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Ecotoxicity and Hemolytic Activity of Fluorinated Ionic Liquids

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Abstract: The task-specific design of ionic liquids (ILs) has emerged in several industrial and pharmaceutical applications. The family of ILs with fluorine tags equal to or longer than four carbon atoms, the fluorinated ionic liquids (FILs), combine the best properties of ILs with the ones of perfluorinated compounds, and are being designed for several specific purposes. In the pharmaceutical field, there is an urgency to search for novel antibacterial agents to overcome problems associated to antimicrobial resistances. Then, the main purpose of this work is to evaluate the environmental impact and the ability of FILs to be used as antibacterial agents against *Pseudomonas stutzeri* bacteria. Beyond its rare pathogenicity, these bacteria are also used as a bioremediation agent to treat several contamination sites. Then, it is important to determine which FILs have antibacterial properties, and which do not impact the bacterial growth. The biocompatibility of FILs was also evaluated through their hemolytic activity and represent a step forward the application of FILs in pharmaceutical applications. The results proved that high concentrations of FILs can have a reduced ecotoxicity and a high biocompatibility. $[C_8C_1Im][CF_3SO_3]$ was identified as the most promising compound to be used as an antibacterial agent since it prevents the growth of bacteria at concentrations compatible with the red blood cells' viability.

Keywords: fluorinated ionic liquids; *Pseudomonas stutzeri*; antibacterial properties; hemolytic activity

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1. Introduction

The task-specific design of materials and the improvement of industrial processes through sustainable and greener ionic liquids (ILs) have emerged in the last decades in many fields of interest, such as catalysis [1–3], energy and electrochemistry [4], lubricants [5], the design of new materials for gas absorption [6], separation and extraction processes [7–9], the design of stimuli-responsive materials and sensors [10,11], and biomedical and pharmaceutical applications [12–17]. The application of ILs in such broad range of fields are supported by their great tuneability, since their final properties are dependent on the cation–anion selected pair. Amongst the most advantageous properties of ILs are their high thermal stability and "green nature", endorsed by their low flammability and reduced vapor pressure [18–21], as well as by their biocompatibility and reduced environmental toxicity associated to several cation–anion combinations [22,23].

The use of ILs in the pharmaceutical field have covered topics such as the screening for antifungal, antiviral, antioxidant and anticancer properties (such as the capacity to inhibit tumor progression), the design of new pharmaceutics and drug delivery systems, as well as the improvement of several analytical techniques and drug synthesis processes [12–17]. Furthermore, the urgent need to evaluate the antibacterial properties of ILs has also been noticed, in order to screen for new substances with potential pharmacological value that may represent a better alternative to traditional treatment and an advantage regarding

the constant increment of antimicrobial resistances that hampers the treatment of several infections. The ability to inhibit the growth of several pathogens has been detected for several common imidazolium, pyridinium, ammonium, piperidinium and pyrrolidinium-based ILs, among others [22,24–30]. Moreover, it is well known that the increment of the alkyl side chain length plays a major role in the antimicrobial potency of ILs, which can be related to a mechanism of cell membrane disruption boosted by the higher hydrophobicity associated with longer alkyl chains-based compounds [27–30]. However, the introduction of hydroxyethyl groups in the IL side chain causes a reduction of the antimicrobial activity [30,31]. Although the anion effect has been generally less studied in comparison to the cation core [24], the selection of metal-based anions has been proved to significantly impact the microbial viabilities, which can be linked to the capacity of these compounds to produce reactive oxygen species and protein denaturation, which can destabilize several cellular pathways [32]. ILs have demonstrated their clinical value as antimicrobial agents through the ability to form antibacterial films towards a broad range of pathogens, to enhance the antibacterial activity of several antibiotics when synthesized in an IL-based platform, such as an ampicillin-based IL, and to avoid the development of biofilms of multidrug-resistant species [33,34]. Moreover, due to these antimicrobial properties, ILs can also be used in industry as protective agents to preserve several materials and cultural heritage [14,23]. During these screening processes, a proper balance between the antimicrobial activity and the hemocompatibility and cytotoxicity of the compounds must occur. Moreover, the screening and selection approaches must be carried out with some caution and must always consider the final objective. For biomedical approaches, the growth inhibition of microbial species is desirable. However, when considering bioremediation and biotechnological processes such as biocatalysis and biotransformation, involved in ethanol production, dairy industry, and biosorption of uranium, this growth inhibition causes a loss in the effectiveness of the process [35–38]. Then, when the main goal is to select ILs to be used in biotechnological processes, their impact on microbial or enzymatic species must be reduced.

