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# Surfactant-mediated enzymatic superactivity in water/ionic liquid mixtures, evaluated on a model hydrolytic reaction catalyzed by α-chymotrypsin

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In this work, the influence of the $C_{12}$-, $C_{14}$-, and $C_{16}$-alkyl chain derivatives of 1-alkyl-3-methyl-imidazole tetrafluoroborate over the hydrolisis of *p*-nitrophenyl trimethylacetate catalyzed by α-chymotrypsin was studied in water and water - ionic liquids mixtures. The ionic liquid used is 1-butyl-3-methyl-imidazole tetrafluoroborate ($BMIMBF_4$).

1-alkyl-3-methylimidazole tetrafluoroborate derived surfactants can increase the catalytic efficiency of α-chymotrypsin on the hydrolysis of *p*-nitrophenyl trimethylacetate. This effect is negatively affected by the decrease on the surfactant's critical micelle concentration (CMC) and the partition of the substrate between the micelles and the external media observed above the critical miscellar concentration (CMC). In water/$BMIMBF_4$ mixtures, the presence of the ionic liquid (IL), render the $C_{14}$ and $C_{16}$ surfactants insoluble, and overrides the effect elicited by the $C_{12}$ alkyl chain surfactant on the activity of α-chymotrypsin. The loss of the surfactants influence on the enzymatic activity due to the presence of $BMIMBF_4$ seems to be a complex process, controlled by a decreased affinity between α-chymotrypsin and the surfactant molecules and, to a larger extent, the appearance of an "acquired resistance" of the enzyme to the influence of the surfactants, related to a more compact and resilient conformation of the protein in the presence of $BMIMBF_4$. The influence of $BMIMBF_4$ is not only limited to the surfactant/enzyme interaction, but also it does modifies the partition of the substrate between the aqueous media and the micellar environment.

## 1. Introduction

Enzymatic catalysis in microheterogenous systems has been extensively studied. In particular, the behavior of enzymatic reaction taking place in the presence of micellar aggregates, [1] due to the physicochemical complexity of these multiphasic systems in relation with their relevance in productive processes involving microemulsions for the extraction, purification or stabilization of enzymes, and the development of suitable media for enzyme catalyzed reactions of economic value [2].

Taking into consideration the Michaelis-Menten description of enzymatic reactions, several models have been proposed for the evaluation of the kinetic parameters for enzymatic reactions in micellar media [1,3]. Special focus has been put on extracting useful kinetic information, able to be contrasted with data obtained in homogeneous media (i.e. aqueous solution), in order to evaluate the extent of the influence of the micellar aggregates.

In aqueous biphasic systems, different mechanistic considerations are evaluated, following the concentration-dependent behavior of the surfactants: at low surfactant concentration, where only surfactant monomer are accompanying the enzymatic reaction, or at surfactant concentrations beyond the CMC, where the presence of direct micelles acquires relevance. It has been reported that some enzymes present enhanced activity in aqueous solution of surfactant monomers, in a phenomenon coined as superactivity. [4–6] This is the case of cationic surfactants with bulky headgroups such as alkylammonium halide surfactants [7], as well as nonionic surfactants such as Brij 35 [8] and Tween 20 [9], whereas some enzymes are readily inactivated in similar conditions, particularly in the presence of anionic surfactants like Sodium dodecilsulface (SDS) [10].

In surfactant/oil/water systems, the phenomenon is more complex [11]. In these systems, an increase of activity is also observed, but now the increase is governed by the water/surfactant molar ratio ($W_0$),

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*Abbreviations:* $BMIMBF_4$, 1-butyl-3-methyl-imidazole tetrafluoroborate; $C_{12}MIMBF_4$, 1-dodecyl-3-methyl-imidazole tetrafluoroborate; $C_{14}MIMBF_4$, 1-tetradecyl-3-methyl-imidazole tetrafluoroborate; $C_{16}MIMBF_4$, 1-hexadecyl-3-methyl-imidazole tetrafluoroborate; α-CT, α-chymotrypsin; PNTMA, *p*-nitrophenyl trimethylacetate; PNP, *p*-nitrophenol; CMC, critical micellar concentration.

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parameter which, in reverse micelles, controls their size, physicochemical properties of the entrapped water [12], and the behavior of the water/surfactant headgroup interface [13]. The observed increase in activity is associated with the interaction of the enzyme molecules with the interfacial region and the compartmentalization of both enzyme and substrate into the reverse micelle aqueous pool [14].

In the last two decades, given the growing interest in the use of alternative solvents, [15] a large number of studies has been developed regarding the use of IL. [16] These are salts usually formed by large asymmetrical ions (commonly an organic cation and an inorganic anion) interacting by weak cation-anion attractive forces, compared to those observed in conventional ionic salts (crystalline solids), giving most of the ILs the ability to remain in liquid form at room temperature. ILs are considered "design solvents" due to the huge number of anions and cations that can be combined in order to control different physicochemical properties such as polarity, viscosity, conductivity, etc. Studies on enzymatic activity in ILs have demonstrated their ability to benefit enzymatic reaction that can take place in conventional organic solvents [17,18], or in media with low water contents [19], in terms of an improvement their selectivity, activity and stability. However, the extent of the influence of ILs is highly dependent on the catalyzed reaction and the nature of the enzyme under consideration. The use of ILs as alternative media for the development of processes involving enzyme catalyzed reactions have led to better results when compared with conventional organic solvents, [20] but their use is still limited for reactions that strictly need aqueous environments in order to properly function. Consider for instance those cases in which the IL needs to be used with equal or larger proportions of water in the solvent mixtures.

The behavior of amphiphilic molecules and the mechanisms for aggregate formation in non-conventional solvents have also been addressed. Particularly both, protic and aprotic ILs, have displayed the ability to act as an amphiphile-assembly media, [21,22] given the fact that some of these ILs share characteristics such as the presence of hydrogen bonding centers, few methylene groups, polar groups in both cation and anion, characteristic also present in conventional molecular solvents that also favors the formation of amphiphilic aggregates. Greaves et al. [22] in a study of the behavior of the non-ionic surfactant, hexa-ethyleneglycol mono-n-dodecyl ether, demonstrated the support of the formation of micellar aggregates in a library of conventional molecular solvents and protic ILs. The observed phenomena, linked to a solvophobic effect, suggested a correlation between the solvent cohesive energy and both the CMC and the free energy of micellization. Similarly, long chain 1-alkyl-3-methylimidazolium based ILs have been tested in the extraction of natural colorants by the use of an aqueous micellar byphasic system based on Triton X-114 and low percentages of the IL as co-surfactant. [23] The incorporation of the IL allows the control, by electrostatic interactions, over the partitioning of the biomolecules between the water and the micellar phase.

In light of the reported behavior of both, enzymes and surfactants in the presence of ILs, it is interesting then to question how does the enzymatic superactivity effect induced by surfactants fares in the presence of ionic liquids. Moniruzzaman et al. [14] reported the improved activity of microencapsulated horseradish peroxidase in sodium-bis-ethylhexylsulfosuccinate (AOT) reverse micellar systems on water-in-oil microemulsions, with a hyperbolic dependence on the catalyzed oxidation of pyrogallol by hydrogen peroxide, with the water to surfactant ratio, and an overall improved performance when compared to AOT/water/isooctane microemulsions. On the other hand, studies in aqueous solution involving cetyltrimethylammonium bromide/IL mixed micelles [24] have shown an improved activity of α-chymotrypsin on the catalyzed hydrolysis of N-succinyl-*l*-phenylalanine-p-nitroanilide. Similar results have also been reported for transglycosilation reaction catalyzed by β-galactosidase in similar mixed micellar systems, using SDS as surfactant. [25]

As an approach to delve further on the study of the surfactant influence over enzymatic activity in the presence of ILs, the present work considers the evaluation of enzymatic activity in complex systems comprising surfactants incorporated in IL/water mixtures. The kinetic analysis of the results and the conclusions derived thereof presented in this paper were done taking into consideration the models proposed by Viparelli et al. [3] for enzymatic reactions taking place in the presence of large concentrations of direct micellar aggregates. Briefly, the kinetic model consider two possibilities for the enzyme/micelle interaction: i) no direct enzyme/micelle interaction, where both the free substrate and the substrate located in the micelle-bound water region is available for the reaction, given the fact that the activity depends now on the contribution of the two substrate forms. An increase in activity can occur when the enzyme is more efficient with the micelle bound substrate. On the contrary, a decrease in activity can be observed when the contribution of the bound substrate is lessened. ii) enzyme/micelle interactions, leading to the presence of two enzymatic forms, free and bound enzyme, with each form being able to interact with the substrate as addressed in i). When enzyme/micelle interactions are operative, the increase or decrease of activity is governed by the differences on the intrinsic activity displayed by both enzyme forms; different proportions of the free/bound species for the enzyme and substrate, and finally, the accessibility to the bound species (particularly the bound substrate) interacting with the micellar aggregates.

