

Thermo-Responsive Microemulsions Containing Deep Eutectic-Based Antibiotic Formulations for Improved Treatment of Resistant Bacterial Ocular Infections

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The rise of antibiotic resistant strains, as methicillin-resistant *Staphylococcus aureus* (MRSA), challenges the current treatment of infections. In the case of ocular infections, antibiotic eye drops are commonly prescribed. However, their efficacy is usually compromised by the low viscosity of these formulations and the eye drainage. To overcome these drawbacks, deep eutectic solvent (DES)-based microemulsions with thermo-responsive character, that increase their viscosity upon contact with the eye have been developed. Using betaine-based DES aqueous solutions, it is possible to increase up to 140-fold the water solubility of the antibiotic chloramphenicol, typically used in ocular infections. The DES solutions containing the antibiotic are applied as water phases in water-in-oil-in-water (w/o/w) microemulsions, being stable up to 3 months. Furthermore, a sustained-release and a higher permeation of the antibiotic through the cornea than that of commercialized eye drops is achieved, while presenting comparable cytotoxicity profiles (cell viabilities > 88%). Higher antimicrobial activity and faster action of the antibiotic in case of infection with MRSA is observed compared to the commercialized formulations (7 log₁₀ of inactivation in 48 h vs 72 h). Overall, these microemulsions comprising DES are a promising strategy to achieve higher antibiotic effectiveness in the treatment of resistant bacterial infections.

1. Introduction

Ocular infections affect people of all ages and genders and are associated with a high degree of visual morbidity and blindness worldwide^[1] Bacteria are the major factor responsible for these infections, particularly Gram-positive bacteria, such as *Staphylococcus aureus*, which usually cause, among many other complications, conjunctivitis.^[2,3] Despite the constant demand for new treatments and novel antimicrobial agents, antibiotic research, development, and industrialization has decelerated in recent years.^[4] Simultaneously, an increase in bacterial resistant strains, such as methicillin-resistance *S. aureus* (MRSA), has been globally observed, boosting the search for alternative therapeutic options.^[5] One of the possible solutions to this challenge is to consider the re-emerging of old antibiotics, that are no longer first-selection in clinical practice, to treat complicated infection cases.^[6] In this context, in the present study, the antibacterial potential of

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chloramphenicol, which remains an alternative in the fight against multidrug resistant pathogens, is explored.

Chloramphenicol presents a bacteriostatic action against *Staphylococcus* species, being used in the treatment of bacterial eye infections.^[7,8] One of the main disadvantages of this drug, when in an ophthalmic solution, is its low water-solubility, that only allows concentrations up to 0.25% (w/v) (pH = 4.5 to 7.5), which is below the usually recommended dosage.^[9] In addition to its low water-solubility, chloramphenicol presents stability issues in aqueous media because it easily hydrolyzes into glycols. As most of the ophthalmic formulations are generally available as solutions, the improvement of these formulations has focused on increasing the drug content by using co-solvents and cyclodextrin complexes.^[10] Such efforts intend to overcome the limited drug concentration in the conjunctival sac and the decreasing therapeutic response; however, such approach also increases the toxicity associated with the drug administration due to the exposure to higher drug dosages.^[8,11] Therefore, the new generation of ophthalmic formulations must focus on enhancing the drug aqueous solubility, the drug concentrations at the site of infection, and extend its residence time in the ocular environment to achieve a higher therapeutic efficacy while lowering the administered dosage.