Within the ILs category, there is a specific family of compounds—the fluorinated ionic liquids (FILs)—characterized by having fluorinated tags equal to or longer than four carbon atoms [39,40] that combine the best properties of ILs with the outstanding properties of conventional perfluorocarbons, such as high surfactant behavior, low surface tension and chemical and biological inertness [41,42]. These compounds have three nanosegregated domains (polar, nonpolar, and fluorinated) enabling the formation of unique structures and the solubilization of different types of compounds [40,43–45]. Few studies were conducted by our group reporting the negligible cytotoxicity of FILs with short hydrogenated and fluorinated side chains in four different human cell lines (Caco-2, HepG2, Ea.hy926 and HaCa-T) [39,46]. Additionally, no acute ecotoxicity was detected for different perfluorobutanesulfonate and perfluoropentanoate-based FILs towards different aquatic microorganisms with different hierarchical orders, such as bacteria (*Vibrio fischeri*), crustaceans (*Daphnia magna*) and an aquatic plant (*Lemna minor*) [31]. These results support the application of FILs for either industrial processes or pharmaceutical applications, as well as for their use as a sustainable alternative for traditional perfluorocarbons displaying low solubility and high toxicity. These properties have endorsed their application in pharmaceutical development, in which they have been tested as delivery and stabilizing agents for two different biomolecules, lysozyme and bovine serum albumin (BSA), without inducing protein conformational changes [47–49]. Imidazolium and pyridinium-based FILs have demonstrated the ability to encapsulate lysozyme with relatively high efficiencies, without affecting the biological activity of the biomolecule [47,48]. Besides, both imidazolium and choline-based FILs were proved to stabilize BSA, and interaction studies between the aromatic FIL and the biomolecule evidenced the encapsulation of BSA, as well as the maintenance of the FIL–protein interaction [49].

The main goal of this study is to evaluate the antimicrobial activity of several FILs, as well as their environmental safety, against the denitrifying *Pseudomonas stutzeri* (*P. stutzeri*)

bacteria. This Gram-negative bacterium is widely distributed in the environment and intensively used for environmental studies [50–53]. *P. stutzeri* is also considered a bioremediation agent for several contaminants in a cost-effective and environmentally friendly approach [52,53]. Its ability to remove metal working fluids, pesticides and polycyclic aromatic hydrocarbons has already been addressed, as well as the removal of dyes after a process of acclimation using ionic liquids [35]. Additionally, these bacteria were also used to produce and isolate new biocorrosion and antibacterial agents [54,55]. Unfortunately, *P. stutzeri* were isolated from hospital environments, and associated with patients suffering from human immunodeficiency virus disease being considered a rare, but opportunistic pathogen [50]. To the best of our knowledge, the research dealing with *P. stutzeri* and ionic liquids was mostly focused on the improvement of bioremediation technologies [35]. This work will be focused on the screening for FIL-based antibacterial compounds, supported by the bacterial growth and hemocompatibility assays. The bacterial growth assays will be also used to select ecofriendly FILs that can be applied in industrial processes or released in the environment without prejudice for the growth of these bacteria present in several bioremediation processes.

2. Materials and Methods

2.1. Materials

The fluorinated ionic liquids used in this work were: 1-ethyl-3-methylimidazolium perfluoromethanesulfonate ($[C_2C_1Im][CF_3SO_3]$, >99% mass fraction purity), 1-methyl-3-octylimidazolium perfluoromethanesulfonate ($[C_8C_1Im][CF_3SO_3]$, >99% mass fraction purity), 1-ethyl-3-methylimidazolium perfluorobutanesulfonate ($[C_2C_1Im][C_4F_9SO_3]$, >97% mass fraction purity), 1-mehtyl-3-octylimidazolium perfluorobutanesulfonate ($[C_8C_1Im][C_4F_9SO_3]$, >98% mass fraction purity), 1-ethyl-3-methylpyridinium perfluorobutanesulfonate ($[C_2C_1py][C_4F_9SO_3]$, ≥99% mass fraction purity) and choline ((2-hydroxyethyl)trimethylammonium) perfluorobutanesulfonate, ($[N_{1112(OH)}][C_4F_9SO_3]$, ≥97% mass fraction purity). These ILs were supplied by IoLiTec GmbH (Salzstraβe, Heilbronn, Germany). On the other hand, 1-ethyl-3-methylimidazolium perfluoropentanoate ($[C_2C_1Im][C_4F_9CO_2]$, >99% mass fraction purity), 1-ethyl-3-methylpyridinium perfluoropentanoate ($[C_2C_1py][C_4F_9CO_2]$, >99% mass fraction purity), and choline perfluoropentanoate ($[N_{1112(OH)}][C_4F_9CO_2]$, >99% mass fraction purity) were previously synthesized and characterized in our laboratory [31,56,57].

