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Article

Imidazolium-based ionic liquids as additives to preserve the Enhanced Green Fluorescent Protein fluorescent activity

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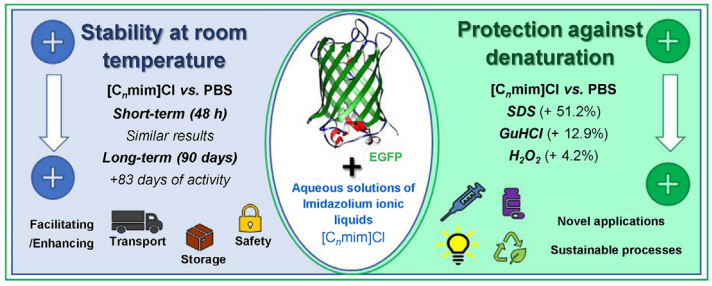
HIGHLIGHTS

- Positive effect of diluted [$C_n$mim]Cl solutions on EGFP fluorescence in the long-term.
- [$C_n$mim]Cl preserved EGFP fluorescence at room temperature (from 7 in PBS to 90 days).
- [$C_n$mim]Cl protected EGFP against chemical denaturation from SDS, GuHCl, and H_2O_2.
- [$C_n$mim]Cl can act as preservatives of EGFP at room temperature and under stress.
- The [$C_n$mim]Cl with n = 2 to 8 were more effective at preserving EGFP fluorescence.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluorescent Proteins (FP) can be applied as biomarkers and biosensors in the industrial and medical fields, but their large-scale use, especially for new industrial applications, is limited due to their low stability. Hence, the discovery of additives capable of preserving the activity of FP at room temperature and under stress conditions can help to expand and facilitate their commercial use. With this goal, we evaluated the application of 1-alkyl-3-methylimidazolium chloride-based ([$C_n$mim]Cl) ionic liquids (ILs) as additives to preserve the activity of Enhanced Green Fluorescent Protein (EGFP) at different storage times and under unfavorable conditions. All [$C_n$mim]Cl solutions (at 0.100 mol L^{-1}) were able to preserve EGFP fluorescence for longer than the phosphate-saline buffer (PBS) and NaCl solutions, increasing its fluorescence manifestation from 1 to 3 months. [$C_n$mim]Cl with shorter to medium cationic alkyl chains were the most effective in preserving EGFP fluorescence. [$C_n$mim]Cl also protected EGFP activity in the presence of the surfactant SDS, the acid guanidine hydrochloride, and H_2O_2. Therefore, [$C_n$mim]Cl can be added to aqueous solutions to preserve EGFP fluorescence activity at room temperature for longer storage times and to reduce the negative impact of denaturing agents on EGFP. Therefore, there is a massive potential for the application of ILs as additives to preserve FP in the long-term without

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refrigeration and under unfavorable conditions, and this is fundamental to enable expansion of FP in industrial and commercial applications.

1. Introduction

Modern biotechnological tools are supporting the discovery and production of novel recombinant proteins such as Fluorescent Proteins (FP), which have remarkable industrial and medical applications as biomarkers and biosensors [1–4]. However, like most proteins, the commercial use of FP is limited due to their low stability, which hinders their distribution, storage, and handling (considering the need for complex cold chains to maintain their biological activity) and, as a consequence, their industrial applications (especially in chemical reactions that require high temperatures or presence of co-solvents) [1,2,5,6]. Therefore, finding additives with the capacity to preserve the activity of FP and other proteins without refrigeration or under unfavorable conditions is crucial not only to widespread access to protein-based biological products, but also to expand their use in more disruptive industrial applications (*e.g.*, energy [7] and textile [8] fields).

The instability of many proteins outside their optimal conditions can hinder their activity and commercial use [9,10]. This is particularly the case for FP, a group of globular proteins with a chromophore located inside a β-barrel maintained by a core helix, where disruption of the cylindrical protein structure that holds the chromophore at its center will cause fluorescence extinction [1,11]. Hence, a variety of external disturbances (*e.g.*, pH, temperature, and certain substances) can quench FP fluorescence [12], hampering or even preventing specific applications for FP. For example, the most popular FP variant, the Enhanced Green Fluorescent Protein (EGFP, mutation F64 L/S65T), is a weak dimer prone to aggregation [2,13], very stable at alkaline pH but susceptible to acidic denaturation [2,14,15]. EGFP does have high thermal stability, with a thermal unfolding transition temperature of 79.5 °C [14]. Many chemical agents can denature EGFP, as well as other GFP variants, such as guanidine hydrochloride (GuHCl) [16,17], sodium dodecyl sulfate (SDS, depending on the buffer pH) [18,19], hydrogen peroxide (H_2O_2) [20], and urea (depending on pH and temperature) [18]. Considering FP can be disturbed by many stress conditions, finding additives capable of preserving their activity in unfavorable conditions is crucial to improve and expand their current commercial applications.

Developing additives to preserve protein activity could potentially allow the creation of new uses for protein-based products, considering their instability under stress conditions (*e.g.*, acidic and basic pH environments, high temperatures, and presence of denaturing substances) is a considerable barrier for disruptive applications [9,10]. Relative to FP-related fields, it would also be fundamentally interesting to find compounds with fluorescent quenching or dequenching abilities, which do not irreversibly damage the protein structure, especially for the development of novel FP-biosensors [2]. In addition, considering the low stability of most proteins (including FP), it is usually necessary to establish a complex cold chain to transport, store, and handle them. Cities and rural settlements with limited access to the electrical grid (particularly in low-income countries) do not have the minimal conditions to guarantee the refrigeration of protein-based products during their transport and storage [21,22]. Hence, finding substances with the ability to preserve the activity of proteins in the long-term outside of cold chains can simplify their distribution and expand access to protein-based biological products.

Researchers have recently investigated ionic liquids (ILs) as potential protein stabilizers or activity enhancers [5,23–26]. ILs are a very diverse group of salts with low melting points, that are easily tailorable to obtain distinct properties and structures [27,28]. Among the IL families, the *n*-alkyl-3-methylimidazolium chloride ILs ([$C_n$mim]Cl) are the most diverse and studied, and are a series of ILs with useful properties for a range of industrial uses (*i.e.*, low vapor pressure, high thermal stability, and good solvation capability) [27–29]. Additionally, there are already reports that successfully demonstrated the capacity of imidazolium-based ILs to stabilize proteins in short-term studies, particularly for [$C_n$mim]Cl ILs [23,27,30]. Together, these aspects encourage us to explore the potential of this class of ILs for long-term protein stabilization, particularly envisaging the expansion of FP in disruptive applications such as optically active centers or fluorophores for manufacturing dye-solar panels [31] and developing Bio-Hybrid Light-Emitting Diodes (Bio-LEDs) [32].

Despite advantageous industrial properties, imidazolium-based ILs do not always favor protein stabilization, and, depending on the chemical structure of the IL and protein type, there are imidazolium-based ILs that can impair protein activity and stability [24]. Furthermore, even the same IL solution can have different interactions with the protein depending on their concentration and medium conditions [24]. Therefore, to effectively understand the interactions between ILs and proteins, it is essential to perform comprehensive studies that consider the intrinsic characteristics of proteins, the properties of the ILs, the contributions of both cations and anions, and different concentrations and conditions of the medium. For example, while many imidazolium-based ILs will have surfactant and salt-like properties, these are dependent on alkyl chain length. As the alkyl chain length increases, the surfactant properties are enhanced, such that some are capable of forming micelles above a critical micelle concentration (CMC). In contrast, the salt-like behavior is a more dominant property for short-chained ILs, having a large effect even at low concentrations [33,34]. This different concentration-dependent behavior will also impact the interactions between ILs and proteins, especially in more complex systems with the presence of other substances (*e.g.*, denaturing agents) and variations of pH and temperature. The present work aims to assess the intricate IL-FP interactions. We used a well-known and highly fluorescent FP (EGFP) to improve our monitoring and quantification of protein alterations. Additionally, EGFP has relevant uses as a biomarker and biosensor (especially in the medical and biological fields) [1], and discoveries regarding its behavior and stabilization could improve existing applications or allow new ones.

Having as a starting point imidazolium-based ILs that maintained or increased the stability/activity of other proteins, we performed a comprehensive study to infer the effect of dilute IL aqueous solutions (*i.e.*, IL with concentrations ≤ 0.5 mol L^{-1}) on EGFP fluorescence, intending to find alternative compounds to be used as additives to preserve protein-based products. Specifically, we aimed to test the ability of different [$C_n$mim]Cl aqueous solutions to maintain the fluorescence of EGFP at room temperature or under unfavorable (denaturing) conditions. With this goal, this FP study evaluated the impact of cationic alkyl chain length and IL concentration on EGFP fluorescence activity, in the presence of denaturing agents, and with different protein storage times (*i.e.*, short and long-term).