In this vein, deep eutectic solvents (DES) have recently arisen as promising options to improve solubility^[12] and stability^[13] of active pharmaceutical ingredients (APIs). Furthermore, they can be advantageously designed for different administration routes. DES are eutectic mixtures that are distinguished by strong interactions occurring between their components, strongly deviating from the ideal thermodynamic solid–liquid phase behaviour in such a degree that it is possible to obtain a liquid mixture at room or human body's temperatures.^[14] These solvents have been studied in the solubilization of antifungal APIs^[15] and applied to improve the chemical stability of β -lactam antibiotics.^[12] Due to their design and application versatility, DES and DES comprising active ingredients have been incorporated in different drug delivery systems, such as ion gels,^[16] hydrogels,^[17,18] eutectogels,^[19] particles,^[20] and nanofibers,^[21] aiming at different administration routes. However, and to the best of our knowledge, their application in ocular drug delivery systems is still unexplored.

In the field of ocular drug delivery, microemulsions and gels have been considered as strategies that intend to overcome the drawbacks associated with conventional eye drops.^[22] Although

DES have also been applied as oil phases in microemulsions,^[23,24] their study towards the development of drug delivery systems has not been pondered. As some of these dispersions still present low viscosity, additional approaches, namely the use of in situ gelling polymers, can be an appealing solution. Gelling polymers allow an easy, safe, and reproducible administration of the system as a liquid drop, while presenting a sol–gel transition in contact with the ocular surface as a result of changes in temperature, pH, or ionic strength.^[25] An example of a thermo-responsive non-ionic in situ gelling polymer that can be used for this purpose is Pluronic F-127 (PF-127), a polymer based on triblock copolymers of poly(ethylene glycol) (PEG) and poly(propylene glycol) (PPG).^[26] The colorless and transparent character of PF-127 solutions, along with its reversible gelation properties occurring at body's temperature, make it optimal for the development of ophthalmic formulations.

Based on the exposed, in the present work, DES aqueous solutions were investigated to improve the solubility of chloramphenicol and then used to prepare thermo-responsive microemulsions. Particularly, we have demonstrated how DES aqueous solutions comprising chloramphenicol can be applied as water phases of w/o/w microemulsions and how these formulations can be designed to present a thermo-responsive character; thus, increasing viscosity upon contact with the ocular environment and enhancing the therapeutic efficacy of the drug toward ocular infections caused by resistant bacteria, namely MRSA. These novel DES-based microemulsions lead to a significant improvement in the drug stability and are capable of effectively eradicating resistant ocular infections in comparison to commercial eye drops, with low cytotoxicity and in a controlled manner.

