are crucial for the transport and delivery of respiratory gases (oxygen and carbon dioxide) (Riess and Krafft 1998). The density of blood (1.05 g·cm^{-3})(Riess 2001)(Owens et al. 2006) and of PFCs (1.5 g·cm^{-3}) are considered as reference values for the analysis of this physical property. The best candidates will be the ionic liquids with a density value closer to the density of PFCs used nowadays in oxygen therapeutic emulsions (1.5g g·cm^{-3})(Fraker, Mendez, and Stabler 2011) because this work explores the possibility of replacement (either partially or totally) of PFCs with

selected fluorinated ionic liquids in oxygen therapeutic emulsions. It is thought that FILs are more able to solubilize respiratory gases than PFC emulsions (increasing fluorinated domain in these emulsions) and they can enhance the stability of PFCs because they can act as surfactants.

Firstly, a comparison between the ionic liquids with carboxilate and sulfonate anions shows that density is lower for ionic liquids based on carboxylate anion:

- $[EtMeIm]^+$ - $[(PFBu)SO_3]^-$ > $[(PFBu)CO_2]^-$
- $[OcMeIm]^+$ - $[(CF_3)SO_3]^-$ > $[(CF_3)CO_2]^-$

In this study, the effect of increasing the hydrogenated alkyl chain length in the imidazolium cation can be evaluated using two different anions, $[(CF_3)SO_3]^-$ and $[(PFBu)SO_3]^-$. This comparison shows that in the case of $[(CF_3)SO_3]^-$ anion, the density decreases with the increasing of hydrogenated alkyl chain and the same behaviour can be observed with the $[(PFBu)SO_3]^-$ anion:

- $[(CF_3)SO_3]^-$ - $[(EtMeIm)]^+$ < $[(OcMeIm)]^+$
- $[(PFBu)SO_3]^-$ - $[(EtMeIm)]^+$ < $[(HexMeIm)]^+$ < $[(OcMeIm)]^+$ < $[(DoMeIm)]^+$

Finally, the increment of fluorinated alkyl chain can be examined for the same cation, imidazolium where the increment of the number of fluoro atoms in the alkyl chain increases the density of these ionic liquids.

- $[(EtMeIm)]+ – [(CF_3)SO_3]^-$ < $[(PFBu)SO_3]^-$

In conclusion, for the application studied in this work, ionic liquids with the density closer to PFCs are $[EtMeIm][(PFBu)SO_3]$, $[EtMeIm][(PFBu)CO_2]$ and $[EtMepy][(PFBu)SO_3]$.

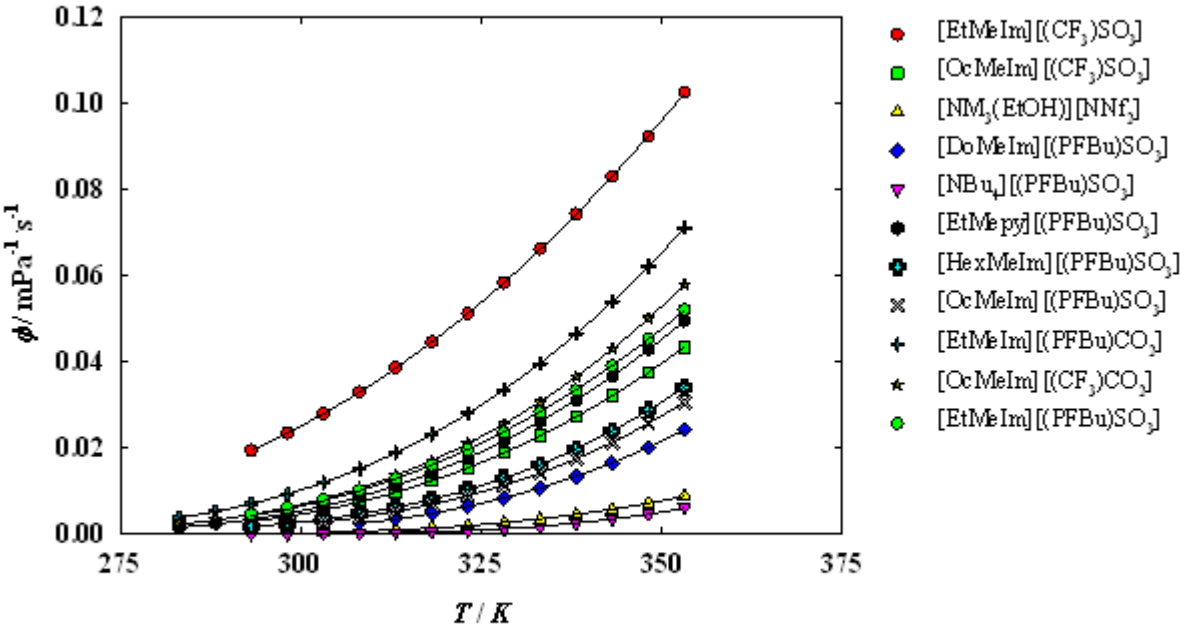


Figure 2.8 - Fluidity (1/viscosity) and fitted curves as a function of temperature for the fluorinated ionic liquids.

The fluidity of a pure ionic liquid is also an important characteristic for biomedical application and this property is very sensitive to temperature The effect of the increment of the hydrogenated alkyl chain length in FILs based on imidazolium cations can be evaluated with $[(CF_3)\ SO_3]^-$ and $[(PFBu)SO_3]^-$ anions. Fluidity of these ionic liquids decreases with the increment of hydrogenated alkyl chain length:

- $[(CF_3)\ SO_3]^-$ - $[(EtMeIm)]^+$ >$[(OcMeIm)]^+$
- $[(PFBu)SO_3]^-$ - $[(EtMeIm)]^+$ > $[(HexMeIm)]^+$ > $[(OcMeIm)]^+$ > $[(DoMeIm)]^+$

The increment of fluorinated alkyl chain can be also evaluate taking into account ionic liquids based on imidazolium cation where the fluidity decreases with the increment of the fluorinated alkyl chain length:

- $[(EtMeIm)]^+$ - $[(CF_3)SO_3]^-$ >$[(PFBu)SO_3]^-$

Moreover, a comparison between the ionic liquids based on carboxilate and sulfonate anions shows that carboxilate anion improve the fluidity of these FILs:

- $[EtMeIm]^+$ - $[(PFBu)SO_3]^-$ < $[(PFBu)CO_2]^-$
- $[OcMeIm]^+$ - $[(CF_3)SO_3]^-$ < $[(CF_3)CO_2]^-$

The best ionic liquids for the application studied in this work are the less viscous such as FILs based on $[(CF_3)CO_2]^-$, [EtMeIm][(PFBu)SO_3] and [EtMeIm][(PFBu)CO_2].

The temperature dependence of the density and the refractive index was studied by applying the following expression:

$$\ln\rho = A_0 + A_1 T \quad (2.1)$$

$$n_D = A_0 + A_1 T \quad (2.2)$$

where T is the absolute temperature and A_0, and A_1 are adjustable parameters. The correlation parameters are given in Table 2.5 together with the standard deviations (S.D.). These deviations were calculated by applying the following expression:

$$\text{S.D.} = \left[\frac{\sum_{i}^{n_{DAT}} \left(z_{exp} - z_{adjust} \right)^2}{n_{DAT}} \right]^{\frac{1}{2}} \quad (2.3)$$

where property values and the number of experimental and adjustable data are represented by z and n_{DAT}, respectively.

Arrenius fitting for fluidity, Ø, and of the ionic conductivity, k, was well carried out using Vogel-Fulcher-Tammann (VFT) equation:

$$P = P_0 \exp\left(\frac{-B}{T - T_0} \right) \quad (2.4)$$

where P designates the thermophysical property fluidity, Ø, or ionic conductivity, k, and P_0, which corresponds to Ø or k_0. B and T_0 are constants. The fitting parameters of the fluidity and ionic conductivity are also summarized in *Table 2.5*. This equation is used to show temperature dependence of ionic conductivity and viscosity.

Table 2.5 - Fitting parameters for the density (equation 2.1), refractive index (equation 2.2), fluidity (equation 2.4) and ionic conductivity (equation 2.4) as a function of temperature for the studied fluorinated ionic liquids. Standard deviations (S.D.) (equation 2.3) are also shown.

[EtMeIm][(CF$_3$)SO$_3$]				
ln ρ /g·cm^{-3}	A_0 = 0.5079	A_1 = - 6.125 · 10^{-4}		S.D. = 1.5 · 10^{-4}
n_D	A_0 = 1.5128	A_1 = - 2.680 · 10^{-4}		S.D. = 9.0 · 10^{-4}
Ø / mPa^{-1}·s^{-1}	ϕ_0 = 6.52	B = 872.90	T_0 = 143.01	S.D. = 8.2 · 10^{-5}
k/ mS·cm^{-1}	k_0 = 1289.68	B = 1700.67	T_0 = 63.72	S.D. = 9.7 · 10^{-3}
[OtMeIm][(CF$_3$)SO$_3$]				
ln ρ /g·cm^{-3}	A_0 = 0.3590	A_1 = - 6.280 · 10^{-4}		S.D. = 2.3 · 10^{-4}
n_D	A_0 = 1.5262	A_1 = - 2.787 · 10^{-4}		S.D. = 9.4 · 10^{-4}
Ø / mPa^{-1}·s^{-1}	ϕ_0 = 3.70	B = 705.42	T_0 = 194.51	S.D. = 2.2 · 10^{-4}
k/ mS·cm^{-1}	k_0 = 1494.13	B = 1170.71	T_0 = 149.30	S.D. = 2.3 · 10^{-3}
[NM$_3$(EtOH)][NNf$_2$]				
ln ρ /g·cm^{-3}	A_0 = 0.7339	A_1 = -6.994 · 10^{-4}		S.D. = 5.7 · 10^{-5}
n_D	A_0 = 1.5223	A_1 = -2.656 · 10^{-4}		S.D. = 1.0 · 10^{-3}
Ø / mPa^{-1}·s^{-1}	ϕ_0 = 1.65	B = 707.68	T_0 = 217.90	S.D. = 8.0 · 10^{-5}
k/ mS·cm^{-1}	k_0 = 1861.53	B = 1547.55	T_0 = 151.42	S.D. = 2.8 · 10^{-3}
[DoMeIm][(PFBu)SO$_3$]				
ln ρ /g·cm^{-3}	A_0 = 0.3827	A_1 = -5.169 · 10^{-4}		S.D. = 8.0 · 10^{-3}
n_D	A_0 = 1.4539	A_1 = -2.584 · 10^{-4}		S.D. = 1.0 · 10^{-3}
Ø / mPa^{-1}·s^{-1}	ϕ_0 = 3.45	B = 707.00	T_0 = 210.66	S.D. = 9.1 · 10^{-5}
k/ mS·cm^{-1}	k_0 = 1914.49	B = 1617.66	T_0 = 136.00	S.D. = 4.0 · 10^{-3}

[EtMeIm][(PFBu)CO_2]				
ln ρ /g·cm^{-3}	A_0 = 0.5990	A_1 = - 6.819 · 10^{-4}		S.D. = 7.5 · 10^{-4}
n_D	A_0 = 0.3942	A_1 = - 2. 000 · 10^{-4}		S.D. = 5.1 · 10^{-5}
Ø / mPa^{-1}·s^{-1}	ϕ_0 = 8.33	B = 875.63	T_0 = 169.36	S.D. = 5.6 · 10^{-5}
k/ mS·cm^{-1}	k_0 = 1289.68	B = 1700.67	T_0 = 63.72	S.D. = 1.7 · 10^{-2}
[OcMeIm][(CF_3)CO_2]				
ln ρ /g·cm^{-3}	A_0 = 0.3045	A_1 = - 6.558. 10^{-4}		S.D. = 1.9 · 10^{-4}
n_D	A_0 = 0.4320	A_1 = - 2. 057 · 10^{-}		S.D. = 1.5 · 10^{-4}
Ø / mPa^{-1}·s^{-1}	ϕ_0 = 8.19	B = 876.26	T_0 = 176.21	S.D. = 8.4 · 10^{-5}
k/ mS·cm^{-1}	k_0 = 1511.97	B = 1113.32	T_0 = 153.72	S.D. = 6.2 · 10^{-3}

2.1.4.3.Free Volume

The refractive index can be used as a measure of the electric polarizability of a molecule and can provide useful information when studying the forces between molecules or their behaviour in solution(Tariq et al. 2009). Polarizability is the measure of the change in a molecule's electron distribution in response to an applied electric field, which can also be induced by electric interactions with solvents or ionic solvents. Polarizabilities determine the dynamical response of a bound system to external fields, and provide insight into a molecule's internal structure (Zhou 2003). The Lorenz-Lorentz equation relates the electronic polarizability, α_e, with the refractive index, n_D, and can also be expressed in terms of the molar refraction, or molar polarizability (Moldover 2007), R_m, using the expression:

$$R_m = \frac{N_A \alpha_e}{3\varepsilon_0} = \frac{n_D^2 - 1}{n_D^2 + 2} V_m \quad (2.5)$$

where the N_A is the Avogadro's constant and V_m the molar volume.

The relation between the polarizability and the refractive index shown above can provide important information about the behaviour of a liquid as a solvent media and constitutes a measure of the importance of the dispersion forces to the cohesion of the liquid. Therefore, solvents with a large index of refraction, and consequently large polarizability, should be capable of enjoying particularly strong dispersion forces(Reichardt and Welton 2011), being also good solvents for species possessing high polarizabilities. Molar refractions can be considered as a measure of the hard-core molecular volumes because the electronic polarizability can be related to a spherical molecular radius(Tariq et al. 2009), a, as follows:

$$\alpha_{\mathrm{e}} = 4\,\pi\,\varepsilon_{0} a^{3} \tag{2.6}$$

and the equation 4 can be expressed in the following form:

$$n_{\mathrm{D}}^{2} - 1 = 3\left(\frac{V_{\mathrm{m}}}{R_{\mathrm{m}} - 1}\right)^{-1} = 3\left(\frac{R_{\mathrm{m}}}{f_{\mathrm{m}}}\right) \tag{2.7}$$

where f_{m} is the free volume defined as:

$$f_{\mathrm{m}} = (V_{\mathrm{m}} - R_{\mathrm{m}}) \tag{2.8}$$

which means that $(n_{\mathrm{D}}^{2} - 1)$ is directly proportional to the occupied part of the molar volume, R_{m} being then considered as the hard-core molecular volume (Deetlefs, Seddon, and Shara 2006).

The molar free volumes can be related to the solubility of low molecular weight solutes that are gaseous at normal conditions (Tariq et al. 2009) The values for the calculated molar refractions (from equation 2.5) and free volumes (from equation 2.8), from Lorentz-Lorenz equation, of all the studied fluorinated ionic liquids were calculated and are listed in *Table 2.6* together with the values for molar volume, that is illustrated in *Figure 2.9* , these results were also comparable with those available in literature (Cortes 2013; Pereiro et al. 2013).

Table 2.6 - Values of calculated molar volume, V_m, molar refraction, R_m, and free volume, f_m, as a function of temperature for the studied fluorinated ionic liquids.

T/K	V_m/cm^3·mol^{-1}	R_m/cm^3·mol^{-1}	f_m/cm^3·mol^{-1}
	[EtMeIm][$(CF_3)SO_3$]		
293.15	187.42	48.86	138.57
298.15	187.99	48.85	139.14
303.15	188.56	48.86	139.70
308.15	189.14	48.87	140.27
313.15	189.70	48.88	140.83
318.15	190.26	48.88	141.38
323.15	190.83	48.89	141.95
328.15	191.41	48.90	142.50
333.15	192.01	48.93	143.09
338.15	192.58	48.87	143.72
343.15	193.17	48.89	144.28
348.15	193.77	48.91	144.86
353.15	194.40	48.94	145.46
	[OctMeIm][$(CF_3)SO_3$]		
293.15	289.13	76.89	212.24
298.15	290.05	76.93	213.13
303.15	290.96	76.96	214.00
308.15	291.87	76.98	214.89
313.15	292.76	76.99	215.77
318.15	293.69	77.00	216.68
323.15	294.59	77.00	217.60
328.15	295.50	77.00	218.50
333.15	296.42	76.99	219.43
338.15	297.31	77.01	220.31
343.15	298.22	77.01	221.21
348.15	299.15	77.04	222.11
353.15	300.09	77.06	223.02
	[NM_3(EtOH)][NNf_2]		
308.15	477.71	109.02	368.69
313.15	478.20	108.76	
318.15	478.65	108.53	370.12
323.15	479.07	108.31	370.76
328.15	479.52	108.07	371.46

T/K	$V_m/cm^3 \cdot mol^{-1}$	$R\_m/cm^3 \cdot mol^{-1}$	$f_m/cm^3 \cdot mol^{-1}$
	$[NM_3(EtOH)][NNf_2]$		
333.15	480.02	107.81	372.20
338.15	480.44	107.59	372.85
343.15	480.90	107.35	373.55
348.15	481.32	107.13	374.19
353.15	481.78	106.88	374.89
	$[DoMeIm][(PFBu)SO_3]$		
303.15	440.56	116.20	324.36
308.15	442.16	116.28	
313.15	443.72	116.34	327.38
318.15	445.23	116.37	328.87
323.15	446.75	116.41	
328.15	448.31	116.44	331.87
333.15	449.85	116.51	
338.15	451.40	116.56	334.84
343.15	452.96	116.65	336.31
348.15	454.57	116.73	337.83
353.15	440.56	116.20	324.36
	$[EtMeIm][(PFBu)CO_2]$		
283.15	249.65	60.74	188.91
288.15			
293.15	250.76	60.62	190.13
298.15	251.66	60.65	191.01
303.15	252.66	60.68	
308.15	253.46	60.70	
313.15	254.35	60.73	193.63
318.15	255.25	60.75	194.51
323.15	256.15	60.77	195.38
328.15	257.05	60.79	196.26
333.15	257.95	60.81	197.14
338.15	258.86	60.84	198.02
343.15	259.77	60.87	198.91
348.15	260.69	60.89	199.80
353.15	261.62	60.92	200.70
	$[OcMeIm][(CF_3)CO_2]$		
283.15	273.83	74.02	199.81
288.15	274.73	74.05	
293.15	275.64	74.09	201.55
298.15	276.54	74.12	
303.15	276.54	74.12	202.42
308.15	277.45	74.15	203.30
313.15	279.27	74.22	205.05
318.15	280.18	74.25	205.93
323.15	281.08	74.28	206.81
328.15	257.05	60.79	196.26
333.15	257.95	60.81	197.14
338.15	283.81	74.37	209.44
343.15	284.72	74.40	210.32
348.15	285.64	74.43	211.20
353.15	286.55	74.47	212.09