α -chymotrypsin (α-CT), selected as model enzyme for our studies, is a serine protease involved on the hydrolytic rupture of peptidic linkage. It has been extensively considered in studies regarding the influence of ionic liquids on the stability and kinetic behavior of enzymes in aqueous and non-aqueous environments [26–32]. The activity of α-CT was monitored by following the catalyzed hydrolysis of the substrate *p*-nitrophenyl trimethylacetate, substrate that has the particularity to allow a simple determination of both the stationary- and pre stationary- state of the reaction mechanism of α-CT [33]. This characteristic may be attributed to a delayed decomposition of the acyl-enzyme complex associated with the steric hindrance introduced by the presence of the methyl groups on the acetate moiety. Among the wide variety of ionic liquids, 1-alkyl-3-methylimidazole derived ionic liquids have reached the spotlight in studies focused on development of alternative media for the tuning and control of enzymatic reactions [34]. 1-butyl-3-methylimidazole tetrafluoroborate ($BMIMBF_4$) is commonly used. Among other ionic liquids, in these kind of studies, particularly in studies involving ionic liquid/water mixtures, where $BMIMBF_4$ displays an excellent miscibility with water [35]. Similarly, long chain 1-alkyl-3-methylimidazole tetrafluoroborate salts were chosen as surfactants.

The use of long chain analogues of $BMIMBF_4$ as surfactants, is not a trivial selection, because when analyzing the surfactant-IL-water interaction, the complexity of this interaction is crucial. To minimize the influence of differences on the nature of the surfactant headgroup and counterion [36,37], preferential solvation effects [38], etc. eases the interpretation of the results, leaving the carbon tail of the surfactants as the main driving force on the observed behavior of the micellar systems studied as well as an important part of their influence over α-CT activity.

The phenomena studied on the present work were addressed from a simple kinetic and thermodynamic approach, taking into consideration the inherent complexity of the studied system. This idea is further expanded into the introduction. Our work encompasses a simple experimental kinetic setup, complimented with a more complex mathematical treatment of the Michaelis-Menten analysis. Moreover, the characterization of the influence of the micellar environment was performed by basic partition assays (physical separation of the unbound substrate) to evaluate the substrate/micelle interaction, as well as thermal stability of the enzyme to assess the direct influence of either the surfactant or the micelles influence on the overall structural behavior of the enzyme. Form both of these analysis it can be ascertain, within certain limits, the effect of the ionic liquid on the studied microheterogeneous system.

## 2. Materials and methods

1-butyl-3-methyl imidazole tetrafluoroborate (Merck), 1-dodecyl-3-metyl imidazole tetrafluoroborate; 1-tetradecyl-3-methyl imidazole tetrafluoroborate, 1-hexadecyl-3-methyl imidazole (Iolitech), Pyrene, *p*-nitrophenyl trymethyl acetate, p-nitrophenol and α-chymotrypsin Type II from bovine pancreas (Sigma) were used as received without further treatment.

Ultrapure water (type I) was obtained from a Merck-Millipore Simplicity water purification system. Buffer Solutions (phosphate buffer 75 mM; pH 7,0), water/$BMIMBF_4$ mixtures were prepared and left to stabilize overnight, and both enzyme and substrate stock solutions were freshly prepared when required.

### 2.1. Reaction rate measurements

The activity of α-CT was evaluated using the catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate at 25 °C in aqueous buffered solutions (75 mM phosphate buffer pH 7.4), following the release of p-nitrophenol by the increase on the absorbance at 400–420 nm of solutions obtained by adding α-CT aliquots to a thermostated cell containing the substrate at the desired concentrations. Spectrophotometric measurements were performed in an Agilent 8453 UV–Vis spectrophotometer.

From plots of the steady-state reaction rates for the α-CT catalyzed reactions vs substrate concentration, the catalytic turnover constant (kcat) and the substrate affinity constant ($K_M$) according to the hyperbolic relationship ascribed to the Michaelis-Menten mechanism (Eq. (1)).

$$rate = \frac{k_{cat}[E]_0[S]}{K_m + [S]} \tag{1}$$

### 2.2. Ultrafiltration experiments

Ultrafiltration experiments were carried out at 25 °C using a Millipore ultrafiltration stirred cell unit with 10 kDa molecular weight cut off ultrafiltration disk membranes. Filtrate volume calibrations were performed as a function of pressure and filtration time in order to obtain the minimum volume of filtrate passed through the membrane. Filtrate volumes were kept under 2% of the total working volume in the ultrafiltration experiments. Controls were performed to ensure that no adsorption of the substrate took place during the experiments.

The concentration of PNTMA was determined by UV-VIS measurements, following the PNP absorbance upon total acid hydrolysis of the feed and filtrate PNTMA solutions. In our experiments, the filtrate correspond to a small portion of the total volume of solution incorporated onto the ultrafiltration chamber, which is extracted and added successive times through the ultrafiltration system and from where the free PNTMA concentration ($[PNTMA]_{free}$) was determined. Similarly, the feed solution correspond to the fixed volume of solution incorporated into the ultrafiltration chamber, and this solution contains the total concentration of PNTMA ($[PNTMA]_0$). From the difference between $[PNTMA]_0$ and $[PNTMA]_{free}$, the micelle-bound PNTMA concentration ($[PNTMA]_{bound}$) can be estimated.

From the free and bound concentrations of PNTMA, the fractions of each "type" of substrate ($F_{free}$ and $F_{bound}$) were evaluated for the different solvent mixtures studied and the partition of the substrate was evaluated by determination of the partition constant (Kp) according to Eq. (2) [7].

$$K_P = \frac{F_{free}}{F_{bound}[Surf]_m} \tag{2}$$

where $[Surf]_m$ correspond to the micellized surfactant concentration, which is determined by subtraction of the corresponding CMC from the total surfactant concentration considered.

To take into consideration the lower aqueous substrate concentration due to the partition of the substrate, the determination of the corrected catalytic efficiency $\left(\frac{k_{cat}}{K_M}\right)_{corr}$ was performed according to Eq. (3).

$$\left(\frac{k_{cat}}{K_M}\right)_{corr} = \frac{\left(\frac{k_{cat}}{K_M}\right)_{app}}{F_{free}} \tag{3}$$

where $\left(\frac{k_{cat}}{K_M}\right)_{app}$ correspond to the catalityc efficiency determined by using the analytical concentration of substrate for the calculation of $k_{cat}$, and $F_{free}$ corresponds to the free substrate concentration.

### 2.3. Micelle size measurements

The hydrodynamic size of the micelles was determined by dynamic light scattering measurements performed in a Zetasizer nano ZS-90 DLS system (Malvern instruments). Measurements were carried out at 25 °C in polystyrene cuvettes, using a viscosity of 0.8872 cP and a refractive index of 1.330, as system defined parameters for measurements in aqueous solutions at the working temperature.