2. Material and methods

2.1. Material

Pure EGFP (> 97.5 wt% of EGFP for total proteins) was produced [35] and purified [36] according to well-established protocols in our research group. The purity of the EGFP sample was confirmed by SDS-PAGE electrophoresis compared with pure EGFP chromatograms previously obtained by us [37].

Phosphate-Saline Buffer (PBS) at pH 7.4 [137.0 mmol L^{-1} of sodium chloride (NaCl), 2.7 mmol L^{-1} of potassium chloride (KCl), 8.0 mmol L^{-1} of sodium hydrogen phosphate (Na_2HPO_4), and 2.0 mmol L^{-1} of potassium phosphate monobasic (KH_2PO_4)] was used to recreate physiological conditions, considering that EGFP is mainly used for medical and

biological purposes (as biosensor and biomarker). PBS was prepared with standard PBS tablets from Sigma-Aldrich® and Milli-Q® water (ultrapure water of Type 1). The pH of the PBS buffer was corrected using 3 mol L^{-1} solutions of sodium hydroxide (NaOH) and HCl, prepared from NaOH ≥ 98.0% and HCl 37 wt%, allowing a maximum variation of 0.1 in pH (to avoid fluorescence quenching or dequenching due to pH alterations, considering EGFP fluorescence is sensitive to pH alterations [2]), both using reactants from Sigma-Aldrich®. The pH and conductivity of all aqueous solutions were monitored and adjusted (if necessary) after their preparation (detailed pH and conductivity values for all solutions are presented in Tables S1 and S2 of the Supporting Information (SI) for the IL-containing solutions with and without the denaturing agents, respectively).

A series of 1-alkyl-3-methylimidazolium chloride ([$C_n$mim]Cl) ILs was selected for the EGFP activity assays (their chemical structures are presented in Fig. S1 of the SI), namely: 1,3-dimethylimidazolium chloride ([C_1mim]Cl); 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl]), 1-hexyl-3-methylimidazolium chloride ([C_6mim]Cl), 1-octyl-3-methylimidazolium chloride ([C_8mim]Cl), 1-decyl-3-methylimidazolium chloride ([C_{10}mim]Cl) and 1-dodecyl-3-methylimidazolium chloride ([C_{12}mim]Cl). All ILs were acquired from Sigma-Aldrich® and Iolitec® (with purity above 98%) and used as received.

For the EGFP protection studies under unfavorable conditions, aqueous solutions were prepared using the following denaturing agents: sodium dodecyl sulfate (SDS, ≥ 99.0%), carbamide (Urea, 99.0%–100.5%), guanidine hydrochloride (GuHCl, ≥ 99%), and hydrogen peroxide (H_2O_2, 35 wt% in water). These compounds were acquired from Sigma-Aldrich® and used as received. Chemical structures of the denaturing agents are shown in Fig. S1 of the SI.

2.2. Experimental methods

2.2.1. Effect of [$C_n$mim]Cl aqueous solutions on EGFP fluorescence

The first study was a screening of the short-term effect of ILs on EGFP fluorescent activity, to select the conditions with the most potential for long-term studies. The effect of IL solutions on EGFP (5.4 μg mL^{-1}) fluorescence was evaluated at different concentrations (0.025, 0.050, 0.100, 0.250 and 0.500 mol L^{-1}) of [$C_n$mim]Cl (Fig. S1 of the SI) in PBS (pH adjusted to 7.4 ± 0.1) using single point fluorescence intensity (FI) analysis over time (0, 0.5, 1, 2, 3, 4, 5, 6, 24 and 48 h) at 25 °C. EGFP fluorescence in PBS and NaCl solutions were evaluated as controls for the same concentrations, times, and at 25 °C (short-term effect of ILs on EGFP activity).

After the short-term screening, the IL concentration of 0.100 mol L^{-1} was selected to evaluate the long-term effect of each IL solution on EGFP fluorescence activity at room temperature (maintained without light exposure, between 22 and 25 °C), for 7, 30, 60, and 90 days (long-term effect of ILs on EGFP activity). Afterward, aqueous solutions (0.100 mol L^{-1}) of four ILs with different cation alkyl chain-lengths (*i.e.*, [C_2mim]Cl, [C_6mim]Cl, [C_{10}mim]Cl, and [C_{12}mim]Cl) were used for the in-depth evaluation of the long-term effect of ILs on EGFP fluorescence using 3D fluorescence spectroscopy. Samples were maintained between 22 and 25 °C with no light exposure and evaluated after 1, 7, and 30 days.

2.2.2. EGFP protection studies using [$C_n$mim]Cl aqueous solutions

The effect of different denaturing stresses was firstly screened to find conditions with a moderate fluorescence quenching (80%–40% decrease of FI in 2 h), namely, to find chemical stressing conditions for the EGFP protection studies using IL solutions with a certain degree of denaturing effect, but without full EGFP denaturation (loss of fluorescence). The EGFP fluorescence was assessed under both physical (temperature) and chemical stress (denaturing agents). For the thermal stress study, EGFP FI in PBS at pH 7.4 (5.4 μg mL^{-1}) was measured at the temperatures of 25, 30, 40, 50, 60, 70, 80 and 90 °C for 0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h. For the chemical stress evaluation, different classes of denaturing agents were selected (GuHCl as acid, urea as base, H_2O_2 as oxidizing agent, and SDS as surfactant). The chemical stress was evaluated after 0, 0.5, 1, 2, 3, 4, 5, 6, and 30 h of exposure at 25 °C, using the following concentrations of denaturing agent: GuHCl – 1, 4, 5, 6, and 8 mol L^{-1}; urea – 4 and 8 mol L^{-1}; H_2O_2 – 0.03, 0.30, 1.50, 3.00, 6.00 mol L^{-1}; SDS – 0.003, 0.035, 0.100 and 0.175 mol L^{-1}.

After selecting the moderate (*i.e.*, drop in FI - 80%–40% decrease of FI after 2 h of exposure) denaturing conditions (SDS at 0.175 mol L^{-1}, GuHCl at 4 mol L^{-1}, and H_2O_2 at 1.50 mol L^{-1}), the potential of each IL solution to protect EGFP fluorescence activity under stress conditions was then evaluated. EGFP fluorescence (5.4 μg mL^{-1}) was evaluated for solutions of 0.100 mol L^{-1} of ILs and NaCl (in PBS, pH corrected to 7.4 ± 0.1) + denaturing agents present at 25 °C for 2 h. Additionally, negative and positive controls were also evaluated of EGFP in IL/salts and EGFP in denaturing agents with no IL present. The interactions between ILs/salts and denaturing agents without the protein were also assessed using Fourier-transform infrared spectroscopy attenuated total reflectance (FTIR-ATR).

2.3. Analytical methods

2.3.1. Fluorescence spectroscopy

For the single-point fluorescence assays, the FI of EGFP solutions was assessed using the EnSpire® multimode plate reader from PerkinElmer® at the point of maximum fluorescence of the protein (F1 λ_{ex} 488, λ_{em} 510 nm), except for the EGFP protection studies using IL solutions, in which an EnSight™ Multimode Plate Reader (PerkinElmer®) was used. Experiments were performed in triplicate with a blank, and results are presented as average ± standard deviation. Statistical differences were determined in Sigma Plot 12.0 with one-way ANOVA with post-hoc test Holm-Sidak for $p < 0.01$. During the assays, pure EGFP (5.4 μg mL^{-1}) in PBS was also evaluated and its FI at 0 h was used as the reference for 100% to calculate relative FI (%). The fluorescence intensity values in the first hour of monitoring were not considered for the statistical analysis or discussion, as they correspond to the initial conditioning period of proteins to their new environment (*i.e.*, conditioning time). During the conditioning time, different peaks and declines in FI are observed, which do not necessarily represent a real increase or decrease in protein stability or activity.

The 3D fluorescence spectra were acquired at 25 °C (maintained with a thermostatic water bath) with a spectrofluorophotometer RF-6000 SHIMADZU®, with an interval of 2.0 nm, scan speed 6000 nm min^{-1}, high sensitivity, λ_{ex} bandwidth 10.0 nm and λ_{em} bandwidth 1.0 nm, ranging from 400 to 516 nm for λ_{ex} and 470–570 nm for λ_{em}. From the 3D spectra, it was possible to extract excitation and emission spectra for EGFP 5.4 μg mL^{-1} in different solutions.

