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Boosting antibiotics performance by new formulations with deep eutectic solvents

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ABSTRACT

The critical scenario of antimicrobial resistance to antibiotics highlights the need for improved therapeutics and/or formulations. Herein, we demonstrate that deep eutectic solvents (DES) formulations are very promising to remarkably improve the solubility, stability and therapeutic efficacy of antibiotics, such as ciprofloxacin. DES aqueous solutions enhance the solubility of ciprofloxacin up to 430-fold while extending the antibiotic stability. The developed formulations can improve, by 2 to 4-fold, the susceptibility of Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) bacteria to the antibiotic. They also improve the therapeutic efficacy at concentrations where bacteria present resistance, without promoting tolerance development to ciprofloxacin. Furthermore, the incorporation of DES decreases the toxicity of ciprofloxacin towards immortalized human epidermal keratinocytes (HaCat cells). The results herein reveal the pioneering use of DES in fluoroquinolone-based formulations and their impact on the antibiotic's characteristics and on its therapeutic action.

1. Introduction

In an era of increased resistance to antibiotics and consequent decreased antimicrobials' susceptibility, the risk of failure of standard therapies has become one of the major health challenges of society (Tillotson and Zinner, 2017). The ongoing emergence of new resistant bacteria has challenged the pharmaceutical industry's research and production; yet, the development of new effective antibiotics has not been sufficiently successful to tackle this challenge. Given this critical scenario, other therapeutic strategies, such as bacteriophages with anti-CRISPR genes, monoclonal antibodies, host modulation and microbioma approaches have started to be investigated (Baker et al., 2018).

Antibiotics regularly face economic drawbacks as older ones remain first in line for use in clinical practice, whereas new options in clinical pipeline are scarcely adopted or safeguarded as the last resort (Årdal et al., 2019; WHO, 2018). To pursue more profitable and effective alternatives, the improvement of the already existing formulations can help boost antibiotic performance for bacteria eradication (Bothwell, Greene and Jones, 2016). Examples of this strategy include the research on nanoparticle-based systems with extended antibiotic release (Gao et al., 2011) and the use of antibiotic adjuvants, which have insufficient antibacterial activity, but when combined can augment or transform the activity of antibiotics (Domalaon et al., 2018).

An example of progressive decrease in the susceptibility of several pathogens to antibiotics and increasing resistance mechanisms corresponds to the clinical use of antibiotics belonging to the quinolone family (Pham et al., 2019). Among these, fluoroquinolones have been increasingly prescribed due to their broad-spectrum of action (Fief et al., 2019). Ciprofloxacin, in particular, is ranked as one of the most important antimicrobials for human medicine applications (WHO, 2018). Although Gram-negative bacteria like enterobacteriaceae are highly susceptible to this antibiotic, as well as *Pseudomonas aeruginosa,* some Gram-positive bacteria, like the *Staphylococcus* species, also present some susceptibility to this fluoroquinolone (Li and Webster, 2018). Unfortunately, due to its wide application, in the last decade an increased resistance rate to ciprofloxacin has been verified for these strains (Haslund et al., 2013). Therefore, the design of novel ciprofloxacin-based formulations must ensure higher efficacy, drug concentration above subinhibitory levels and prevent the development of resistance that will limit therapeutic efficacy (Gill et al., 2015).

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However, ciprofloxacin, as many other antibiotics, is poorly water-soluble, being its application in different dosage forms limited (Varanda et al., 2006). Therefore, the possibility to design formulations that can not only improve the solubility of the antibiotic in water but also improve its antimicrobial action is a mandatory demand.

In the present work, we propose an innovative formulation for antibiotics based on aqueous solutions of deep eutectic solvents (DES), shown with ciprofloxacin, that significantly boosts the drug's performance by overcoming its solubility, stability, susceptibility and resistance drawbacks. DES are eutectic mixtures that present interactions between its components, strongly deviating from the ideal thermodynamic solid–liquid phase behavior in such a degree that can be liquid at room or human body's temperature (Martins, Pinho and Coutinho, 2018). These alternative biodegradable solvents have been applied in the solubilization of analgesic (Berton et al., 2017), anti-inflammatory (Lu et al., 2013) and antifungal (Li and Lee, 2016) agents, surpassing the drug solubility achieved by the use of organic solvents and even co-solvents (Nerurkar, Beach and Jun, 2005). Nevertheless, the therapeutic efficacy of drugs solubilized in DES is still shortly addressed and the potential brought by the numerous possibilities of design is not fully explored. To the best of our knowledge, no reports on the application of DES to improve the antimicrobial activity of antibiotics have been found nor their potential to avoid bacterial resistance. Furthermore, DES were mainly investigated as neat compounds, being aqueous solutions of DES less studied in the field of pharmaceuticals and active pharmaceutical ingredients (APIs) (Nava-Ocampo et al., 2021; Pereira et al., 2019).

Herein, we demonstrate the possibility to achieve remarkable solubility enhancements for ciprofloxacin in aqueous solutions of DES, while providing detailed insights towards the understanding of the simultaneous influence of the adequate DES design and the formulation of antibiotics in DES aqueous solutions in tackling therapeutic efficacy and antibiotic resistance. To this purpose we demonstrate the improved therapeutic effect achieved with these novel formulations on Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus*) bacteria.

2. Experimental section

2.1. DES preparation

The DES investigated were prepared by mixing the respective precursors (HBA and HBD) in sealed glass vials with constant heating and stirring, until a homogeneous transparent liquid was formed (maximum temperature of 90 °C). The mixtures were then kept for one hour at this maximum temperature before returning to room temperature. DES were prepared at the following molar ratios: [Ch]Cl:urea:malonic acid ([Ch]Cl:U:MA), 1:2:0.05; proline:urea:malonic acid (Pro:U:MA), 1:1:0.05; and citric acid:xylitol (CA:Xyl), 2:1. The respective DES components' integrities were confirmed by ^{13}C and ^{1}H NMR. The ^{1}H NMR and 13C NMR spectra were recorded using a Bruker Avance 300 at 300.13 MHz and 75.47 MHz, respectively, by previously dissolving the mixtures in deuterated water and using trimethylsilyl propanoic acid (TMSP) as an internal reference. The respective list of chemicals, their purity and source are provided in Supplementary Information.

2.2. Ciprofloxacin's solubility assays

Ciprofloxacin was added in excess to 2.0 g of each DES aqueous solution (0–60% (w/w) of DES) and pure water, and placed in sealed glass vials with a stirring bar. These mixtures were allowed to equilibrate in a specific aluminum disk placed on a stirring plate with heat control, at constant temperature (25 and 37 ℃) and stirring (900 rpm) over 72 h. Due to the high viscosity of the obtained solutions, it was only possible to study ciprofloxacin's solubility up to 60% (w/w) of DES in an aqueous solution. After saturation of the DES aqueous solutions, the samples were centrifuged and a supernatant aliquot was taken and diluted in water. After this, the sample was carefully filtered with a 0.20 μm syringe filter to remove any solid from the liquid phase and subsequently quantified by HPLC-DAD (Shimadzu, model PROMINENCE). HPLC analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), Kinetex 5 μm C18 100 Å, from Phenomenex. The mobile phase consisted of 25% (v/ v) of acetonitrile and 75% (v/ v) of ultra-pure water with 0.3% (v/v) of orto-phosporic acid. The separation was conducted in isocratic mode, at a flow rate of 0.8 $mL{\cdot}min^{-1}$ and using an injection volume of 10 μL. The column oven and the auto-sampler operated at 35 °C. The wavelength was set at 290 nm and each sample was analyzed at least in duplicate form. Calibration curves were obtained using the pure ciprofloxacin dissolved in the mobile phase. Under these conditions, ciprofloxacin displays a retention time of 3.3 min.