### 2.4. Fluorescence measurements

Critical micelle concentration was determined by monitoring the change of the fluorescence spectra of pyrene, particularly, the change on the ratio between the first and third peak ($I_I/I_{III}$), observable from the hyperfine structure of the spectra [39–41]. Pyrene steady state fluorescence measurements were recorded in a Perkin Elmer LS55 fluorescence spectrometer, using 1 cm path length quartz cuvettes and an excitation wavelength of 405 nm. Briefly, different masses of surfactant were dissolved in aqueous solutions of pyrene (6 μM), in order to achieve surfactant solutions in a wide range of concentrations. The obtained solutions were stabilized overnight in a thermostated bath at 25 °C, and the temperature was kept constant during the fluorescence recordings. The values of the $I_I/I_{III}$ ratio were determined from the pyrene spectra averaged from 20 recordings. The CMC value was determined at the midpoint of the sigmoidal curve obtained from $I_I/I_{III}$ vs. surfactant concentration plots.

Tryptophan (TRP) fluorescence spectra were recorded in a Perkin-Elmer LS-55 fluorescence spectrometer, using 1 cm path length quartz cuvettes and an excitation wavelength of 295 nm with a spectral bandpass of 2,5 nm for both excitation and emission beams. For the protein thermal stability experiments. α-CT solutions were equilibrated overnight and the measurements were performed by increasing the temperature of the cuvettes using a peltier type thermostated holder. The solutions were heated for 20 min after the measurements, to allow the thermal equilibrium of the samples. The fluorescence emission maxima reported are the result of 20 averaged readings.

The thermal stability of the enzyme was analyzed through the determination of the fraction of native enzyme for each temperature step [42]. The fraction of native protein ($f_N$) was calculated according to Eq. (4).

$$Native\ fraction = \frac{[F - F_U]}{[F_N - F_U]} \tag{4}$$

where F correspond to the fluorescence intensity measured at each temperature; $F_N$ is the fluorescence of the folded conformation and $F_U$ the fluorescence of the unfolded state of the protein.

The determination of the thermodynamic parameters for the denaturation process of α-CT was performed by the calculation of the equilibrium constant of the denaturation ($K_D$), [43] by using Eq. (5).

$$K_D = \frac{f_D}{1 - f_D} \tag{5}$$

where $f_D$ correspond to the denatured α-CT fraction, calculated from $f_D = 1 - f_N$. Upon the calculation of $K_D$, the Gibbs free energy of denaturation ($\Delta G_D$) was calculated, for the set of temperatures studied, according to Eq. (6). ΔH and ΔS were determined analytically from a plot of $\Delta G_D$ vs temperature, using the transition temperature (Tm) for the calculation of ΔS (the intercept of the plot with the x axis, where $\Delta G_D = 0$), [43] based on Eq. (7).

$$\Delta G_D = -RT \ \ln K_D \tag{6}$$

$$\Delta G_D = 0 = \Delta H_D - T\Delta S_D \tag{7}$$

## 3. Results and discussion

In order to ascertain the extent of the influence of 1-alkyl-3-methylimidazole tetrafluoroborate surfactants in aqueous solutions, the catalytic efficiency of the enzyme, calculated as the ratio between $k_{CAT}$ and $K_M$, were plotted against the analytical concentration of surfactants with $C_{12}$, $C_{14}$, and $C_{16}$ alkyl chains (Fig. 1). The effect elicited by the surfactants follows a bell-shaped trend, with an increase on the catalytic efficiency at low surfactant concentrations, up to a maximum value near the surfactants CMC, followed by a decay at larger surfactant concentrations. Among the studied surfactants, $C_{12}MIMBF_4$ induces the larger change on the catalytic efficiency (nearly 30 times larger than that in water), followed by $C_{14}MIMBF_4$ with a 10 fold increase, and finally $C_{16}MIMBF_4$ which induces a small effect (two-fold increase), instead leading up to an overall decrease on enzymatic activity.

Table 1 shows a comparison between the CMC values for the studied surfactants, and the surfactant concentration at the peak value of α-CT catalytic efficiency. From this data, a good correspondence between the CMC values and the maximum catalytic efficiency achieved with the addition of surfactants was observed. Note that it is also possible to distinguish a relationship between the shift of the peak on the kcat/$K_M$ vs surfactant concentration profiles towards lower surfactant concentration, associated with the decrease on the CMC values, in the range of 13 to 0.6 mM going from the $C_{12}$ to $C_{16}$ derivatives, thereby displaying a nearly linear decrease with the increase in the number of carbon atoms on the alkyl chain of the surfactants. On the contrary, for the resulting micelles formed at values above the CMC, it is also possible to observe a size increase with the alkyl chain length. Table 1 also displays the hydrodynamic radii for the micelles of $C_{12}$, $C_{14}$ and $C_{16}$, where measured values in the interval from 8 to 12 nm, going from $C_{12}$ to $C_{16}$ surfactants. These figures are in agreement with the expected size of the micellar aggregates, considering surfactant lengths and hydration layer surrounding these systems.

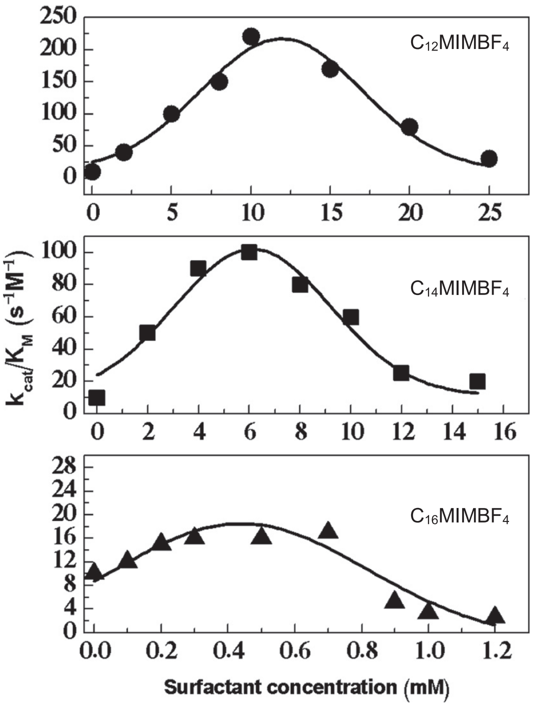


**Fig. 1.** Catalytic efficiency (kcat/Km) of α-CT for the catalyzed hydrolysis of PNTMA, as a function of the concentration of long alkyl chain surfactants from the 1-alkyl-3-methylimidazole family, in aqueous solution (pH = 7.0; 25 °C) and [α-CT] = 10 μM. $C_{12}BMIMBF_4$ (●); $C_{14}BMIMBF_4$ (■); $C_{16}BMIMBF_4$ (▲). (Data on Table S1 of Appendix A).

**Table 1**
Concentration of long alkyl chain surfactants from the 1-alkyl-3-methylimidazole family at the maximum value of catalytic efficiency determined from the data of Fig. 1, compared with the CMC and micellar hydrodynamic radii of the studied surfactants.

| | $[C_nMIM^+BF_4^-]$ (mM) | | Size (nm) |
|---|---|---|---|
| | At peak $k_{cat}/K_M$ | CMC (Pyrene)[a] | |
| $C_{12}$ | 13.1 ± 0.7 | 12.8 ± 0,2 | 8.1 ± 1.2 |
| $C_{14}$ | 5,0 ± 0,6 | 4,0 ± 0,5 | 10.5 ± 0.6 |
| $C_{16}$ | 0,6 ± 0,2 | 0,8 ± 0,1 | 11.8 ± 0.8 |

[a] CMC determination data on Fig. S1 on Appendix A.