To reduce volatile chemicals and water contents, all FILs were dried under vacuum (4 Pa) with vigorous stirring at about 40 °C for at least 48 h before their use. The purity of all FILs was checked by ^1H and ^{19}F NMR. The chemical structures of the fluorinated ionic liquids used in this work are presented in Table 1.

The bacterial strain *Pseudomonas stutzeri* (ATCC 17588) was purchased from ATCC (Manassas, VA, USA). Tablets for the phosphate buffered saline (PBS) solution preparation were purchased from PanReac Applichem ITW Reagents Division (Chicago, IL, USA). Milli-Q water (obtained from a Milli-Q Integral water purification system from Merck, Darmstadt, Germany) was used for the PBS and medium preparation of samples to be used in the microbial and hemocompatibility studies.

Table 1. Chemical structure and acronyms of the fluorinated ionic liquids (FILs) used in this work.

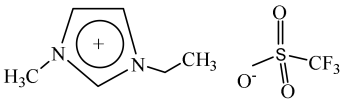
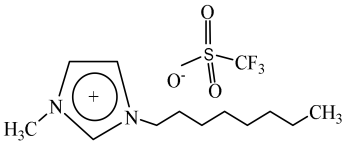
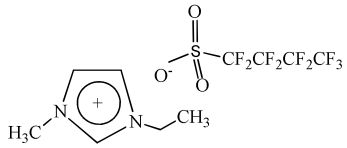
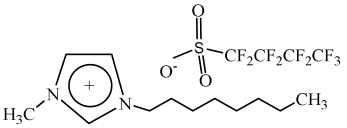
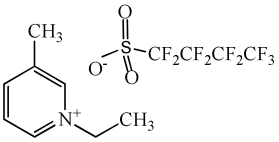
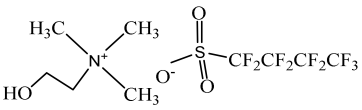
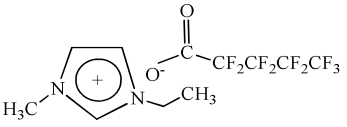
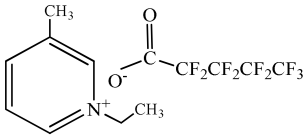
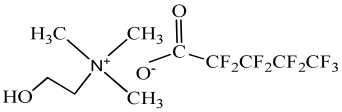
FIL Designation	Chemical Structure
1-Ethyl-3-methylimidazolium perfluoromethanesulfonate $[C_2C_1Im][CF_3SO_3]$	

Table 1. *Cont.*

FIL Designation	Chemical Structure
1-Methyl-3-octylimidazolium perfluoromethanesulfonate [C_8C_1Im][CF_3SO_3]	
1-Ethyl-3-methylimidazolium perfluorobutanesulfonate [C_2C_1Im][$C_4F_9SO_3$]	
1-Methyl-3-octylimidazolium perfluorobutanesulfonate [C_8C_1Im][$C_4F_9SO_3$]	
1-Ethyl-3-methylpyridinium perfluorobutanesulfonate or 1-ethyl-3-picolinium perfluorobutanesulfonate [C_2C_1py][$C_4F_9SO_3$]	
Choline perfluorobutanesulfonate [$N_{1112(OH)}$][$C_4F_9SO_3$]	
1-Ethyl-3-methylimidazolium perfluoropentanoate [C_2C_1Im][$C_4F_9CO_2$]	
1-Ethyl-3-methylpyridinium perfluoropentanoate [C_2C_1py][$C_4F_9CO_2$]	
Choline perfluoropentanoate [$N_{1112(OH)}$][$C_4F_9CO_2$]	