2.3. Stability of DES formulations

To evaluate the stability of the drug in the DES aqueous solutions and in pure water, the drug was dissolved below its solubility limit (3.00×10^{-5} $mol{\cdot}dm^{-3}$) in water. The same amount of drug was solubilized in each DES aqueous solution. An aliquot of each solution was collected and analyzed by HPLC-DAD to obtain the initial profile for each formulation. Then, the formulations were kept in the dark at (25 ± 2) ℃ and 75–80% relative humidity during 30 days. After this period new samples were collected and analyzed. The drug concentration in water and in the DES' formulations was quantified by HPLC-DAD using the method described above. The stability of the DES aqueous solution in the absence of the antibiotic was conducted by Fourier Transform Infrared with Attenuated Total Reflectance (FTIR-ATR) to evaluate changes in the functional groups of the DES components. The spectrum of each sample was acquired in a FTIR system Spectrum BX, PerkinElmer, equipped with a diamond crystal and a single horizontal Golden Gate ATR cell. The analyses of the solutions at 40% (w/w) of DES were performed at room temperature (25 ± 2) ℃ with controlled relative humidity (75–80%). Samples were collected on the day of preparation of the DES solutions and after 30 days at the storage conditions. All data were recorded in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and by accumulating 32 scans with and interval of 1 cm^{-1}. For all the spectra acquired, the background air spectrum was subtracted and the results were recorded as transmittance values.

2.4. Bacterial strains and culture conditions

The strains of *Echerichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 6538 were grown on solid medium Trypic Soy Agar (TSA) (Liofilchem) at 37 °C during 24 h and posteriorly kept at 4 °C. Each bacterium strain was inoculated whenever necessary in liquid medium Trypic Soy Broth (TSB) and grown aerobically at 37 °C for 24 h under stirring (100 rpm). For each assay, an aliquot of this culture (300 μL) was transferred twice into a new fresh TSB medium (subcultured in 30 mL) and grew overnight at 37 °C under stirring.

2.5. Minimum inhibitory concentration (MIC) of ciprofloxacin formulated in DES aqueous solutions

Minimum Inhibitory Concentration (MIC) determination for *E. coli, P. aeruginosa* and *S. aureus* was achieved by broth dilution method (Wiegand et al., 2008). Dilutions of standardized microbial suspension adjusted to 0.5 McFarland scale in TSB were prepared and dispensed in a 96-well microtitration plate. Ciprofloxacin solutions in water and formulated in DES were freshly prepared for a starting concentration of 10 $\mu g{\cdot}mL^{-1}$ of ciprofloxacin, and each were two-fold diluted along the 96-well microtitration plate (12 concentrations for each ciprofloxacin formulated in DES were evaluated in the range 10–0.00244 $\mu g{\cdot}mL^{-1}$). DES alone were also tested in the same concentration to infer their

influence in the antimicrobial effect. After well-mixing, the optical density (O.D.) at 600 nm was read, and 96-well microtitration plate was incubated under suitable conditions at 37 ℃ for 24 h. After this time, O. D. at 600 nm was read and the MIC was achieved by the lowest concentration of antimicrobial agent that inhibits growth of each microorganism.

2.6. Antimicrobial susceptibility to ciprofloxacin in DES aqueous solutions

Antimicrobial activity of ciprofloxacin and DES formulations with the antibiotic was assessed by dissolving the pure antibiotic in water and in the DES [Ch]Cl:U:MA, Pro:U:MA and CA:Xyl aqueous solutions against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 6538 strains. The susceptibility test was performed using the modified Kirby-Bauer disk diffusion method. Each bacterial suspension in physiological saline solution (PBS), with a turbidity of 0.5 on the McFarland scale, was prepared by peaking up 1–2 colonies from pure cultures. The suspension was spread plated using a swab on Mueller-Hinton Agar. Antimicrobial-impregnated disks were (BD BBL, Sensi-Disc) placed onto the cultures medium surface. Disks containing 0.5 and 5 $\mu g \cdot mL^{-1}$ of the drug were used for *E. coli*, *P. aeruginosa* while disks with 5 and 10 $\mu g \cdot mL^{-1}$ were used for *S. aureus* evaluation. The disks with the antibiotic in water and in aqueous solutions of DES with the antibiotic were placed and incubated at 37 °C for 18–24 h. Each DES was evaluated without the drug to determine its impact on the antimicrobial susceptibility. The antimicrobial efficacy of each formulation was determined by measuring the diameter of the zones of inhibition and compared with the zone diameter breakpoint established by EUCAST European Committee on Antimicrobial Susceptibility Testing and accordingly to the Clinical and Laboratory Standards Institute [CLSI] (CLSI, 2020; EUCAST, 2020).

2.7. Bacterial inactivation efficacy by ciprofloxacin in DES solutions

The bacterial cultures (*E. coli*, *P. aeruginosa* and *S. aureus*) were grown overnight and were diluted and adjusted to 0.5 Macfarland scale in phosphate buffered saline (PBS), pH 7.4. The bacterial suspensions were equally distributed in 2 mL tubes. Afterwards, appropriate volumes of ciprofloxacin, CA:Xyl and Pro:U:MA alone and both deep eutectic solvent formulations with ciprofloxacin were added to achieve a final concentration of 0.5 $\mu g \cdot mL^{-1}$ of the antibiotic. A bacterium positive control (Control) containing only the bacterial inoculum in PBS was also carried out. The inactivation efficiency of ciprofloxacin, each DES and DES formulations with ciprofloxacin were evaluated by quantifying the number of colony forming units (CFU) per volume ($CFU \cdot mL^{-1}$). Aliquots of samples and control were taken at different incubation times (0, 1, 2, 3, 4, 6, 12 and 24 h). Each solution was serially diluted in PBS and each sample dilution was pour-plated TSA. The plates were incubated at 37 °C for 24 h and the $CFU \cdot mL^{-1}$ was counted. Experiments were carried out in duplicate and repeated three times.

2.8. Bacterial tolerance to ciprofloxacin in DES formulations

In order to verify the development of tolerance to the treatment with ciprofloxacin and the antibiotic formulated in the DES solutions, 7 cycles of inactivation under the conditions previously described were performed. The concentration of ciprofloxacin was equal to the previous ones used in the inactivation profile (0.5 $\mu g \cdot mL^{-1}$). The time of exposure used was chosen based on the reduction of ca. ~ 50% in the CFU levels, 4 h for *E. coli* and 6 h for *P. aeruginosa*. After each cycle of treatment with pure ciprofloxacin and ciprofloxacin formulated in DES solutions, the *E. coli* or *P. aeruginosa* colonies that survived to the previous cycle of inactivation were aseptically removed from the TSA plates and re-suspended in PBS, and then undertook the same inactivation protocol. The optical density of both bacterial suspensions, before each assay, was measured to prevent differences in the treatment efficiency. Three independent assays in duplicate were performed for each strain.

2.9. Keratinocytes cell culture and cytotoxicity assay

Immortalized human epidermal keratinocytes (HaCaT cells) obtained from Cell Lines Services (Eppelheim, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal bovine serum (FBS) and 1 % of L-glutamine, penicillin–streptomycin and fungizone (Life Technologies, Grand Island, NY, USA) and incubated in a humidified atmosphere at 37 °C and 5 % CO_2. Cytotoxic effect of ciprofloxacin aqueous solution, of ciprofloxacin formulated in DES aqueous solutions and of the DES formulations without the drug was assessed on human epidermal keratinocytes (HaCat) cells by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. For this purpose, HaCaT cells were seeded in 96-well plates at a concentration of 15 000 $cells \cdot mL^{-1}$ and allowed to adhere for 24 h. After adhesion, cells were exposed to a range of five concentrations (0.05–0.75 $\mu g \cdot mL^{-1}$), of the tested compounds diluted in DMEM medium (these previously sterilized with a 0.22 μm syringe filter), and incubated for 72 h at 37 °C in 5% of CO_2. After this period, the wells were washed with PBS, and 100 μL of fresh medium was placed in each well, as well as 50 μL of MTT solution (1 $mg \cdot mL^{-1}$ in PBS, pH 7.2) was added to each well. After 4 h of incubation, the medium was replaced with 150 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Posteriorly, the plate was shaken for approximately 2 h, protected from light. Cell viability was measured through the optical density of reduced MTT at 570 nm using a microplate reader (Synergy HT from BioTeK Instruments Inc., Winooski, VT, USA). The percentage of viable cells was calculated as the ratio between the absorbance of treated versus control cells.