2.3.2. FTIR-ATR analysis

The FTIR-ATR analysis was performed at 25 (±1) °C using a PerkinElmer® spectrum 100/Universal diamond attenuated total reflectance (FTIR/ATR). The spectra were obtained in the wavelength range from 4000 to 400 cm^{-1}, with a data interval of 1.0 nm, 64 accumulation scans, and resolution of 4 cm^{-1}. For the FTIR-ATR, the first step of the analysis was to run a "blank" to subtract the scattering of the environment. Then, one drop of the liquid sample was deposited on the diamond window of the equipment and the analysis was executed again, providing the infrared spectra of the sample. Triplicate samples of ILs (0.100 mol L^{-1}), denaturing agents (SDS 0.175 mol L^{-1}, GuHCl 4 mol L^{-1}, and H_2O_2 1.50 mol L^{-1}), and IL + denaturing agents aqueous solutions were analyzed. The spectra of the ILs were subtracted from their respective

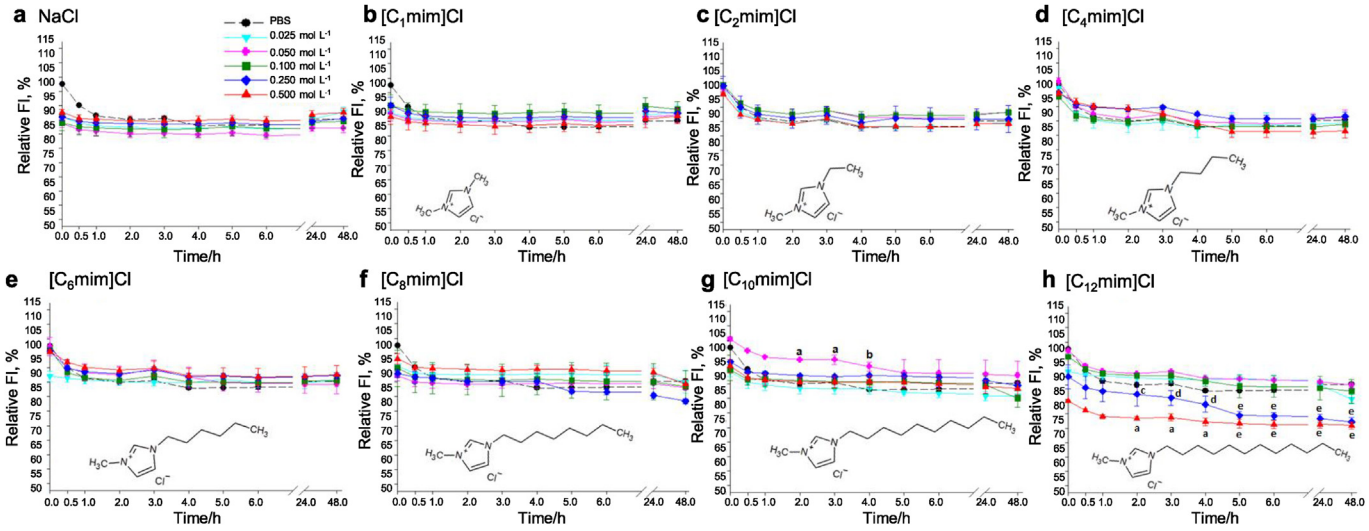

Fig. 1. Effect of NaCl and 1-alkyl-3-methylimidazolium chloride-based ILs ([$C_n$mim]Cl) aqueous solutions (concentrations: 0.025, 0.050, 0.100, 0.250 and 0.500 mol L^{-1}) on EGFP (5.4 μg mL^{-1}) relative fluorescence intensity [FI (%)] over time (48 h) at 25 °C. The pH of all solutions was maintained at 7.4 (±0.1). PBS is presented as a control and EGFP in PBS pH 7.4 at 0 h is the reference for 100%. Results were evaluated in triplicate with a blank and presented as average ± standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. Statistical differences between the concentrations for every hour after the 1 h equilibrium (one-way ANOVA with post-hoc test Holm-Sidak for $p < 0.01$, Sigma Plot 12.0) are demonstrated by the following letters: (a) different to all other groups; (b) different to PBS and 0.025 mol L^{-1}; (c) different to 0.025, 0.100 and 0.500 mol L^{-1}; (d) different to 0.025, 0.050 and 0.500 mol L^{-1}; and (e) different to PBS, 0.025, 0.050 and 0.100 mol L^{-1}.

IL + denaturing agents aqueous solutions (resulting in a spectrum of the denaturing agent in an aqueous solution) and presented as the average of the triplicate.

3. Results and discussion

3.1. Short-term effect of [$C_n$mim]Cl aqueous solutions on EGFP fluorescence

The first set of experiments was a 48 h screen to evaluate the effect of each [$C_n$mim]Cl aqueous solution on EGFP fluorescent activity at 25 °C. This was to study the influence of time and IL concentration, as well as to define the conditions with the highest potential for long-term stability and EGFP protection studies.

Before screening using IL solutions, the influence of ionic strength on EGFP relative FI was determined by evaluating if diluted solutions of a common (high melting) inorganic salt (namely, NaCl) could change EGFP fluorescence activity. Hence, EGFP FI was monitored for 48 h (at 0, 0.5, 1, 2, 3, 6, 24, and 48 h) in NaCl aqueous solution at 0.025, 0.050, 0.100, 0.250, and 0.500 mol L^{-1}. As depicted in Fig. 1a, independent of the time of exposure, all diluted aqueous solutions of NaCl (from 0.025 to 0.500 mol L^{-1}) had no significant impact (decrease of less than 5.4%) on EGFP relative FI (%) when compared to EGFP fluorescence in PBS at pH 7.4. It confirms that the ionic strength by itself at these concentrations does not affect EGFP fluorescence.

The following experimental set of studies evaluated the effect of increasing the concentration and the cationic alkyl chain length of [$C_n$mim]Cl on EGFP fluorescence activity for 48 h. The results in Fig. 1b to 1 h show that, similar to NaCl aqueous solutions, and independent of the concentration and time, almost all [$C_n$mim]Cl had no significant impact (mostly not greater than ± 5% variations, with a maximum variation of 6.8%) on EGFP relative FI when compared with PBS in the short-term (48 h). The exceptions were the [C_{10}mim]Cl solution at 0.050 mol L^{-1} after 2, 3, or 4 h, and [C_{12}mim]Cl solutions at 0.250 and 0.500 mol L^{-1}, which caused considerable quenching of the EGFP fluorescence. For the [C_{10}mim]Cl solution, the increase of relative FI of EGFP was only observed at the start of the monitoring, suggesting a longer conditioning time for the EGFP. On the other hand, the EGFP fluorescence quenching observed with the two most concentrated solutions of [C_{12}mim]Cl studied seems to be a result of the amphiphilic (*i.e.*, surfactant) nature of [$C_n$mim]Cl due to its longer alkyl chain length [38], considering they both are well above the CMC of [C_{12}mim]Cl (CMC around 0.013–0.020 mol L^{-1}) [39]. Although the other concentrations of [C_{12}mim]Cl were also above its CMC, the effect of surfactants on EGFP is also enhanced or accelerated with increasing surfactant concentration, as will be seen in section 3.3 (*EGFP fluorescence under chemical stress conditions,* Fig. 5*)* with different concentrations of SDS.

Despite the evident negative effect of [C_{12}mim]Cl, molecular dynamics (MD) studies are still required to reveal which are the unfavorable self-assembly structures created, and mainly, how these are quenching the fluorescence of EGFP.

With this initial short-term (48 h) study, we aimed to define the most appropriate IL concentration for the long-term and under chemical stress EGFP protection studies. Therefore, from the overall analysis of Fig. 1, we selected the IL concentration of 0.100 mol L^{-1} for further studies because it caused no significant changes in EGFP fluorescence activity, independent of which [$C_n$mim]Cl solution.

3.2. Long-term effect of [$C_n$mim]Cl aqueous solutions on EGFP fluorescence

The relative FI of EGFP was determined after 0, 7, 30, 60, and 90 days of exposure of the protein to different [$C_n$mim]Cl aqueous solutions (at 0.100 mol L^{-1}) to assess the long-term effect of ILs on EGFP fluorescence activity at room temperature (*i.e.,* 22–25 °C). The results obtained are depicted in Fig. 2, which compares the effect of each IL solution with PBS buffer and NaCl aqueous solutions (both used as control groups).