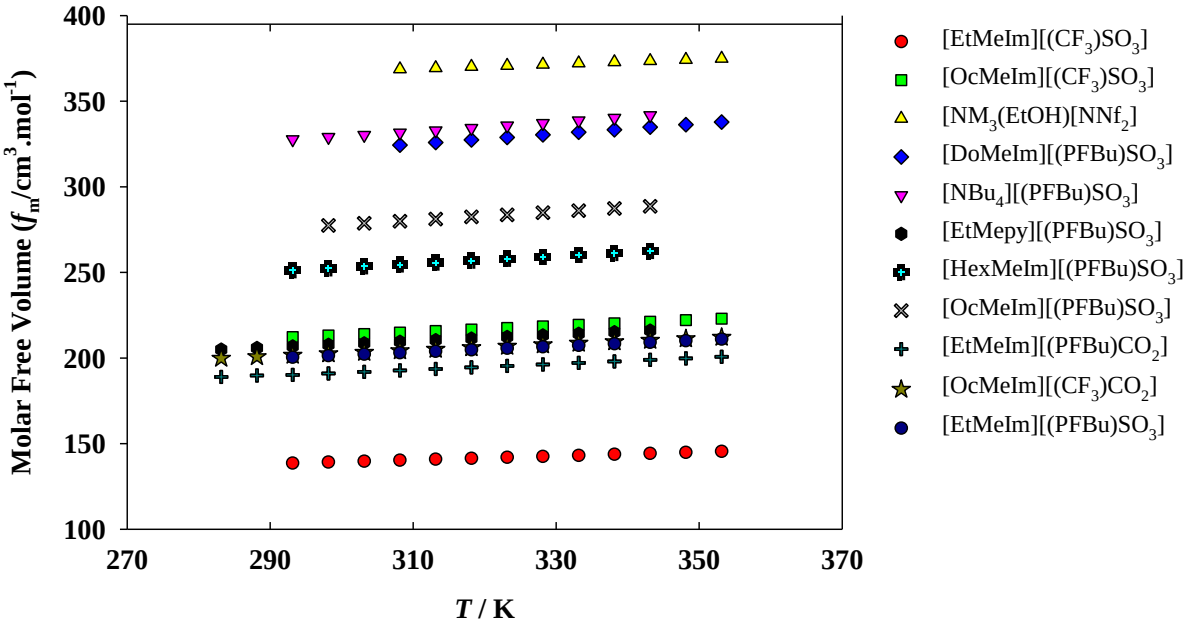


Figure 2.9- Molar free volume as a function of temperature (*T*) for the fluorinated ionic liquids.

Taking into account the nature of FIL's, molar free volume is one of the most important properties for their use in gas separation and delivery.

A comparison between ionic liquids with carboxilate and sulfonate anions shows that the sulfonate anion improves the molar free volume in these FILs:

- $[EtMeIm]^+$ - $[(PFBu)SO_3]^-$ > $[(PFBu)CO_2]^-$$[OcMeIm]^+$ - $[(CF_3)SO_3]^-$ > $[(CF_3)CO_2]^-$

From the analysis of experimental results obtained in this work for two different anions, $[(CF_3)CO_2]^-$ and $[(PFBu)SO_3]^-$, it can conclude that molar free volume increase with the increment of hydrogenated alkyl chain in imidazolium cation:.

- $[(CF_3)CO_2]^-$ - $[EtMeIm]^+$ < $[OcMeIm]^+$
- $[(PFBu)SO_3]^-$ - $[EtMeIm]^+$ < $[HexMeIm]^+$ < $[OcMeIm]^+$ < $[DoMeIm]^+$

The increment of fluorinated alkyl chain, in FILs with imidazolium cation, also increases the molar free volume of these ionic liquids:

- $[(EtMeIm)]^+$ - $[(CF_3)SO_3]^-$ < $[(PFBu)SO_3]^-$

Relatively to the family type of cation and take in to account the $[(PFBu)SO_3]^-$anion, the molar free volume trend is to increase accordingly to that order:

Pyridinium < Imidazolium < Ammonium

To conclude, taking into account the free molar volume analysed in this discussion, $[NM_3(EtOH)\ ][\ NNf2]$, $[NBu_4][(PFBu)SO_3]$ and $[DoMeIm][(PFBu)SO_3]$, are the most appropriate ionic liquids for this application that involve diffusion of gases. The very strong C−F bonds existent in FILs cause an increase in rigidity of the molecular structure and a decrease in polarity. This fact justifies the increment of molar free volumes and molar volumes of FILs with long fluorinated chains (Pereiro et al. 2013). The $[\ NNf2]^-$ anion of $[NM_3(EtOH)\ ][\ NNf2]$ has a higher number of fluors than the others, so like it was expected that ionic liquid due to his high molar volume is very good for gas separation processes like diffusion of O_2 and CO_2. $[NBu_4][(PFBu)SO_3]$ and $[DoMeIm][(PFBu)SO_3]$ have also high molar volumes, the first case is realated to the structure of the cation and the second case is related to the hydrogenated chain lengt, these features make an increase in the molar free volume.

2.1.4.4.Walden Plot

The Walden plot is a convenient and versatile tool to measure the ionicity of ionic liquids since establish a relationship between the molar conductivity and the viscosity or fluidity of a fluid. A way to determine the efficiency of an ionic liquid as an electrolyte, solvent, or reaction medium, has been proposed Walden rule. The Walden rule (Walden 1906) gives information on relationship between ion mobility and viscosity. In the case of an ideal ionic liquid conductor, conductivity is only influenced by the viscosity (Papaiconomou et al. 2012) in the form:

$$\text{Molar conductivity} = \frac{C}{\eta} \qquad (2.9)$$

where the molar conductivity is expressed in S cm^2 mol^{-1} and the viscosity (η) is expressed in poise, and C is a constant. “The fractional” Walden model added an exponent α (Harris 2010).

$$\text{Molar conductivity} \propto \left(\frac{1}{\eta}\right)^{\alpha} \quad (2.10)$$

For ionic liquids it has been shown that the molar conductivity can be calculated by(Papaiconomou et al. 2012):

$$\text{Molar conductivity} = \frac{k}{\rho \mathrm{M_w}} \quad (2.11)$$

with the k conductivity in S cm^{-1}, the ρ the density in g cm^{-3} and M_w the molecular weight in g mol^{-1}. The eq. (2.11) can be expressed by:

$$\log(\text{Molar conductivity}) = \alpha \log\left(\frac{1}{\eta}\right) + \log C \quad (2.12)$$

The Walden-plot is usually represented by the $\log$ Molar conductivity as a function of $\log\ 1\ \eta$ (Harris 2010) and is a good and versatile tool to measure ionicity of inorganic salts solutions and ionic solvents. The straight line of *Figure 2.10 and Figure 2.11*fixes the position of the "ideal" electrolyte, where the system is known to be fully dissociated and to have ions of equal mobility. Usually, the behaviour of diluted aqueous potassium chloride solutions are taken as the behaviour of an ideal electrolyte (Miran et al. 2012). For the unit chosen, the ideal line runs from corner to corner of a square diagram (Xu, Cooper, and Angell 2003).*Figure 2.10* shows the classification proposed by Angell and co-workers (Xu et al. 2003) for "good" and "poor" ionic liquids.

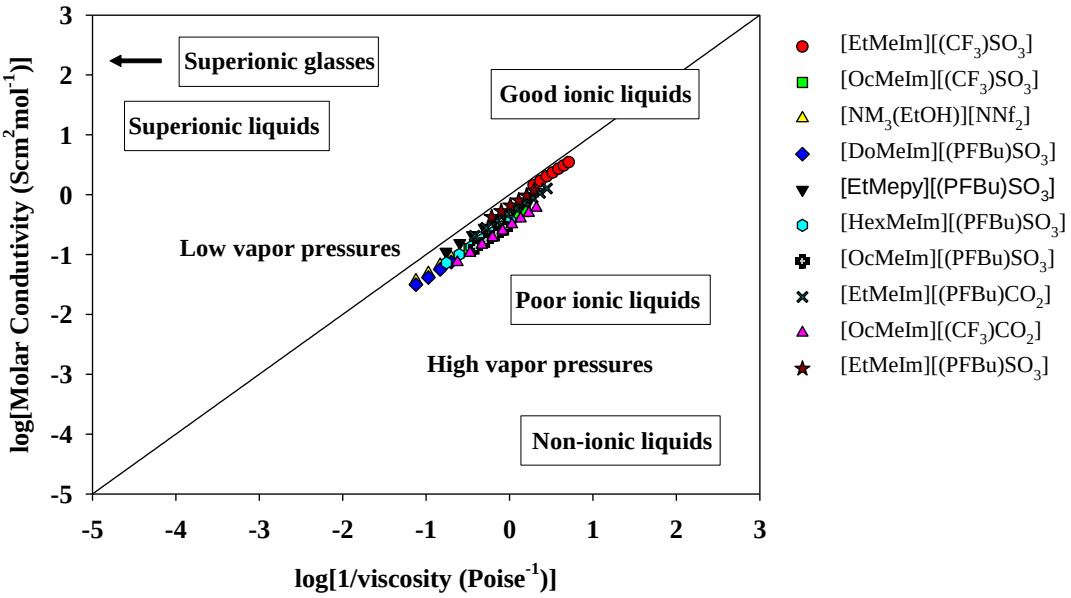


Figure 2.10 - Classification proposed by Angell and co-workers for "good" and "poor" ionic liquids.

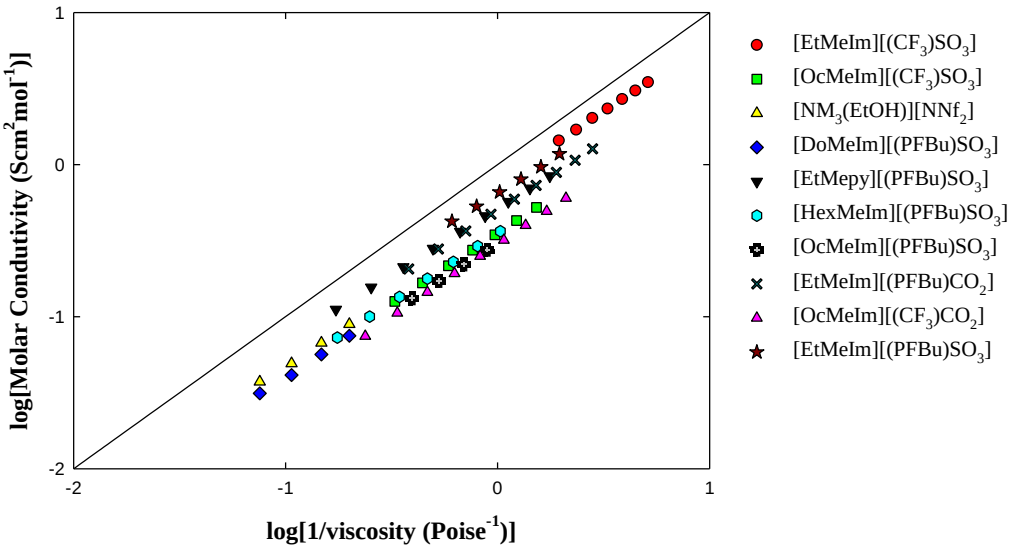


Figure 2.11 - Walden plot for the fluorinated ionic liquids.

Ionic conductivity and its relation with viscosity, known as ionicity, provide useful information about transport properties, mobility and the possible formation of aggregates in ionic liquids. The formation of aggregates could decrease the mobility of these FILs and increase its viscosity, obtaining a ionicity behaviour away from ideal electrolyte. Clearly, the

increment of ionicity improves the physical properties of FILs that could be used in artificial blood substitutes. Experimental information on ionicity of fluorinated ionic liquids is extremely scarce in literature review and experimental data are available for only six FILs (Pereiro et al. 2013). The data obtained for the fluorinated ionic liquids studied in this work and the presents in literature (Cortes 2013; Pereiro et al. 2013) are represented in *Figure 2.10* and *Figure 2.11* together with the classification proposed by Angell and co-workers (Xu et al. 2003). For each fluorinated ionic liquid, the Walden plot has a linear behaviour with the temperature.

In the walden plot, a comparison between our fluorinate ionic liquids with the ideal electrolyte is carried out.

Fluorinated ionic liquids with a sulfonate group shows high iconicity than with a carboxylate group, for the same imidazolium cation, $[(EtMeIm)]^+$.

- $[(EtMeIm)]^+$: $[(PFBu)SO_3]^-$ > $[(PFBu)CO_2]^-$

By the analysis of this data it can be concluded that the increment of hydrogenated alkyl chain in imidazolium cation is related to a decrease of ionicity in $[(PFBu)SO_3]^-$ anion.

- $[(PFBu)SO_3]^-$: - $[(EtMeIm)]^+$ > $[(HexMeIm)]^+$ > $[(OcMeIm)]^+$ > $[(DoMeIm)]^+$

A decreasing in ionicity is also related to the increment of fluorinated length chain, for imidazolium cations.

- $[(EtMeIm)]^+$: $[(CF_3)SO_3]^-$ > $[(PFBu)SO_3]^-$
- $[(OcMeIm)]^+$: $[(CF_3)SO_3]^-$ > $[(PFBu)SO_3]^-$

The difference in ionicity for two different cation families, imidazolium and pyridinium could be evaluated with $[(PFBu)SO_3]$ anion. In these comparison, the higher iconicity in the presence of imidazolium cation is verified.

- Imidazolium > Pyridinium

For all above mentioned reason, and by the direct analysis of *Figure 2.11* the best candidates to use in oxygen therapeutic emulsions are $[(EtMeIm)][(CF_3)SO_3]$, and $[(EtMeIm)][(PFBu)SO_3]]$ which present a behaviour closest to ideal electrolyte.

2.1.5.Conclusions

The final goal of this chapter is to measure the thermodynamic and thermophysical properties of 13 FILs in order to expand knowledge of FILs and understand its behaviour as pure fluid so that they can be used in oxygen therapeutics based on PFCs-emulsions. Here some comparisons with the values available in literature were also presented to establish of guidelines for selecting the most suitable FILs for this application.

The viscosity of PFC-based emulsions should increase when is added a good surfactant with aim to enhance the stability of emulsions. All the fluorinated ionic liquids have higher viscosity than the PFCs and can increase the stability of actual oxygen therapeutics. However, the FILs with higher viscosity should not be selected because can present problems to promote dispersion of fluorinated compounds in the water and make difficult the intravenous administration of artificial blood substitutes.

Mapping the behaviour of thermodynamic and thermophysical properties, it can be conclude that [EtMeIm][(PFBu)SO_3] and [EtMepy][(PFBu)SO_3] are a good election because it has a viscosity and a density similar to that of PFC-based emulsions and thus would not present any problems in the preparation or stability of the emulsion. However, these FILs present lower values of molar free volume than other FILS, which might be hindered the solubility of respiratory gases. Nevertheless, the fluorinated domains of these emulsions are expected to increase with the addition of these FILs, increasing the capacity for solubilizing respiratory gases. On the other hand, [EtMeIm][(CF_3)CO_2] present a good candidate for biomedical applications, because are very stable at a wide range of temperatures, has a high fluidity and show a behaviour very similar to ideal electrolyte. However, it present the smallest number of fluoro atoms indicating that it is impossible the formation of a fluorinated nanodomains. It is expected that the fluorinated alkyl chain present in FILs will also control the formation of these fluorinated nanodomains easing the accommodation of fluorinated solutes (gases or liquids) (Almantariotis et al. 2010; Bara et al. 2009; Muldoon et al. 2007). In this sense, it is expected that the length of the alkyl chain will also play a role on the aggregation.

2.2 Cytotoxicity of Fluorinated Ionic Liquids

2.2.1.Introduction

The development of a new product for biomedical applications requires the execution of several processes until the commercialization. The first step passes through the research and development of the product. Since toxicological effects of ILs are not well known, the screening of cellular toxicity for some FILs need to be addressed (Ranke et al. 2007) with aim to accomplish our goal of develop an artificial blood substitute with fluorinated ionic liquids and to other possible biomedical applications. This screening is important not only for predict *in vivo* toxicity from *in vitro* studies but it also provides important information about the possible mechanism of action involved in toxicity (Malhotra and Kumar 2010).

Recent studies report the influence of cation and anion in the toxicity of the ionic liquids and most of these publications establish parameters to predict the cytotoxicity (García-Lorenzo et al. 2008; Kumar et al. 2008; Pereiro et al. 2013; Petkovic et al. 2011; Ranke et al. 2007). However, just one article performed in our laboratory refer the toxicity of this specific family of ionic liquids, the fluorinated ionic liquids (Pereiro et al. 2013). Due to the scarce information available, the study of the toxicity and biocompatibility of these fluorinated ionic liquids will be very useful for their application in numerous fields, such as biomedical applications.