3. Results and discussion

3.1. Selection and preparation of DES for ciprofloxacin solubilization

The DES components used in this work were carefully selected given the intended purpose, i.e., of improving the antibiotic's therapeutic action and to allow different administration routes for drug delivery. After an initial screening of potential biocompatible DES components, we have selected three DES with different hydrogen-bond acceptors and donors, namely [Ch]Cl:urea ([Ch]Cl:U (1:2)), proline:urea (Pro:U (1:1)) and citric acid:xylitol (CA:Xyl (2:1)). [Ch]Cl was selected since it is a strong hydrogen-bond acceptor and one of the most extensively explored compounds to create DES (Abranches et al., 2019). Urea was selected since it has been one of the most investigated hydrogen-bond donor species coupled to [Ch]Cl to create DES (Abbott et al., 2003), whereas citric acid was selected due to its effective results reported for the control of skin and soft tissue infections (Nagoba et al., 2017), The additional hydrogen-bond acceptor, proline, was chosen based on the reported capability of proline-based DES to improve the *in vivo* pharmacokinetic parameters of nutraceuticals (Faggian et al., 2016), with the DES Pro:U allowing a direct comparison with [Ch]Cl:U. Finally, xylitol was considered due to its antibacterial and antioxidant properties (Mäkinen, 2017). All these DES were prepared by the heat method, being in a jellified form at body's temperature, with the exception of [Ch]Cl:U that is liquid at this temperature.

It is known that the pH of wounds can influence the effectiveness and performance of antibiotics and antiseptics or potentially alter the metabolic state of bacteria (Percival et al., 2014). Alkaline media allow bacterial growth and acquired resistance; in this sense, an acidic environment is more promising for the effective treatment of infections. When considering a topical application purpose, for instance, the alkaline pH associated with the urea-based DES used in this work could compromise the therapeutic action of the formulation. Thus, the pH of the investigated DES was adjusted to a more suitable value to fulfil the requirements for a wide range of applications. For this purpose, we used

malonic acid at a fixed molar ratio in the DES [Ch]Cl:urea:malonic acid ([Ch]Cl:U:MA (1:2:0.05)) and proline:urea:malonic acid (Pro:U:MA (1:1:0.5)) to adjust the pH of the mixtures to a final pH near 4.5.

3.2. Ciprofloxacin solubility in aqueous solutions of DES

The three selected DES ([Ch]Cl:U (1:2), Pro:U (1:1) and CA:Xyl (2:1)) were tested allowing to appraise the effect of their hydrogen-bond donors (urea, citric acid) and hydrogen-bond acceptors ([Ch]Cl, proline, xylitol) on the solubilization of ciprofloxacin. Solubility was tested up to 60% (w/w) of DES due to the high viscosity of the solvent above these concentrations. To better understand which concentrations should be applied in the formulations, the ciprofloxacin's solubility enhancement in each DES aqueous solution was assessed at both room (25 °C) and human body (37 °C) temperatures. Fig. 1 depicts the results in solubility enhancement (S/S_0, where S corresponds to the solubility of ciprofloxacin in the DES aqueous solution and S_0 to the solubility of ciprofloxacin in water at the same temperature and at the same pH of 4.5). The respective solubility curves are provided in Supplementary Information (Fig. S1) along with the values of solubility determined for each DES aqueous solution, in mol.dm^{-3} (M) and molar fraction, at both temperatures (Tables S1, S2 and S3).

Ciprofloxacin shows low solubility in common organic solvents such as ethanol, 2-propanol or acetone (Caço et al., 2008) and notably in water (<100 μg·mL^{-1} at pH = 4.5), limiting its formulation. In fact, ethanol seems to exhibit lower capability than water and acetone to solubilize this antibiotic, and even lower for its hydrochloride form (Caço et al., 2008). Due to the pH-dependent solubility of ciprofloxacin in aqueous media, its solubility is higher at pH values below 5.5 (Yu et al., 1994). Generally, improvements in solubilization are achieved by addition of diluted hydrochloric acid (as used in this work), and in case of intravenous formulations, using lactic acid achieving pH values up to 4.6 (FDA, 2011). Although DES are known for their high solvation ability for some APIs, such as anti-inflammatory, analgesic or antifungal drugs (Li and Lee, 2016; Lu et al., 2013), the study of the influence of DES aqueous solutions solubilization of APIs has only been recently been described (Cysewski et al., 2021; Jeliński et al., 2021, 2019). The pH adjustment of these mixtures for a specific application has not been fully explored for pharmaceutical purposes.

As determined in the present study, the ciprofloxacin solubility in water was found to be (2.277 ± 0.007) × 10^{-4} mol·dm^{-3} and (3.607 ± 0.197) × 10^{-4} mol·dm^{-3} at room and human body temperatures, respectively (which is in the range of the values mentioned above). Remarkably, the studied aqueous solutions of DES allowed to enhance the solubility of ciprofloxacin in aqueous media up to 430 ± 21-fold achieved with the DES Pro:U:MA at 30% (w/w) at room temperature (25 °C, Fig. 1a) and at 40% (w/w) at body's temperature (37 °C, Fig. 1b) (9.691 ± 0.200) × 10^{-2} mol·dm^{-3} at room temperature and (1.500 ± 0.039) × 10^{-1} mol·dm^{-3}at body's temperature, respectively). The Pro:U:MA DES is not only among the best DES aqueous solutions studied, but also allows a similar to higher solubilization ability for ciprofloxacin than its hydrochloride form (by 2 orders of magnitude) (Caço et al., 2008). This DES, allows a non-monotonic solubility enhancement in aqueous media at both temperatures, with a solubility behavior indicative of an hydrotrope-mediated mechanism (Shimizu and Matubayasi, 2016). At room temperature, is possible to enhance ciprofloxacin's solubility from 160 ± 8-fold (using 10 % (w/w) of DES) to 430 ± 21-fold at 30% (w/w) of DES. The solubility enhancement then decreases when higher DES concentrations are used, achieving a 44 ± 1-fold increase at 60% (w/w) of DES (Fig. 1a). Considering the results at body's temperature, a similar behavior is observed, solubility improvements from 117 ± 5-fold, at 10% (w/w) of DES, to 430 ± 21-fold at 40% (w/w) of DES are observed, reaching also a minimum at 60% (w/w) of DES, where a 238 ± 8-fold increase in ciprofloxacin's solubility could be obtained (Fig. 1b).

Aqueous solutions of the DES CA:Xyl present a monotonic increase of solubility within the studied DES concentration range, with a minimum at 10% (w/w) of DES (10 ± 1-fold, at body's temperature (Fig. 1a) and 8 ± 1-fold, at body's temperature (Fig. 1b)). The maximum in the solubility enhancement occurred at 60% (w/w) of DES for both room (50 ± 3-fold, Fig. 1a) and body's temperature (35 ± 2-fold, Fig. 1b). The observed behavior is in accordance with a co-solvency mechanism (Millard et al., 2002). Aqueous solutions of [Ch]Cl:U:MA exhibited the lower ciprofloxacin solubility enhancement among the studied DES, presenting a maximum of approximately 40 ± 1-fold increase at 40% (w/w) at 25 °C (Fig. 1a) and up to 20 ± 3-fold at 37 °C at 20% (w/w) of DES (Fig. 1b). The [Ch]Cl:U:MA presents the same mechanism of solubilization of Pro:U:MA, hydrotrope-mediated. It is possible to increase the solubility of the antibiotic from 24 ± 1-fold (using 10 % (w/w) of DES) to its maximum at 40% (w/w) of DES, and similarly to the Pro:U:MA behavior find a minimum at 60% (w/w) of DES (19 ± 1-fold, Fig. 1a) at room temperature. At body's temperature, the solubility enhancements at 10% (w/w) of DES are similar to those obtained using a 60 % (w/w) DES solution (10 ± 1-fold, Fig. 1b). This DES presents the lowest ability of all DES aqueous solutions to solubilize the antibiotic. Although [Ch]Cl and proline seem to play a significant role in this mechanism, the [Ch]Cl had a lower impact on the solubilization ability.