At low surfactant concentrations, the observed behavior for the catalytic efficiency is related to an increase on $k_{CAT}$ (Fig. 2A) coupled with an important decrease of $K_M$ (Fig. 2B). After the CMC, $k_{CAT}$ tends to decrease, whereas $K_M$ reaches a constant value. Albeit in a different extent, a similar response is observed in all three surfactants. In the case of $C_{12}MIMBF_4$, $k_{CAT}$ readily increases with the addition of surfactant, going from $4 \times 10^{-3}$ to $2 \times 10^{-2}$ $s^{-1}$ at the CMC value. In the same surfactant concentration interval, $K_M$ shows a steep decrease, from $4 \times 10^{-4}$ M in water to $1 \times 10^{-4}$ M at 5 mM $C_{12}MIMBF_4$ (nearly half of the CMC value). Beyond this concentration, a very moderate decrease of $K_M$ is observed, remaining almost constant up to 25 mM $C_{12}MIMBF_4$, with a value of $K_M$ equal to $8 \times 10^{-5}$ M. For $C_{14}MIMBF_4$. Note that $k_{CAT}$ displays an increase from $4 \times 10^{-3}$ to $1 \times 10^{-2}$ $s^{-1}$ at the CMC value (quite similar to that observed for the $C_{12}$ surfactant), whereas $K_M$ diminishes from $4 \times 10^{-4}$ M in water, to $1 \times 10^{-4}$ M at 4 mM $C_{14}MIMBF_4$, exactly the same as the $C_{12}$ derivative, but much more closer to the actual $C_{14}$ CMC value. Finally, $C_{16}MIMBF_4$, shows an overall similar behavior compared to $C_{12}$ and $C_{14}$ surfactants. However, the magnitude of its influence over $k_{CAT}$ and $K_M$ is by far less significant. For $k_{CAT,}$ an increase from $4 \times 10^{-3}$ to $6 \times 10^{-3}$ $s^{-1}$, close to the CMC value of the surfactant, beyond this point a decrease is observed. At 1.6 mM $C_{16}MIMBF_4$, a value of $k_{CAT}$ four times lower than that measured in the absence of surfactant is observed. Similarly, in the surfactant concentration interval, $K_M$ displays a marginal decrease, from $4 \times 10^{-4}$ to $3 \times 10^{-4}$ M.

For the commonly accepted model of surfactant-induced enzymatic superactivity, the bell shaped profile of activity is explained by the conformational changes on the enzyme due to its interaction with the surfactant molecules. According to the behavior observed by the Michaelis-Menten parameters measured for the set of surfactants studied, the shape of the catalytic efficiency profile is mainly governed by the magnitude of the increase in kcat, which might be associated to an enhancement on the rate of the deacylation step of the α-CT mechanism. On the other hand, the moderate decrease of Km, albeit of being indicative of an increased affinity of the enzyme or the synthetic substrate, does not significantly changes from the $C_{12}$ to $C_{16}$ surfactants in order to be relevant for the obtained profiles observed in the kcat/$K_M$ vs surfactant concentration plots.

To follow up with the study on the influence of $BMIMBF_4$ on the aforementioned phenomena, $C_{12}MIMBF_4$ was considered as our subject of study, in part due to its larger influence on α-CT activity, and also due to solubility issues of both the $C_{14}$ and $C_{16}$ derivatives in the presence of the IL. Sorted this out, the assays of enzymatic activity versus surfactant

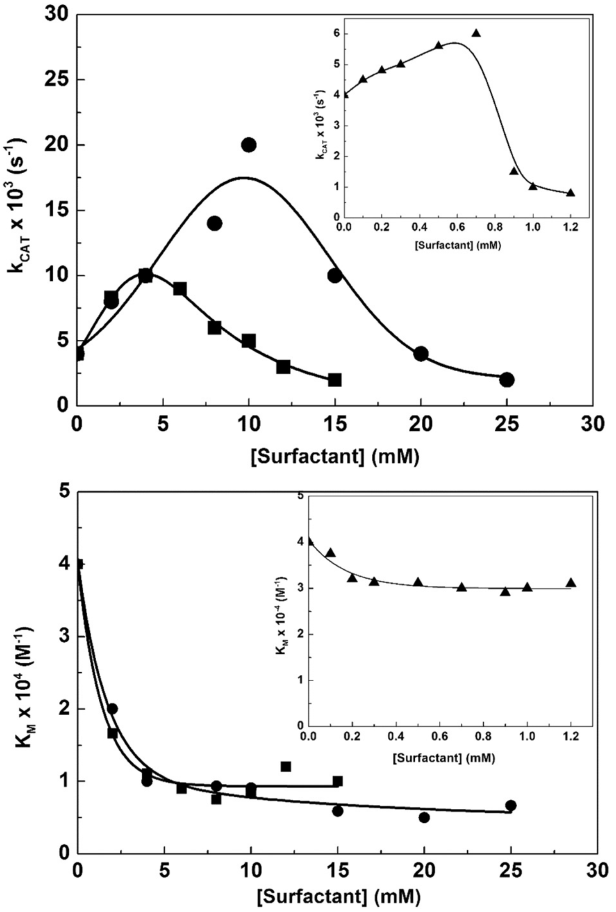


**Fig. 2.** Variation of kcat (A) and $K_M$ (B) upon the addition of long alkyl chain surfactants from the 1-alkyl-3-methylimidazole family, in aqueous solution (pH = 7.0; 25 °C) and [α-CT] = 10 μM. $C_{12}BMIMBF_4$ (●); $C_{14}BMIMBF_4$ (■); $C_{16}BMIMBF_4$ (▲). (Data on Table S2 on Appendix A).

concentration were successfully performed in water/$BMIMBF_4$ mixtures up to a surfactant concentration of 30% w/w. In terms of the relative activity of the enzyme in the water/$BMIMBF_4$ mixtures, the activity of α-CT remained constant up to a 30% w/w of IL, followed by a steep decrease of activity up to a value of 80%, concentration value from which a complete loss of activity is observed. This loss of activity has been attributed to a withdrawal of the water molecules from the active site of the enzyme due the increase in IL concentration.

A relevant aspect to further consider as a starting point when analyzing the enzymatic activity modulation mediated by surfactant molecules in the presence of $BMIMBF_4$ is associated with the fact that the presence of the IL might affect the partition of the substrate molecules between the micelles and the external media. Table 2 shows PNTMA partition data obtained by ultrafiltration experiments. The presence of the IL, evaluated at the maximum concentration considered in our experiments, does modifies in a significant way the partition of PNTMA, leading to an increase on the partition constant, probably due to the withdrawal of the substrate from the micellar media. These results suggest the possibility that the influence of $BMIMBF_4$ might operate by directly modifying the behavior of the substrate in the microheterogenous media provided by the presence of the micelles. Taking into account that the partition process is mediated by differences on the substrate solubility between water and the micellar environment, the concentrations of IL considered might introduce important changes between the initial polarity of pure water and the polarity changes introduced by the addition of $BMIMBF_4$, which polarity has been reported as comparable to that of ethanol [44].

**Table 2**
PNTMA partition data measured by ultrafiltration in water and water/$BMIMBF_4$ mixtures, in the presence of micelles of 1-dodecyl-3-methylimidazole tetrafluoroborate.

| [$BMIMBF_4$] (w/w %) | [Surfactant] (mM) | [Surfactant]m (mM) | $F_{free}$ | Kp |
|---|---|---|---|---|
| 0 | 15 | 2.2 | 0.78 | 0.13 |
| | 20 | 7.2 | 0.44 | 0.18 |
| | 25 | 12.2 | 0.21 | 0.31 |
| 5 | 15 | 3.6 | 0.76 | 0.09 |
| | 20 | 8.6 | 0.52 | 0.11 |
| | 25 | 13.6 | 0.27 | 0.20 |
| 10 | 15 | 4.2 | 0.84 | 0.05 |
| | 20 | 9.2 | 0.61 | 0.07 |
| | 25 | 14.2 | 0.43 | 0.09 |
| 20 | 15 | 5.5 | 0.88 | 0.02 |
| | 20 | 10.5 | 0.70 | 0.04 |
| | 25 | 15.5 | 0.57 | 0.05 |
| 30 | 15 | 5.9 | 0.94 | 0.01 |
| | 20 | 10.9 | 0.78 | 0.03 |
| | 25 | 15.9 | 0.73 | 0.02 |

More information on Table S2 on Appendix A.