In this work, cytotoxicity tests were carried out to determine dose-dependent toxicity curves and the concentration of sample necessary to decrease 50% of cell viability (cytotoxic effective concentration, EC_{50} value) for each FIL using the MTS colorimetric cytotoxicity assay. These tests were performed using four different human cell lines: human colon carcinoma cells, Caco-2; human hepatocellular carcinoma cells, HepG2; spontaneously immortalized human keratinocyte cell line, HaCaT; and human umbilical vein cell line, EA.hy926.

Caco-2 cells were chosen for this work since these cells represent a prototype of the human epithelial cells, and they are commonly used as physiological model for intestinal transport and toxicity studies (Engle, Goetz, and Alpers 1998; García-Lorenzo et al. 2008; Sambuy et al. 2005). Other reports regarding ILs and some FILs from our studies use this model and then comparisons take into account Caco-2 cells can be achieved in this work.
HepG2 cell was also used by other authors to determine the toxicity of the compounds due to the fact that this model preserves both liver-specific functions and morphology over

prolonged periods of time and have a metabolic capacity higher than Caco-2 (Pereiro et al. 2013; Wilkening, Stahl, and Bader 2003). The selection of EA.hy926 and HaCaT cell lines is related to the goal of the project, development of artificial blood substitutes based on fluorinated ionic liquids. In this biomedical application, FILs are incorporated in new improved oxygen therapeutic emulsions with aim to replace, totally or in part, the inert PFCs presently used as oxygen carriers in these emulsions. Since administration of emulsion is intravenous, the evaluation of toxic profile in skin layers is mandatory. Keratinocytes (HaCaT cells) are the predominant cell type in the epidermis, constituting 95% of the cells found there (Rousselle et al. 2009). The endothelial cells (EA.hy926) belong to blood vessels and this type of cells coats the inside of blood vessels, particularly blood capillaries, forming part of its wall. When artificial blood substitute is administrated, the FILs-based emulsions will be in contact with blood vessels and the diffusion of respiratory gases will also occur here. Then, it is expected that the compounds, FILs and FILs-based emulsions, presents no toxicity in endothelial cells. The cytotoxic study was carried out using EA.hy926 cell line which is one of the most used and best characterized human vascular endothelial cell lines (Bouïs et al. 2001; Wei et al. 2010).

Some parameters like the concentration tested, exposure time, the media composition, the ion-pairing or other possible synergetic effects, like the model of mixture toxicity (where the individual compounds, cation and anion, will exhibit similar or different modes of action, leading to concentration dependent or independent mode of action, respectively), interactions between the compound and the cells could also occur and could influence the final response.

Finally, the complexity of living organisms and all the physical and biochemical processes that simultaneously occur could also interfere with toxicity mechanism of action (Petkovic et al. 2011). Then, it is important to highlight that this study shows results with different variants that must be considered. The information obtained in this work allows us to predict the behaviour of the ionic liquids taking into account the own characteristics of FILs, the characteristics of the living model studied and the parameters established for the assay, like exposure time.

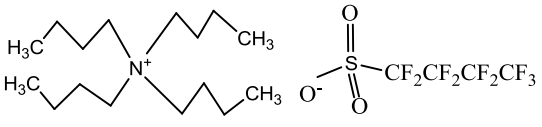
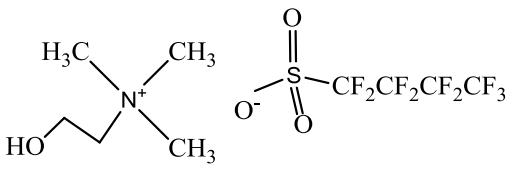
2.2.2 Materials

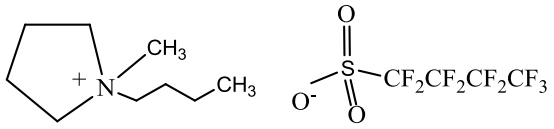
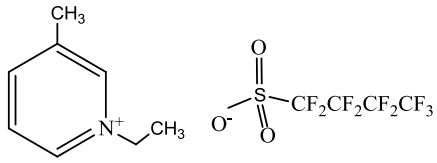
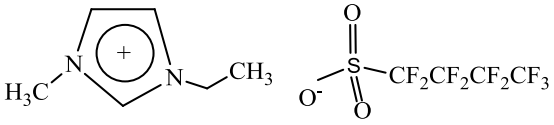
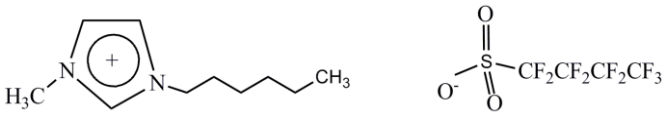
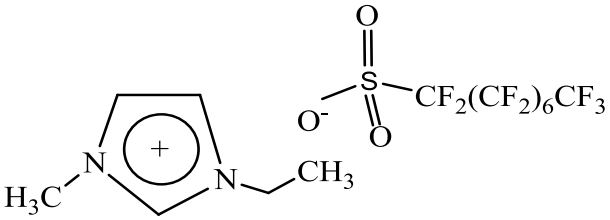
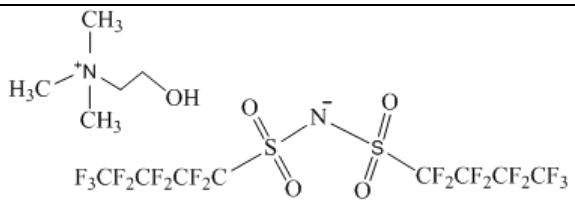
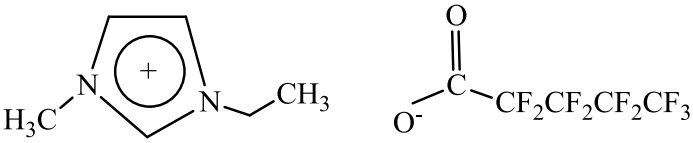
Tetrabutylammonium perfluorobutanesulfonate, >97% mass fraction purity, (2 hydroxyethyl)trimethylammonium perfluorobutanesulfonate, >97% mass fraction purity, 1-butyl-1-methylpyrrolidinium perfluorobutanesulfonate, 98% mass fraction purity, 1-ethyl-3-methylpyridinium perfluorobutanesulfonate, >97% mass fraction purity, 1-ethyl-3-methylimidazolium perfluorobutanesulfonate, >97% mass fraction purity, 1-hexyl-3-methylimidazolium perfluorobutanesulfonate, >99% mass fraction purity; 1-ethyl-3-methylimidazolium perfluorooctanesulfonate, >98% mass fraction purity; (2-hydroxyethyl)trimethylammonium bis(nonafluorobutylsulfonyl)imide, >97% mass fraction purity were acquired at Iolitec. The purity of the commercial ionic liquids was checked by ^{1}H-NMR.

1-Ethyl-3-methylimidazolium perfluoropentanoate was synthesized in Molecular Thermodynamic Lab by ion exchange resin methods, as develop by Ohno *et al* (Fukumoto, Yoshizawa, and Ohno 2005). The purity of the final products was checked by ^{1}H, ^{13}C and ^{19}F NMR and elemental analysis.

All fluorinated ionic liquids were dried under vacuum (3.10^{-2} Torr) and vigorous stirring at 323.15K for at least 2 days, immediately prior to their use. This step is crucial in order to reduce volatile reduce volatile impurities, as well, as water, which can influence the ionic liquids properties. The water content, determined by Karl Fisher titration, was less than 100 ppm for all the studied ionic liquids. No further purification was carried out. Structures and acronyms of all fluorinated ionic liquids are shown in *Table 2.7*.

Table 2.7.- Chemical structure and respective abbreviation of fluorinated ionic liquids in study.

FIL designation	Chemical Structure
Tetrabutylammonium perfluorobutanesulfonate **[NBu_4][(PFBu)SO_3]**	
(2-hydroxyethyl)trimethylammonium perfluorobutanesulfonate **[NM_3(EtOH)][(PFBu)SO_3]**	

FIL designation	Chemical Structure
1-Butyl-1-methylpyrrolidinum perfluorobutanesulfonate **[BuMepyr][(PFBu)SO_3]**	
1-Ethyl-3-methylpyridinium perfluobutanesulfonate **[EtMepy][(PFBu)SO_3]**	
1-Ethyl-3-methylimidazolium perfluobutanesulfonate **[EtMeIm][(PFBu)SO_3]**	
1-Hexyl-3-methylimidazolium perfluorobutanesulfonate **[HexMeIm][(PFBu)SO_3]**	
1-Ethyl-3-methylimidazolium perfluorooctanesulfonate **[EtMeIm][(PFOc)SO_3]**	
(2-Hydroxyethyl)trimethylammonium bis(nonafluorobutylsulfonyl)imide **[NM_3(EtOH)][NNf_2]**	
1-Ethyl-3methylimidazolium perfluoropentanoate **[EtMeIm][(PFBu)CO_2]**	

For cell culture, human colon carcinoma cells, Caco-2, were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen(DSMZ; Germany), human hepatocellular carcinoma cells, HepG2, were obtained from the European Collection of Cell Culture (ECACC; UK), spontaneously immortalized human keratinocyte cell line, HaCaT, were purchased from Cell Lines Service GmbH(CLS; Germany), and human umbilical vein cell line, EA.hy926, were purchased from American type Culture Collection (ATCC, USA). Cell culture medium , RPMI 1640 medium, MEM medium, DMEM medium and supplements,

fetal bovine serum (FBS), L-Glutamine, penicillin-streptomycin solution, MEM nonessential amino acids (MEM-NEAA), sodium pyruvate and trypsin-EDTA solution were purchased from Gibco (Invitrogen Corporation, Paisley, UK).

For the *in vitro* cytotoxicity assays, CellTiter 96® Aqueous One Solution Cell Proliferation Assay was purchased from Promega (CA, USA)

2.2.3 Experimental Procedure

In the cell culture, human colon carcinoma cells, Caco-2, were routinely grown in RPMI medium 1640 supplemented with 10% of inactivated fetal bovine serum (FBS), 2mM of glutamine and (1%) of penicillin-streptomicin. Human hepatocellular carcinoma cells, HepG2, were cultured in MEM medium with 10% of inactivated FBS, 2mM glutamine, 1% MEM-NEAA and 1% sodium pyruvate. Spontaneously immortalized human keratinocyte cell line, HaCaT, were routinely grown in DMEM medium supplemented with 10% of FBS, 2mM of glutamine, 1% of penicillin-streptomicin. Finally, human umbilical vein cell line, EA.hy926, were routinely grown in DMEM medium supplemented with 10% FBS and 1% of penicillin-streptomicin. Cells were kept at 37 ºC in a humidified incubator with 5% CO_2 and routinely grown 175 cm^2 culture flasks. The cell lines were split once or twice a week, the morphology and growth of cells were monitored daily.

Caco-2 cells were seeded at a density of $2x10^4$ cells per well, in 96-well plates and their media was replaced every 48 hours. The experiments were performed using cells after reaching confluence, 96 hours after seeding. HepG2 cells were seeded at a density of $6x10^4$ cells per well and experiments were performed at a confluence of 80%, 24 hours after seeding. HaCaT cells were seeded at a density of $1x10^4$ cells per well and experiments were performed at a confluence of 90%, 24 hours after seeding. EA.hy926 cells were seeded at a density of $2x10^4$ cells per well and experiments were performed after reaching confluence, 24 hours after seeding.

Stock solutions of the FILs were prepared in dimethyl sulfoxide (DMSO), the FILs were studied in different ranges of concentrations due to the poor solubility of some of them in cell culture medium. All the ILs were homogenous in solution and diluted in 0.5% FBS culture medium with a maximum of 1% DMSO. All cell lines were incubated for 24 hours with the FILs in a 0.5% FBS medium.

Positive control cells were prepared containing culture medium with the minimum percentage of DMSO used (1% DMSO) and negative control cells were prepared containing only DMSO. After 24 hours of incubation, the samples were removed and 100 µl of a CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (containing MTS and PES) was added to each well and left to react for 4 hours. This solution reagent contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron-coupling reagent, phenazine ethosulfate (PES). PES has an enhanced chemical stability which allows it to be combined with MTS, leading to a stable solution. MTS is bio-reduced by cells into a coloured formazan product by mitochondrial reductase enzymes active in viable cells, thus the amount of formazan product that is soluble in tissue culture medium, is proportional to the number of viable cells. Formazan was quantified spectrophotometrically at 490 nm in a BioTek FLx800 microplate reader (BioTek, USA). Each sample was incubated in three different wells and the obtained value was the average of three independent assays. Cell viability was determined by the ratio between the measured absorbance of FILs-contacted cells and the measured absorbance of control cells with 99% of cellular medium. Dose-dependent toxicity curves were determined presenting the toxicity trends for each FIL. The concentration of sample necessary to decrease 50% of cell viability (cytotoxic effective concentration, EC_{50} value) were obtained from dose-response curves using the GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) software.

2.2.4 Results and Discussion

The information about the toxicity and biocompatibility of fluorinated ionic liquids is of crucial importance for all applications that incorporate ionic liquids, biomedical applications especially. There are only one publication in literature for Caco-2 and HepG2 performed in our lab using fluorinated ionic liquids (Pereiro et al. 2013) but was only carried out assays with 4 h of exposure (digestion time). It is important to emphasize that, to our knowledge there are as yet no cytotoxicity data for fluorinated ionic liquids in HaCaT and EA.hy926. The combination of data from literature and the experimental measurements of this work will provide the useful information to choose the best fluorinated ionic liquid for biomedical applications, such as artificial blood substitutes.

The toxicity profiles, *Figures 2.12-2.17*, and the EC_50 values, *Table 2.8*, calculated for FILs studied in this work were analysed taking into account the following parameters: the cell line, the difference between sulfonate and carboxylate FILs, the increment of hydrogenated chain length, the increment of fluorinated chain length, the nature of cation and the nature of anion.

Table 2.8 - Cytotoxic effective concentration (EC_{50}) values for fluorinated ionic liquids in cells

	Log EC_{50}			
FIL designation	**CaCo-2**	**HepG2**	**EA.hy926**	**HaCaT**
$[NBu_4][(PFBu)SO_3]$	>4	>4	>4	
$[NM_3(EtOH)][(PFBu)SO_3]$	>4	3.98	>4	
[BuMepyr][$(PFBu)SO_3$]	>4	>4	>4	
[EtMepy][$(PFBu)SO_3$]	>4	3.88	>4	3.85
[EtMeIm][$(PFBu)SO_3$]	>4	>4	>4	3.82
[HexMeIm][$(PFBu)SO_3$]	3.84	>4	3.81	3.32
[EtMeIm][$(PFOc)SO_3$]	2.18	1.90		
$[NM_3(EtOH)][NNf_2]$	1.74	1.83		
[EtMeIm][$(PFBu)CO_2$]	>4	>4		

By analysis of the experimental toxicity profiles (*Figure 2.12*) for two different

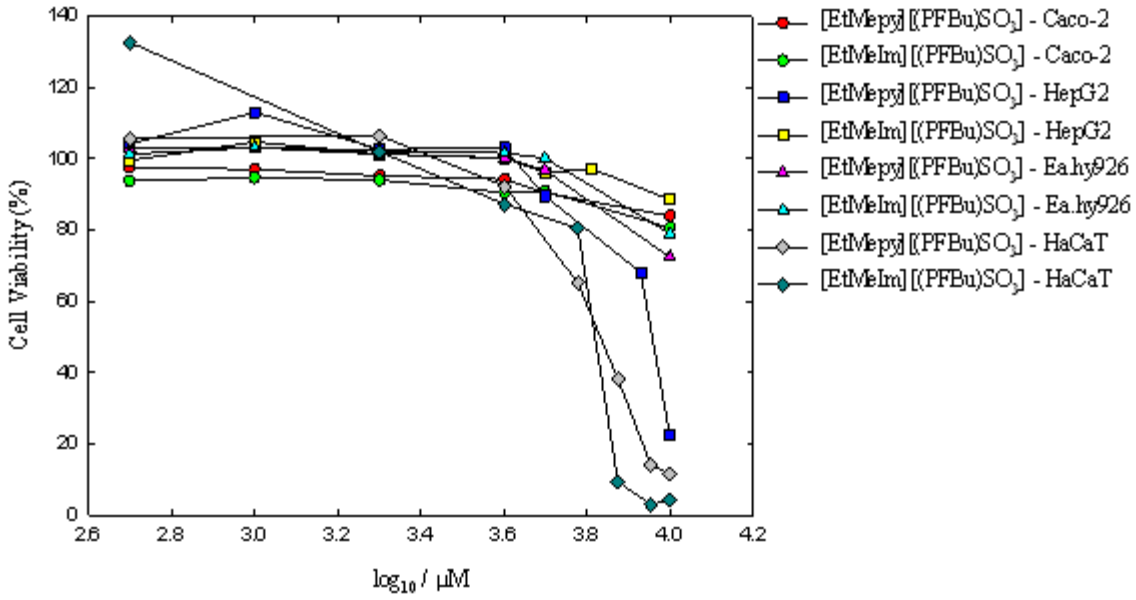


Figure 2.12 - Cell viability in all cell lines for [EtMepy][$(PFBu)SO_3$] and [EtMeIm][$(PFBu)SO_3$]

families of cations, pyridinum $[EtMepy]^+$ and imidazolium $[EtMeIm]^+$ with the same anion, $[(PFBu)SO_3]^-$ in the four cell lines studied in this work, Caco-2, HepG2, EA.hy926 and HaCaT, it is possible to obtain the following conclusions: HaCaT cell line shows a drastically decrease in cellular viability since the lower concentration. A possible explanation to this behaviour in both ionic liquids is related to the hypothesis that HaCaT cells have lost some of their protection mechanism against DNA-damage through mutation of the *p53* gene *in vivo* (Rekus 2000). If these ionic liquids cause DNA damage, in absence of that protection mechanism, cells could lose her viability until death. The EC_{50} values reported in *Table 2.8* for HaCaT reveal the toxicity behaviour of the compounds in this cell line which can be considered the most sensitive one.