Recent studies have been dedicated to understanding the influence of amino acid-based DES on antibiotics' solvation, namely β-lactam ones (Atilhan et al., 2020). These findings highlight the strong solute − solvent intermolecular interactions along with a slight volume expansion. In the case of ciprofloxacin solubilization, the key for the observed efficacy of the studied DES relies also in the interactions with the DES components taking advantage of the different types of HBA/HBD sites in

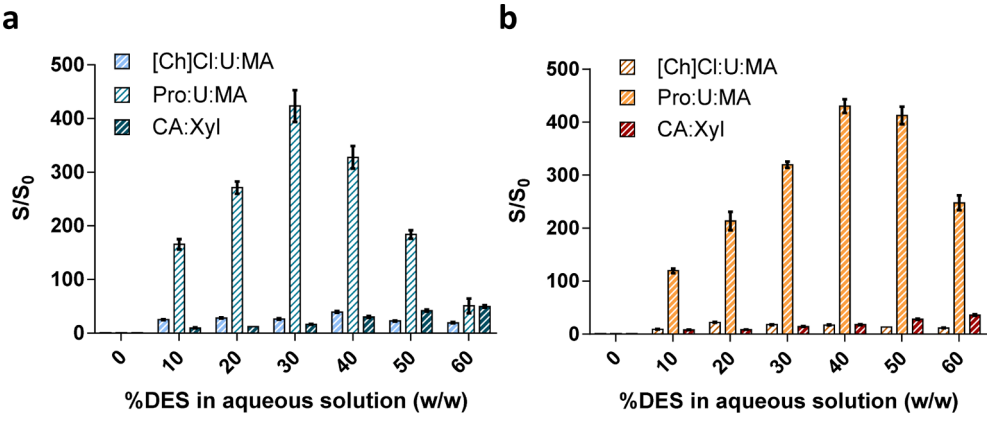

Fig. 1. Solubility enhancement of ciprofloxacin in DES aqueous solutions. Effect of the different DES and their concentrations in the antibiotic solubility at room temperature (a) and human body's temperature (b). The results are expressed as the mean ± SD of three independent measurements.

the antibiotic structure, that lead to highly effective interactions in the case of Pro:U:MA. Given the high solubility enhancements achieved at 40% (w/w) of all DES in aqueous solution at room temperature, this DES concentration was selected for further studies.

3.3. Ciprofloxacin stability in aqueous solutions of DES

The hydrogen-bond donors/acceptors integrity was maintained over the DES' preparation; the respective chemical stability of the components was confirmed for the remaining DES formulations based on ^{1}H and ^{13}C NMR (in SI Figs. S2.1, S2.2 and S2.3). The ^{1}H and ^{13}C NMR results reveal the components' integrity by the presence of the characteristic resonances of the individual components. Prior to the solubilization of the components in aqueous media, the physical appearance of the three DES with solubilized ciprofloxacin was monitored over one week. These formulations were monitored for changes in color, homogeneity and drug precipitation. No physical changes were verified for the formulations at room temperature conditions (25 °C) as depicted in Supplementary Information (Fig. S3).

To determine the shelf-life of ciprofloxacin in the novel aqueous formulations, the stability of the solvents (DES aqueous solutions at 40% (w/w)) and of the API formulated in the DES aqueous solutions was accessed. Stability experiments were carried out at storage conditions of 25 ± 2 °C and 75–80% relative humidity, in the dark. The drug concentration in the DES formulations, was determined during a one-month period (T_{30}) at the same storage conditions. DES formulations containing the API were prepared by dissolving ciprofloxacin below its solubility limit, namely 3.00×10^{-5} mol·dm^{-3}, in aqueous solutions of DES and by direct dissolution in water at the same pH = 4.5 for direct comparison purposes. Table 1 presents the ciprofloxacin content in each DES formulation and when solubilized in water (T_{30}), relatively to initial drug concentration (at T_0).

It is known that ciprofloxacin aqueous solutions have limited stability at room temperature, thus being stored at low temperatures (-18 to 4 °C) to decrease degradation (Kussmann et al., 2019). Alternatively, in aqueous solution, the antibiotic can be stable for 14 days at room temperature with addition of either 5% dextrose or 0.9% sodium chloride (Donnelly, 2011; FDA, 2011); the stability of the mentioned solutions can be further improved up to one month, through their storage in polyvinylchloride minibags (Donnelly, 2011). DES have also been explored to improve the chemical, thermal, and photostability of drugs, being proved to be effective pharmaceutical excipients to enhance the stability of formulations during the storage (Daneshjou et al., 2017; Olivares et al., 2018).

The results obtained here for the drug content in pure water, after 30 days, show a decrease in the concentration of ciprofloxacin to 52.3 ± 3.1 %, reinforcing the reported instability of this antibiotic in water and the need to develop improved formulations. The chromatograms shown in Fig. 2, demonstrate that no significant degradation peaks of ciprofloxacin appear and only a slight decrease in drug concentration was observed after 30 days. More specifically, the DES aqueous solutions used here allowed to preserve 98.4 ± 0.5 % and 94.7 ± 0.6 % of ciprofloxacin in aqueous media, using only 40% (w/w) of Pro:U:MA and CA:Xyl, respectively, well above the required 90% as reported in the literature (Donnelly, 2011). The stability values are similar to ones achieved by the formulation of the antibiotic in lipidic-nanoparticles and nanoemulsions, designed to protect the drug from aqueous media and avoid the hydrolysis of APIs (Youssef et al., 2020, 2021). In the case of [Ch]Cl:U:MA aqueous solution only 80.7 ± 0.7 % of the drug was found in the solution at day 30, highlighting the importance of the nature of the DES in the stability of the drug. Furthermore, the stability of the studied DES aqueous solutions was analyzed by Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR–ATR) after the end of 30 days (in SI, Fig. S4), and as far as the technique can ascertain, no significant changes in the DES functional groups vibrations specific were verified at the end of this period.

Table 1
Effect of the solvent on ciprofloxacin's stability in aqueous media when stored at room temperature for 30 days.

Ciprofloxacin content (%±SD)		
Solvent	**T_0**	**T_{30}**
Water	100 ± 0	52.3 ± 3.1
Water + 40% [Ch]Cl:U:MA	100 ± 0	80.7 ± 0.7
Water + 40% Pro:U:MA	100 ± 0	98.4 ± 0.5
Water + 40% CA:Xyl	100 ± 0	94.7 ± 0.6

3.4. Susceptibility of bacteria to formulations of ciprofloxacin in DES aqueous solutions

The antimicrobial activity of ciprofloxacin formulated in DES aqueous solutions was evaluated towards Gram-negative bacteria, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, and Gram-positive bacteria, *Staphylococcus aureus* ATCC 6538. The results achieved were compared to the antibiotic in water at the same concentration. For all the antimicrobial assays, the potential pH interference was eliminated by adjusting the pH of all solutions to more suitable values for bacterial growth, namely to pH = 6.8. The study involved firstly the determination of the minimum inhibitory concentration (MIC) by the dilution method, whose results are shown in Table 2. The strains used in this work can be classified as susceptible to ciprofloxacin and the drug susceptibility results are in agreement with the EUCAST classification (EUCAST, 2020). As expected, these bacteria are not susceptible to the pure DES solutions in the range of the studied concentrations.

For *E. coli*, the results obtained show that the MIC values of the formulations of ciprofloxacin in DES aqueous solutions are similar to those observed for the antibiotic in water. This fact demonstrates that the solubilization of ciprofloxacin in DES aqueous solutions does not affect the efficacy of the drug towards this bacterium. In what concerns the *P. aeruginosa* and *S. aureus* bacteria, an increase in growth inhibition was observed with ciprofloxacin formulated in the DES aqueous solutions comparing to the antibiotic in water at the same pH. Furthermore, it is noticeable that Pro:U:MA and CA:Xyl aqueous solutions present higher ability to improve the antimicrobial activity against *P. aeruginosa* than [Ch]Cl:U:MA.