Fig. 3 shows the α-CT catalytic efficiency profiles, corrected considering the substrate partition obtained in water/$BMIMBF_4$ mixtures. At pre-CMC surfactant concentrations, low concentrations of IL (5% w/w), induces negligible differences on the influence exerted by the surfactant molecules on the enzyme activity. Further increase on $BMIMBF_4$ concentration, from 10 to 30% w/w induces an almost tenfold decrease on the catalytic efficiency value, compared to water. From these results at least two interesting observations can be made. First, it seems to be that a certain threshold of ionic liquid concentration is required in order to modify in a significant fashion the influence exerted by the surfactant molecules over α-CT. Second, the near exponential decrease on kcat/$K_M$, at a given surfactant concentration, with the increase on the $BMIMBF_4$ concentration, suggests that the effect of the IL goes beyond a simple modification on the surfactant/α-CT interaction (changes in the ratio of surfactant molecules per enzyme molecules), leading to the possibility that the influence of the ionic liquid might operate directly over the enzyme, and the mechanism by which the surfactant is able to increase the catalytic rate constant, thus becoming less efficient in the presence of $BmimBF_4$. These phenomena are in line with the aforementioned threshold of IL concentration, thereby suggesting that a critical amount of IL is required in the microenvironment of the enzyme in order for the IL to induce a noticeable influence on the process under study.

In the vicinity of the CMC value of $C_{12}MIMBF_4$ in water, the maxima of catalytic activity achieved displays an important decrease, with a

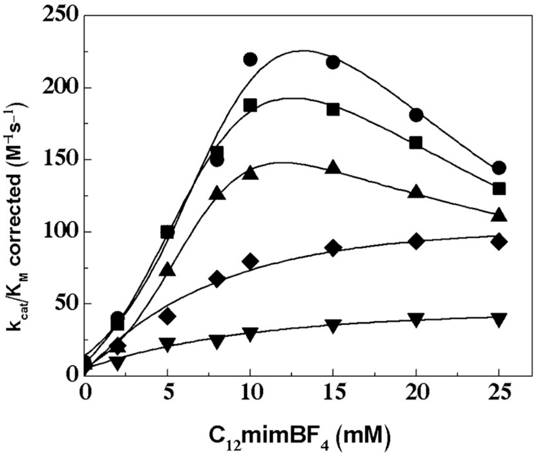


**Fig. 3.** Substrate partition corrected catalytic efficiency (kcat/Km) of α-CT as a function of the concentration of $C_{12}BmimBF_4$, [α-CT] = 10 μM. Water (●); 5% w/w $C_{12}MIMBF_4$ (■); 10% w/w $C_{12}MIMBF_4$ (▲); 20% w/w $C_{12}MIMBF_4$ (♦); 30% w/w $C_{12}MIMBF_4$ (▼) (Data on Table SM3 on Appendix A).

moderate increase in the IL content, ranging from a value of $k_{CAT}/K_M$ of 225 in water, to 150 at 10% of BMIMBF$_4$. Moreover, with the increase on the IL content, the maximum value of the catalytic efficiency is displaced towards lower BMIMBF$_4$ concentrations. Strikingly, at concentrations above 10% w/w IL, the maxima of $k_{CAT/}K_M$ near the CMC disappears, leading to a change on the shape of the $k_{CAT/}K_M$ vs surfactant concentration profiles, from the bell shaped curve observed in water, to an hyperbolic increase (albeit a moderate increase compared to water) on the catalytic efficiency with the surfactant concentration, at 30% w/w BMIMBF$_4$.

The observed loss of the peak catalytic efficiency near the surfactant CMC, with the increase on the IL content in aqueous media, could be linked to a lessened efficiency of the surfactant molecules to influence the enzyme behavior. Initially, at IL concentrations near 10% w/w, the initial stage of the influence of the surfactant molecules observed at pre-CMC surfactant concentrations, is now coupled with the micelle formation phenomena, which might affect in a more impactful way the effect elicited by the surfactant, leading to decrease on the peak values of $kcat/K_M$. The observed behavior correspond to an interesting result when compared to the behavior of the CMC values measured for $C_{12}$MIMBF$_4$ on the studied BMIMBF$_4$/water mixtures. Particularly the shift of the peak catalytic efficiency towards lower surfactant concentrations. From the data displayed in Table 3, it can be seen a progressive decrease on the CMC values of the surfactant, measured by changes on the I/III band ratio from the hyperfine structure of the fluorescence spectra of pyrene, probably associated to a "salting out" of the surfactant molecules from the aqueous environment due to the presence of BMIMBF$_4$.

The second question to be addressed rests on the disappearance of the peak catalytic efficiency at BMIMBF$_4$ concentrations beyond 10% w/w. When the magnitude of the catalytic efficiency at the respective CMC values, measured at each BMIMBF$_4$/Water is compared to that measured at surfactant concentrations past the CMC, interesting observations can be made regarding the data shown in Fig. 3. It shows a particular response of the post-CMC α-CT activity with the addition of BMIMBF$_4$. With the increase on the IL concentration, namely up to 20% w/w BMIMBF$_4$, the decrease on α-CT activity post CMC is preserved, albeit with an smaller decrease at the highest surfactant concentration studied, in contrast to the major activity decrease observed at the CMC in the mentioned IL/water compositions, reaching an almost constant $kcat/K_M$ value past the CMC value of $C_{12}$MIMBF$_4$ in 20% w/w BMIMBF$_4$ in water. Interestingly, at 30% w/w BMIMBF$_4$, the observed trend leads to an important change on the catalytic efficiency vs. surfactant concentration profiles, displaying a hyperbolic behavior, where the value of $kcat/K_M$ at the CMC is now lower than those evaluated at high surfactant concentrations, values that remain constant but are lower than those observed at 20% in mass of BMIMBF$_4$.

Specifically, the post-CMC results might be indicative that the presence of the ionic liquid has influence on a variety of levels on the behavior of α-CT in surfactant-based microheterogeneous systems. From surfactant concentrations from the CMC and onwards, it was established that the partition of the substrate plays a relevant role on the decrease of activity observed upon the apparition of micelles into the system. The decrease of activity, even after correction of $Kcat/K_M$ to take into account the substrate partition, points towards a loss on the efficiency of the enzyme to catalyze the hydrolysis of the free substrate, even though the partition of the substrate towards water is improved in the presence of the IL. However, the substrate partition does not fully account for the differences observed at- and beyond the CMC. The fact that the catalytic efficiency of α-CT readily decreases at the CMC, but seem to be less affected by the presence of the IL, when there is a large concentration of micelles, leads the possible explanation to the theory that supports the presence of unbound and bound forms of the enzyme, and the shift in balance between these two forms due to the increase in surfactant concentration and the presence of the IL.

**Table 3**
Concentration of 1-dodecyl-3-methylimidazole tetrafluoroborate at the maximum value of catalytic efficiency, compared with the critical micellar concentration (CMC) of the surfactants under consideration and the hydrodynamic diameter of the resulting micelles.

| Solvent | $[C_{12}MIM^+BF_4^-]$ (mM) | | | |
|---|---|---|---|---|
| | At peak $k_{cat}/K_M$ | CMC (DLS) | CMC (Pyrene)* | Size (nm) |
| Water | 13.1 ± 0.7 | 12.0 ± 0,5 | 12.8 ± 0,2 | 9.4 ± 0,8 |
| 5% BMIMBF$_4$ | 12.5 ± 0,6 | 12.1 ± 1,1 | 11,4 ± 0,5 | 9.8 ± 0,3 |
| 10% BMIMBF$_4$ | 11.3 ± 0,2 | 11.8 ± 0,4 | 10,8 ± 0,1 | 10.1 ± 0,5 |
| 20% BMIMBF$_4$ | NA | 10.3 ± 1,0 | 9,5 ± 0,5 | 10.0 ± 0,8 |
| 30% BMIMBF$_4$ | NA | 9.8 ± 0,5 | 9,1 ± 0,8 | 10.9 ± 0,7 |

NA = data not available; *More information on Fig. S3 on Appendix A.