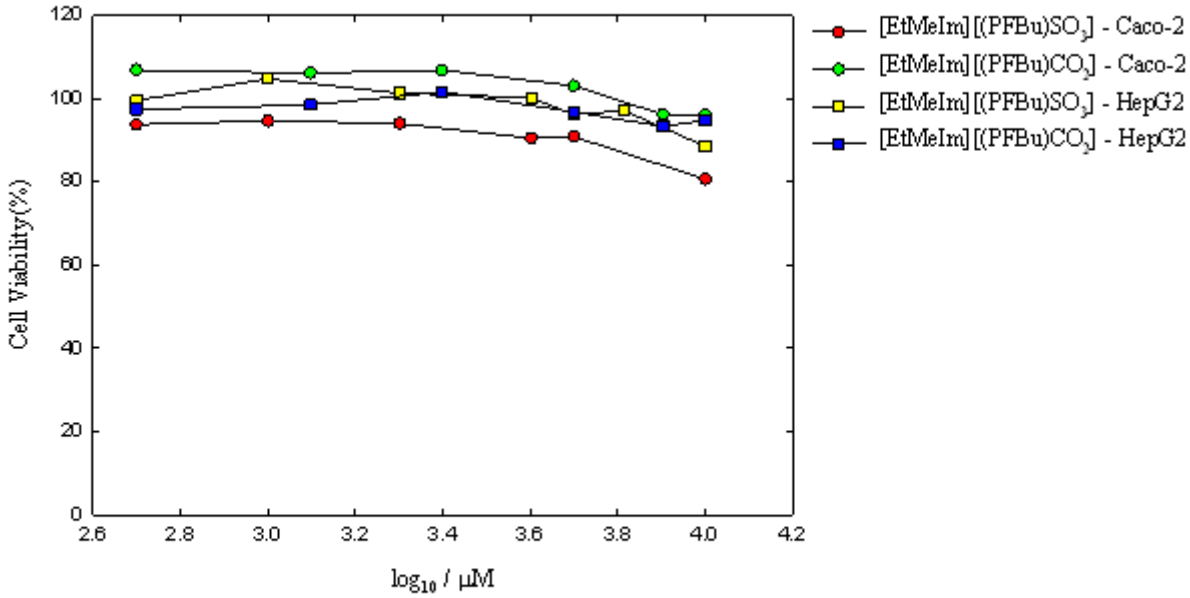


Figure 2.13 - Cell viability in Caco-2 and HepG2 cell lines for [EtMeIm][(PFBu)SO_3] and [EtMeIm][(PFBu)CO_2]

In general, the toxicity of these FILs, [EtMepy][(PFBu)SO_3] and [EtMeIm] [(PFBu)SO_3], was similar in the studied cell lines, with EC_{50} values upper 10000µM (*Table 2.8*).The exception was found for HaCaT cell line and for [EtMepy][(PFBu)SO_3] in HepG2. The toxicity of pyridinium in HepG2,with a EC_{50} value equal to 7586 µM (*Table 2.8*), shows an opposite behaviour to the other lines (the same ionic liquid shows high viability in Caco-2 and EA.hy926) because this cell line has a higher metabolic capacity than other cell lines. In this case, the toxicity could be linked to the low expression of some phase I enzymes in this cellline which could be involved in the metabolism of pyridinium compound (Pereiro et al.

2013; Wilkening et al. 2003). A comparison between ionic liquids based on $[(PFBu)SO_3]^-$ sulfonate and carboxylate $[(PFBu)CO_2]^-$ anions with the same imidazolium cation, $[EtMeIm]^+$, for Caco-2 and HepG2 cell lines (EC_{50} values from *Table 2.8*, upper than 10000µM and the experimental toxicity profiles from *Figure 2.13*) shows that both ionic liquids in both cell lines are viable in the range of concentrations studied. Only, a slight difference between the carboxylate and sulfonate anion can be observed in Caco-2 cells, a decrease of 20% of viability for the sulfonate anion (*Figure 2.13*).

The increment of hydrogenated chain length in imidazolium cation, from $[EtMeIm]^+$to $[HexMeIm]^+$ for the same anion $[(PFBu)SO_3]^-$, shows a descent of the viability in all cell lines. This information is supported by the analysis of toxicity profiles (*Figure 2.14*) and EC_{50} values (*Table 2.8*).The same behaviour is observed in previous published reports with these same cations (García-Lorenzo et al. 2008; Pereiro et al. 2013; Ranke et al. 2004). Despite that the increment of the hydrogenated alkyl chain do not significantly change the cell viablility in HepG2 cell line, in other cell lines $[HexMeIm]^+$ cation decrease drastically cellular viability.

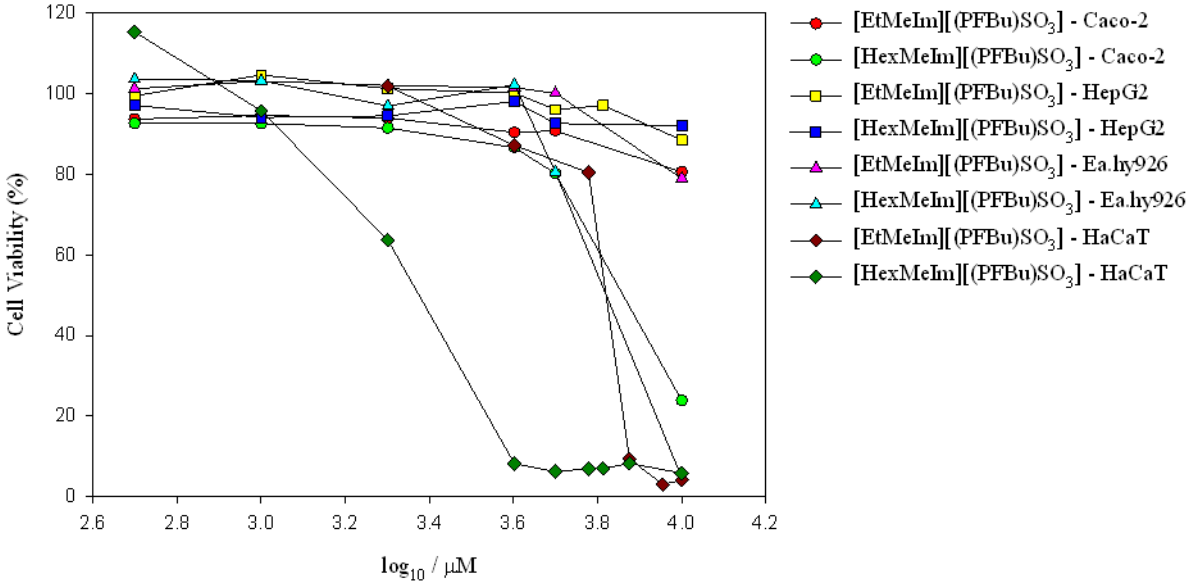


Figure 2.14 - Effect of the increment of hydrogenated length chain in Caco-2, HepG2, EA.hy926 and HaCaT cell lines

In the case of Caco-2 cell, viabilities are around 90% to 80% in all range of studied concentrations (500 µM to 10000µM) for $[EtMeIm]^+$ cation. However, when the hydrogenated chain increases, the viability decrease until a cell viability of 20% in the highest concentration, 10000 µM, for example the case of $[HexMeIm]^+$ cation. This ionic liquid has

an EC_{50} value of 6918 µM (*Table2.8*) consistent with the toxicity curve shown in *Figure2.14*. For EA.hy926 cells, FILs based on $[EtMeIm]^+$ cation show that the viability drops from 100% to 80%, between 500 µM and 10000µM. However, the viability of $[HexMeIm]^+$ sharply decreases until 5% in higher concentration, 10000 µM. This ionic liquid has an EC_{50} value of 6457µM (*Table 2.8*) consistent with the toxicity curve shown in *Figure 2.14*. In HaCaT, both ionic liquids show a toxicity profile, however in the presence of $[HexMeIm]^+$ the EC_{50} value decreases to 2089µM while in $[EtMeIm]^+$ the EC_{50} is around 6606µM.

Through the obtained results (*Table 2.8*, *Figure 2.12*, *Figure 2.14* and *Figure 2.16*) it is possible to conclude that the toxicity profile for FILs with short hydrogenated alkyl chains depends on the nature of the cation and the anion. However, the cellular viability decreases in both compounds with longer hydrogenated chains. In the HepG2 cell line this decrease is almost insignificant whereas for other cell lines like Caco-2, EA.hy926 and HaCaT this descent is linked to a sharp decline in cellular viability. However the cytotoxicity of ionic liquids should be measured accordingly to the system, cation-anion, and not only based on the summation of the effects of cation and anion. The information obtained in this study could be a tool for the design of the pretended ionic liquid, but other aspects associated to thermophysical properties should be also considered.

Some previous reports studied the anion influence in the toxicity of ionic liquids in different cell lines. However just one report performed in our lab, evaluated the increase of the fluorinated alkyl chain length, from $[(PFBu)SO_3]^-$ to $[(PFOc)SO_3]^-$ for FILs based on the amonium cation with Caco-2 cell line (Pereiro et al. 2013) during 4 hours of exposure (digestion time).

In this work, the increment of fluorinated alkyl chain was studied for the anions mentioned above, but for FILs based on imidazolium cation,$[EtMeIm]^+$. The examination of *Figure 2.15* shows that for different cell lines, Caco-2 and HepG2, the increment of fluorinated alkyl chain leads to an increase in cells toxicity. These results are agreed with those previously obtained for the ammonium cation. Due to the poorly solubility of $[(PFOc)SO_3]^-$ anion in cell culture medium, the range of tested concentrations was slightly different for the both FILs in this study. In the case of FILs based on $[(PFBu)SO_3]^-$, the studied concentrations range was between 500µM and 10000 µM, whereas that for $[(PFOc)SO_3]^-$ anion, the range of concentrations varies from 4µM to 1000 µM in both cell lines. A reduction in order of 250 times in concentration of FILs was carried out in the last FILs to obtain an identical behaviour in cytotoxic profiles, with viability close to 100% in Caco-2 and HepG2 cell lines. And then the EC_{50} values were calculated and a value of 150µM was obtained for Caco-2 cells and of

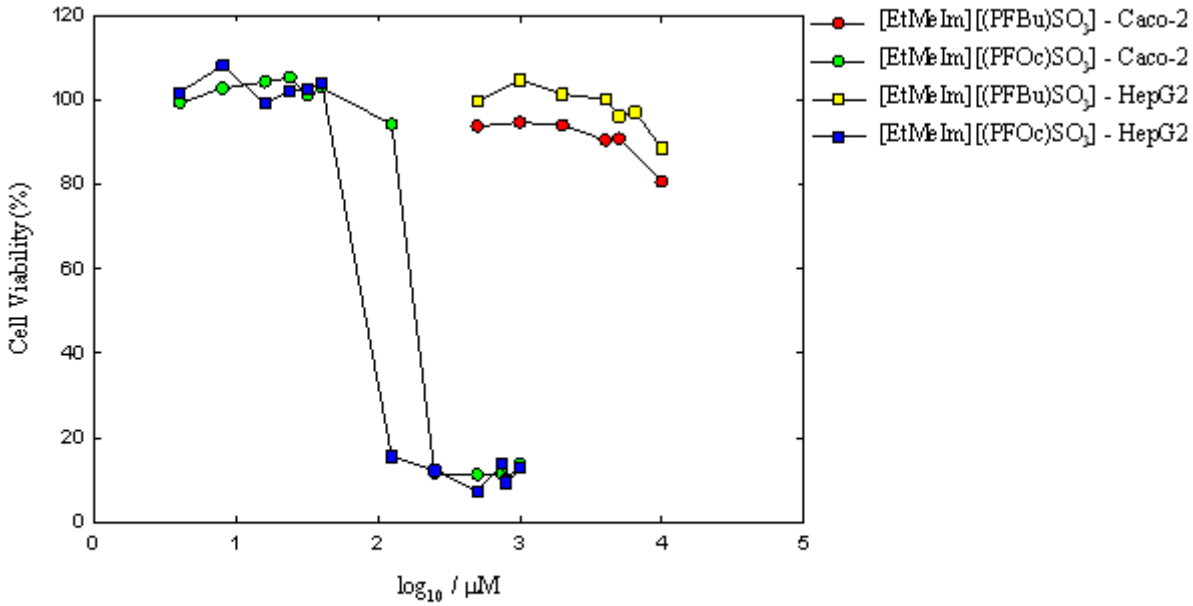


Figure 2.15 - Effect of the increment of fluorinated length chain in Caco-2 and HepG2 cell lines

79 µM for HepG2 cells. This increasing in toxicity with the increment of the fluorinated chain could be linked to the formation of free fluoride ions, by hydrolytic cleavage. These chemical species may exhibit a high intrinsic reactivity leading to cytotoxic effects. Once they are potent inhibitors of Na^+-K^+-ATPase located at the cells surface and could influence cellular mechanisms, leading them to death (Kumar et al. 2008). On the other hand, the increment of cytotoxicity could be also linked to the increment of hydrophobicity of FIL when the fluorinated alkyl chain increases (Petkovic et al. 2011; Ranke et al. 2004).

In order to select the fluorinated ionic liquid with less toxicity in human cell lines, the evaluation of the cation is an essential factor. In this work five different cations, ammonium, cholinium, pyrrolidinium, pyridinium and imidazolium were studied for Caco-2, HepG2 and EA.hy926 cell lines and the experimental results are plotted in *Figure 2.16*. Some studies were

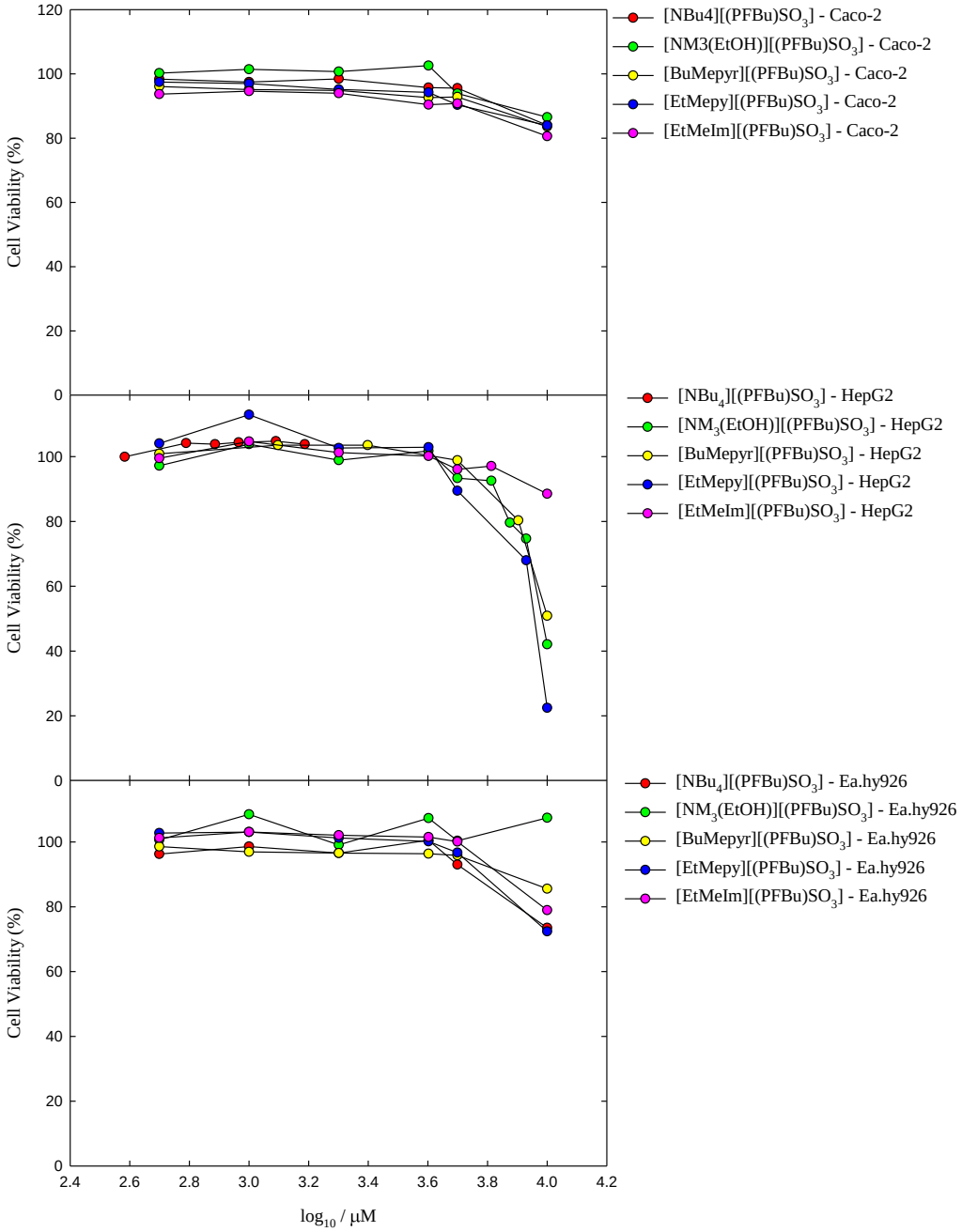


Figure 2.16 - Cell viability of different cation families in Caco-2, HepG2, EA.hy926 and HaCaT cell lines

published indicating the toxicity of these cations, however there is no information in endothelial cell lines (Frade et al. 2009; García-Lorenzo et al. 2008; Petkovic et al. 2011).