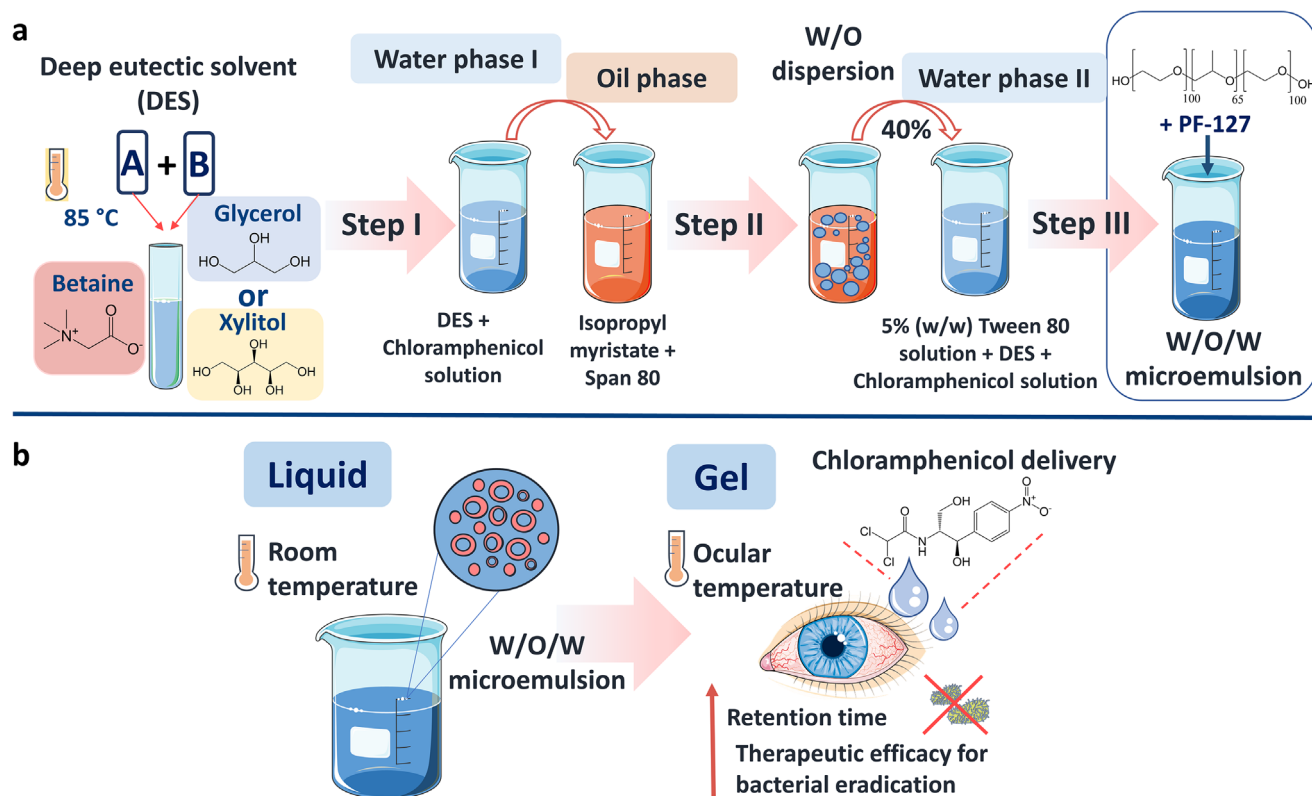
2. Results and Discussion

This work explores an innovative approach to improve antibiotic based ophthalmic formulations by using DES-based microemulsions comprising chloramphenicol with a thermo-responsive character for the treatment of ocular infections. The DES components were selected to ensure biocompatibility for ocular administration. In this context, betaine was selected as a hydrogen-bond acceptor, and glycerol and xylitol were considered as hydrogen-bond donors. All these components present osmoprotectant properties and are suitable to be applied in eye drops.^[27,28] Betaine:glycerol (Bet:gly) and betaine:xylitol (Bet:xyl) DES were prepared in 1:2 and 1:1 molar ratios, respectively, based on previous solubility studies (data not shown). The composition of the DES and the integrity of the single components were confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy—Figures S1 and S2, in the Supporting Information. Aqueous solutions of DES were studied for the solubilization of chloramphenicol, and then used in the preparation of microemulsions (Scheme 1a). The DES-based microemulsions were characterized in terms of pH, droplet size, and viscosity. The stability of these parameters and of the antibiotic in the microemulsions were also assessed. The in vitro cytotoxicity in retinal cells, the in vitro drug release, and the permeation across corneal tissue were conducted to evaluate the effect of the thermo-responsive character of these microemulsions in the overall drug delivery and its influence on the therapeutic action of the antibiotic. Last, the antimicrobial efficacy was evaluated against MRSA to assess the potential of these

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Scheme 1. a) Schematic representation of the preparation procedure of the thermo-responsive deep eutectic solvent (DES)-based microemulsions containing chloramphenicol. b) Thermo-responsive behavior of the w/o/w microemulsions: the ophthalmic formulations are liquid at room temperature, increasing the viscosity upon contact with ocular environment and improving the drug retention time and therapeutic efficacy in the treatment of ocular infections. Image made with Servier Medical Art and adapted by the authors according to Servier under the CC-BY 3.0 License (at <https://smart.servier.com/>).