First, as was previously discussed, the surfactant monomers induce an important increase on $kcat/K_M$ up to the CMC. Under these conditions, the presence of the IL affects the surfactant/enzyme interaction, leading to a decrease on the maximum $kcat/K_M$ achieved, at this point, only the behavior of the "free" enzyme is observed. Second, beyond the CMC, substrate partition becomes relevant, leading to the important decrease of activity observed (which is lessened when kcat is calculated considering the free substrate concentration), at the same time, α-CT molecules begin to interact with the micellar aggregates, purportedly, these enzyme molecules are less active than their free counterparts (which still possess their "surfactant enhanced" activity), leading to the observed decrease on activity even when the substrate partition is accounted. At this stage, the role of the IL is either associated to: i) changes in the way that the micelles are able to modify the activity of the bound enzyme or ii) the inability of the IL to affect the micelle-bound α-CT.

Regarding point i), it could be tempting to think that the increase on the IL proportion might be able to modify the interfacial characteristics of the micelles (particularly with the changes on hydrodynamic radii observed in the presence of IL), with a direct impact on the activity of the bound enzyme, which could lead to a preservation of the enhanced activity or a further increase (which might be limited due to the partition behavior of the substrate), with the increase on the micellar concentration. However, the most likely explanation, according to the presented data, correspond to ii), may be as follows: while the IL is able to negatively influence the monomeric surfactant/free enzyme interaction, relevant to the decrease of activity at the CMC (CMC value also decreases with the addition of IL), it is unable to affect the enzyme molecules in direct contact with the micelles. Further binding experiments to evaluate the interaction between the enzyme and the micelles are required to delve into this phenomenon.

Also in Table 3, the hydrodynamic radii of the $C_{12}$MIM BF$_4$ micelles, determined by dynamic light scattering measurements, shows that the topography and integrity of the micelles in BMIMBF$_4$/water mixtures is quite comparable to those in pure water, with no significant size changes that might indicate differences in shape or aggregation state of the micelles, thereby reinforcing the fact that the decrease of the maximum catalytic efficiency of α-CT, with comparable amounts of surfactant, and the concomitant peak displacement towards lower surfactant concentration, is related to the presence of fewer surfactant molecules able to interact with the enzyme. A response probably due to the early formation of $C_{12}$MIMBF$_4$ micelles as a consequence of the addition of BMIMBF$_4$.

The kinetics of the pre-stationary conditions of the catalytic mechanism of α-CT were also considered. In order to do this, the mathematical treatment proposed by Bender et al. [45] was used, enabling the study of the individual steps of the kinetic mechanism (for more details, please refer to Appendix A). In particular, the evaluation of the ratio $k_{-1}/k_1$, commonly associated to the classical equilibrium constant (Ks) for the formation of the enzyme/substrate complex, as well as $k_2$, the rate constant for the transformation of the substrate-enzyme complex into the acyl-enzyme complex, as is considered in a three-step Michaelis-Menten mechanism, as shown in Scheme 1.

Fig. 4 shows the variation of the aforementioned kinetic parameters as a function of both surfactant concentration and IL content. At low surfactant concentrations, the variation observed for $k_{-1}/k_1$ (Fig. 4A), show a decrease of the ratio with the surfactant concentration, further supporting the influence of the surfactant over the formation of the substrate-enzyme complex. The lessened influence of the surfactant on $k_{-1}/k_1$ with the increase on $BMIMBF_4$ content goes in the direction of the diminished interaction between the enzyme and the surfactant.

The progression of both $k_2$ and $k_3$ rate constants with the addition of surfactant show a bell-shaped profile for both parameters. The variation of both rate constants shows an overall decrease over the range of $BMIMBF_4$ concentrations considered. It is interesting to note that $k_3$ values, albeit smaller than $k_3$, seems to have a considerable impact on the decrease observed on $K_M$, according to the relationship $K_M = (k_{-1}/k_1)[k_3/(k_2 + k_3)]$. Therefore, the influence of the surfactant on the observed $k_3$ change, diminished by the addition of $BMIMBF_4$, suggest that the IL has direct influence over the mechanism by which the surfactant molecules increase (or decrease) the rate of the acyl-enzyme complex formation, evidenced in the increased rate of release of PNP. Taking into consideration that, in the absence of surfactant, $k_2$ is not significantly modified in the water/$BMIMBF_4$ mixtures considered (up to 30% w/w $BMIMBF_4$). These results might support the fact that the interaction of the enzyme with the IL may supersede its interaction with the surfactant molecules, therefore inducing the loss of effectiveness of the surfactant to modify the enzyme acylation process. Hence, the relevance of the modification of $k_{cat}$, the rate limiting step of the reaction, is related to the formation of the acylated enzyme as a consequence of the interaction between the “empty” enzyme molecules and the substrate, which leads to the fast release of PNP observed at this stage, up to a point in which all of the enzyme molecules are acylated. As a result, a slow progressive PNP release follows on the stationary conditions, where now the release of PNP is dependent of the deacylation of the enzyme molecules, and their ability to restart the catalytic cycle.

Based on the analysis of the pre-stationary kinetics condition, the antagonizing effect between $C_{12}MIMBF_4$ and $BMIMBF_4$, can be summarized by two considerations: i) in aqueous solution, the influence of the surfactant is greater in the later steps of the catalytic mechanism, than in the earlier steps. This result may be traced to the change of $k_3$ and $k_2$ as well as $K_M$ and $k_{-1}/k_1$ with surfactant. ii) On the other hand, in the presence of $BMIMBF_4$, the influence of the surfactant molecules is affected by the IL. This effect may be based on the values of the $k_2$ and $k_3$ rate constants.

In order to evaluate the influence of the IL on the structure of the enzyme, we studied the thermal behavior of α-CT in the presence of $BMIMBF_4$, by monitoring the change on the intrinsic fluorescence of the enzyme (associated to the presence of TRP residues on its amino acid sequence) with the increase of temperature. Fig. 5A shows the denaturation profiles of α-CT at different $BMIMBF_4$ weight percentages, where, from the midpoint of the denaturation profiles, the enzyme main transition temperature (Tm) can be determined. The obtained profiles display a progressive shift of Tm towards higher values with the increase of IL in the aqueous solution, ranging from a value of 48 °C in water, 52 °C at 30% w/w and up to 66 °C in pure $BMIMBF_4$ (Table 4; Fig. 5B). The observed behavior of the enzyme Tm values indicates a further stabilization of the enzyme structure due to its interaction with the IL, [31,46–48] indicated by the increased amount of energy required to achieve a given degree of denaturation of the protein ensemble, as presented in Fig. 5A, where the fraction of denaturation is correlated with the IL content. Interestingly, the U-shaped profile obtained from the derivative of the native fraction with the temperature are preserved, irrespective of the $BMIMBF_4$ content in the aqueous environment, thereby suggesting that the denaturation pathway followed by the enzyme is well preserved, irrespective of the potential interactions (favorable/unfavorable) introduced by the IL.

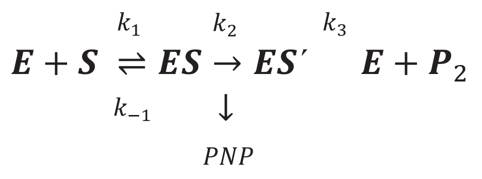
Form the plot presented in Fig. 5C, the thermodynamic parameters determined for the melting transition of α-CT (Table 3) reveal an enthalpy-driven process, with an increase on the enthalpy of the process with the addition of ionic liquid, with little to null variation in the entropy of the process, which might be associated with an entropic stabilization where the denaturation process of α-CT is coupled with another process that we are not able to monitor with the performed experiment (i.e entropy changes due to structural changes in other sector of the protein that does not influence the Trp fluorescence monitored and/or entropy changes associated to the complexity of the solvent due to the presence of the ionic liquid). The $BMIMBF_4$ interaction seems to be inducing a “stiffening” of the protein, which may have direct relationship with the decrease of the influence due to surfactant molecules over the activity of the enzyme in the presence of $BMIMBF_4$. This observation may be traced to direct interactions of the ionic liquid with the protein/water interface or potential rearrangement of the solvent cage surrounding the protein molecules, which leads to changes in the exposure of polar/non polar regions of the protein.