Table 3 and Fig. 3 show the results of the antibiotić susceptibility in the three DES aqueous solutions and in water (see Supplementary Information Fig. S5 for additional antibiogram results on the remaining bacteria). Overall, the diffusion of the drug into the media is not affected by DES aqueous solutions and can match or even increase the growth inhibition zones of the pure antibiotic when compared to ciprofloxacin in water. As expected, aqueous solutions of DES (without ciprofloxacin) do not present antimicrobial activity in the concentrations used for ciprofloxacin's solubilization, being in accordance with the results obtained for the MIC analysis.

All bacteria are susceptible to ciprofloxacin and to the antibiotic formulated in the DES aqueous solutions according with EUCAST classifications, and at the same (5 μg·mL^{-1}) and even at lower (0.5 μg·mL^{-1}) concentrations. The only exception to this trend occurs with *S. aureus* where higher doses are required to observe bacterial susceptibility (in SI, Fig. S5c and Fig S5d). Similarly, at lower drug concentrations (0.5 μg·mL^{-1}) *P. aeruginosa* seems to be resistant to the antibiotic solubilized in water. However, a noticeable enhanced effect is observed when the DES solutions are combined with the antibiotic. Considering the results obtained for *P. aeruginosa* (Fig. 3b and 3c), a higher impact on the antibiotic's growth inhibition activity is noticed with the formulations comprising Pro:U:MA acid and CA:Xyl, at both concentrations (0.5 and 5.0 μg·mL^{-1} for *P. aeruginosa*). In this case, a prominent increase on the susceptibility of the bacterium to the antibiotic occurs when using aqueous solutions of Pro:U:MA acid and CA:Xyl (5 ± 2 mm and 7 ± 2 mm, respectively, comparatively with the antibiotic in water that has no

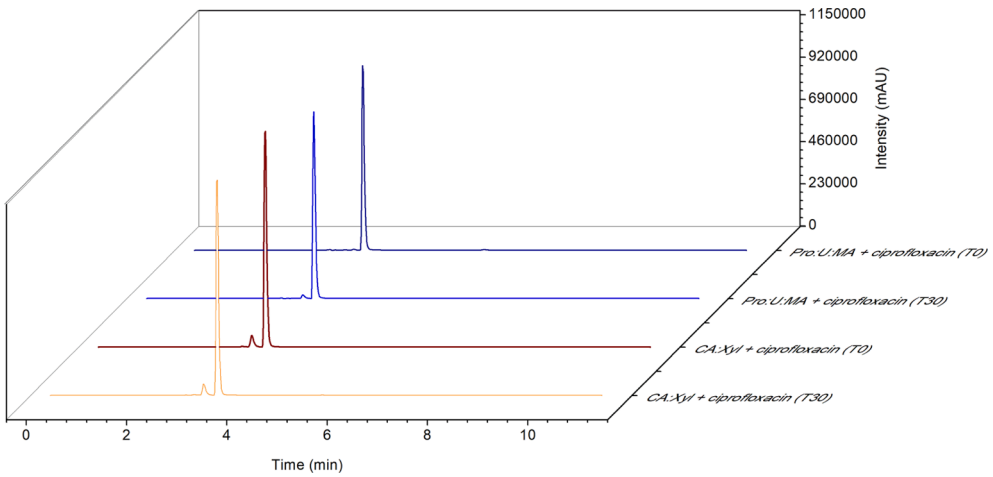

Fig. 2. Stability of ciprofloxacin in DES formulations (Pro:U:MA and CA:Xyl). HPLC chromatograms of ciprofloxacin at the day of preparation of the formulations (T_0) and after 30 days (T_{30}) of storage at (25 ± 2) °C. Ciprofloxacin's peak retained its shape and retention time after 30 days.

Table 2
Minimal inhibitory concentrations of ciprofloxacin in water and in DES aqueous solutions for *E. coli* ATCC-25922, *P. aeruginosa* ATCC-27853 and *S. aureus* ATCC 6538. Results obtained for DES aqueous solutions (results without ciprofloxacin are also included for comparative purposes). Results are of three independent concordant experiments for each formulation and for each strain.

Minimum inhibitory concentration (μg·mL^{-1})			
	E. coli	*P. aeruginosa*	*S. aureus*
Ciprofloxacin	0.00488	0.00488	0.00977
[Ch] Cl:U:MA + ciprofloxacin	0.00488	0.00488	0.00244
Pro:U:MA + ciprofloxacin	0.00488	0.00244	0.00244
CA:Xyl + ciprofloxacin	0.00488	0.00244	0.00244
[Ch] Cl:U:MA	–	–	–
Pro:U:MA	–	–	–
CA:Xyl	–	–	–

activity).

For *E. coli* and *S. aureus*, the effect of the antibiotic in DES aqueous solutions is less pronounced. For *E. coli* the susceptibility to the antibiotic is similar when solubilized in water or in DES aqueous solutions, with the exception of the formulation with Pro:U:MA in which a slight effect is denoted (35 ± 2 mm vs. 31 ± 2 mm for ciprofloxacin in water at 5.0 μg·mL^{-1}, in SI Fig. S5b). Regarding *S. aureus*, and despite the higher dosages required, the formulations with Pro:U:MA and CA:Xyl are also slightly more effective than the ones with the antibiotic in water (7 ± 2 mm and 6 ± 2 mm vs. 4 ± 2 mm for ciprofloxacin in water at 5.0 μg·mL^{-1} – cf. in SI Fig. S5c). Accordingly, for all strains, the antibiotic formulation with [Ch]Cl:U:MA has equal or lower effect than the antibiotic in water.

It is always relevant to address different DES combinations since, as demonstrated in this work, each DES presents a different impact in the therapeutic efficacy of the antibiotic. This trend is specially observed for the antimicrobial activity of the antibiotic formulated in [Ch]Cl:U. [Ch]Cl:U is amongst the most studied DES, with antimicrobial activity studies against similar microorganisms in the absence of APIs already reported (Radošević et al., 2018). Nonetheless, this DES does not represent an effective alternative to improve ciprofloxacin performance, has seen for its effect on the studied bacteria in comparison to the remaining formulations, and additionally for its higher toxicity (Macário et al., 2019). Thereby, the two best formulations correspond to ciprofloxacin in aqueous solutions of Pro:U:MA and CA:xyl, which were selected for further experiments. Also, 0.5 μg·mL^{-1} was the concentration selected for further studies since it corresponds to the lowest one presenting antimicrobial activity against the bacteria, while envisaging the reduction of the antibiotic dosage.

The inactivation efficiency was evaluated by exposure of the three bacteria strains, *E. coli* (Fig. 4a), *P. aeruginosa* (Fig. 4b) and *S. aureus* (Fig. 4c) to 0.5 μg·mL^{-1} of the antibiotic in water and to the antibiotic formulated in the selected DES aqueous solutions during 24 h. Bacterial controls without these formulations were also evaluated to guarantee the bacterial viability during the time and conditions of the experimental procedure. As previously described, the results of the new formulations were compared with the activity of ciprofloxacin in water at the same concentration and pH in aqueous media. The DES formulations were tested in the same concentrations present in the formulations with ciprofloxacin. The gathered results (Fig. 4) demonstrate different profiles according with the formulation type and strain considered.

The results obtained for the inactivation of *E. coli* (Fig. 4a) with Pro:U:MA and CA:Xyl demonstrate that there is no influence or efficacy of the DES aqueous solutions (without ciprofloxacin) in the inactivation of

Table 3
Results on antimicrobial susceptibility of *E. coli* ATCC-25922, *P. aeruginosa* ATCC-27853 and *S. aureus* ATCC 6538 to ciprofloxacin in water and to the antibiotic formulated in DES aqueous solutions at different concentrations.