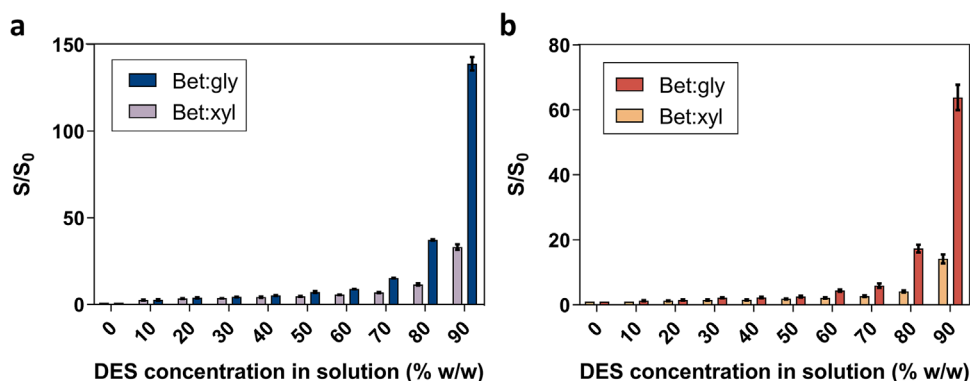


Figure 1. Profile of the solubility enhancement (S/S_0) of chloramphenicol enabled by DES aqueous solutions (% w/w). The effect of the studied DES and their concentrations in the antibiotic solubility are provided at a) 25 °C and b) 32 °C.

formulations to improve the antibiotic capacity to eradicate ocular infections caused by multidrug-resistant bacteria (Scheme 1b).

2.1. Chloramphenicol Solubility in DES Aqueous Solutions

The solubility of chloramphenicol in aqueous solutions of Bet:gly and Bet:xyl, in the range of 0–90% (w/w) of DES, was initially

evaluated. For a better prediction of the solubility, while considering the intended ophthalmic application, chloramphenicol's solubility was studied at room (25 °C) and ocular surface (32 °C) temperatures. **Figure 1** depicts the solubility enhancements achieved by the two types of DES solutions at both temperatures (S/S_0 , where S corresponds to the solubility of chloramphenicol in the DES aqueous solution and S_0 to its solubility in water). The solubility curves for all studied DES

and temperatures are presented in Figure S3a,b, Supporting Information.

The solubility of chloramphenicol was found to be 4.26×10^{-3} mol L⁻¹ and 1.45×10^{-2} mol L⁻¹ in water at room and ocular surface temperatures, respectively. The DES aqueous solutions investigated in this work allowed to considerably enhance the aqueous solubility of chloramphenicol. Both types of DES provide a monotonic increase in the solubility, with the increment of the percentage of DES, in agreement with a co-solvency mechanism.^[29]

The use of 90% (w/w) of Bet:gly DES should be highlighted because it allows a remarkable 140-fold increase in the chloramphenicol solubility at room temperature (Figure 1a) and a 65-fold increase at ocular's surface temperature (Figure 1b) when compared to the antibiotic's water solubility. This corresponds to formulations with 180 to 280 mg mL⁻¹ of chloramphenicol solubilized at room and body's temperature. The solubility of chloramphenicol in the Bet:gly aqueous solution at room temperature (6.20×10^{-1} mol L⁻¹) is even more promising when compared to its solubility in ethanol, 6.05×10^{-1} mol L⁻¹ (data not shown), a common organic solvent used for the solubilization of this drug. The use of 90% w/w of Bet:xyl, even though not as effective as the previous DES solution, also enables to enhance the water solubility of the drug by 30-fold at room temperature (Figure 1a) and by 15-fold at ocular temperature (Figure 1b).