Due to the poor solubility of ammonium cation, the range of studied concentrations for this ionic liquid is different to the others, from 380µM to 1513µM.By the analysis of experimental data in (*Figure 2.16*), it can be showed that in Caco-2 cells none of the studied compounds are toxics in the range of concentrations studied. All ionic liquids present values of cellular viability around 100% in lower concentrations and 80% in higher concentrations. Despite the similarity of the curve- dose response in all ionic liquids, these data are according with the previous studies presents in literature where the minor toxicity to cholinium and pyrrolidinium cation is demonstrated (Frade et al. 2007; Petkovic et al. 2011).

Furthermore, FILs based om imidazolium and pyrrolidinium cations present cellular viability in all range of studied concentration. The FIL based on ammonium cation shows 100% of cellular viability in all possible studied concentrations (miscible composition range), but all FILs also shows same values of cellular viability in this same range of concentrations. However, cholinium cation ($[NM_3(EtOH)]^+$) described as one of the less toxics cations (Frade et al. 2007; Petkovic et al. 2011; Weaver et al. 2010) shows an EC_{50} value of 9550 µM for this cell line. Pyrridinium cation also shows some toxicity in this cell line with an EC_{50} value of 7586 µM. These results also suggest a dependence between the exposure time and the toxicity, since that all cation families in a exposure time of 4 hours did not induce any toxicity for the highest concentrations, 10000 µM (Pereiro et al. 2013), this same conclusion was reported in previous studies and in our lab (Pereiro et al. n.d.; Petkovic et al. 2011). The results from EA.hy926 are similar to those seen in Caco-2 cells, in these cell lines no toxicity

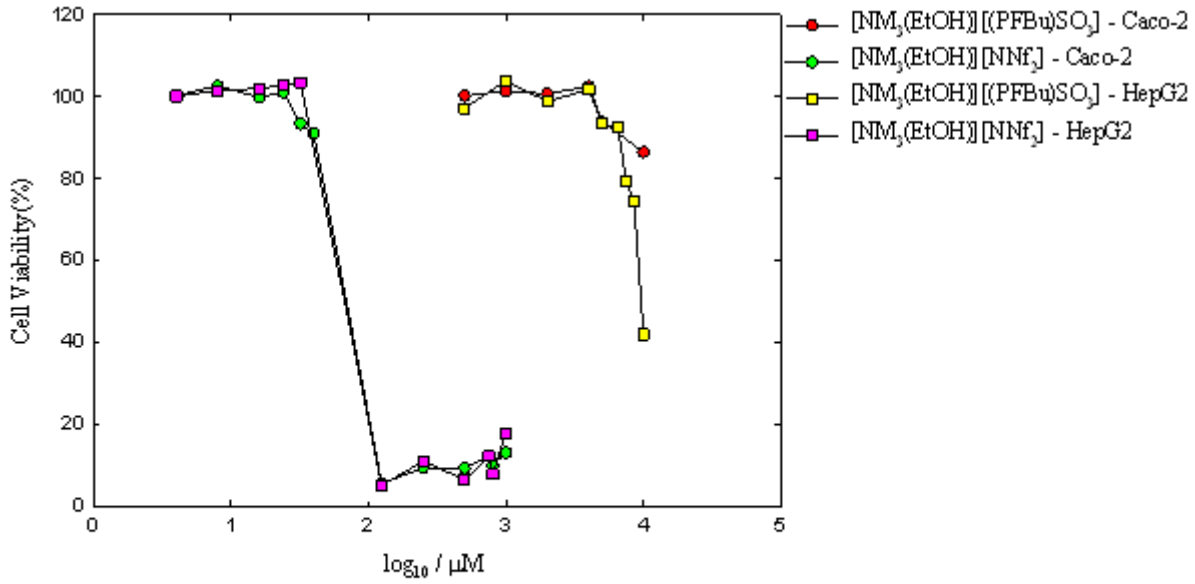


Figure 2.17 - Cell viability for FILs based in Cholinium cation with different anion families in Caco-2 and HepG2 cell lines

was registered and the higher viability is verified with cholinium cation, followed by pyrrolidinium cation.

The effect of the anion in cellular viability is apparently secondary compared to the cation, and it is more evident for the less toxic cations (Petkovic et al. 2011). In this study, cholinium cation, one of the less toxic described in literature, was tested with two different anions, $[(PFBu)SO_3]^-$ and $[NNf_2]^-$, *Figure 2.17*. The fluorinated nature of these anions makes them the very lipophilic and/or unstable and this characteristic play a major role in cytotoxicity of these FIL_S, due to the formation of fluoride ions by hydrolytic cleavage (Kumar et al. 2008; Petkovic et al. 2011) and then the increment of hydrophobicity. When the fluorinated domains increase significantly, a drastic decrease in the cell viability is reported. This effect can be verified in the case of $[NNf_2]^-$ anion when it is compared with $[(PFBu)SO_3]^-$ anion in both cell lines . Besides, the $[NNf_2]^-$ anion decreased the solubility of cholinium cation in cellular medium and a lower concentration range was tested, from 4µM to 1000µM. This ionic liquid, $[NM_3(EtOH)]$ $[NNf_2]$, presents EC_{50} values of 55µM and 68µM for Caco-2 and HepG2 cells, respectively. Besides, this anion is much more toxic than $[(PFBu)SO_3]^-$ which is viable in all range of studied concentrations, 500µM to 10000µM, for Caco-2 cells. In the case of HepG2 cells, this last anion, $[(PFBu)SO_3]^-$, displays a high EC_{50} value, 9550µM.

2.2.5 Conclusion

All the results that were experimentally determined in this work contribute to better understand the behaviour of FILs in human cell lines . Furthermore, these studies represent an increasing in data bases for FILs that are still very scarce in the literature. In this study, it was demonstrated the toxicity of FILs based on two different cations families, imidazolium and pyridinium, in HaCaT cell lines. This toxicity could be linked to the own characteristics of the cell line. However, some future studies should be carried out, because these imidazolium (with short alkyl chain length) and pyridinium cations show lower toxicity in the others cell lines (Caco-2, HepG and HaCaT). More studies should be done in this line in order to better understand the cell death mechanisms and who it could be solved. Cations like cholinium and pyrrolidinium should be also tested for HaCaT due to their low toxicity in other cell lines. Artificial blood substitutes are marketed in the form of emulsions which will be into contact with all the skin layers. Cytotoxicity assays in keratinocytes cells such as HaCaT cell line should be studied in order to support this information.

On the other hand, it can be concluded that the difference between sulfonate and carboxylate anions is insignificant. The increment of the hydrogenated and fluorinated alkyl chain length is related to an increment of toxicity. Furthermore, the cations with lower toxicity profile are cholinium and pyrrolidinium. However, pyridinium cation and imidazolium with short hydrogenated chain also present good results when are combined with $[(PFBu)SO_3]^-$ anion.

Taking all results into account, the best ionic liquids for the development of new artificial blood substitutes based on fluorinated ionic liquids are [BuMepyr][$(PFBu)SO_3$], [NM_3(EtOH)][$(PFBu)SO_3$], [EtMepy][$(PFBu)SO_3$] and [EtMeIm][$(PFBu)SO_3$]. Finally, after the choice of the FILs, it should be explain that cytotoxicity studies of the final product (emulsion of water + PFC + FIL in the best formulation) will be needed in order to evaluate the viability of these products in biomedical market.

2.3. Partition properties of Fluorinated Ionic Liquids

2.3.1.Introduction

One of the most important parameter in pharmacological and toxicological research is the partitioning of a chemical, also known as hydrophobic/hydrophilic balance, which is used to model blood / lipid partition (Lee and Lee 2009).

This parameter is mandatory to understand the ecological effects of fluorinated ionic liquids, but it is also important to evaluate the tendency of a chemical to cross biological membranes and to understand their behaviour *in vivo*. This information is correlated with the differences in thermophysical properties, bioviability as well as toxicity and half-life features (Florindo et al., 2013).

The octanol-water partition coefficient (K_{ow}) is the most common method to access the hydrophobic/hydrophilic balance of a compound, due to the characteristics of octanol (organic solvent) that mimics the biological membranes (Florindo et al. 2013).

By definition, the activity of a compound in the water-rich phase and in the octanol-rich phase must be the same when equilibrium is reached. If the compound is sufficiently dilute in both phases, it can be considered at "infinite dilution", namely extremely dilute concentration (C_i). In these conditions, the activity coefficients should not change even with small variation in the concentrations. The K_{ow} value were calculate through de ratio between the concentration of the test compound in octanol phase(C_i^o) and the concentration of compound in water phase (C_i^w) (Ropel et al. 2005)

$$K_{ow} = \frac{C_i^o}{C_i^w} \quad (2.12)$$

The most common methods to determine K_{ow} are estimation by high performance liquid chromatography (HPLC), the generator column method, the "*Shake Flask"* and "*Slow Stirring"* method (Florindo et al. 2013; Ropel et al. 2005; Sangster 1989) In the HPLC technique, the retention time of the compound is compared with chemical references. However this type of measurement should only be used for K_{ow} values greater than one. For generator column method, water presaturated with octanol is slowly passed through a column which is packed with a solid support that has been coated with octanol containing a small amount of the test chemical. The liquid solution leaving the column is assumed to be in equilibrium with the octanol on the packing. The

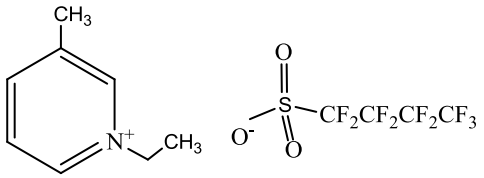
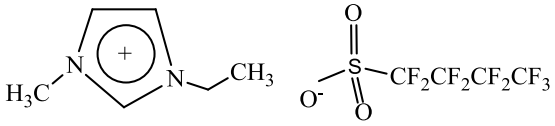
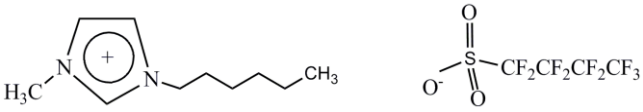
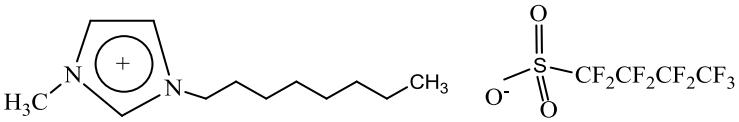
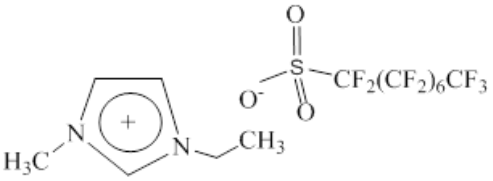
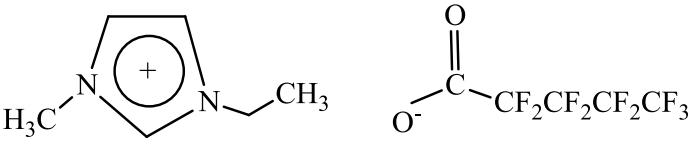
analysis of aqueous phase provides the K_{ow} value. However, some error could occur due to the interaction between the test chemical and the column packing. In "*Shake Flask"* method, the sample is vigorously stirred for approximately 1 hour at a constant temperature, 25ºC, the adequate amount of time for the equilibrium to be reached. Some errors can be detected due to the presence of microdroplets of octanol in water (Ropel et al. 2005).

In this study, the measurements were made with *Slow Stirring* method. In this method, after the pre-saturation of octanol and water and vice versa, a small amount of test chemical is mixed with octanol saturated. Then this mixture and the same volume of water saturated in octanol are placed in a glass vial. The sample is stirred slowly to minimize the stagnant diffusion layer between the phases while preventing emulsification. When the concentrations in each phase have stabilized, the chemical concentrations are measured in each phase to determine the K_{ow}. This method had the advantage to evaluate the diffusion of the compound during the time with diverse samplings (Ropel et al. 2005).The choice of this method is also related to the reliability of the method. Furthermore, a verification with the reported results on literature for conventional ionic liquids similar to FILs (ionic liquids based on bis(trifluoromethylsulfonyl)imide) confirms that this methods is the best for the study of FILs.

2.3.2.Materials

1-Ethyl-3-methylpyridinium perfluorobutanesulfonate, >97% mass fraction purity, 1-ethyl-3-methylimidazolium perfluorobutanesulfonate, >97% mass fraction purity, 1-hexyl-3-methylimidazolium perfluorobutanesulfonate, >99% mass fraction purity, 1-mehtyl-3-octylimidazolium perfluorobutanesulfonate,], > 98% mass fraction purity, 1-ethyl-3-methylimidazolium perfluorooctanesulfonate, >98% mass fraction purity were acquired at Iolitec. The purity of the commercial ionic liquids was checked by ^{1}H-NMR.

Table 2.9 - Chemical structure and respective abbreviation of fluorinated ionic liquids in study.

FIL designation	Chemical Structure
1-Ethyl-3-methylpyridinium perfluobutanesulfonate **[EtMepy][(PFBu)SO_3]**	
1-Ethyl-3-methylimidazolium perfluobutanesulfonate **[EtMeIm][(PFBu)SO_3]**	
1-Hexyl-3-methylimidazolium perfluorobutanesulfonate **[HexMeIm][(PFBu)SO_3]**	
1-Mehtyl-3-octylimidazolium perfluorobutanesulfonate **[OcMeIm][(PFBu)SO_3]**	
1-Ethyl-3-methylimidazolium perfluoroctanesulfonate **[EtMeIm][(PFOc)SO_3]**	
1-Ethyl-3methylimidazolium perfluoropentanoate **[EtMeIm][(PFBu)CO_2]**	

1-Ethyl-3-methylimidazolium perfluoropentanoate was synthesized in Molecular Thermodynamic Lab by ion exchange resin methods, as develop by Ohno et al (Fukumoto, Yoshizawa, and Ohno 2005). The purity of the final products was checked by ^{1}H, ^{13}C and ^{19}F NMR and elemental analysis.

All fluorinated ionic liquids were dried under vacuum (3.10^{-2} Torr) and vigorous stirring at 323.15K for at least 2 days, immediately prior to their use. This step is crucial in order to reduce volatile reduce volatile impurities. Structures and acronyms of all fluorinated ionic liquids are shown in *Table 2.3.1*. 1-Octanol ACS reagent, ≥ 99% purity, was acquired at Sigma-Aldrich and Milli-Q ultrapure water (Milli-Q Integral Water Purification System) was used in all experiments throughout the work.

2.3.3.Experimental Procedure

Slow Stirring Method

The first step of process was the mutual saturation of solvents, *Figure 2.18*, octanol and water. An equal quantity of both substances was vigorously mixed and when two different phases (water saturated in octanol and octanol saturated in water) were distinguishable, approximately after 72 hours, the separation was made.

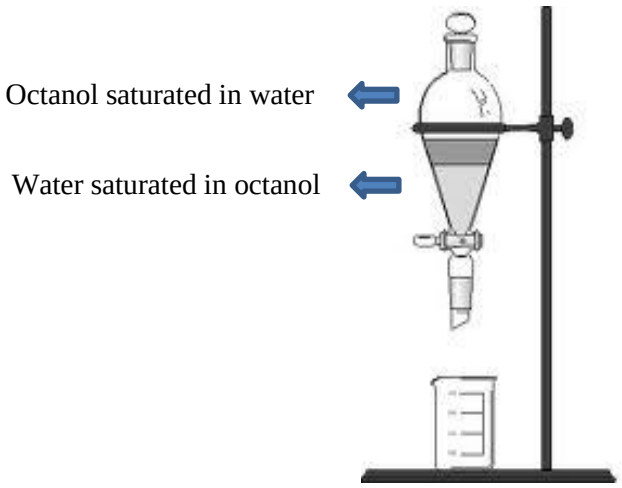


Figure 2.18 – Mutual saturation of solvents, octanol and water.

In a glass vial, with an open top screw-cap sealed with a septum of silicone and a magnetic stir bar, approximately 5 mL of water saturated in octanol was added. The same volume of solution containing the mixture of FIL and octanol was added carefully to the vial, to prevent the emulsification of solutions. The starting concentration of FILs in octanol phase varied from 0.25×10^{-3} mol/L to 3.88×10^{-3} mol/L.

To prevent the contamination during the sampling, a sealed syringe was inserted in the system before the addition of solution containing octanol and FIL.. The vials were stirred slowly to prevent emulsification and were maintained at room temperature ($25 \pm 2\ ^{0}$C). Samples were taken from the water-rich phase at least three times in a period between 24 and 48 days. The sampling process is illustrated in *Figure 2.19* and the systems are presented in *Figure 2.20*. The last sampling occurs when the concentrations in both phases were stabilized. At least three different concentrations were tested for each FIL

Figure 2.19 – *Slow Stirring* method, with samples at 25ºC

After sampling, octanol-rich phase was centrifuged for 1 hour at 3270 rpm and 4 ^{0}C using an Allegra® X-12R Centrifuge, *Beckmn CoulterECKAMN COULTER*, with aim to ensure the complete separation of residues that could come from water phase. This centrifugation also eliminates possible octanol micelles presents in the sample. The sampling occurs until the K_{ow} values no longer changed with time.