Concentration	**Inhibition zone diameter (mm)**			
[Cipro] (μg.mL^{-1})	**Ciprofloxacin**	**[Ch] Cl:U:MA + ciprofloxacin**	**Pro:U:MA + ciprofloxacin**	**CA:Xyl + ciprofloxacin**
E. coli				
0.5	26	26	28	26
5.0	31	32	35	32
P. aeruginosa				
0.5	–	2	5	6
5.0	15	14	18	23
S. aureus				
5.0	4	3	7	6
10	13	12	15	14

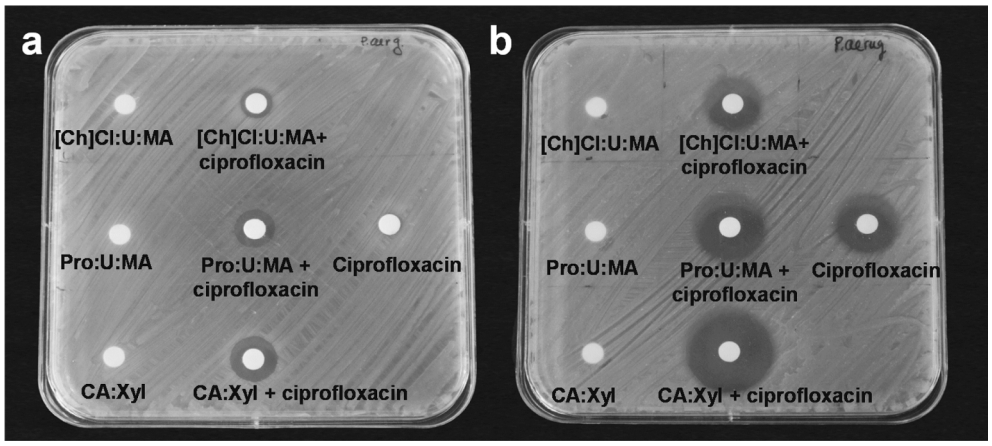

Fig. 3. Antimicrobial susceptibility of bacteria to formulations of ciprofloxacin in DES aqueous solutions. a and b Representative inhibition zone assays on *P. aeruginosa* ATCC-27853 cultures using disks impregnated with ciprofloxacin's formulations with [Ch]Cl:U:MA, Pro:U:MA, CA:Xyl, at concentrations of 0.5 (a) and 5.0 (b) $\mu g \cdot mL^{-1}$, respectively. Ciprofloxacin in water and the DES aqueous solutions (without ciprofloxacin) were evaluated in the same concentrations. Results are expressed as mean of three experimental replicate measurements.

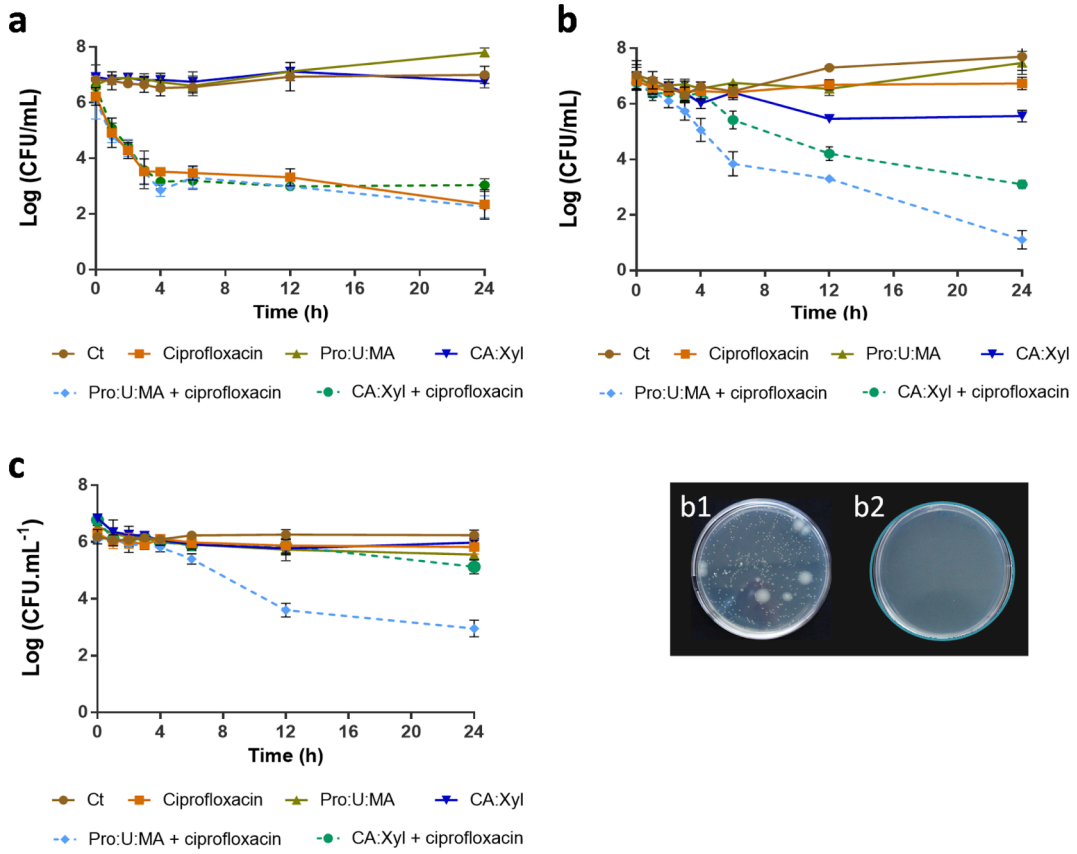

Fig. 4. Effect of different times of exposure of bacteria to ciprofloxacin in water, to DES solutions (without ciprofloxacin) and to the formulations of ciprofloxacin in DES solutions in the inactivation efficiency bacteria. Inactivation profiles were determined for (a) *E. coli*, (b) *P. aeruginosa* and (c) *S. aureus* strains. The number of colony forming units per volume ($CFU \cdot mL^{-1}$) from samples collected over 24 h are shown. An antibiotic dose of 0.5 $\mu g \cdot mL^{-1}$ was used in all experiments. (b1) and (b2) demonstrate the difference between ciprofloxacin's ability to inactivate *P. aeruginosa* in water and formulated in a aqueous solution of Pro:U:MA, respectively, after 24 h. Data are presented as mean ± SD values of three independent studies for each sample and for each strain.

this bacterium. Nevertheless, the antibiotic in water and the ciprofloxacin formulated in the DES aqueous solutions (Pro:U:MA and CA:Xyl) are effective on the inactivation of *E. coli*. These formulations cause a decrease of 3.9 Log10 (ANOVA, $p < 0.0001$) in the viability of the bacterium after 24 h of incubation. In all cases, the bacterial viability decreased with the exposure time. However, no significant differences on the activities are observed between the two ciprofloxacin DES formulations tested and the antibiotic in water. The marked decrease in the bacteria's viability after 4 h of incubation is in agreement with previous antimicrobial studies for *E. coli* response to ciprofloxacin (Adamus-białek et al., 2019; Machuca et al., 2017).

Regarding the effect of ciprofloxacin DES formulations in the inactivation of *P. aeruginosa* (Fig. 4b), a completely different profile was achieved. While aqueous solutions of Pro:U:MA had no influence on the bacterial viability, the aqueous solution of CA:Xyl without ciprofloxacin showed a small effect on *P. aeruginosa* inactivation. This is in accordance with the antimicrobial activity of citric acid and xylitol (Burel et al., 2021; Mäkinen, 2017; Nagoba et al., 2017), causing a decrease of 2.1 Log10 (ANOVA, $p < 0.0001$) in the bacterium viability after 24 h of incubation. However, when the antibiotic is formulated in DES aqueous

solutions of Pro:U:MA and CA:Xyl, the inactivation of *P. aeruginosa* has a distinct profile when compared to the antibiotic in water. Such differences highlight the improved efficacy of the inactivation rates of the antibiotic when formulated in DES aqueous solutions. In fact, ciprofloxacin alone was not able to inactivate *P. aeruginosa* after 24 h of incubation. Nevertheless, when formulated with the selected DES aqueous solutions the antibiotic's effect is enhanced. This potentiated effect is verified only after 6 h of incubation, where a decrease in the viability of *P. aeruginosa* of 1.0 and 2.6 Log10 (ANOVA, $p < 0.0001$) was achieved for ciprofloxacin formulated in aqueous solutions of Pro:U:MA and CA:Xyl, respectively. After 24 h of incubation, the pronounced effect of the DES formulations with ciprofloxacin is more notorious, achieving *P. aeruginosa* inactivation values of 5.6 and 3.6 Log10 (ANOVA, $p < 0.0001$) for formulations of Pro:U:MA and CA:Xyl with ciprofloxacin, respectively.