2.2. Methods

2.2.1. Ecotoxicity–Antibacterial Activity

Bacteria were firstly cultured in a specific broth for 24 h at 37 °C in an orbital shaker at 150 rpm. The flasks were inoculated (3% v/v) with previously obtained cell pellets. The composition of the broth media for the bacteria growth is (g/L): 1.0 beef extract; 2.0 yeast extract; 5.0 casein peptone; and 5.0 sodium chloride. The bacterial growth was then evaluated in a mineral medium (MM) with the following composition (g/L): Na_2HPO_4 6.78, KH_2PO_4 3.0, NaCl 0.5, NH_4Cl 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2$ 0.0147. Trace elements are also included in MM (g/L): $CuSO_4$ 0.4, KI 1.0, $MnSO_4{\cdot}H_2O$ 4.0, $ZnSO_4{\cdot}7H_2O$ 4.0, H_3BO_3 5.0, 1.6, $FeCl_3{\cdot}6H_2O$ 2.0. Glucose, 10 g/L, were included in the culture medium as a carbon source. Several FILs were firstly diluted in miliQ water to obtain the final test concentrations ranging from 500 to 800,000 µM. The antibacterial activity of these FILs

was determined incubating the microorganism, the MM and the FILs solutions in 96-well plates. The bacteria growth was measured photometrically at 600 nm in a Multiskan™ GO microplate reader purchased from Thermo Scientific (Waltham, MA, USA) after incubation at 37 °C for 9 days. Negative controls containing only *P. stutzeri* and the MM and positive controls containing only the MM were conducted under the same conditions. The screening along the different FIL concentrations enabled the determination of the minimal inhibitory concentration (MIC) defined as the lowest concentration that inhibits the visible growth of the bacteria. For an accurate determination of these values, the bacterial growth was determined for all the tested samples relative to the maximum growth (100%) obtained for the samples containing only bacteria and the MM. Calculations were made based on the maximum growth achieved after 9 days for each FIL concentration. For samples with a relative growth under 10%, the MIC was selected. The experiments were performed at least twice with a maximum standard deviation of 12%.

2.2.2. Hemolytic Activity

The hemolytic activity of the different FILs was determined according to the method optimized by Gaspar and co-workers using ethylene diamine tetraacetic acid (EDTA)-preserved peripheral human blood obtained from voluntary donors used in the same day of experiments [58,59]. To separate the serum from the erythrocytes, a centrifugation was performed at 1000× *g* for 10 min. Then, the erythrocyte suspension was washed three times in a phosphate buffered saline (PBS) solution. All FILs were diluted in PBS with concentrations ranging from 166 to 266,092 µM. The assay was performed in 96-well plates, in which 100 µL/well of sample were diluted with 100 µL of the erythrocyte suspension. The microplates were incubated at 37 °C for 1 h followed by a centrifugation at 800× *g* for 10 min. The absorbance of supernatants was measured at 570 nm and 630 nm. The percentage of the hemolytic activity for each sample was calculated by subtracting the values at 630 nm to the values at 570 nm and comparing each individual sample to a positive control (100% hemolysis, erythrocytes in distilled water), and to a negative control (0% hemolysis, erythrocytes in PBS), according to following formula:

$$\text{Hemolytic Activity } (\%) = \frac{\text{AbsS} - \text{AbsN}}{\text{AbsP} - \text{AbsN}} \times 100 \quad (1)$$

where AbsS is the average absorbance of the sample, AbsN is the average absorbance of the negative control and AbsP is the average absorbance of the positive control.

3. Results

3.1. Ecotoxicity–Antibacterial Activity

It is well known that the IL toxicity is highly dependent and variable with the biologically tested system [22]. Then, this work provides critical insights about FIL ecotoxicity and antibacterial properties towards the highly dispersed Gram-negative *P. stutzeri* bacteria. These bacteria are easily maintained at laboratorial conditions, which eases the experimental procedure. Afterwards, several structure–activity relationships will be considered for analysis, such as the effect of the: (a) cation family (imidazolium, pyridinium and choline); (b) anion nature (perfluorobutanesulfonate and perfluoropentanoate); (c) alkyl side chain length; and (d) fluorinated side chain length. The bacterial growth curves used to determine the MIC value were stablished for all tested FIL concentrations regarding the maximum growth of negative controls, and some examples are depicted in Figures S1–S4 (in Supplementary Materials). The MIC values of the tested compounds are shown in Figures 1 and 2 and indicated in Table S1. These values range from >2930 to 400,000 µM, which corroborates the already demonstrated ecofriendly nature of FILs [31].