In contrast to the low or no significant changes in EGFP fluorescence observed in the short-term studies, the long-term evaluation of the effect of aqueous IL solutions showed substantial differences among the ILs, the buffer (PBS), and the salt (NaCl) solutions. Fig. 2 shows that all [$C_n$mim]Cl-based ILs have a remarkable ability to preserve EGFP fluorescence activity at room temperature, with relative FI of EGFP higher than 60% after 90 days of exposure to IL solutions. From a more detailed analysis, it is possible to infer that solutions composed of [$C_n$mim]Cl with cationic alkyl chain length (n) from 1 to 8 guaranteed complete maintenance (or

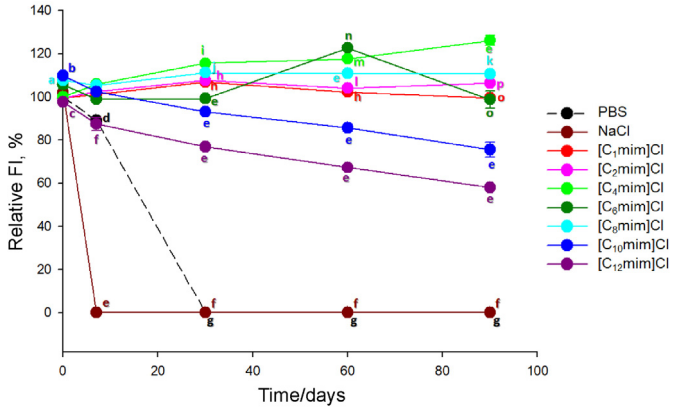

Fig. 2. EGFP (5.4 μg mL^{-1}) relative fluorescence intensity [FI (%)] for 90 days (3 months), between 22 and 25 °C and absent of light, in the presence of 1-alkyl-3-methylimidazolium chloride-based ILs ([$C_n$mim]Cl) aqueous solutions (0.100 mol L^{-1}), buffer (PBS) and conventional salt (NaCl 0.100 mol L^{-1}) controls. The pH of all solutions was maintained at 7.4 (±0.1). The results were evaluated in triplicate with a blank sample and presented as average ± standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data. Statistical differences between the groups for every day evaluated (one-way ANOVA with post-hoc test Holm-Sidak for $p < 0.01$, Sigma Plot 12.0) are demonstrated by the following letter with the color of every group: different to – (a) PBS, [C_1mim]Cl, [C_2mim]Cl and [C_{12}mim]Cl; (b) PBS, [C_1mim]Cl, [C_2mim]Cl, [C_4mim]Cl and [C_{12}mim]Cl; (c) [C_6mim]Cl, [C_8mim]Cl and [C_{10}mim]Cl; (d) All, except [C_{12}mim]Cl; (e) All; (f) All, except PBS; (g) All, except NaCl; (h) PBS, NaCl, [C_4mim]Cl, [C_6mimCl], [C_{10}mim]Cl, [C_{12}mim]Cl; (i) All, except [C_8mim]Cl; (j) PBS, [C_6mim]Cl, [C_{10}mim]Cl and [C_{12}mim]Cl; (k) All, except [C_2mim]Cl; (l) All, except [C_1mim]Cl; (m) All, except [C_6mim]Cl; (n) All, except [C_4mim]Cl; (o) PBS, NaCl, [C_4mim]Cl, [C_8mim]Cl, [C_{10}mim]Cl and [C_{12}mim]Cl; and (p) PBS, NaCl, [C_4mim]Cl, [C_{10}mim]Cl and [C_{12}mim]Cl.

even enhancement) of the EGFP fluorescence (*i.e.*, relative FI ≥ 100%) during the 90 days of study. This result is particularly relevant since the EGFP completely lost its fluorescence after seven days of exposure to a NaCl aqueous solution, and after 30 days of storage in a PBS solution. Interestingly, despite the decrease in EGFP relative FI observed after 30 days of exposure in aqueous solutions of [$C_n$mim]Cl with longer cationic chains (*i.e.*, $n = 10$ and 12), these IL solutions were better at preserving the EGFP activity at room temperature than the two controls (PBS and NaCl). The rank of the effectiveness of the [$C_n$mim]Cl solutions to preserve EGFP relative FI was the following (highest to lowest): [C_4mim]Cl > [C_8mim]Cl = [C_2mim]Cl ≈ [C_1mim]Cl ≈ [C_6mim]Cl > [C_{10}mim]Cl > [C_{12}mim]Cl. This rank is in line with the trend observed in the short-term study and in protection experiments (as will be discussed in the next section), in which increasing the length of the cationic alkyl chain of the IL decreases its ability to preserve EGFP fluorescence.

Most of the protein-IL studies in the literature reported a stronger anion influence on protein stability [40–43], but, as demonstrated in Fig. 2, it is evident that the IL cation can also generate a strong stabilization of proteins in dilute aqueous environments. Although there were no reports using FP and diluted IL solutions, the favorable cation effect of imidazolium ILs were reported for hen egg-white lysozyme (HEWL), single-chain antibody fragment ScFvOx [30], and *Aspergillus niger* lipase [23], while the impact of the cations for ammonium ILs was demonstrated for α-chymotrypsin [44].

To further verify the long-term effect of [$C_n$mim]Cl solutions (0.100 mol L^{-1}) on EGFP fluorescence, EGFP in [C_2mim]Cl, [C_6mim]Cl, [C_{10}mim]Cl, and [C_{12}mim]Cl were evaluated by 3D fluorescence spectroscopy after 1, 7, and 30 days at room temperature (*i.e.,* 22–25 °C). This test was performed to check if there were shifts in the peak of the EGFP fluorophore under these conditions, as fluorescence is very sensitive to changes in the fluorophore environment, structure, and conformation [37]. Particularly, a previous study with a GFP variant (sfGFP) showed certain ammonium-based ILs can induce shifts in GFP fluorescence, and this could be a parameter to monitor IL-GFP interactions [45]. The 3D spectra of the blanks of the [$C_n$mim]Cl IL solutions are presented in Fig. S3 of the SI, while the spectra of EGFP in different IL solutions at 25 °C can be seen in Fig. S4 of the SI. For a more straightforward visualization, Fig. 3 presents the emission and excitation spectra at the wavelengths corresponding to the EGFP highest intensity peak, extracted from the 3D spectra. Specifically, the emission spectrum was performed for the excitation wavelength (λ_{ex}) of 488 nm, and the excitation spectrum for the emission wavelength (λ_{em}) of 510 nm.

As previously observed in the single point-fluorescence long-term study (Fig. 2), there were minor differences between the spectra of EGFP in PBS, [C_2mim]Cl, [C_6mim]Cl, [C_{10}mim]Cl after 1 and 7 days, with a decrease in fluorescence intensity in only the [C_{12}mim]Cl. Note that for the [C_{12}mim]Cl, the additional peak observed in the excitation spectra of this IL (around λ_{ex} 400–440 nm and λ_{em} at 510 nm) was due to the fluorescence of [C_{12}mim]Cl, but importantly, this fluorescence peak of [C_{12}mim]Cl did not overlap with the EGFP fluorophore, as can be confirmed by comparing Figs. S3 and S4 of the SI. After 30 days of storage at room temperature, there was complete quenching of EGFP fluorescence in PBS solution, with a maintenance of the activity of the protein in all the [$C_n$mim]Cl solutions evaluated. Again, the ILs with shorter cationic alkyl side chains ([C_2mim]Cl and [C_6mim]Cl) were better at preserving EGFP fluorescence than the ones with longer alkyl chains ([C_{10}mim]Cl and [C_{12}mim]Cl). However, it is important to emphasize that despite the changes in EGFP fluorescence intensity, as can be confirmed in Figs. 3 and S4, there were no shifts in the EGFP peak of more than ±2 nm for either λ_{ex} or λ_{em}, which is within expected variations, considering ±2 nm variations were observed even for the PBS control.

3.3. EGFP fluorescence under chemical stress conditions

In addition to understanding the stabilizing effects of dilute IL solutions on EGFP fluorescent activity, this work also aimed to evaluate if [$C_n$mim]Cl ILs can be used to protect the EGFP structure under unfavorable denaturing conditions. Therefore, as a first step, the effect of unfavorable conditions on the EGFP was determined by measuring the fluorescence activity of EGFP solutions (5.4 μg mL^{-1}, pH 7.4 ± 0.1) at high temperatures and in the presence of distinct chemical denaturing agents, namely, acidic, alkaline, oxidizing, and surfactant agents. The pH and concentrations used are described in Table S2 of the SI. Therefore, an initial investigation was carried out to find "mild but unfavorable" stress conditions (*i.e.*, denaturing agents nature and concentration, and time of exposure) in which a moderate fluorescence quenching (relative FI between 40% and 80%) of EGFP occurred. An intermediate quenching was desired to avoid major and irreversible changes in the EGFP structure since a complete denaturation of EGFP could overshadow the positive impact of ILs on protecting EGFP fluorescence activity. For that purpose, we started by assessing the effect of high temperatures on EGFP fluorescence, namely, measuring the relative FI (%) over 24 h in a temperature range from 25 to 90 °C (Fig. 4).