Concentrations of FILs were measured using UV-vis spectroscopy, model Shimadzu UV-1800, *Pharma-Spec spectrophotometer,* illustrated in *Figure 2.20*, at specific wavelengths (see *Table 2.10*) since this technique detects the aromatic ring on the cation. The uncertainty of the method is around 1%

Table 2.10 - λ_{max} of FILs used for absorbance determination

FIL designation	λ_{max} in water/nm	λ_{max} in octanol/nm
[EtMepy][(PFBu)SO_3]	265	266
[EtMeIm][(PFBu)SO_3]	211	211
[HexMeIm][(PFBu)SO_3]	211.5	210.5
[OcMeIm][(PFBu)SO_3]	212	212.5
[EtMeIm][(PFOc)SO_3]	212	211
[EtMeIm][(PFBu)CO_2]	210.5	211.5

Figure 2.20 – Shimadzu UV-1800 Pharma-Spec spectrophotometer

Samples were diluted in order to obtain absorbance values less than 1. For quantification in water phase the reference cell contained water saturated with octanol. However, for quantification in octanol phase the reference cell is filled with octanol saturated in water. The K_{ow} was measured at very dilute concentrations with aim to the best representation of the concentrations of FILs in contact with organism, and to avoid the formation of micelles, which difficult the measurement of quantification . This experimental method was validated by measuring three different stock solutions of two conventional ionic liquids acquired at *Iolitec*, 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide and 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, and comparing the results with previous literature values(brenecke). These commercial ionic liquids are the more similar to fluorinated ionic liquids, due to the number of fluoros in anion (bis(trifluoromethylsulfonyl)imide).

2.3.4.Results and Discussion

For biomedical applications, the behaviour of a chemical in contact with cell membranes is crucial. In this work, the K_{ow} values of different FILs were measured with aim to determine the affinity of these compounds to cross the cell membrane. This capacity needs to be low for artificial blood substitutes in order to promote the transport of respiratory gases through blood vessels without entry in cells. In *Table 2.11*, the concentration of FILs in water phase is showed as also the respective K_{ow} values. The uncertainty of the Kow value is

5%. This uncertainty could be linked to the variation in room temperature and to the fact that octanol and water form an emulsion that could lead to error in K_{ow} measurements. Furthermore, the solubility of water in octanol at 25ºC is quite larger than octanol in water and that fact could influence the partitioning of FILs between two solvents (Ropel et al. 2005).

Table 2.11 – K_{ow} values and concentration of FIL in water phase

FIL designation	K_{ow}	Concentration range in water phase/mol L^{-1}
[EtMepy][(PFBu)SO_3]	0.270 – 0.664	2.95×10^{-3} - 1.19×10^{-4}
[EtMeIm][(PFBu)SO_3]	0.3195	2.98×10^{-3} - 2.44×10^{-3}
[HexMeIm][(PFBu)SO_3]	4.27	7.07×10^{-4} - 5.23×10^{-4}
[OcMeIm][(PFBu)SO_3]	7.68	2.44×10^{-4}
[EtMeIm][(PFOc)SO_3]	2.96 − 4.35	2.5×10^{-4} − 1.87×10^{-4}
[EtMeIm][(PFBu)CO_2]	0.513 – 0.889	2.84×10^{-3} − 1.82×10^{-3}

In general the final concentrations of FILs in water rich phase vary from 1.87×10^{-4} $molL^{-1}$ to 2.98×10^{-3} $molL^{-1}$, *Table 2.11*. In octanol rich phase concentrations are between 6.17×10^{-4} and 3.30×10^{-3} $molL^{-1}$. For [EtMeIm][(PFBu)SO_3], [HexMeIm][(PFBu)SO_3] and [OcMeIm][(PFBu)SO_3] substantial differences in K_{ow} values were not observed, then it can be concluded that the dilute limit was reached for these FILs.In the case of [EtMepy][(PFBu)SO_3], [EtMeIm][(PFOc)SO_3] and [EtMeIm][(PFBu)CO_2] , K_{ow} were slightly dependent of concentration, even at dilute regions, with concentrations less than 3.88×10^{-3}mol/L. In this case *Table 2.11* shows the range of K_{ow} measured for the different concentrations. The same behaviour were verified in literature for some convencional ionic liquids (Ropel et al. 2005). In order to select the most appropriated FIL to develop an artificial blood substitute, the partition coefficients octanol-water must be analysed. The influence of the anion nature, carboxylate $[(PFBu)CO_2]^-$ and sulfonate $[(PFBu)SO_3]^-$ anions, in K_{ow} value for FILs with the same imidazolium cation $[EtMeIm]^+$ is shown in *Figure 2.21*. The analysis of the results for two FILs shows that the K_{ow} values for [EtMeIm] [(PFBu)CO_2] are approximately two times higher (0.684) than for [EtMeIm] [(PFBu)SO_3] (0.320). This result suggests that the carboxylate anion increase the hydrophobicity of the compound. However, a comparison between these K_{ow} values and the ones of some common

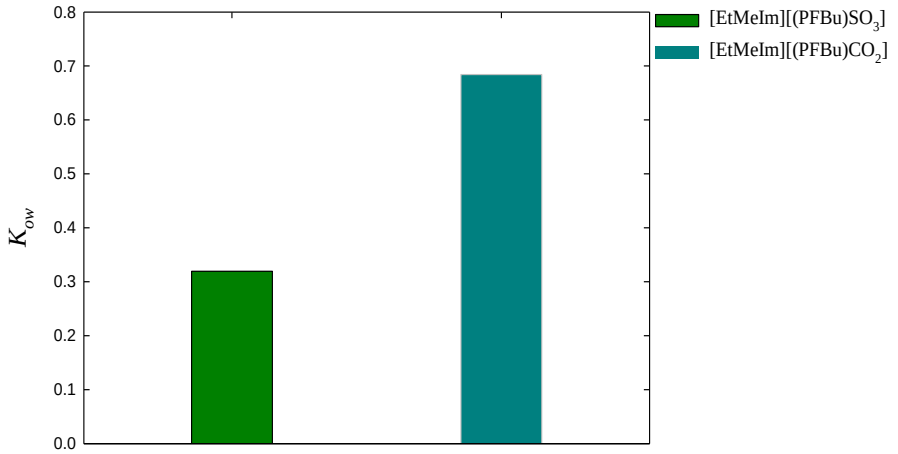


Figure 2.21 – K_{ow} values for sulfonate and carboxylate anion

organic compounds like caffeine (0.85), benzene (135) and cyclohexane (2754) shows that both values of these FILs are lower (Ropel et al. 2005). As a consequence both FILs can be considered extremely hydrophilic.

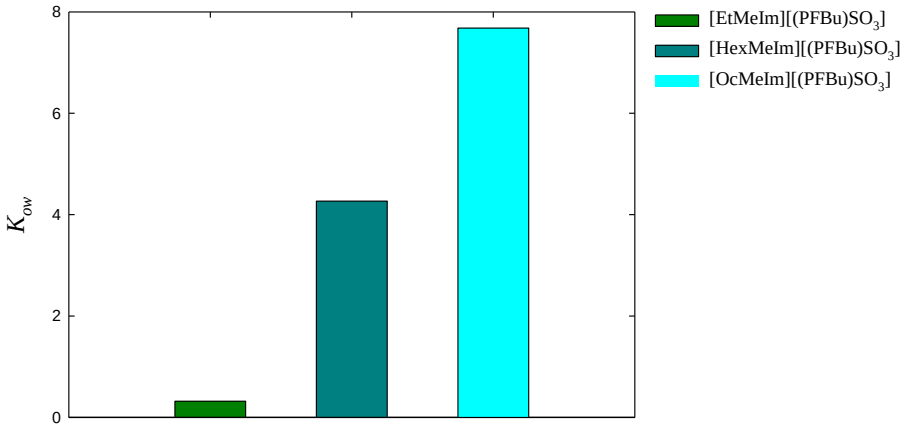


Figure 2.22 – K_{ow} values due to the increment of hydrogenated length chain

Another comparison was made in order to understand how the increment of hydrogenated length chain could influence the behaviour of FILs in contact with cell membranes (see *Figure 2.22*). Taking the analysis of results into consideration, it is possible to verify that the increment of the chain provides an increasing of K_{ow} values.

The obtained results are consistent with literature . In these papers, it was reported that solubility of imidazolium based ionic liquids in alcohols increases with alkyl chain length. These study sugest that greater van der Waals interactions between

FILs alkyl chain and octanol are responsible for this evidence (Ranke et al. 2007; Ropel et al. 2005).

Accordingly to these results [OcMeIm][(PFBu)SO_3] had a more hydrophobic behaviour than other imidazolium FILs showing an increasing of the ability of entry in cell membranes.

Another parameter that should be evaluated is also the increment of fluorinated alkyl chain length, *Figure 2.23*. Taking into account the same imidazolium cation, $[EtMeIm]^+$, the hydrophobicity of the FIL increases with the fluorinated alkyl chain length. In the case of [EtMeIm][(PFOc)SO_3], the K_{ow} value is much higher (3.15) than for [EtMeIm][(PFBu)SO_3] (0.320). In a same way that happened with the increment of hydrogen alkyl chain, van der Walls interactions between FILs fluorinated chain and octanol could promote the linkage or interaction between the FIL and cell membrane facilitating the entry in the cells.

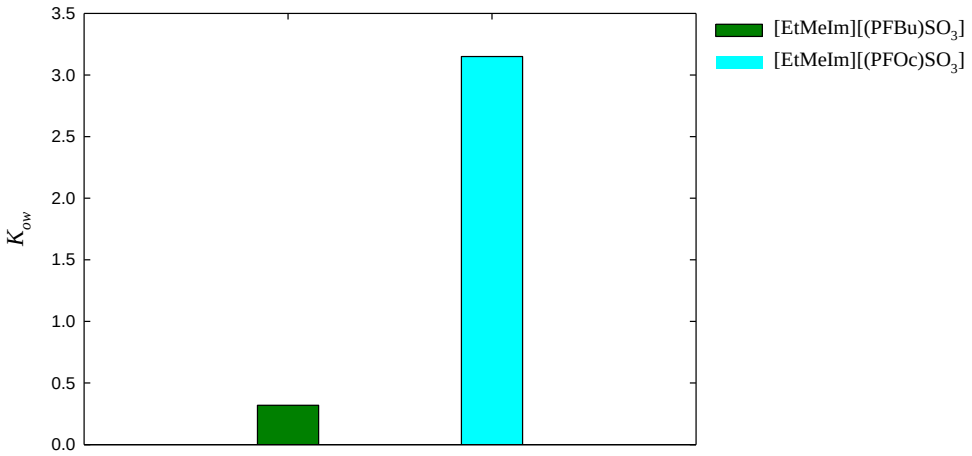


Figure 2.23 – K_{ow} values due to the increment of fluorinated length chain

In order to choose the best fluorinated ionic liquid, the cation nature was also evaluated (see *Figure 2.24*). Taking into account the same $[(PFBu)SO_3]^-$ anion, imidazolium $[EtMeIm]^+$ and pyridinium $[EtMepy]^+$ cations were compared. Both ionic liquids are hydrophilic, with low K_{ow} values and could be used for artificial blood substitutes. However $[EtMeIm]^+$ cation is the most appropriate. As mentioned above, these FILs have a low K_{ow} value in all range of studied concentrations whit values lower than some organic solvents. K_{ow} values of $[EtMepy]^+$ cation were slightly dependent of concentration even at very dilute concentrations. This is a disadvantage comparatively with $[EtMeIm]^+$ cation.

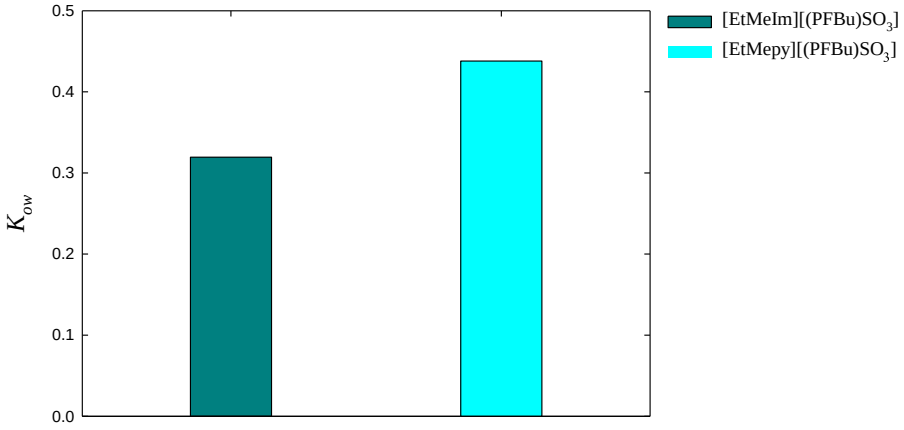
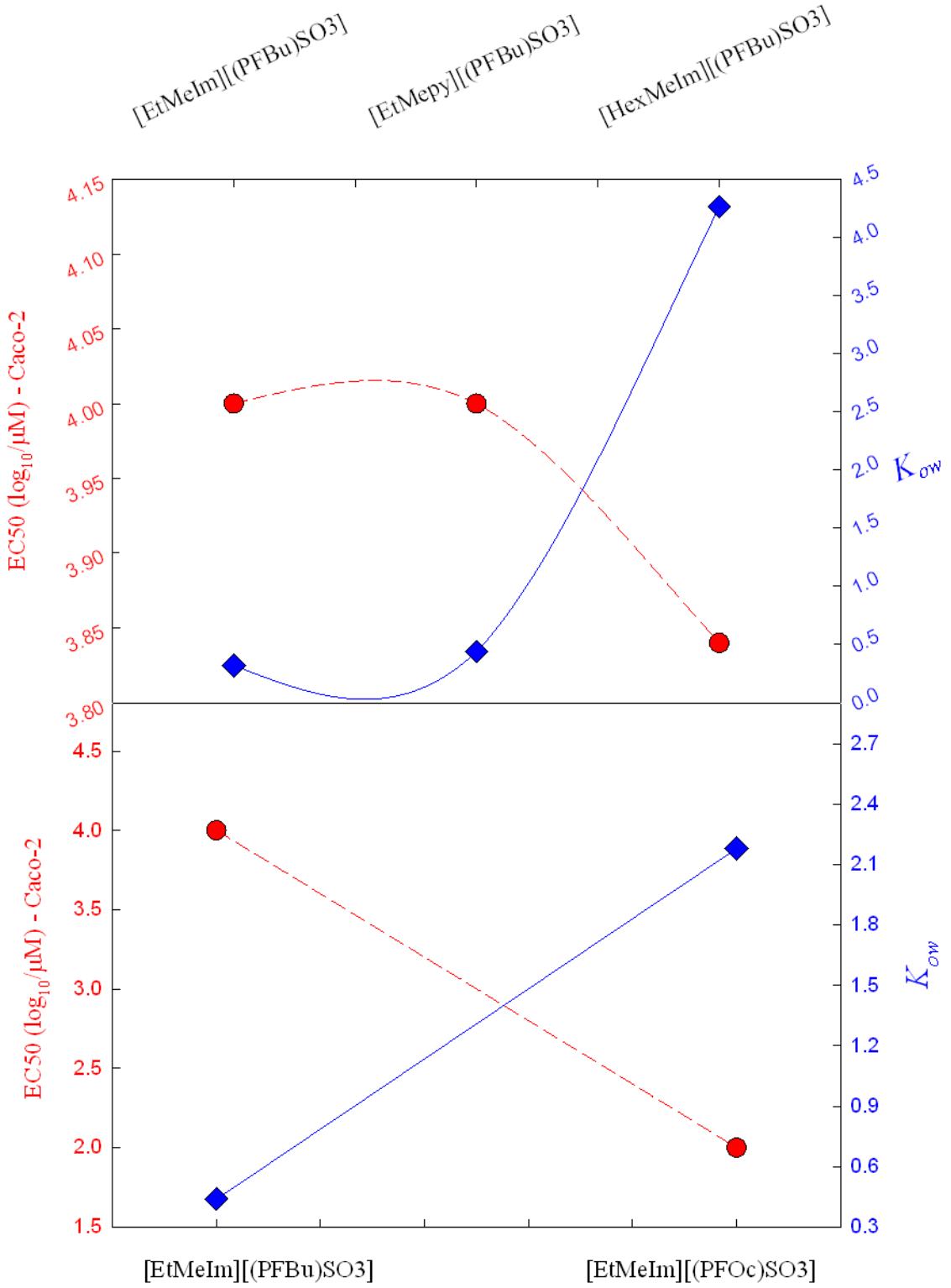


Figure 2.24 – K_{ow} values for different cations families

Finally, to compare data of cytotoxicity assays and Kow values, Figure 2.25 shows a possible correlation between the hydrophobicity/hydrophilicity of FILs and the EC50 values in Caco-2 cell line, cytotoxicity assays. The increasing of hydrophobicity, higher values of Kow, shows a decreasing in cell viability. These conclusions were in line with previous reports (Petkovic et al. 2011) . Furthermore, it is also possible to verify the influence of the increment in hydrogenated and fluorinated length chain in the K_{ow} values and consequently in the corresponding cytotoxicity. With longer length chains, the affinity of compound to entry in cell increases as also the cytotoxicity. Take into account this conclusion, the most appropriate ionic liquid for our goal application is [EtMeIm] [(PFBu)SO_3].


[EtMeIm][(PFBu)SO3]
[EtMepy][(PFBu)SO3]
[HexMeIm][(PFBu)SO3]
EC50 (log10/μM) - Caco-2
Kow
EC50 (log10/μM) - Caco-2
Kow
[EtMeIm][(PFBu)SO3]
[EtMeIm][(PFOc)SO3]

2.3.5.Conclusion

In order to select the most appropriate FIL to use in emulsions of artificial blood substitutes, several comparisons were made in this work. It was essential that blood substitutes kept in blood circulation to ensure the effective respiratory gases transport. Then, a hydrophilic behaviour plays an important role in this matter. This hydrophilic behaviour is connected with low K_{ow} values showing that low K_{ow} values are associated to a hydrophilic behaviour. The opposite behaviour was verified for higher values

Taking the partition coefficient octanol-water into consideration, it was possible to conclude that imidazolium cation is the more hydrophilic cation. Besides, it was also confirmed that the increment of both hydrogentated and fluorinated alkyl chain length was a disadvantage because it increases the hydrophobicity of FILs. Also, sulfonate anions are more hydrophilic than carboxylate anions.