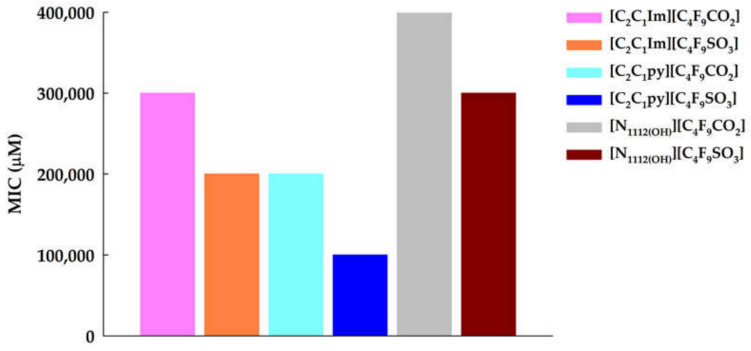


Figure 1. Minimal inhibitory concentration (MIC) values determined for choline, and short hydrogenated alkyl side chain imidazolium and pyridinium cations conjugated with perfluorobutanesulfonate ($[C_4F_9SO_3]^-$) or perfluoropentanoate anion ($[C_4F_9CO_2]^-$).

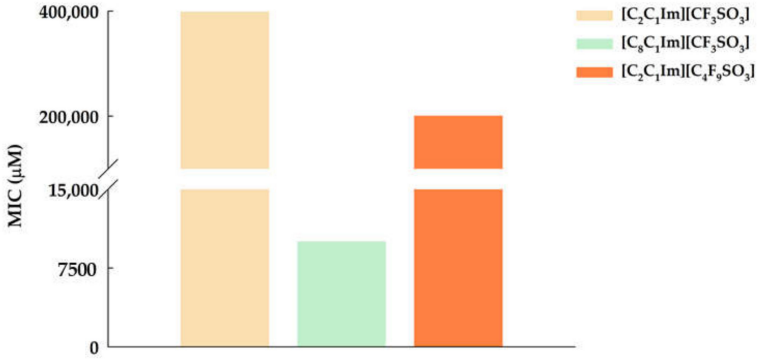


Figure 2. MIC values determined for imidazolium-based fluorinated ionic liquids (FILs) conjugated with the sulfonate-based anion with the increment of both the hydrogenated and fluorinated alkyl side chain length.

The effect of the choline and the short chain-based imidazolium and pyridinium cations conjugated with either perfluorobutanesulfonate ($[C_4F_9SO_3]^-$) or perfluoropentanoate ($[C_4F_9CO_2]^-$) anions on the bacterial growth and in the determined MIC value, are shown in Table S1 and depicted in Figure 1.

According to the obtained results, when conjugated with the $[C_4F_9SO_3]^-$ the choline cation is the least toxic cation, with a MIC value of 300,000 μM, followed by the imidazolium-based cation in which the visible growth of bacteria is limited at a concentration equal or higher than 200,000 μM. Lastly, the higher toxicity is achieved with the pyridinium cation, in which the MIC is achieved at a concentration of 100,000 μM. The same trend was observed with the $[C_4F_9CO_2]^-$, however, the values of the MICs for the carboxylate-based FILs were consistently 100,000 μM higher than those obtained for the $[C_4F_9SO_3]^-$, which means that compounds based on this anion are relatively less toxic than the $[C_4F_9SO_3]^-$-based FILs. The differences amongst the choline, imidazolium and pyridinium cations are in the same order as the differences between the carboxylic and the sulfonic-based fluoroorganic anions. Both imidazolium and choline compounds conjugated with $[C_4F_9SO_3]^-$ and $[C_4F_9CO_2]^-$ were previously tested against the Gram-negative bacteria *V. fischeri*, with lower toxicities achieved for the choline-based cation, and for the carboxylate-based anions [31]. In this previous study, the pyridinium cation was the most toxic compound amongst the $[C_4F_9CO_2]^-$-based FILs, following the same trend observed for *P. stutzeri*, although the differences between pyridinium, choline and imidazolium compounds are less evidenced for *P. stutzeri* than for *V. fischeri*, which can be associated to intrinsic differences between the microorganisms [31]. The increment of the alkyl side chain length from $[C_2C_1Im]^+$ to $[C_8C_1Im]^+$ in both $[CF_3SO_3]^-$ and $[C_4F_9SO_3]^-$ caused a significant reduction on the