As shown in Fig. 4, EGFP presented no significant changes in fluorescence when exposed to temperatures from 25 to 70 °C, in a period of 24 h. However, at 80 °C, an increase in quenching of EGFP fluorescence within the exposure time was observed, exhibiting a ≈ 50% relative FI after 3 h of exposure, and only 10% of residual fluorescence activity after 6 h. Increasing temperature up to 90 °C enhanced the thermal denaturation of EGFP, leading to a decrease of 85% of the relative FI after 1 h, and a complete quenching of EGPF fluorescence after 2 h of exposure at that temperature. These results are consistent with previous data obtained by Scheyhing et al. [14], which found a thermal unfolding transition temperature for GFP_{mut1} (*i.e.,* EGFP) at 79.5 °C. Although GFP and its variants initially were thought to unfold by two states under thermal denaturation [46], recent studies have suggested otherwise [12,16,17,47]. The experimental investigations and molecular simulations indicated the presence of intermediary states during the denaturing of GFP.

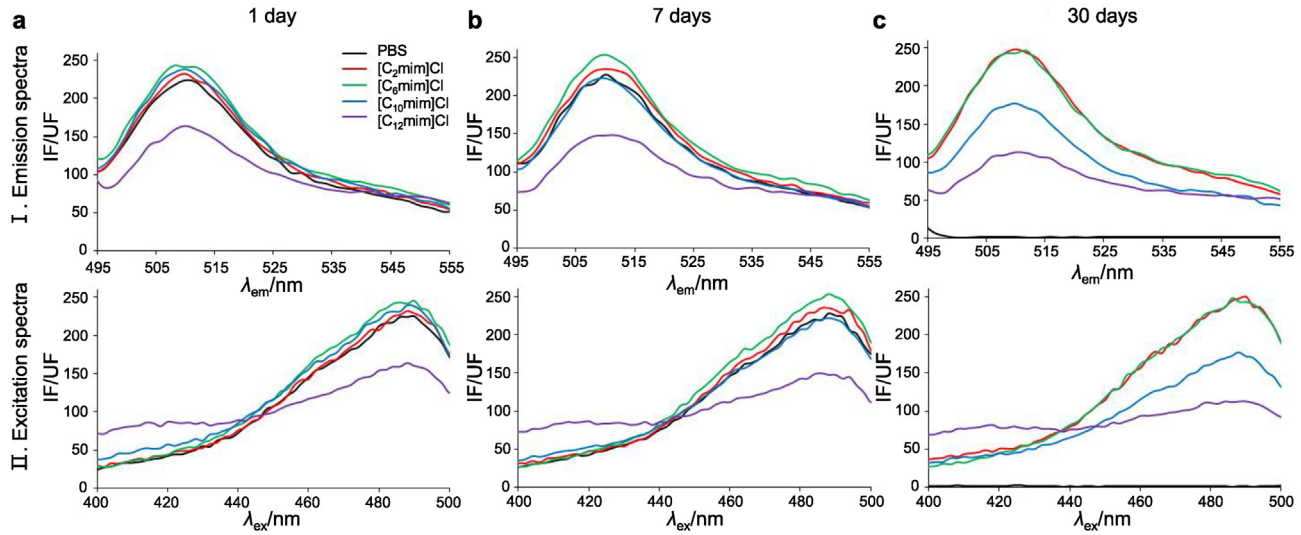

Fig. 3. (I) Emission spectra at λ_{ex} 488 nm and (II) Excitation spectra at λ_{em} 510 nm of EGFP (5.4 μg mL^{-1}) in PBS and 1-alkyl-3-methylimidazolium chloride-based ILs ([$C_n$mim]Cl) aqueous solutions (0.100 mol L^{-1}) after (a) 1 day, (b) 7 days and (c) 30 days at 25 °C. The pH of all solutions was maintained at 7.4 (± 0.1). Data presented in fluorescence intensity (FI) in unities of fluorescence (UF). Spectra extracted from the 3D fluorescence spectra represented in Fig. S4 from the SI.

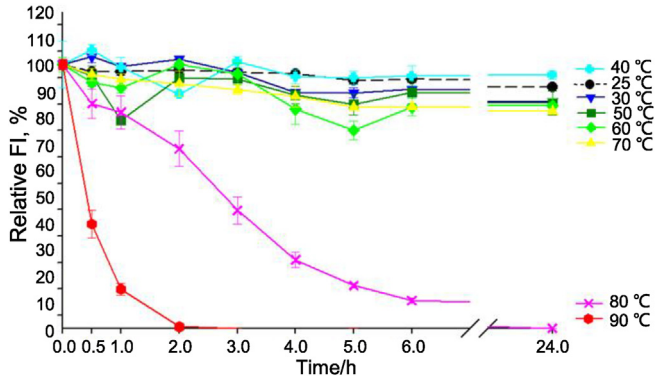

Fig. 4. Effect of temperature (25–90 °C) on EGFP (5.4 μg mL^{-1}) relative fluorescence intensity [FI (%)] over time (48 h). EGFP in PBS pH 7.4 0 h is the reference for 100%. Results were evaluated in triplicate with a blank and presented as average ± standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.

These intermediary states were not apparent at first because they were stabilized by the chromophore [12,16,17,47].

After evaluating the stability of EGFP at different temperatures, the effect of chemical denaturing agents on EGFP fluorescence was evaluated. EGFP was exposed to four distinct classes of denaturing agents in different concentrations, for 30 h at 25 °C, namely: GuHCl as an acidic agent; urea as an alkaline agent; H_2O_2 as an oxidizing agent; and SDS as a surfactant denaturing compound. The results of EGFP relative FI for each denaturing solution are presented in Fig. 5.

Fig. 5a shows that GuHCl (acidic denaturing agent) had little impact on EGFP relative FI (%) at 1 mol L^{-1} concentration, while above 5 mol L^{-1}, the fluorescence was completely suppressed immediately after the addition of the denaturation agent (*i.e.*, 0 h). In between, namely at a concentration of 4 mol L^{-1}, there was a slower fluorescence quenching, with a decline of the relative FI from 37.5 ± 11.2% at 0 h to 23.4 ± 2.4% at 2 h, and complete fluorescence suppression after 30 h. Contrarily, in the presence of the alkaline denaturing agent, as depicted in Fig. 5b, the fluorescence activity of EGFP was maintained, demonstrating that EGFP is very stable in the presence of urea, even at the highest concentration (*i.e.,* 4 and 8 mol L^{-1}) of this denaturing agent. These results confirm that EGFP is very resistant to alkaline conditions but sensitive to acids, which is in accord with previous literature [2,14,15]. Similar to the thermal denaturation of GFP, the protein also presents two apparent states with GFP denaturation with GuHCl with intermediary states concealed by the stabilization of its chromophore [16,17].

The oxidizing agent H_2O_2 (Fig. 5c) had a concentration-dependent fluorescence quenching effect on EGFP. The quenching was enhanced as the concentration of H_2O_2 increased until complete suppression of the fluorescence at 6.00 mol L^{-1}. It is important to highlight that there was a gradual decrease in EGFP fluorescence over time for concentrations of 0.30 mol L^{-1} and 1.50 mol L^{-1} of H_2O_2, which presented an intermediate FI suppression at around 1–2 h. The denaturing effect of this oxidizing agent is associated with the free radical species of H_2O_2, which can damage the EGFP, and even denature it, *via* oxidative reactions [48]. A similar denaturing effect was previously observed for EGFP variant Super Glow GFP (sgGFP, mutation I167T), which loses its fluorescence in the presence of reactive oxygen species such as H_2O_2 [20].

As depicted in Fig. 5d, a reduction in the relative FI of EGFP over time was observed with the addition of SDS, with full suppression after 30 h. Although the fastest EGFP fluorescence quenching was observed with an SDS concentration of 0.175 mol L^{-1} (*i.e.*, in comparison to the concentrations of 0.035 and 0.100 mol L^{-1}), overall, a similar fluorescence quenching with the SDS concentration was obtained, namely: a decrease of 40%–50% of EGFP fluorescence after 3 h, and complete suppression after 30 h of exposure, even for the lowest surfactant concentration (0.003 mol L^{-1}). The CMC of SDS is 0.007–0.010 mol L^{-1} (20–25 °C) according to Sigma-Aldrich®. Hence, even at 0.003 mol L^{-1}, the loss of EGFP fluorescence is likely caused by the amphiphilic nature of SDS [49]. The general mechanism of denaturation of proteins with SDS follows a model called "pearl necklace" because the proteins wrap around the micelles of SDS in a fluid configuration that resembles a pearl necklace. By interacting with the SDS micelles, the protein can drastically change its structure and denature [50]. Denaturation studies of EGFP variant sgGFP showed the protein can be denatured by SDS; however, this effect is dependent on the solution conditions, for example, the pH of the buffer, with the most pronounced effects observed at acidic pH values [18,19].