Finally, taking all of these conclusions into account, [EtMeIm][(PFBu)SO_3] is the most suitable FIL to be used in the development of artificial blood substitutes.

2.4. General Conclusion

This work is part of a project focused on the development of a new and improved generation of artificial blood substitutes. Evaluations of several thermodynamic and thermophysical properties were carried out in order to select the most appropriate fluorinated ionic liquid to apply in this biomedical application.

The target FIL should be stable and liquid at a considerable range of temperatures, human body temperature included ($\approx$ 37 ^{0}C). Melting point was measured to determine the fusion temperature of these FILs. However, this value is not a limiting factor because the emulsion is also composed of water and perfluorocarbons, and the FIL could be solubilized in the corresponding emulsion. Fluorinated ionic liquids based on cholinium cation are an example of these compounds, their melting points cannot be determined because their decomposition temperature is lower than fusion temperature. However, the high solubility in water of these compounds makes them very useful.

Density, fluidity, iconicity and ionic conductivity of FILs were also evaluated. One disadvantage of PFCs-emulsions used nowadays as artificial blood substitutes is their low stability. Fluorinated ionic liquids increase the viscosity of PFCs-emulsion leading to a higher stability. Nevertheless once that blood substitutes are intravenous administrated that increment should not exceed values of blood fluidity. Also free molar volume and the increasing of fluorinated domains in PFCs-emulsion was evaluated to better understand the capacity of FIL to solubilize respiratory gases.

Another important factor is the toxicity of FILs in human cell lines. The results obtained provide us useful information to predict the less toxic anions and cations. Thus, it is possible to select the best FIL to apply in PFCs-emulsions. Several cell lines were evaluated at different concentrations. Expected concentrations of FILs in emulsions were lower than the tested for pure ionic liquids. Then, for biomedical applications, the highest concentrations linked to EC50 values will be never achieved.

Finally, the ability of FILs to cross cell membranes was evaluated through octanol-water partition coefficients. This parameter is directly linked with cytotoxicity. More hydrophobic compounds lead to a decrease in cell viability.

To sum up, all parameters considered in this work show that some cations and anions should be discarded for the development of a new and improved generation of artificial blood substitutes. The thermophysical and thermodynamic properties together

cytotoxicity tests show that $[NNf_2]^-$ anion should be excluded due to its high toxicity, high melting point and lower solubility in water. The increment of fluorinated and hydrogenated chain were also evaluated and all these parameters proved to be a disadvantage. Some families of different cations were analysed and imidazolium and pyridinium present the best results to be used in PFCs-emulsions. However, the utilization of imidazolium is restricted to short hydrogenated and fluorinated alkyl chains. FILs based on pyrrolidinium cation are also one of less toxic, but these FILs are practically insoluble and the high melting point is also a limiting factor. Cholinium cation is a good candidate too for biomedical applications due to its low toxicity and to its high water solubility described in literature (Araújo et al. 2014)Until now it can be conclude that pyridinium and cholinium cation, as also imidazolium with short hydrogenated chain are the most promising FILs when combined with perfluobutanesulfonate anion. However, more studies need to be done in order to evaluate the behaviour of PFCs-emulsions with FILs.

Finally, to conclude, this chapter present new and important data for scientific community and for industrial application of fluorinated ionic liquids. Until this work, previous reports with fluorinated ionic liquids are extremely scarce. To our knowledge, the cytotoxicity of these compounds was only evaluated in one report with FILs are available in our laboratory that have been used in this work with aim to compare the different anions and cations. Besides, there are yet no octanol-water partition coefficients data for fluorinated ionic liquids, defined as ionic liquids with fluorine tags equal or longer than four carbon atoms, in the open literature. This low number of previous reports reflects the innovative character of this study based on neoteric fluorinated ionic liquids. Furthermore, these experimental results provide relevant information for other biomedical applications.

3. Ionic Liquids with Pharmaceutical Active Anions

3.1.Introduction

Likewise as was mentioned in general introduction section, the use of ionic liquids in pharmaceutical research is in exponential growing. The unique characteristics of ionic liquids could be used with a lot of active pharmaceutical ingredients to improve their pharmacological activity. This concept of pharmacological design enables the balance between the desired pharmacological effect and the reduction of adverse side effects (Florindo et al. 2013). Also physical properties required for industrial applications and pharmaceutical manufacturing could be managed (Ferraz et al.,2011). Some previous studies reinforce the potential of ILs to dissolve poorly soluble APIs, as well as biochemical compounds, such as DNA and nucleic acid bases(Ferraz et al. 2011; Hough et al. 2007)In this work, API-ILs, compounds with cholinium cation and different active pharmaceutical ingredients, nalidixic acid (NAL), niflumic acid (NIF) and pyrazinoic acid (PYR) were synthetized, characterized and their toxicity and bioavailability were also evaluated. Once that our goal is the development of an efficient drug delivery system, that increase the efficiency of the pattern active pharmaceutical ingredients. All of these studies were performed in comparison with parent active pharmaceutical ingredients, available in the market.

First of all, the relevance of thermophisical behaviour for ionic liquids in biomedical applications was clarified. The addition of specific cations or anions leads to a modification in some properties of the original compounds. Melting and decomposition temperatures are crucial to determine the range of temperatures were APIs and API-ILs could be used at a liquid state without decompose.

Secondly, toxicity data is of full importance to the implementation of a drug delivery system in the market. All potential pharmaceutical formulation need to pass by *in vitro* tests to predict the *in vivo* results until get approved for commercialization. These studies were performed in order to determine the cytotoxicity of API-IL comparatively with parent API in two human cell lines, namely Caco-2 and HepG2 cells.

Finally and to analyse the absorption of API-ILs and APIs in cell membranes, the hydrophobic/hydrophilic balance was measured by octanol-water partition coefficients through Slow Stirring Method. This parameter is related to the

thermophysical properties and influences the bioavailability of compounds as well as its toxicity and half-life features(Florindo et al. 2013). In contrast with artificial blood substitutes, for this delivery application, a hydrophobic character is desired once that to have a pharmacological effect the compound need to entry in the cell.

Briefly, the goal of this work is to determine if the addition of cholinium in pharmaceutical active principles improves their therapeutic action.

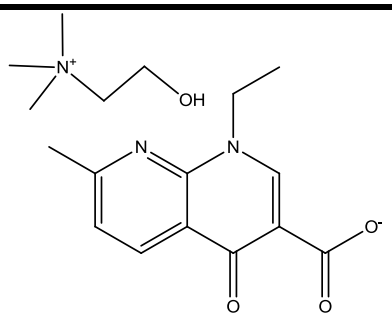
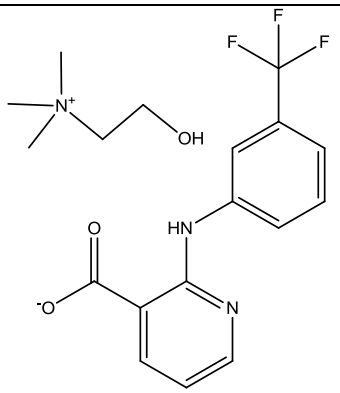
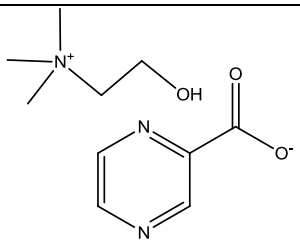
3.2.Materials

3.2.1.Parent APIs, ILs and API-ILs

Nalidixic acid, ≥98% mass fraction purity, niflumic acid, ≥98% mass fraction purity and pyrazinoic acid, 99% mass fraction purity were purchased from Sigma-Aldrich. The parent APIs were used without further purification. Cholinium chloride ((2-hydroxyethyl) trimethylammonium chloride), ≥98% mass fraction purity was purchased from Sigma-Aldrich. API-ILs were synthesized in Molecular Thermodynamic Lab by ion exchange resin method, as develop by Ohno *et al (Fukumoto, Yoshizawa, & Ohno, 2005).* The purity and structures of the final products was checked by ^{1}H and ^{13}C NMR, ESI mass spectra and elemental analysis.

Ionic liquid was dried under vacuum (3.10^{-2} Torr) at 323.15 K for at least 2 days, immediately prior to their use. This step is crucial in order to reduce volatile impurities, as well as water, which can influence the ionic liquids properties. The water content, determined by Karl Fisher titration, was less than 500 ppm. The chloride content (halide impurities), quantified by Chloride Ion Selective Electrode, and was found to be less than 500 ppm. Chemical structure of APIs are available in General Introduction, Figure 1.Whereas chemical structure and designation of API-ILs are summarized in Table 3.1

Table 3.1. Chemical structure and respective abbreviation of APIs and API-ILs in study

Cholinium based API-IL	Chemical Structure
Cholinium nalidixate	
Cholinium niflumate	
Cholinium pyrazinate	

3.2.2.Toxicity Assay

For cell culture, human colon carcinoma cells, Caco-2, were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen(DSMZ; Germany), human hepatocellular carcinoma cells, HepG2, were obtained from the European Collection of Cell Culture (ECACC; UK). Cell culture medium , RPMI 1640 medium, MEM medium and supplements, fetal bovine serum (FBS), L-Glutamine, penicillin-streptomycin solution, MEM nonessential amino acids (MEM-NEAA), sodium pyruvate (100x) and trypsin-EDTA solution were purchased from Gibco (Invitrogen Corporation, Paisley, UK).

For the *in vitro* cytotoxicity assays, CellTiter 96® Aqueous One Solution Cell Proliferation Assay was purchased from Promega (CA, USA)

3.2.3.Partition Coefficients – Solvents

1-Octanol ACS reagent, ≥ 99% purity, was acquired at Sigma-Aldrich and Milli-Q ultrapure water (Milli-Q Integral Water Purification System) was used in all experiments throughout the work.

3.3.Experimental Procedure

3.3.1.Thermal Properties

Thermogravimetric analyses (TGA) were carried out with a TA instrument Model TGA Q50, shown in *Figure 2.1*, where the thermal stabilities and decomposition temperatures of the APIs and cholinium based API-ILs were measured. Experimental procedure was detailed in Section 2.1.2. Samples were placed inside of aluminium pans and heated to 873.15 K at a rate of 274.15 K until complete thermal degradation was achieved.

A Differential Scanning Calorimeter (DSC), shown in *Figure 2.2*, was used to measure the thermal properties of the cholinium based API-ILs. Procedure was also detailed at *Chapter 2*, *Section 2.1.2*. Melting temperature was selected at a rate of 1 ºC/min for cholinium nalidixate and cholinium niflumate and 5 ºC/min for cholinium pyrazinate.

3.3.2.In vitro cytotoxicity assays

Cell culture procedure for Caco-2 and HepG2 cells was described in *Section, 2.2.3* as well as *MTS* assay. The major difference between cytotoxicity studies with FILs and API-ILs is the range of tested concentrations. Due to poor solubility of APIs in cellular medium, all samples of APIs and API-ILs were firstly prepared in Milli-Q water and then the minimum dilution with culture medium was reached to avoid precipitation. Similar concentrations were tested for APIs and API-ILs in order to evaluate the influence of cholinium in cellular viability of these cell lines.

3.3.3.Octanol-water partition coefficients

Partition coefficients of APIs and API-ILs were measured according *Slow Stirring* method described in *Section 2.3.3*. For parent APIs and API-ILs, the started concentration in octanol phase varied from 0.007 g/L to 0.014g/L. Samples were

measured until 42 days, when the concentrations in both phases were stabilized. The last sampling occurs when the concentrations in both phases were stabilized.

Glass vials with the samples were kept isolated of light, due to photosensitizer characteristic of nalidixic and niflumic acid. Concentrations of compounds in water phase were measured using UV-vis spectroscopy (Shimidzu UV-1800, *Pharma-Spec spectrophotometer, Figure 2.20*), at specific wavelengths, *Table 3.2*.

Table 3.2 - λ_{max} of APIs and API-ILs used for absorbance determination

Parent API designation	**λ_{max} in water/nm**	**λ_{max} in octanol/nm**
Nalidixic acid	285	258
Niflumic acid	286	293
Pyrazinoic Acid	268	268
API- ILs designation	**λmax in water/nm**	**λmax in octanol/nm**
Cholinium nalidixate	258	258
Cholinium niflumate	287	293
Cholinium pyrazinate	268	268

3.4.Results and discussion

3.4.1.Thermal properties

With the analysis of melting and decomposition temperatures, it is possible to define the liquid range of the fluids and their range of application. The thermal properties (melting point, thermal stabilities, decomposition temperature and glass transition temperature) of API-ILs are presented in *Table 3.1*. Additionally, a comparison of melting temperatures with literature values for parent API is illustrated in *Figure 3.1* (Araújo et al. 2014a; Bustamante et al. 2002).

Table 3.3 – Melting temperature, T_m, decomposition temperature, T_{dec}, and glass transition temperature, T_g of API-ILs in study

API- ILs designation	**T_m / K**	**T_{dec} / K**	**T_g / K**
Cholinium nalidixate	349.22	434.14	256.34
Cholinium niflumate	268.45	461.62	253.70
Cholinium pyrazinate	354.46	452.56	226.00

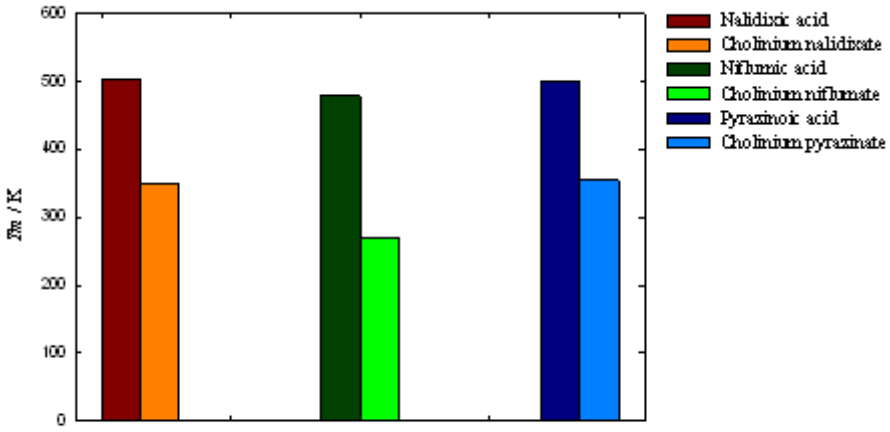


Figure 3.1 – Melting temperature of parent APIs and API-ILs
(Araújo et al., 2014)

One of the major problems in pharmaceutical industry has been attributed to physical properties of APIs. Most of APIs are marketed in solid state, however, there are some disadvantages related to this kind of formulation. The conversion of these APIs to a liquid formulation could be a great step to pharmaceutical industry(Ferraz et al. 2011; Hough et al. 2007).

In *Table 3.3*, thermal properties of API-ILs formulations are summarized and it is possible conclude that the presence of cholinium cation directly influences the thermal stability of compounds, once that all compounds had approximately the same temperature of decomposition, 400K.

The major advantage for thermal properties in incorporating cholinium cation with APIs is related to the decrease in melting temperature, *Figure 3.1,* in comparison to the parents APIs. All melting points decrease bellows 373.15 K, and the major decrease was verified for cholinium niflumate that are now liquid at room temperature.

Besides cholinium pyrazinate have a higher melting point than room and body temperature, this API-IL remains liquid upon cooling until it reaches a glass transition at low temperature, 226.00 K. Upon heating above glass transition, had an exothermic crystallization peak (294.19 K), cold crystallization temperature, followed by an endothermic melting peak at 354.46 K. Thus, depending on the cooling-heating cycles this compound can be easily handled as liquid at room temperature. This represents an advantage to development of new drug delivery systems (Araújo et al. 2014).

3.4.2.In vitro cytotoxicity assays

Biomedical application of API-ILs requires cytotoxicity measurements, in order to establish the biocompatibility of compounds in the organism. The aim of this study is to evaluate the impact of cholinium based APIs in comparison with parent APIs in cell lines mentioned above at different ranges of concentrations. Figure 3.2, Figure 3.3 and Figure 3.4 illustrates the cellular viability of the three parents APIs and API-ILs in study, in both cell lines.The range of study concentration varied from 10 μM to 6000 μM depending of the compound. All tested concentrations are in accordance with pharmacokinetic parameter maximum plasma concentration of the parent APIs, which is above possible intracellular concentrations. For nalixidic acid and cholinium nalixidate, Figure 3.2, the tested concentration varied from 20 μM to 57.5 μM.

Nevertheless, the maximum plasma range of concentrations accepted for nalidixic acid is between 53.82 μM and 61.58 μM.

For niflumic acid and cholinium niflumate, Figure 3.3, the tested concentrations varied from 10 μM to 35 μM. Nevertheless, the maximum plasma range of concentrations accepted for niflumic acid is between 20.25 μM and 25.16 μM.