MIC value, as shown in Table S1, Figure 2 and Figures S1–S4. $[C_2C_1Im][CF_3SO_3]$ have a MIC value of 400,000 μM, which is reduced to 10,000 μM when the side chain increases to $[C_8C_1Im][CF_3SO_3]$. The same trend was expected to be achieved with the $[C_4F_9SO_3]^-$, however, at the range of tested concentrations, it was not possible to determine the MIC value. Concentrations higher than 2930 μM were not tested due to the poor solubility of $[C_8C_1Im][C_4F_9SO_3]$ in the bacterial growth medium. Nevertheless, as depicted in Figures S3 and S4, for $[C_8C_1Im][C_4F_9SO_3]$ at this maximum tested concentration (2930 μM), the achieved bacterial growth is slightly lower than 50%, whereas in $[C_2C_1Im][C_4F_9SO_3]$ for concentrations up to 5000 μM the bacterial growth is slightly higher than 95%, which is in accordance to the results achieved for the $[CF_3SO_3]^-$-based FILs. This higher antibacterial activity caused by the elongation of the hydrogenated side chain was linked to the increment of the hydrophobicity and is consistently reported in several cell cultures and microorganisms, including several Gram-negative bacteria [22,39,46,60].

The increment of the fluorinated chain from $[CF_3SO_3]^-$ to $[C_4F_9SO_3]^-$ also impacted the ecotoxicity of the FILs towards *P. stutzeri* bacteria, with higher toxicities obtained for the longer fluorinated anions. Even so, the increment of the fluorinated chain has a less pronounced effect on the MIC values of FILs than the increment of the hydrogenated side chain length of the cation.

For the short chain-based imidazolium ($[C_2C_1Im]^+$), the increment of the fluorinated chain from $[CF_3SO_3]^-$ to $[C_4F_3SO_3]^-$ caused a decrement of the MIC value from 400,000 to 200,000 μM. For the $[C_8C_1Im]^+$-based FIL, the increment of the fluorinated chain for $[C_4F_3SO_3]^-$ impacted the solubility of the FIL. As illustrated in Figure S4, and as mentioned before, for the maximum tested concentration of $[C_8C_1Im][C_4F_9SO_3]$, the relative bacterial growth is slightly below 50%, whereas for a concentration of 5000 μM in $[C_8C_1Im][CF_3SO_3]$, as illustrated in Figure S2, the bacterial growth is 80%. Although less is known about the anion effect on ILs, it was also proved that the anion elongation also increases the hydrophobicity of the compounds, and consequently its toxicity towards different organisms, including human pathogenic bacteria [39,46,61,62]. Based on these results, the better antibacterial potential was achieved for $[C_8C_1Im][CF_3SO_3]$, which inhibits the growth of these microorganisms at concentrations approximately 10 times lower than the other tested FILs, and can represent a treatment alternative to the antibiotics commonly administered. Even so, all determined MICs correspond to very high concentrations which corroborates the ecofriendly nature of these compounds, as already demonstrated for other aquatic microorganisms. This reduced toxicity towards *P. stutzeri* can be related to the structure of the Gram-negative bacteria which possess several membrane layers that confers them an additional protection against the external stimuli, such as FILs [31,63,64].

3.2. Hemolytic Activity

An in vitro hemolysis study must be performed prior to any pharmaceutical application of FILs in order to guarantee their biocompatibility and to follow the Food and Drug Administration (FDA) recommendations for the development of compounds for intravenous or parenteral administration [59]. This study evaluates the red blood cell lysis through the hemoglobin release in the plasma, for FIL concentrations up to 266,092 μM. The hemolytic activity of the different FILs was analyzed regarding the: (a) cation family (imidazolium, pyridinium and choline); (b) anion nature (perfluorobutanesulfonate and perfluoropentanoate); (c) alkyl side chain length; and (d) fluorinated side chain length.

As shown in Table S2 and as depicted in Figures 3 and 4, the cytotoxicity towards the red blood cells was achieved at very high concentrations of FIL. Furthermore, as shown in Figure 3, contrary to the expected and noticed in the antibacterial, acute aquatic toxicity and prior cytotoxicity studies [31,46], a higher hemolytic activity was obtained for the choline-based FILs, particularly for $[N_{1112(OH)}][C_4F_9CO_2]^-$. These results can be associated to the type of structures formed by these specific choline-based FILs, at concentrations higher than the critical micellar concentration (CMC) [43,45], that may interact with the cellular membrane, causing its disruption and leading to a higher hemolysis in comparison to the

other FILs. The lower CMC of $[N_{1112(OH)}][C_4F_9CO_2]$ in comparison to $[N_{1112(OH)}][C_4F_9SO_3]$ justifies the higher hemolytic activity of $[N_{1112(OH)}][C_4F_9CO_2]$ at similar concentrations than the correspondent perfluorobutanesulfonate FIL [43,45]. Nevertheless, a considerable hemolysis was only achieved at extremely high FIL concentrations (see Table S2).