Based on the results from Figs. 4 and 5, for the EGFP protection studies using IL aqueous solutions, we selected: SDS at 0.175 mol L^{-1}; GuHCl at 4 mol L^{-1}; and H_2O_2 at 1.50 mol L^{-1}. These conditions were

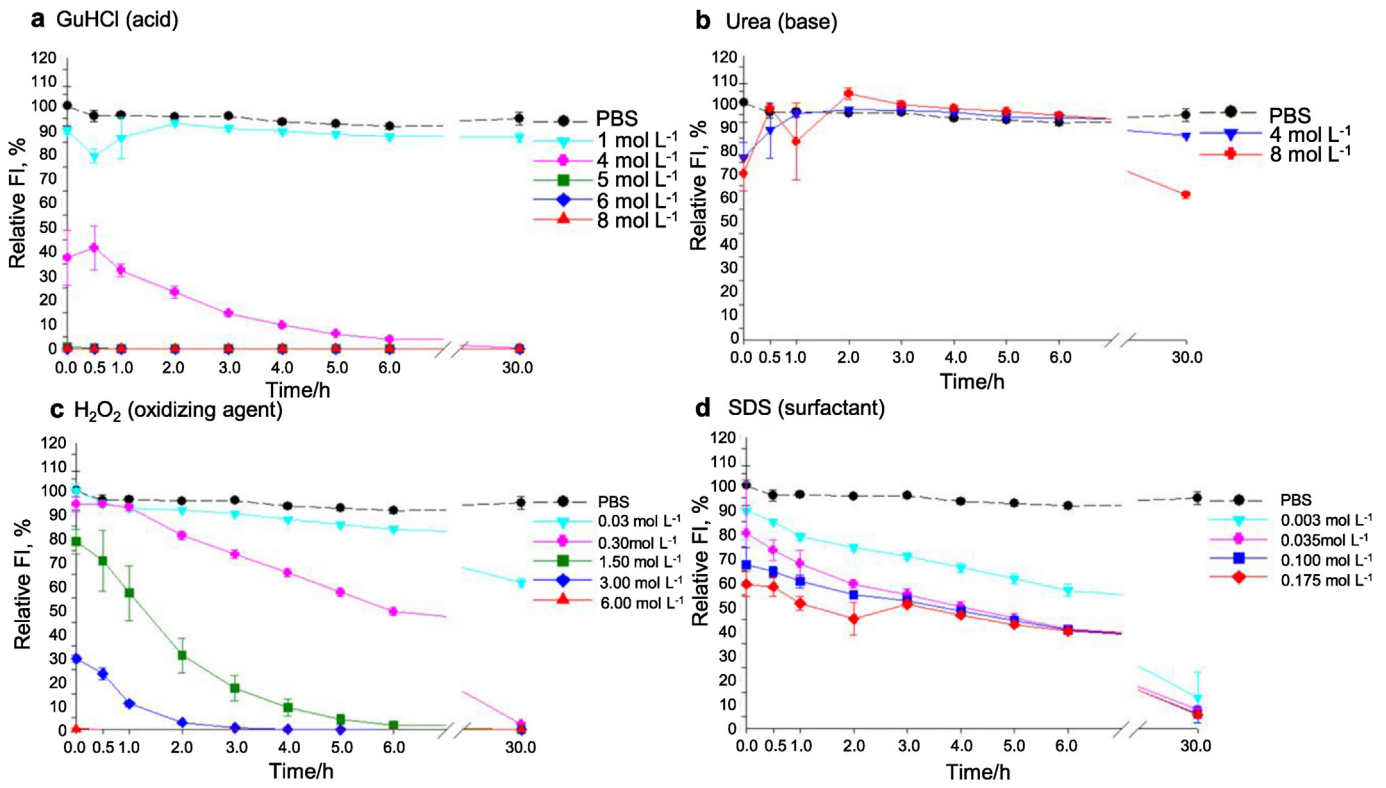

Fig. 5. Effect of chemical denaturing agents on EGFP (5.4 μg mL^{-1}) relative fluorescence intensity [FI (%)] over time (48 h) at 25 °C. (a) GuHCl (acid), (b) Urea (base), (c) H_2O_2 (oxidizing agent) and (d) SDS (surfactant). EGFP in PBS pH 7.4 0 h is the reference for 100%. PBS is presented as a control. Results were evaluated in triplicate with a blank and presented as average ± standard deviation. The lines are represented as eye guidelines to facilitate the comparison between the experimental data.

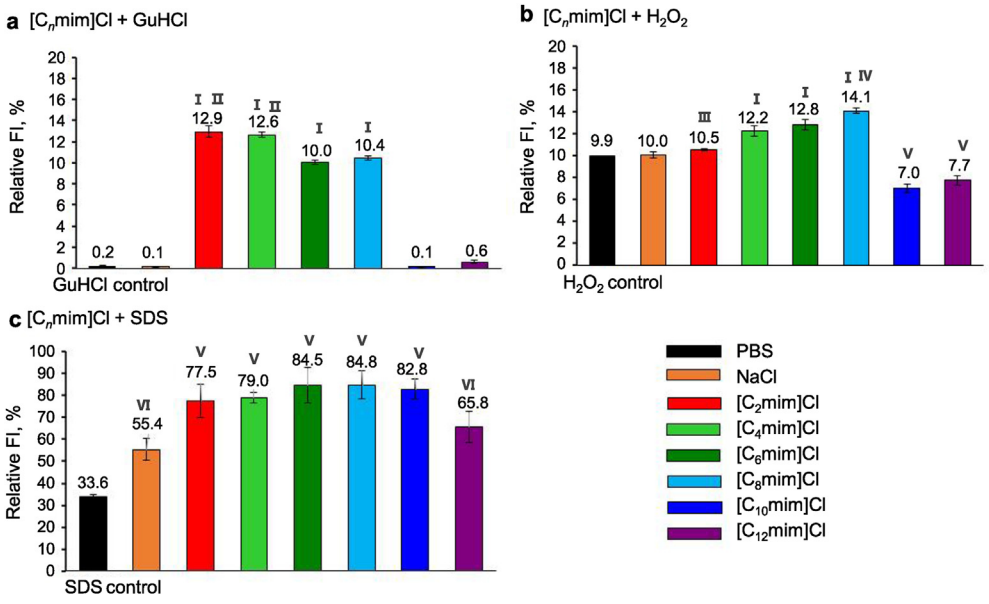

Fig. 6. Effect of 1-alkyl-3-methylimidazolium chloride-based ILs ([$C_n$mim]Cl) aqueous solutions (0.100 mol L^{-1}) on EGFP (5.4 μg mL^{-1}) relative fluorescence intensity [FI (%)] after 2 h of exposure and at 25 °C in the presence of (a) acid GuHCl (4 mol L^{-1}), (b) oxidizing agent H_2O_2 (1.50 mol L^{-1}) and (c) surfactant SDS (0.175 mol L^{-1}). The pH of the ILs and salt solutions before the addition of the denaturing agents were maintained at 7.4 (±0.1). Data are presented as relative FI (%) of EGFP. A buffer (PBS pH 7.4) and conventional salt (NaCl 0.100 mol L^{-1}) in the presence of GuHCl, H_2O_2 and SDS are control groups. The scale of relative FI goes from 0 to 20% in (a) and (b), and from 0 to 100% in (c). Results were evaluated in triplicate with a blank and presented as average ± standard deviation. Statistical differences between the groups for each time were evaluated (one-way ANOVA with post-hoc test Holm-Sidak for $p < 0.01$, Sigma Plot 12.0) are demonstrated by the following letters: different from (I) PBS, NaCl, [C_{10}mim]Cl and [C_{12}mim]Cl; (II) [C_6mim]Cl and [C_8mim]Cl; (III) [C_4mim]Cl, [C_6mim]Cl, [C_8mim]Cl, [C_{10}mim]Cl and [C_{12}mim]Cl; (IV) [C_4mim]Cl and [C_6mim]Cl; and (V) PBS and NaCl; (VI) PBS.

chosen based on their moderate EGFP fluorescence quenching (40%–80%) after 2 h of exposure. As EGFP already has a very high resistance to high temperatures and alkaline substances, both urea and thermal denaturation were not considered for subsequent protection studies.