Interestingly, for the Gram-positive bacterium *S. aureus*, the effect of ciprofloxacin formulated in CA:Xyl is in contrast with the one obtained for Pro:U:MA. Both the antibiotic in aqueous solution and the ciprofloxacin formulated in CA:Xyl have no significant effect on the inactivation of the bacterium for an incubation period of 24 h. However, after 12 h of exposure to the antibiotic formulated in Pro:U:MA, a decrease in the viability of 2.4 Log10 (ANOVA, $p < 0.0001$) was achieved. The exposure of the bacterium to this formulation was more effective than to the aqueous solution of the antibiotic and the antibiotic formulated in aqueous solution of CA:Xyl, causing a decrease of 3.1 Log10 (ANOVA, $p < 0.0001$) in the inactivation of *S. aureus* after 24 h.

3.5. Ciprofloxacin activity after storage in DES formulations

The drug activity was assessed based on the results for the MIC value and inactivation efficiency. To evaluate the stability of the drug in the DES formulations after storage, the assays were carried out on the day of preparation (T_0) and after a month (T_{30}) period, stored at (25 ± 2) ℃ and 75–80% relative humidity. An antibiotic dosage of 0.5 μg·mL^{-1} was used for the inactivation efficiency experiments and for all the bacteria studied (Fig. 5). The MIC values of ciprofloxacin at T_0 and after 1 month are similar, showing a maintenance of the antimicrobial activity of the drug after storage (see Supplementary Information Table S4). The bacterial inactivation for *E. coli* (Fig. 5a), *P. aeruginosa* (Fig. 5b) and *S. aureus* (Fig. 5c) was analyzed for the results obtained after 24 h of incubation. The obtained data show that the inactivation efficiencies for ciprofloxacin formulated in the DES aqueous solutions after 1 month storage are comparable to those obtained with freshly prepared formulations.

3.6. Evaluation of the development of bacterial tolerance to ciprofloxacin in DES formulations

The effective eradication of bacteria has become challenging due to their remarkable ability to resist antibiotics, as seen for ciprofloxacin (Pankuch et al., 2008; Ermertcan et al., 2001). Given the medical prescription of this antibiotic to tackle infection conditions caused by Gram-negative bacteria (Troughton et al., 2011), we demonstrated the relevance of ciprofloxacin DES formulations. To evaluate the potential development of bacterial resistance to ciprofloxacin formulated in the DES aqueous solutions, we simulated a 7-day treatment with specific dosages (0.5 μg·mL^{-1}) administered at each 4 h (*E. coli* (Fig. 6a)) and 6 h (*P. aeruginosa* (Fig. 6b)). We have monitored this behavior in both strains and selected the administration intervals according to the inactivation profiles previously presented. Bacterial controls were also conducted in the same conditions, being subcultured in the same number of cycles but in the absence of the antibiotic and DES.

According to the results depicted in Fig. 5a, there is no significant increase in resistance of *E. coli* to ciprofloxacin in water or to the antibiotic formulated in DES aqueous solutions after 7 days of consecutive administrations at each 4 h treatment. A similar behavior is seen with *P. aeruginosa* (Fig. 6b) since no significant increase in the inactivation efficiency was detected after 7 cycles of administration at each 6 h using the antibiotic formulated in the DES aqueous solutions at the same concentration.

Ciprofloxacin's resistance in *P. aeruginosa* is of complex and multifactorial nature still, it has been mostly attributed to target-site modifications and upregulation of efflux pumps (Breidenstein et al., 2008; Kureishi et al., 1994; Nakano et al., 1997; Rehman et al., 2019). In addition to numerous *gyrAB* and *parCE* genes mutations, these bacterium stand out for their low permeable outer membrane (1/100 of the permeability of *E. coli*'s) (Oh et al., 2003). Usually drug combinatory therapies are selected, attempting to avoid drug resistance and improve the treatment success rate (Kiser et al., 2010). However, due to ciprofloxacin's resistance mechanisms, the resistance to other antibiotics might also be increased, being this one of the major disadvantages of the use of such strategy (Tanimoto et al., 2008). The reduced susceptibility of ciprofloxacin is usually associated with mutations in regulatory genes of efflux pumps and their resulting overexpression, which increases the ciprofloxacin's expulsion from the bacterial cells, decreasing the intracellular concentration (Pagès, 2013; Westbrock-wadman et al., 1999). In this work we have taken advantage not only of the DES' ability to enhance antibiotic's solubility in several orders of magnitude but also its capacity to promote variations in the cellular envelope permeability (Zhang et al., 2020) and manipulated the concentration as an advantage to improve the efficacy of ciprofloxacin. By increasing the bacterial cell permeability, and the ciprofloxacin concentration in the media, is not only possible to increase the intracellular concentration of antibiotic, as well as to compromise the bacterial integrity improving the susceptibility of the bacterium to this formulation.

In general, and as seen for the inactivation efficiency rates depicted in Fig. 6, the formulation of ciprofloxacin in DES aqueous solutions do not induce the development of tolerance to the treatment or potentiate resistance mechanisms of these bacteria to the antibiotic. The use of DES formulations in combination with ciprofloxacin are able to maintain an enhanced efficacy of bacterial inactivation during 7 days of treatment of infections with both strains.

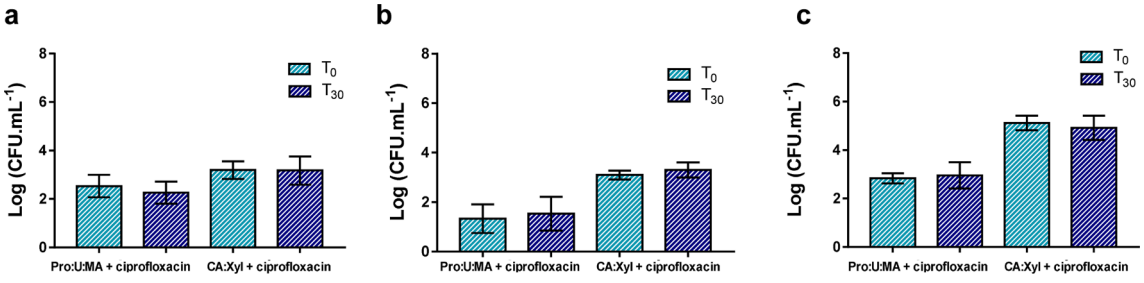

Fig. 5. Activity of ciprofloxacin after 1 month storage in DES formulation. The antibiotic activity in the DES formulations was evaluated at the day of preparation (T_0) and after 1 month storage at 25 °C (T_{30}). The inactivation efficiency was studied in (a) *E. coli*, (b) *P. aeruginosa* and (c) *S. aureus* strains. Activity was evaluated in 3 independent experiments.