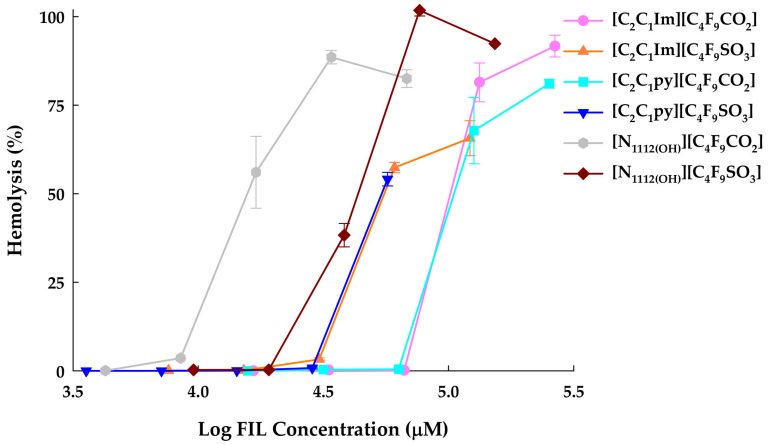


Figure 3. Hemolysis (%) determined for choline, and short hydrogenated alkyl side chain imidazolium and pyridinium cations conjugated with perfluorobutanesulfonate ($[C_4F_9SO_3]^-$) or perfluoropentanoate anion ($[C_4F_9CO_2]^-$).

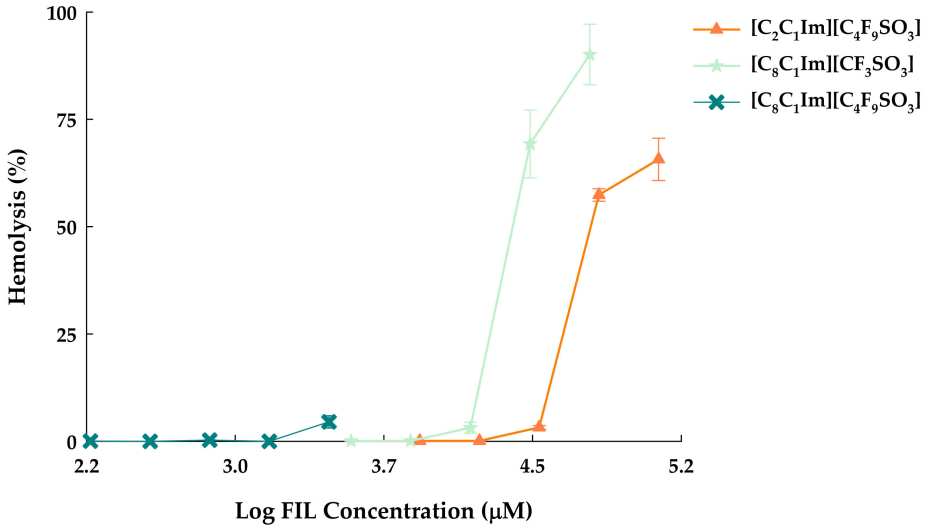


Figure 4. Hemolysis (%) determined for imidazolium-based FILs conjugated with the sulfonic-based anion with the increment of both hydrogenated and fluorinated alkyl side chain length.

No substantial differences were noticed between $[C_2C_1Im]^+$ and $[C_2C_1py]^+$ cations when conjugated with both perfluorobutanesulfonate or perfluoropentanoate anions. Even so, the lowest hemolytic activity was achieved for $[C_2C_1py][C_4F_9CO_2]$, followed by $[C_2C_1Im][C_4F_9CO_2]$, without significant variances amongst the two compounds.

The hemolysis was achieved at lower concentrations for $[C_4F_9SO_3]^-$-based FILs than for the ones based on the $[C_4F_9CO_2]^-$ anion, except for choline-based FILs, which demonstrated an opposite trend. Due to the poor solubility of $[C_8C_1Im][C_4F_9SO_3]$ in PBS, it was not possible to determine at which concentration a considerable hemolytic activity occurs, which makes a proper evaluation about the influence of alkyl and fluorinated side chain length in the FILs toxicity difficult. However, based on the profiles depicted in Figure 4, and on the knowledge about the effect of the cationic and anionic side chain elongation, it is expected that the increment from $[C_2C_1Im][C_4F_9SO_3]$ or $[C_8C_1Im][CF_3SO_3]$ to