3.4. EGFP protection effects of [$C_n$mim]Cl aqueous solutions under chemical stress conditions

For EGFP protection studies using IL solutions, the relative FI (%) of

[$C_n$mim]Cl aqueous solution (0.100 mol L^{-1}) was determined for three distinct chemical stress conditions (*i.e.*, surfactant SDS at 0.175 mol L^{-1}, acid GuHCl at 4 mol L^{-1} and oxidizing H_2O_2 at 1.50 mol L^{-1}), immediately after the addition of the denaturing agent (0 h) and after 2 h of exposure. EGFP in PBS at pH 7.4 was measured as a reference for 100%, while the following samples were measured as control groups: EGFP in PBS + denaturing agents aqueous solutions (buffer control); EGFP in NaCl (0.100 mol L^{-1}) + denaturing agents aqueous solutions (salt control); EGFP in ILs + denaturing agents aqueous solutions (blank controls). All solutions were compared in terms of relative FI (%), with the respective results for the denaturing agents GuHCl, H_2O_2 and SDS presented in Fig. 6a-c, respectively.

Fig. 6a presents the protective effect of each [$C_n$mim]Cl solution against the acidic denaturing agent GuHCl. Although ILs were able to grant extra protection to EGFP fluorescence when compared to the buffer and NaCl solution controls, this effect was not very intense (around 10%–13% of relative FI of EGFP, with a maximum increase of 12.9% when compared to PBS) and was only observed for [$C_n$mim]Cl with shorter cationic alkyl chain lengths ($n = 2$ to 8).

Similarly, the results of the protection study using H_2O_2 as an oxidizing denaturation agent (Fig. 6b) revealed that there was higher protection using [$C_n$mim]Cl with shorter cation alkyl chain lengths ($n = 2$ to 8) with a clear decrease of protective effects for $n = 10$ and 12, following the rank: [C_2mim]Cl ≈ PBS and NaCl; [C_4mim]Cl, [C_6mim]Cl, and [C_8mim]Cl > PBS and NaCl; and [C_{10}mim]Cl and [C_{12}mim]Cl < PBS and NaCl. There was a maximum increase of only 4.2% in the relative FI when comparing the [$C_n$mim]Cl with PBS.

As for the surfactant protection study, it can be seen in Fig. 6c that even the common inorganic salt (NaCl) solution reduced the quenching effect of SDS on EGFP fluorescence, maintaining 55.4% of EGFP relative FI in comparison with the control PBS + SDS sample (which has a residual relative FI of 33.6%). However, the [$C_n$mim]Cl solutions contributed a higher protective effect towards EGFP fluorescence activity in the presence of SDS (except [C_{12}mim]Cl, which had no significant difference compared to the NaCl). Aqueous solutions of [$C_n$mim]Cl with $n = 2$, 4, 6, 8, and 10 maintained the EGFP relative FI above 75% (between 77.5% and 84.8%, with an increase of up to 51.2% when compared to PBS), showing grant additional protection to the protein against the denaturing surfactant compound. The ability of [$C_n$mim]Cl ILs to preserve EGFP fluorescence from SDS quenching was enhanced by increasing the cationic chain length from $n = 2$ to 8, with a minimal decrease for $n = 10$ and a considerable decrease for $n = 12$. This outcome reinforces the results from the long-term studies in Fig. 2, with all [$C_n$mim]Cl protecting EGFP fluorescence in comparison with PBS and salt controls, but with [C_{12}mim]Cl presenting the lowest aptitude to preserve EGFP relative FI, probably due to the amphiphilic nature of this IL. The remarkable protection of EGFP fluorescence with ILs against surfactants could be exploited to facilitate the development of EGFP formulations with emulsions and encapsulations, for creating new types of biosensors or during drug delivery/release processes using FP as biomarkers. Particularly, during the encapsulation of proteins, significant amounts of the target-biomacromolecule can undergo denaturation due to the presence of disruptive solvents and substances (such as surfactants) in the formulating solutions or even because of the harsh encapsulation process (*e.g.*, shear and shaking; variations in temperature, pH and protein concentration) [51,52]. Therefore, the addition of a low amount of [$C_n$mim]Cl could help to maintain EGFP fluorescence activity in the presence of the surfactants required to form emulsions and capsules for biosensing/biomarking applications.

From this experiment, we can conclude that [$C_n$mim]Cl aqueous solutions can overall protect EGFP fluorescence against different denaturing agents, although their performance is considerably better against SDS. Several studies [53–55] have demonstrated that favorable interactions can occur between [$C_n$mim]$^+$-based ILs and SDS detergent molecules, with more pronounced effects with increasing cation alkyl chain length ($n > 6$). These interactions result in a different local environment around the EGFP protein structure, generally beneficial for the protein in most of the ILs studied. However, for [C_{10}mim]Cl and [C_{12}mim]Cl, the complex between SDS and these ILs may result in bulkier species with lower protective aptitude towards EGFP [56]. The lower pH of the GuHCl 4 mol L^{-1} (6.09) and H_2O_2 1.50 mol L^{-1} (6.70) (Table S2 from the SI) may explain the lower protective aptitudes of the ILs for these conditions, considering EGFP changes states (from deprotonated to protonated) between pH 7 and 6 [2]. The protonated state of EGFP variants is more sensitive to denaturation (*e.g.*, heat, urea, SDS) [2,18,19] and could explain why the [$C_n$mim]Cl ILs were less effective in protecting EGFP in more acidic conditions.

Regarding the mechanisms involved for the protective effect of [$C_n$mim]Cl solutions on EGFP fluorescence against denaturing agents, it would be complex to isolate the variables responsible for this phenomenon, considering these protective effects could be not only due to specific interactions between the protein and ILs, but also from specific interactions between denaturing agents and ILs, or even a combination of different mechanisms from all the compounds in solution. For example, there is the possibility of the ILs chemically reacting or degrading in the presence of GuHCl and H_2O_2. Additionally, depending on their concentrations, studies are using imidazolium-based ILs (particularly [C_8mim]Cl) to form mixed-micelles with SDS in water [34], and hence the addition of these ILs could lead to different interactions with proteins when compared to systems solely composed of SDS micelles in water. To verify these possibilities, the FTIR-ATR spectra of the ILs + denaturing agents were obtained, since IR spectroscopy can be used to verify acid neutralization [57], H_2O_2 concentration and degradation [58], and to study micelle alterations [59,60].

Fig. S5 in the SI shows the FTIR spectra of the aqueous solutions of denaturing agents + ILs [(a) GuHCl, (b) H_2O_2, and (c) SDS] after the subtraction of the NaCl or IL contribution to the spectra. Fig. S5b-c of the SI show there were no alterations in the GuHCl and H_2O_2 FTIR spectra, suggesting the ILs did not act to degrade the acid or oxidizing agent. In Fig. S5c of the SI, it is possible to see that the ILs did not alter the SDS FTIR spectra, which would be expected to change if there was a formation of mixed micelles [34]. It is important to note that the FTIR does not rule out possible surface interactions between the ILs and the denaturing agents. However, it indicates that the ILs do not neutralize GuHCl, degrade H_2O_2 or form mixed micelles with SDS. Additionally, considering the ILs were able to triple the maintenance time of EGFP fluorescence at room temperature, it is likely that there are interactions between the protein and the ILs.

Considering the combined results of this study, it is clear that imidazolium-based ILs have a higher potential to preserve EGFP when compared to conventional buffer and salt solutions (most commonly used in several protocols with proteins). However, a clear picture of all the mechanisms occurring at a molecular level between ILs and EGFP is not easily obtained, requiring further experimental and computational structural evaluations (usually combining several techniques) for a complete reveal of the interactions behind the IL protective mechanisms in diluted solutions. It is important to consider that several interactions can occur between ILs and proteins (*e.g.*, hydrogen bonding, hydrophobic interactions, strong Coulomb interaction, dispersion forces) [61], making the process of isolating each variable very long and complex. A telling example of the complexity of IL-protein systems was demonstrated in a previous study using sfGFP with pyrrolidinium and imidazolium-based ILs, where a multi-technique approach was required to solve their molecular-level mechanisms of these complex systems [45]. It was only possible to understand (at least partially) the site-specific IL-sfGFP interactions after combining several structural characterization methods, such as circular dichroism, fluorescence, ultraviolet–visible, nuclear magnetic resonance spectroscopy, and small-angle X-ray scattering. Because certain fluorescent proteins such as EGFP and sfGFP are weak dimers, they could be found as monomers, dimers, or as larger aggregates [2,13,62]. The presence of many groups with different sizes, sometimes not related to FP loss of activity or structure, increases the complexity of

analyzing samples of weak dimeric and trimeric proteins such as EGFP and sfGFP.