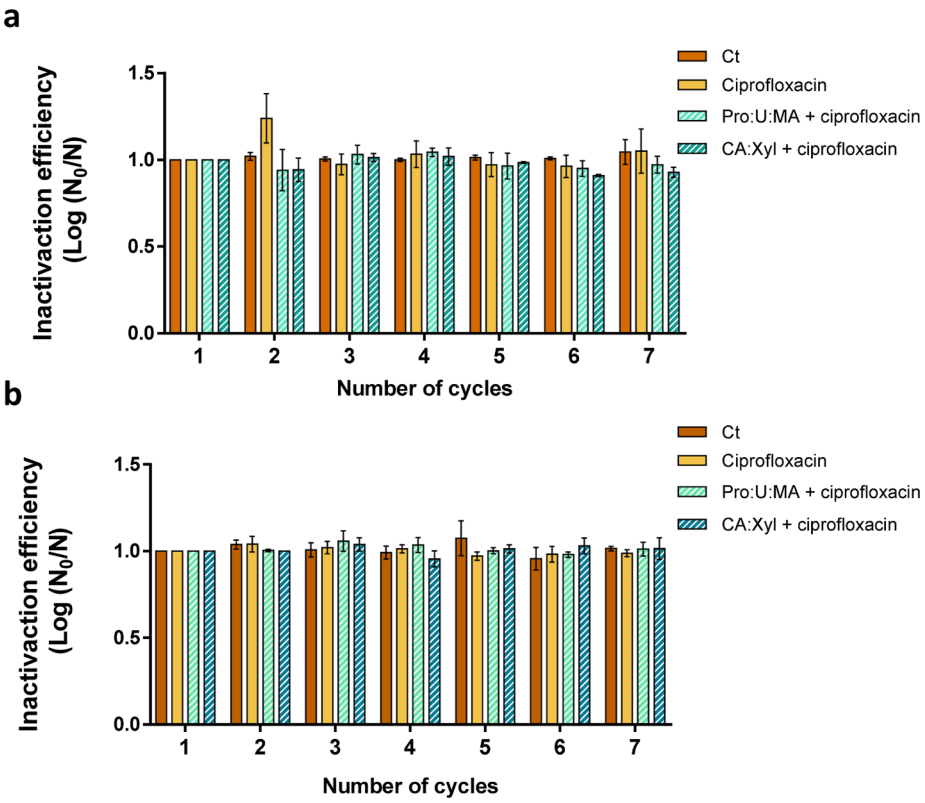

Fig. 6. Inactivation efficiency of seven consecutive cycles of exposure to ciprofloxacin in water and in DES formulations. Data on (a) *E. coli*, and (b) *P. aeruginosa*. Efficiency was determined after 4 and 6 h of incubation, respectively, with ciprofloxacin in water and ciprofloxacin formulated in the DES solutions at 0.5 $\mu g.mL^{-1}$. N_0 and N represent, respectively, the number of colony forming units per volume ($CF{\cdot}mL^{-1}$) for the first treatment with each formulation and the values after the respective cycle. The results are expressed as mean ± SD of three independent experiments for each formulation and for each strain.

3.7. Cytotoxicity of DES formulations

Envisaging the application of the novel DES formulations comprising ciprofloxacin for human use, the cytotoxicity of ciprofloxacin and of ciprofloxacin formulated in DES solutions was determined towards Immortalized human epidermal keratinocytes (HaCat cells) after 72 h of exposure. The respective results are given in Fig. 7.

Since anti-topoisomerase drugs like ciprofloxacin can inhibit the expression of topoisomerase I (Pessina et al., 2001), some toxicity is expected for HaCat cells at high concentrations. In fact, the pure ciprofloxacin was found to be slightly toxic for these cells at concentrations higher than 0.5 $\mu g{\cdot}mL^{-1}$. On the other hand, both DES aqueous solutions used, namely Pro:U:MA and CA:Xyl, exhibit an increase of more than 20% in cell viability in the range of concentrations studied (ANOVA, $p < 0.0002$). Notwithstanding, when the antibiotic is formulated in DES aqueous solutions, its toxicity is reduced, even at higher concentrations. Although, at the concentration of 0.5 $\mu g{\cdot}mL^{-1}$, ciprofloxacin presents slight toxicity (<75% cell viability), a significant increase of more than 25% in cell viability can be noticed when the antibiotic is formulated in both DES aqueous solutions (ANOVA, $p < 0.025$, $p < 0.0002$).

4. Conclusions

In the current study, we have designed DES formulations comprising ciprofloxacin to tackle antibiotic's drawbacks. We have demonstrated how the rational selection of the DES components can go further from

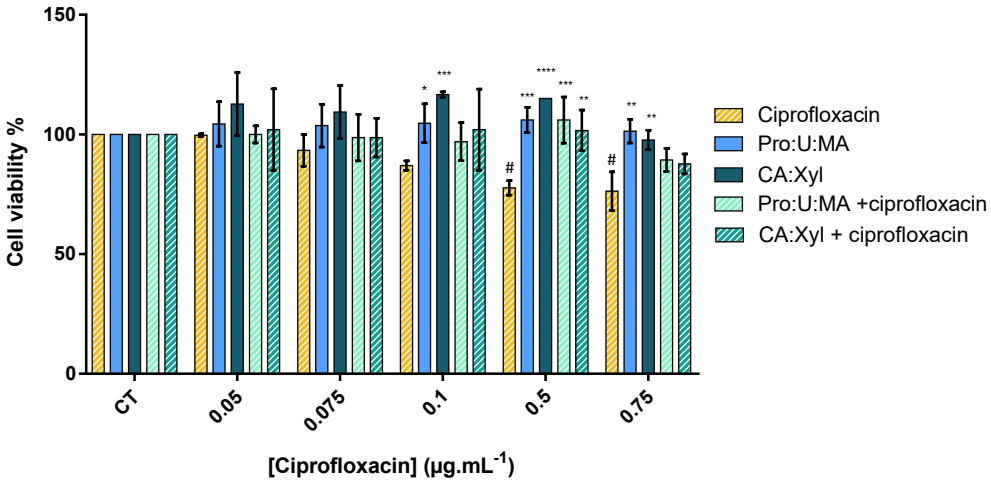

Fig. 7. Effect of ciprofloxacin and DES formulations in the viability of HaCaT cells. Cytotoxicity profile after 72 h of exposure vs control cells (CT). Results are expressed as mean ± SD of three independent experiments. # $p < 0.0015$ viability increase compared to the control and *$p < 0.05$, **$p < 0.0025$, ***$p < 0.0002$, ****$p < 0.0001$ viability increase compared to ciprofloxacin's effect on HaCat cells.

their common solvent applications to the simultaneous improvement of solubility, stability, and therapeutic efficacy of antibiotics, such as ciprofloxacin, without tolerance development or cell toxicity. Our findings demonstrate that the studied DES in aqueous media can be successfully used as common pharmaceutical co-solvents and hydrotropes in antibiotics solubilization, allowing to achieve solubility enhancements up to 430-fold. While being capable of enhancing ciprofloxacin's solubility, these formulations are also able to improve the stability of the antibiotic solutions by almost 50% in comparison to its formulation in water at a similar pH. Our results reveal the long-term stability (up to 1 month) of the aqueous DES solutions as well as of the antibiotic in this novel media. The DES formulations not only preserve the fluoroquinolone's activity, but are capable of increasing the antibiotic's action against both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus*) strains. The possibility to produce a higher effect on the antimicrobial activity than ciprofloxacin in water (up to 2-fold for *P. aeruginosa* and 4-fold for *S. aureus*) might allow to decrease the drug dosage required to achieve the same therapeutic efficacy, thus decreasing the associated side-effects upon administration of higher doses. Additionally, the bacterial susceptibility to the antibiotic is enhanced by the formulation of the antibiotic in DES aqueous solutions, allowing to even produce effect in concentrations where the pure API in water presents no antimicrobial activity (0.5 $\mu g \cdot mL^{-1}$). This effect can be mainly attributed not only to an improvement in the solubility of the antibiotic, but also to the ability of DES formulations to increase bacterial cell wall permeation, allowing a higher drug content available intracellularly to exert antimicrobial action. Such an effect can be achieved by the proper design of DES and the careful selection of drug and DES concentrations to the final formulation. To the best of our knowledge, this is the first report on DES aqueous solutions where insights on the therapeutic efficacy of DES formulations with fluoroquinolones are addressed. These results reveal the pioneering use of including DES aqueous solutions in antibiotic-based formulations and their remarkable impact on improving fluoroquinolones solubility, stability and therapeutic action and on lowering bacteria resistance with low cytotoxicity associated. Given the versatility of DES formulations comprising antibiotics, they can be directly incorporated in a vast number of materials to develop a wide range of drug delivery systems.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2022.121566.

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