The implications of the IL-induced stabilization of the enzyme structure and its influence over its interaction with the surfactants under study, might be associated to an “acquired resistance” of the enzyme to interact with the surfactant molecules, associated with a more compact state of the globular structure of the enzyme, which might hinder the incorporation of the surfactant to the hydrophobic pockets present on the structure of the enzyme. Also, it is worth considering that the surface interactions between $BMIMBF_4$ and the ionizable side chains of the protein, may lead to fewer sites of interaction between the enzyme and the charged moiety of the surfactants. Based on these observations, it is possible to address the lesser influence of the surfactants on the activity of α-CT to the $BMIMBF_4$/enzyme interaction which renders the enzyme less susceptible to the surfactants influence, particularly in the vicinity of the critical micellar concentration.

Fig. 6 shows the variation of the wavelength of the fluorescence maxima of α-CT with temperature. In water there is a progressive, albeit moderate, shift towards longer wavelength with the increase of temperature in the 20–40 °C interval, thereby indicating the initial steps of an overall exposure of the enzyme TRP residues to the more polar environment provided by water. Beyond 50 °C, there is a considerable red shift (˜10 nm) in a 5 °C temperature interval, probably associated with an important loss of tertiary structure of the more labile segments of the protein that bear TRP residues. Past 60 °C, the change on the maximum position leads to a constant value at 360 nm.

In the presence of $BMIMBF_4$, there are particular differences and similarities with the response observed in water. At 30% w/w $BMIMBF_4$, there is no significant change in the position and variation of the fluorescence maxima up to 50 °C. Past 50 °C, the change in wavelength is less steep than that observed in water, leading to a value of 356 nm from 70 °C onward, indicating that at this IL composition, pointing towards a lesser exposure of the TRP residues or/and that these residues are now, fully exposed, but are subjected to a less polar environment due to the addition of the IL to the aqueous environment. This change is not evident when the protein is in a more compact conformation. In pure $BMIMBF_4$, the observed emission wavelength is overall lower than their water counterparts. In addition, the change of the position of the fluorescence band does displays a smoother variation with the increase of temperature, following a progressive red shift at temperatures up to 70 °C, followed by an increased rate of change of the wavelength at higher temperatures, leading to a maxima located at 347 nm at 90 °C.

The influence of the IL and its relevance on the interaction between $C_{12}MIMBF_4$ and α-CT becomes patent when the enzyme fluorescence is



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} ES' \overset{k_3}{\phantom{\rightarrow}} E + P_2$$
$$\qquad\qquad\qquad\qquad \downarrow$$
$$\qquad\qquad\qquad\qquad PNP$$

**Scheme 1.** Michaelis-Menten mechanism for the hydrolysis of PNTMA catalyzed by α-chymotrypsin.

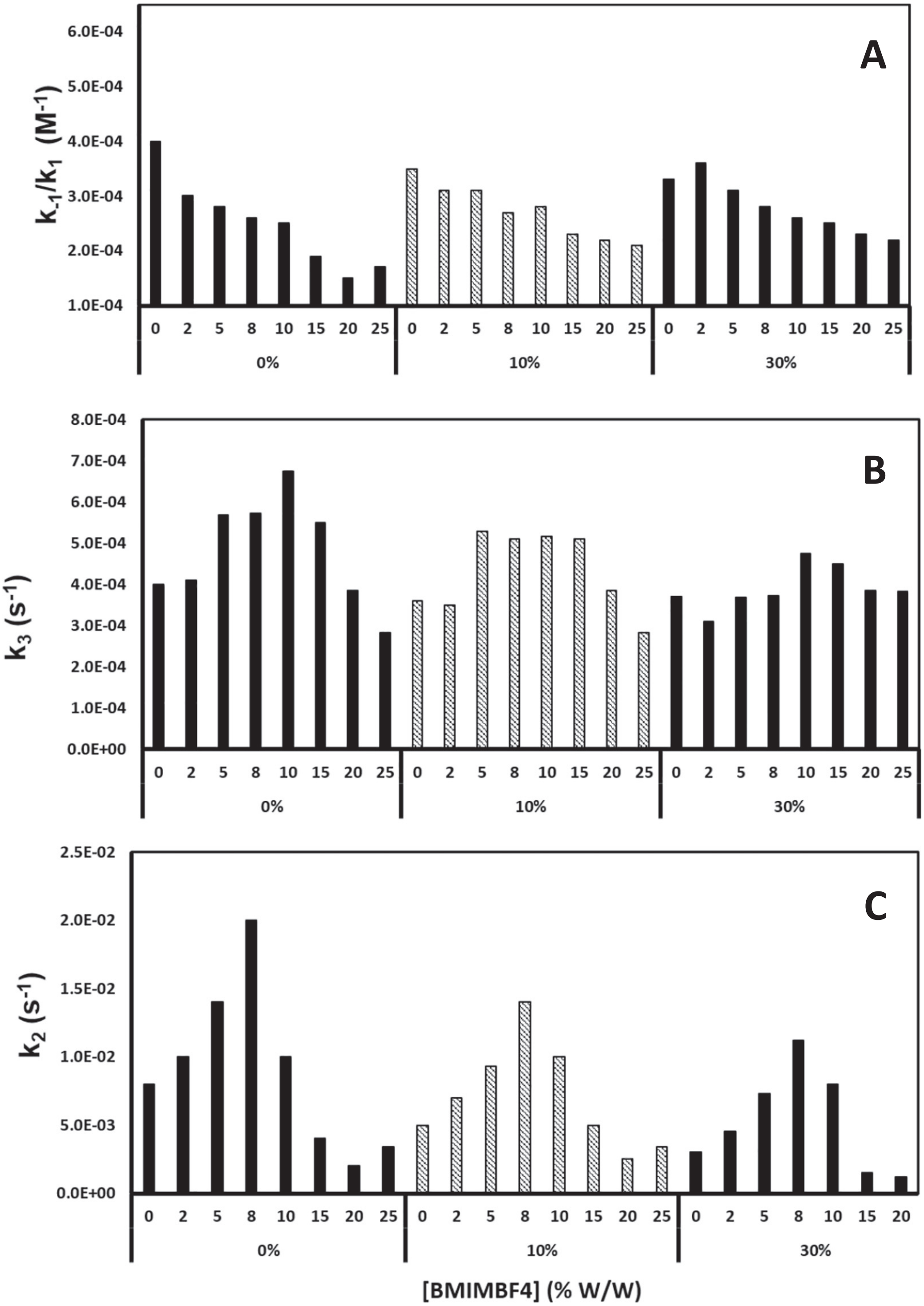


**Fig. 4.** Variation of the catalytic rate constants. A) $k_{-1}/k_1$ ratio; B) $k_3$; C) $k_2$, for the α-CT catalyzed hydrolysis of PNTMA, with the $C_{12}MIMBF_4$ surfactant concentration, in water and water/$BMIMBF_4$ mixtures (Data on Table S4 on Appendix A).

registered as a function of the surfactant concentration in aqueous solution (Fig. 7). In water, the surfactant molecules are able to induce a moderate change in the measured fluorescence intensity of α-CT, leading up to a 30% decrease of fluorescence a the maximum $C_{12}MIMBF_4$ concentrations considered in the experiment (25 mM). When $BMIMBF_4$ is added to the aqueous media, from the results on Fig. 7, it is observed that even at low percentages of $BMIMBF_4$ (5% w/w) a decrease on the change of the fluorescence intensity of α-CT is observed, with the addition of similar amounts of surfactants. At 30% w/w $BMIMBF_4$, the efficiency of the surfactant molecules to induce an equivalent change on the fluorescence intensity of the protein is dramatically lessened by the presence of the IL.