As a hypothesis for explaining the protective effect of [$C_n$mim]Cl solutions, we believe that ILs with shorter cationic alkyl chain lengths can decrease EGFP aggregation, considering that this protein is a weak dimer prone to aggregation, which is mainly associated with its instability [2, 13]. For example, replacing hydrophobic residues at the crystallographic interface of the EGFP dimers with positively charged residues can reduce their aggregation, as shown for the monomeric version of EGFP, mEGFP (mutations A206K, L221K, or F223R) [63]. These mutations did not alter the spectral properties of EGFP but decreased its aggregation. Hence, the interactions between the hydrophobic residues at the interface of the EGFP dimers with the ILs could decrease protein aggregation, considering it would reduce the attraction between the hydrophobic residues from the proteins in the solution. Consequently, the reduced aggregation would then improve the long-term maintenance of EGFP activity at room temperature. For example, Liem et al. found that nitrate-based ILs (*e.g.*, ethylammonium nitrate 1 mol%–17 mol%, butylammonium nitrate 1 mol%–6 mol%, and ethanolammonium nitrate 1 mol%–10 mol%) could reduce sfGFP aggregation; however, this phenomenon was also associated with a slight fluorescence quenching in short-term studies [40]. These authors also suggested that ILs can form weak hydrogen bonds with the protein surface and bury hydrophobic groups away from water molecules, hence better hydrating sfGFP and stabilizing the water-protein interface [40]. A similar is possibly occurring between the [$C_n$mim]Cl ILs and EGFP in our study. It would be necessary to monitor EGFP aggregation in IL solutions and perform molecular simulations to confirm this hypothesis.

Other relevant information to consider is that the IL-EGFP interactions are improved with increasing the alkyl chain length of [$C_n$mim]Cl with n from 2 to 8; but further increasing the cationic alkyl chain to $n = 10$ or 12 is detrimental, and corresponds to other interactions possibly occurring [*e.g.*, self-assembly of [C_{10}mim]Cl (CMC around 0.040–0.060 mol L^{-1}) and [C_{12}mim]Cl (CMC around 0.013–0.020 mol L^{-1})] [39] as a result of the intrinsic amphiphilic nature of the longer chained ILs. The hypothesis of this surfactant-like effect is reinforced considering the other IL solutions used do not assemble or are below the respective CMC values, namely: [C_2mim]Cl and [C_4mim]Cl do not self-assemble; [C_6mim]Cl has a CMC of 0.900 mol L^{-1} - considerably above the concentrations of this study; [C_8mim]Cl has a CMC around 0.090–0.235 mol L^{-1} - close to the two highest concentrations evaluated in this study [39].

There are many possible mechanisms for how ILs can stabilize or destabilize proteins in aqueous solutions, including salting-in and salting-out aptitudes, changes in the relative strength of H-bonds, hydrophobic or ionic interactions, self-assembling or nano-structuring mechanisms, and even a combination of two or more of them [56]. Considering these mechanisms are generally very dependent on the type and concentration of both the IL and protein, it is difficult to provide a clear mechanistic picture. As for the protective effects, this is even more difficult considering we are adding a third component (*e.g.*, the denaturing agents GuHCl, H_2O_2, or SDS in this work) to an already complex IL-protein system. Nonetheless, previous findings can help us provide some hypotheses for the experimental results obtained in our study.

The study of Yamaguchi et al. [64] using [$C_n$mim]Cl aqueous solutions (concentrations from 0.001 to 2 mol L^{-1}) to stabilize lysozyme reinforces our hypothesis that these ILs can improve protein stability by reducing their aggregation. Specifically, these authors evaluated the effect of different [$C_n$mim]Cl ILs on the folding rates during the refolding of denatured lysozyme. In their work, they observed that ILs with short cation alkyl chain lengths (*i.e.*, $n = 2$ or 4) significantly enhanced the lysozyme refolding yield, while the ILs with longer chains ($n > 5$) were less effective. To understand the effect of the [$C_n$mim]$^+$, these authors compared the kinetics of the refolding and aggregation of denatured lysozyme. Interestingly, and in agreement with our current study, increasing the alkyl chain length of the cation reduced the improvement of the aggregation rate. Yamaguchi et al. [64] suggested that this effect is probably a balance between the change in the salting-in properties of the [$C_n$mim]$^+$ with the increase of alkyl chain length or because [$C_n$mim]$^+$ with longer alkyl chains are more prone to self-assembly, decreasing favorable interaction with the hydrophobic portions of the protein. However, we agree with Yamaguchi et al. [64] that it is necessary to combine experimental work (as ours) with further computational studies at a molecular level (*e.g.*, molecular dynamics) to define the extent of the contribution of each mechanism (*e.g.*, salting-in, hydrophobic interactions, and self-assembly) for IL-proteins interactions.

Nonetheless, it is evident for us that there is a fine balance between the concentration of the IL and cationic alkyl chain length (n) that will define if the [$C_n$mim]Cl will improve protein stability by decreasing its aggregation or impair it due to surfactant effects, with subsequent high or low protection aptitude towards EGFP fluorescence. This hypothesis is reinforced by analyzing the study of Heller et al. [65] with [C_4mim]Cl aqueous solutions (concentrations of 1.56 and 3.12 mol L^{-1}) and wtGFP. In particular, these authors observed that the addition of [C_4mim]Cl favored not only the monomeric state of wtGFP and its low aggregation (decrease in its radius of gyration) but also made the protein structure less compact and more prone to thermal denaturation. This phenomenon was clearer at the highest IL concentration (3.12 mol L^{-1}), with an evident concentration-dependent denaturing effect. Hence, [$C_n$mim]Cl can improve the stability of aggregation-prone proteins at lower concentrations and shorter cation alkyl-chain lengths by reducing its aggregation, but at high IL concentrations, they could lead to less compact protein structures, which will contribute to protein denaturation. Computational studies are necessary to elucidate the complex details in these systems, firstly to determine the main IL-proteins interactions and later to provide information about which mechanisms are responsible for the IL protective effects against denaturing agents.

Considering the complexity of the evaluation of IL-protein interactions, in this work, we focused solely on screening and revealing the most suitable ILs, respective concentrations, and processual conditions for more applied studies. In other words, this research aimed to identify initial parameters for further physical chemistry fundamental studies, which would require a multi-technique approach to elucidate the different interactions responsible for each outcome. Therefore, we are comfortable affirming that the imidazolium-based ILs are chemicals with great potential to preserve EGFP fluorescence in the long-term at room temperature and to protect EGFP from chemical denaturation. Additionally, this study shows that dilute aqueous solutions of [$C_n$mim]Cl ILs can act as additives to preserve protein activity without refrigeration and under unfavorable conditions.

4. Conclusions

The potential of [$C_n$mim]Cl ILs to maintain EGFP fluorescence over long-term storage at room temperature, and to protect the protein against denaturing agents was demonstrated. The protection of EGFP with [$C_n$mim]Cl ILs depended on the cation alkyl chain length and concentration of the ILs, being impaired for [$C_n$mim]Cl with longer alkyl chains ($n = 10$ and 12). The [$C_n$mim]Cl with $n = 2$, 4, 6, and 8 were particularly effective in protecting EGFP fluorescence from denaturation-based surfactant quenching, presenting a remarkable potential for applications in the development of FP formulations (*e.g.*, emulsions, encapsulations).

Considering these promising results, imidazolium-based ILs could be used as preservatives to increase the shelf-life of protein-based products at room temperature and could also be used as additives to protect proteins from different chemical stresses. Our work also revealed that novel imidazolium IL-based formulations have the potential to improve the transport, storage, and handling of proteins-based products, and expand their industrial applications in unfavorable conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

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Abbreviations

λ_{em}	Wavelength of emission
λ_{ex}	Wavelength of excitation
EGFP	Enhanced GFP
F1	EGFP fluorescence peak at λ_{ex} 488 nm and λ_{em} at 510 nm (highest intensity peak)
FI	Fluorescence intensity
PBS	Phosphate-buffered saline
UF	Units of fluorescence

Author's contributions

JFBP and NVV proposed and designed the study; NVV and JFBP drafted the paper; NVV and CFS conducted the experiments; NVV, CFS, TLG, and JFBP planned the experiments and analyzed data; TMR helped with discussion and analysis of results; TLG and JFBP were responsible for funding acquisition and supervision. All authors collaborated with the discussions of results, review, and editing of the original manuscript.

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