$[C_8C_1Im][C_4F_9SO_3]$ induces an increment on the FIL hemolytic activity, and consequently on the FIL toxicity. This increased toxicity is associated to the increment of the hydrophobicity that may contribute to a higher permeation or disruption of cell membranes, and the destabilization of the cellular viability mechanisms [46,65–68]. The overall obtained results support the application of FILs in the pharmaceutical field (such as stabilizing or delivery agents), since a considerable red blood cell lysis only occurs at concentrations higher or close to the CMC [43–45]. These CMCs are used as reference for the design of delivery systems. For the imidazolium and pyridinium-based FILs conjugated with the perfluorobutanesulfonate anion and for $[C_2C_1Im][C_4F_9CO_2]$, the hemolysis occurs at concentrations higher than two times the CMC, in which the encapsulation of lysozyme was already demonstrated [47]. Furthermore, based on the hemolytic activity of the different FILs, it becomes clear that $[C_8C_1Im][CF_3SO_3]$ is the only compound with the potential to be used as an antibacterial agent without affecting the integrity of red blood cells. The growth of *P. stutzeri* bacteria is inhibited at 10,000 μM, whereas at concentrations up to 13,697 μM, the hemolysis caused by this FIL is equal or lower than 3.15%. For the other tested compounds, the MIC concentration is associated to a hemolysis always higher 50%. Nevertheless, it must be reinforced that all compounds present relatively lower hemolytic activities up to high concentrations, which is a good indicator for their application in either biopharmaceutical or industrial applications, without prejudice of public health.

4. Conclusions

The results obtained in this work are vital for the characterization of the FIL family, for the design of new antibacterial compounds for the treatment of infections caused by *P. stutzeri*, as well as for the selection of ecofriendly FILs with low environmental impacts to be used in other pharmaceutical and industrial purposes.

Firstly, the results obtained support the ecofriendly nature of these compounds since the inhibition of the bacterial growth is only achieved at very high concentrations. The choline cation conjugated with the perfluoropentanoate anion exhibited the lowest ecotoxicity amongst all the tested FILs, as well as the perfluoropentanoate demonstrated to be the most ecofriendly anion. Besides, the increment of both hydrogenated and fluorinated alkyl side chain induced an increased ecotoxicity.

The hemolytic activity of these FILs reveals that the red blood cell lysis only occurs at very high concentrations, which corroborates their biocompatible nature. An atypical behavior was noticed with a highest toxicity associated to the choline-based FILs, namely $[N_{1112(OH)}][C_4F_9CO_2]$, so further studies must be carried out to better understand the mechanisms of cell lysis associated to these FILs. The biocompatibility of imidazolium and pyridinium-based FILs were very similar, and for these two cations a higher hemolytic activity was achieved with the perfluorobutanesulfonate anion. The higher toxicity expected with the elongation of the hydrogenated and fluorinated alkyl side chain was not fully proved due to the poor solubility of $[C_8C_1Im][C_4F_9SO_3]$. However, based on the obtained hemolysis profiles, it is clearly expected that $[C_8C_1Im][C_4F_9SO_3]$ will have the higher hemolytic activities at similar concentrations to the other imidazolium-based compounds.

Finally, $[C_8C_1Im][CF_3SO_3]$ was selected as the most promising FIL to be used as an antibacterial compound. This FIL can inhibit the bacterial growth at a lower concentration than the other tested compounds, without causing the hemolysis of red blood cells. Even so, the results obtained from the other tested FILs reveal the low ecotoxicity and the reduced cytotoxicity of FILs up to high concentrations, ensuring their safe application in other pharmaceutical and industrial applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/2673-4079/2/1/8/s1, Table S1. MIC value of different FILs against *Pseudomonas stutzeri* in μM, Table S2. Hemolysis (%) as function of concentration determined for several FILs, Figure S1. *Pseudomonas stutzeri* growth curves for different concentrations of 1-ethyl-3-methylimidazolium perfluoromethanesulfonate $[C_2C_1Im][CF_3SO_3]$, Figure S2. *Pseudomonas stutzeri* growth curves for different concentrations of 1-methyl-3-octylimidazolium perfluoromethanesulfonate $[C_8C_1Im][CF_3SO_3]$, FigureS3.

Pseudomonas stutzeri growth curves for different concentrations of 1-ethyl-3-methylimidazolium perfluorobutanesulfonate [C_2C_1Im][$C_4F_9SO_3$], Figure S4. *Pseudomonas stutzeri* growth curves for different concentrations of 1-methyl-3-octylimidazolium perfluorobutanesulfonate [C_8C_1Im][$C_4F_9SO_3$].

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