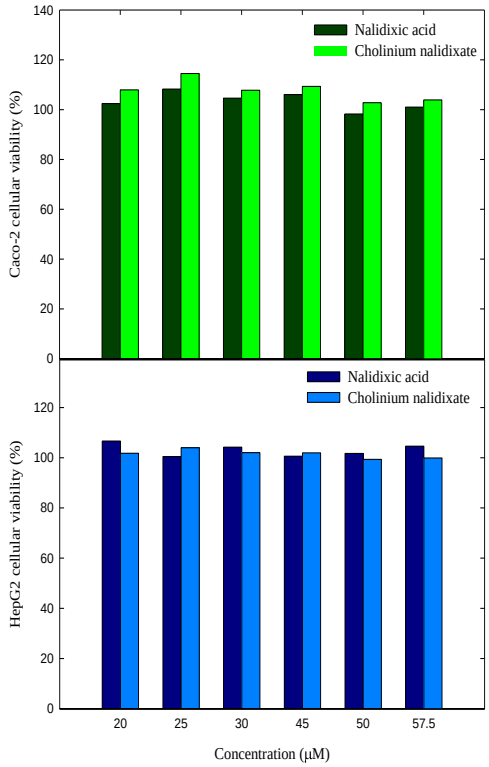


Figure 3.2 – Cellular viability for nalidixic acid and cholinium nalidixate in Caco-2 and HepG2 cell lines

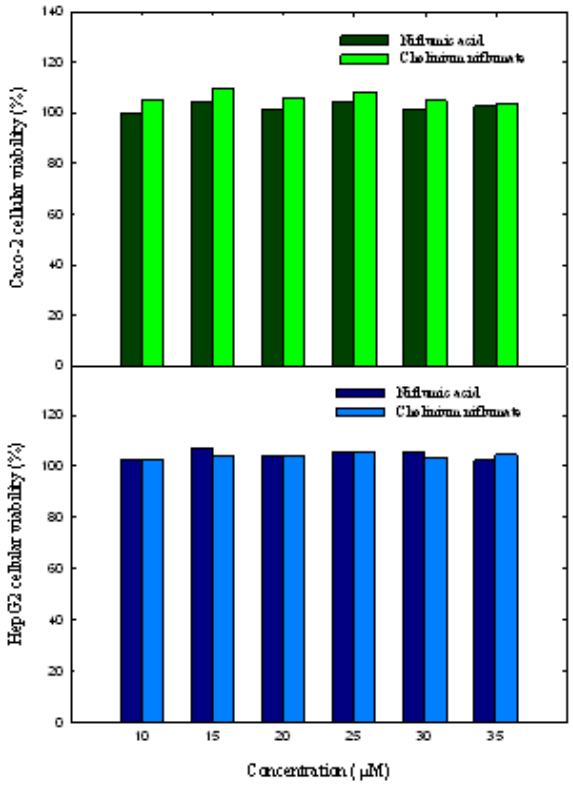


Figure 3.3 – Cellular viability for niflumic acid and cholinium niflumate in Caco-2 and HepG2 cell lines

Finally, for pyrazinoic acid and cholinium pyrazinate, the tested concentrations varied from 500 μM to 6000 μM (*Figure 3.4*). Nevertheless, the maximum plasma range of concentrations accepted for pyrazinoic acid is between 170.03 μM and 202.26 μM.

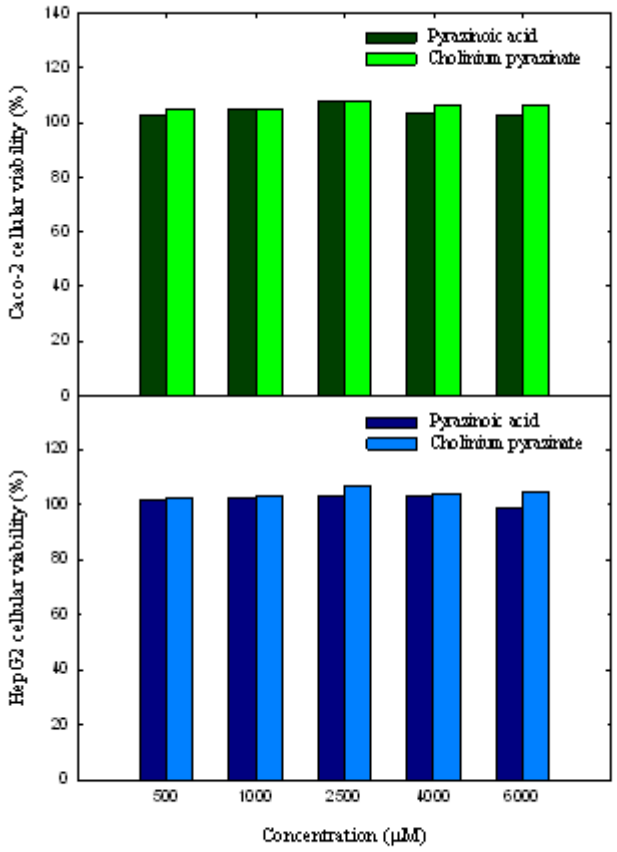


Figure 3.4 – Cellular viability for pyrazinoic acid and cholinium pyrazinate in Caco-2 and HepG2 cell lines

The cellular viability for both cell lines does not change significantly. Values of cellular viability in all concentrations for both APIs and API-ILs are around 100%. Sometimes higher values than 100% were achived due to the hormetic response, characterized by low-dose stimulation and a high-dose inhibition in the presence of the test compound (Calabrese and Baldwin 2003) *Figure 3.2*, *3.3* and *3.4*). Cholinium is an essential nutrient presents in diverse functions of organism then it was supposed to have a high biocompatibility in human cell lines. (refseddo). This result confirms the behaviour that was expectable due to the nature of cholinium. Thereby no cytotoxicity was detected, as also none significance difference was observed in the metabolic activity of cells due to the incorporation of cholinium cation.

In conclusion, the design of API-ILs formulations does not show biocompatibility problems in comparison to the classical approach of APIs.

3.4.3.Octanol-water partition coefficients

The partition of active pharmaceutical principles represents the trend of the chemical to keep in circulation or passes through cells membranes. According to the mentioned in introduction and experimental procedure sections, the concentrations of APIs and API-ILs in water and octanol were measured to determine the affinity of the test compound to cross the cell membrane. Cholinium were described as a benign and high hydrophilic cation (Petkovic et al.2011) and the addition of cholinium to parent APIs greatly increases the solubility of these compounds (Araújo et al., 2014).In this work, it was essential to evaluate if the addition of cholinium could interfere with the partition of APIs. In *Table.3.4,* the concentration of APIs and API-ILs in water phase is shown, as also the respective K_{ow} values. In *Figure 3.5*, is represented the average of K_{ow} values for both compounds, parent APIs and API-ILs. The uncertainty of the quantitive method was around 1% and the uncertainty of K_{ow} values are 10%. This last value like was mentioned in *Section 2.3.1* could be related to diverse factor like the variation in room temperature, the different solubilities between octanol and water at room Moreover, octanol and water form an emulsion that could lead to error in K_{ow} measurements (Ropel et al.,2005).

Table3.4 – K_{ow} values and concentration of compound in water phase

API	**K_{ow}**	**Concentration range in water phase/g L^{-1}**
Nalidixic acid	2.39	2.3×10^{-3}-4.9×10^{-3}
Niflumic acid	9.77-11.2	1.1×10^{-3}-1.4×10^{-3}
Pyrazinoic Acid	0.344	6.6×10^{-3}-1.4×10^{-2}
API- ILs designation	**K_{ow}**	**Concentration range in water phase/g L^{-1}**
Cholinium nalidixate	2.18	2.3×10^{-3}-4.8×10^{-3}
Cholinium niflumate	4.13-7.32	1.4×10^{-3}-1.7×10^{-3}
Cholinium pyrazinate	0.20-0.32	1.0×10^{-3}-1.1×10^{-3}

To niflumic acid, cholinium niflumate and cholinium pyrazinate the K_{ow} value are dependent of concentration. Furthermore, *Figure 3.5* illustrates the differences between Kow values with parent APIs and API-ILs. These results suggest that no significant changes occur with this new approach.

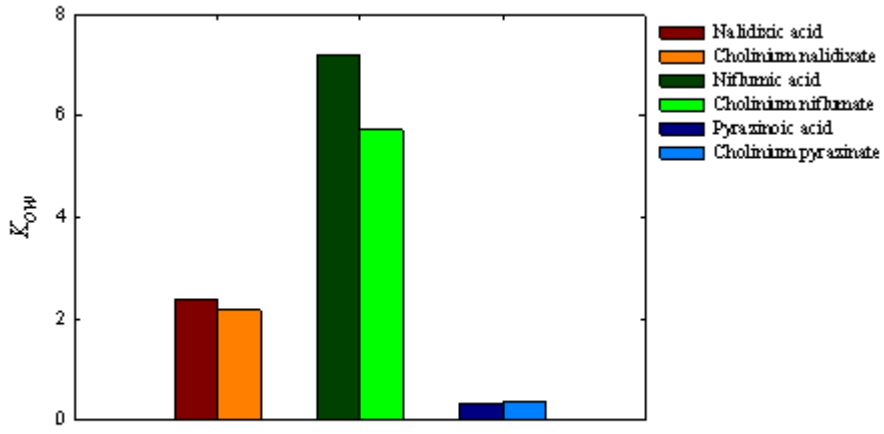


Figure 3.5 – Kow values for APIs and API-ILs

Taking into account the increment in solubility and the changes no significant in partition properties with addition of cholinium (Araújo et al., 2014) , it was possible to conclude that there will be an increasing of bioavailability of these compounds.

3.5.Conclusion

Finally is possible to conclude that this new cholinium based ILs approach with active pharmaceutical anions (nalidixic acid, niflumic acid and pyrazinoic acid) could effectively improve the solubility of parents APIs without affect cytotoxicity and penetrance into cell membranes, reducing the major problems in pharmaceuticals manufacturing. Additionally the low cost of cholinium cation and their own properties (biocompatibility, low toxicity and high water solubility) make this new design strategy a great opportunity to pharmaceutical industry to reuse some chemicals previous rejected during development process and turn the acceptance of novel APIs candidates easily.

4. Final Remarks

4.1 General Conclusion

This work is initially part of a project FCT: PTDC/EQU – FTT/118800/2010 focused on the development of a new and improved generation of artificial blood substitutes containing fluorinated ionic liquids. However due to the collaborations opportunities in Thermodynamics laboratory there was the possibility to evaluate other pharmaceutical opportunities using ionic liquids, namely cholinium based API-ILs.

The overall of the work is to evaluate thermophysical properties, toxicity profiles and octanol-water partition coefficients of some FILs. Besides, the comparison between parent APIs and cholinium based APIs were also achieved for the same properties mentioned before.

Take into account the first part of the work, the FILs who better accomplish the characteristics of a blood substitute, conjugating all the results of thermophysical properties, cytotoxicity and partition properties were [EtMeIm][(PFBu)SO3], [EtMepy][(PFBu)SO3] and [NM3(EtOH)][(PFBu)SO3].

Finally, it was also possible to conclude that the formulation of API-ILs with the cholinium cation represent an advantage for pharmaceutical research. Once that API-ILs present a higher solubility in water than APIs and the toxicity and the partition for cell membranes does not alter. With a higher solubility and the same partition the bioavailability of the original compouns increase a lot.

4.2 Future Work

Fortunately the numerous applications for ionic liquids make this area very attractive for different search fields and future work will always be innovative.

As future work and in order to continue our project some FILs recently synthetized should also be characterized for these properties (thermophysical properties, cytotoxicity and bioavailability). The results obtained in this work will help in the design of the most suitable FILs for application in artificial blood substitutes and had a great importance also in a fundamental research as in an application level.

Firstly and to evaluate the partition properties of FILs without aromatic ring the HPLC technique will be optimized. Some toxicity tests will be done to understand the mechanism involved in cellular death.

After select the FIL to incorporate in current PFCs emulsions all the tests made until now should also be done, in order to evaluate the systems behaviour, to discard some possible side effects of the mixture.

The development of this new cholinium based ILs give the opportunity too many pharmaceutical discarded by lack of solubility came to the market. The evaluation of more possible combination and the formulations of new API-ILs that improve the characteristics of pattern drugs are also in our pipeline.

5. References

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6. Scientific Publications

Papers

- Published:

1. "Cholinium-Based Ionic Liquids with Pharmaceutically Active Anions". João M. M. Araújo, Catarina Florindo, Ana B. Pereiro, Nicole S. M. Vieira, Ana Matias, Catarina M. M. Duarte, Luís P. N. Rebelo, Isabel M. Marrucho. RSC Adv., 2014,4.

- In preparation:

1. "Phase Equilibria and Surfactant Behaviour of Fluorinated Ionic Liquids with water". Fabiana S. Teixeira, Nicole Vieira, Olga A. Cortes, J. M. M. Araújo, I. M. Marrucho, L. P. N. Rebelo, A. B. Pereiro, Nicole S. M. Vieira

2. "Perspectives on fluorinated ionic liquids: properties and applications". Nicole M. Vieira, Patrícia M. Reis, Olga A. Cortes, João M. M. Araújo, Isabel M. Marrucho, José N. C. Lopes, J. M. S. S. Esperança, Ana B. Pereiro, Luis P. N. Rebelo

3. ***"Evaluation of cytotoxicity and partition properties of fluorinated ionic liquids***". Nicole M. Vieira, João M. M. Araújo, Sara Nunes, Ana Matias, Isabel M. Marrucho, Catarina M. M. Duarte, Ana B. Pereiro, Luís P. N. Rebelo

Posters in Scientific Meetings

- **Accepted for Presentation:**

1. ***"Ionic Liquids for Biological Applications"***. João M. M. Araújo, Ana B. Pereiro, Nicole S. M. Vieira, Ana Matias, Luís P. N. Rebelo, Isabel M. Marrucho. The Green Chemistry Gordon Research Conference, July 27 – August 1, 2014, Hong Kong, China.

2. ***"Evaluation of Cytotoxicity and Partition Properties of Fluorinated Ionic Liquids"***. Nicole S. M. Vieira, João M. M. Araújo, Sara Nunes, Ana Matias, Isabel M. Marrucho, Catarina M. M. Duarte, Ana B. Pereiro, Luís P. N. Rebelo. EUCHEM2014, Molten Salts and Ionic Liquids XXV, 6-11 July 2014, Tallinn, Estonia.

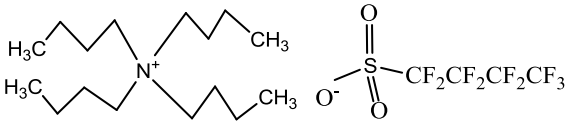
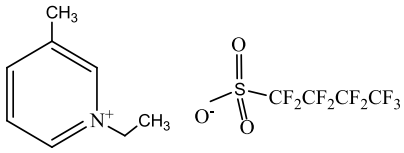
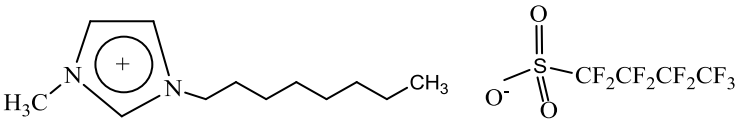
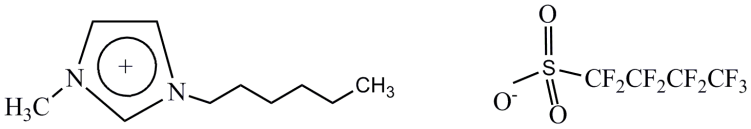
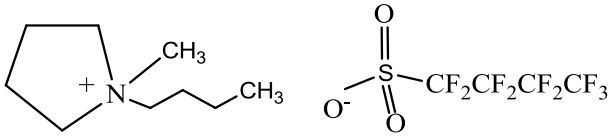
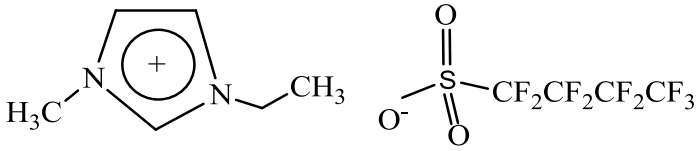
3. ***"PFCs Environmental Impact Mitigation using Fluorinated Ionic Liquids"***. Nicole S. M. Vieira, Patrícia M. Reis, Olga A. Cortes, João M. M. Araújo, Isabel M. Marrucho, José N. C. Lopes, J. M. S. S. Esperança, Ana B. Pereiro, Luis P. N. Rebelo. EUCHEM2014, Molten Salts and Ionic Liquids XXV, 6-11 July 2014, Tallinn, Estonia.

4. ***"Liquid-Liquid, Solid-Liquid Equilibria and Ionic Conductivity of Binary (Fluorinated Ionic Liquids + Water) Systems"***. Fabiana T. Sousa, Olga A. Cortes, Nicole S. M. Vieira, João M. M. Araújo, Isabel M. Marrucho, Luís P. N. Rebelo, Ana B. Pereiro. EUCHEM2014, Molten Salts and Ionic Liquids XXV, 6-11 July 2014, Tallinn, Estonia.

5. ***"Pharmaceutically Active Cholinium-Based Ionic Liquids combined with Acid-Derived Anions"***. João M. M. Araújo, Catarina Florindo, Ana B. Pereiro, Nicole S. M. Vieira, Ana Matias, Catarina M. M. Duarte, Luís P. N. Rebelo, Isabel M. Marrucho. EUCHEM2014, Molten Salts and Ionic Liquids XXV, 6-11 July 2014, Tallinn, Estonia.

Appendix

Table A.1 - Chemical structure and respective abbreviation of fluorinated ionic liquids in literature (Cortes, 2013; Pereiro et al., 2013)

FIL Designation	Chemical Structure
Tetrabutylammonium perfluorobutanesulfonate **[NBu_4][$(PFBu)SO_3$]**	
1-Ethyl-3-methylpyridinium perfluobutanesulfonate **[EtMepy][$(PFBu)SO_3$]**	
1-Mehtyl-3-octylimidazolium perfluorobutanesulfonate **[OcMeIm][$(PFBu)SO_3$]**	
1-Hexyl-3-methylimidazolium perfluorobutanesulfonate **[HexMeIm][$(PFBu)SO_3$]**	
1-Butyl-1-methylpyrrolidinum perfluorobutanesulfonate [**BuMepyr][$(PFBu)SO_3$]**	
1-Ethyl-3methylimidazolium perfluorobutanesulfonate **[EtMeIm][$(PFBu)SO_3$]**	
1-Butyl-N-methylpyrrolidinium bis(nonafluorobutylsulfonyl)imide **[BuMepyr][NNf_2]**