Taking into consideration that both, $BMIMBF_4$ and $C_{12}MIMBF_4$, share the imidazole moiety in their chemical structures, and that this group is the most likely responsible for the fluorescence quenching observed, we are positive that the behavior described in Fig. 7, can support the

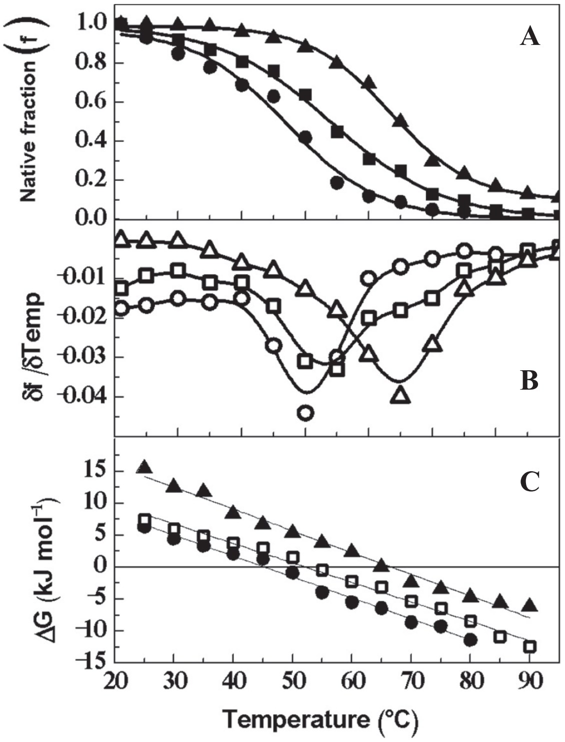


**Fig. 5. A:** Denaturation curve of α-CT solutions represented as fraction of native protein (calculated according Eq. (1)) as a function of temperature. Solvents: Water (●); Water/30% $BMIMBF_4$ (■); $BMIMBF_4$ (▲) **B:** First derivative with respect to temperature of the native fraction of α-CT in different solvents. Water (○); Water/30% $BMIMBF_4$ (□); $BMIMBF_4$ (△). **C:** Free energy of denaturation vs temperature profiles. Water (●); Water/30% $BMIMBF_4$ (□); $BMIMBF_4$ (▲) (Data on Tables S5–7 and S8–10 on Appendix A).

hypothesis that the interaction $C_{12}MIMBF_4$/α-CT is affected by the ionic liquid, hence the surfactant induced enzymatic superactivity is affected in $BMIMBF_4$/water mixtures.

The results herein presented, particularly regarding the prediction of the influence of IL on the interaction between the enzyme/substrate and surfactant/micelle open up interesting venues and potential research lines devoted to try to establish the dependence of the described phenomena with the nature of both the cation and anion of the ionic liquid, particularly taking into consideration the vast collection of IL series known. If we only draw our attention to the enzyme/substrate pair, the complexity of the interaction with the surfactants and the ionic liquids, can lead us to believe that the overall phenomena might be conserved (at least for hydrolytic enzymes such as α-CT) but the magnitude of the influence of the ionic liquids might largely vary between different enzyme families.

## 4. Conclusions

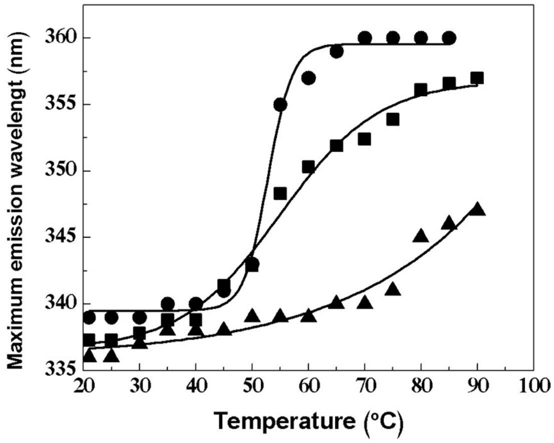


**Fig. 6.** Wavelength of the fluorescence maxima of α-CT as a function of temperature. Water (●); Water/30% $BMIMBF_4$ (■); $BMIMBF_4$ (▲). (See Fig. S4 on Appendix A).

1-alkyl-3-methylimidazole tetrafluoroborate derived surfactants can increase the catalytic efficiency of α-chymotrypsin on the hydrolysis of *p*-nitrophenyl trimethylacetate. This effect is negatively affected by the decrease on the surfactant's CMC and the partition of the substrate between the micelles and the external media observed above the CMC. From the series of surfactants tested, $C_{12}MIMBF_4$ was the more efficient one, regarding the increase of the catalytic efficiency of α-chymotrypsin, both in water and water/$BMIMBF_4$ mixtures, $C_{14}$ and $C_{16}$ surfactants displayed solubility issues in the presence of the ionic liquid.

In water/$BMIMBF_4$ mixtures, the presence of the ionic liquid overrides the effect elicited by the surfactants on the activity of α-CT. This phenomenon takes place, up to a different extent, below and at the CMC, thereby modifying the bell shaped profile of the catalytic efficiency vs. surfactant concentration plots. The loss of the surfactant molecules influence on the enzymatic activity due to the presence of $BMIMBF_4$ seems to be a complex process, primarily controlled by a decreased affinity between α-chymotrypsin and the surfactant molecules and, to a larger extent, the appearance of an “acquired resistance” of the enzyme to the influence of the surfactants, related to a more compact and resilient conformation of the protein in the presence of $BMIMBF_4$. In addition, the influence of $BMIMBF_4$ is not only limited to the surfactant/enzyme interaction, but also it does modifies the partition of the substrate between the aqueous media and the micellar environment, increasing the aqueous concentration of substrate.

In the presence of micelles, enzyme/micelle interactions would lead to a population of enzyme molecules abstracted from the influence of the IL, where the overall influence of $BMIMBF_4$ on the overall catalytic

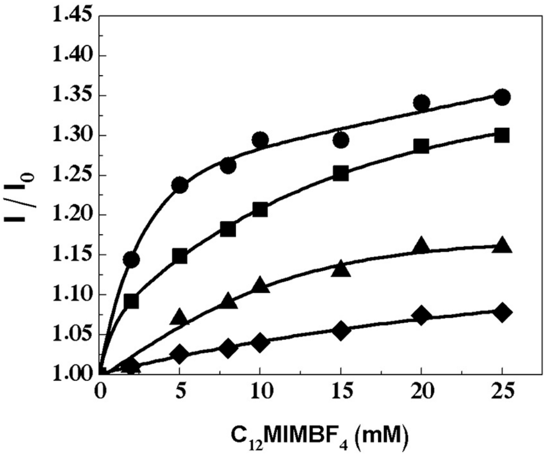


**Fig. 7.** Variation of tryptophan intrinsic fluorescence upon the addition of $C_{12}MIMBF_4$ to solutions of α-CT (10 μM) in water and water/ionic liquid mixtures. Water (●); water/5% $BMIMBF_4$ (■); water/10% $BMIMBF_4$ (▲); water/30% $BMIMBF_4$ (♦). (See data on Table S11 on Appendix A).

**Table 4**
Thermodynamic parameters for the thermal denaturation of α-CT, estimated at the thermal transition temperature (Tm) in water and in the presence of $BMIMBF_4$.

| Solvent | ΔHm (kJ/mol) | ΔSm (kJ/mol K) | ΔH/ΔS | Tm |
|---|---|---|---|---|
| Water | 14.8 ± 0.5 | 7.3 ± 0.7 | 2.0 | 48.0 ± 2.0 |
| 30% BMIMBF4 | 17.9 ± 0.8 | 6.5 ± 0.2 | 2.8 | 52.1 ± 1.5 |
| Pure BMIMBF4 | 22.7 ± 1.0 | 5.4 ± 1.1 | 4.2 | 66.3 ± 0.8 |

efficiency profiles is dependent on the fluctuation of the media between solution and a microheterogeneous biphasic system.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2019.03.106.

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