The described solubility enhancements greatly surpass the solubilities achieved with existing commercial strategies, which enable formulations with 4 to 10 mg mL⁻¹ of chloramphenicol.^[30] The high solubilization ability of the DES aqueous solutions proposed herein allows easy manipulation of the DES concentrations and the drug content according to the intended application. Moreover, these DES present higher solubilization capacity than the strategies already commercialized, such as the use of β -cyclodextrin complexes.^[10] Although 90% (w/w) of aqueous DES provided the best result in terms of solubility enhancement, due to the intended application of the DES aqueous solutions as water phases in the preparation of microemulsion, solutions with 70% w/w of DES in water were used in the subsequent experiments with the goal of increasing the water content. At this DES concentration, solubility enhancements ranging from 7- to 15-fold can be obtained using Bet:xyl and Bet:gly aqueous solutions, still enabling the preparation of delivery systems with high drug content.

2.2. Characterization and Stability of the Aqueous DES-Based Microemulsions

Aiming to obtain systems that can be compared with commercial formulations, DES-based microemulsions comprising 4 mg mL⁻¹ of chloramphenicol were prepared and characterized in terms of rheological and physicochemical properties. Microemulsions without the drug were equally prepared and characterized to infer the DES influence on the properties of these systems. The visual aspect of both microemulsions comprising the DES and chloramphenicol is depicted in Figure 2a and their thermo-responsive behavior portrayed in Figure 2b. For this purpose, the viscosity, pH, and particle size of the microemulsions, stored at

4 °C and protected from light to avoid degradation, were evaluated in the day of preparation (day 0) and after 90 days of storage.

Tear fluid has extremely low buffering ability because pH fluctuations depend mostly on the opening time of the eyelids; therefore, the formulations should be in the range of the tears' pH (6.5–7.6) to avoid ocular damage.^[31] Following this notion, the pH of the microemulsions was monitored to evaluate the need for additional excipients to control pH variations. As depicted in Figure 2c, all DES-based microemulsions have suitable pH values for ophthalmic administration, namely 7.62 ± 0.02 for Bet:gly and 7.55 ± 0.02 for Bet:xyl. A slight decrease in pH of the microemulsions comprising chloramphenicol (7.38 ± 0.02 vs 7.26 ± 0.01 , respectively) was verified, especially after 90 days of storage at 4 °C in the dark (Figure 2c). However, these values are still in the ocular tolerable range.^[32]

Regarding the droplet size of the internal oily phase of the multiple w/o/w microemulsion, the DES-based microemulsions presented a droplet diameter of 86.1–112.5 nm after their preparation, as shown in Figure 2d. The droplet size of the Bet:xyl-based microemulsions was slightly lower than that of Bet:gly ones ($\approx 86.1 \pm 1.2$ nm and 101.7 ± 3.8 , respectively). A slight increase in the droplet size of both microemulsions was verified with the addition of chloramphenicol with values up to 110.0–111.0 nm, confirming its incorporation inside the droplets of the internal phase (Figure 2d). After 90 days of storage, the microemulsions presented a homogeneous appearance without phase separation and no significant differences in the droplet size of the internal oily phase. The droplet size influences not only the permeation process but also the stability of the product. Microemulsions in a size range from 20 to 200 nm present good thermodynamic stability and low surface tension, promote mucoadhesion, and are suitable for ocular administration.^[33] Therefore, the DES-based microemulsions developed in this work not only have internal oily phase dimensions appropriate for ocular delivery but also have good size stability under long-term storage conditions.

The DES-based microemulsions were formulated to present a thermo-responsive character, which will translate into an increase in the viscosity upon contact with the ocular media as ocular surface temperature (32 °C) is higher than the room temperature (25 °C). To study the viscosity response of these systems, we tested the influence of the DES concentration and of the responsive polymer, that was PF-127, by dispersing the polymer in DES aqueous solutions (at 10% and 30% w/w) (see Figure S4, Supporting Information for additional information). As dispersions with concentrations of PF-127 above 15% w/w have been reported to present adequate viscosities for ophthalmic application and a sol–gel transition below 35 °C,^[34] we started by testing this polymer concentration. DES aqueous solutions with PF-127 dispersed at 15% w/w were initially evaluated, showing an increased viscosity at ocular temperature; however, the DES concentration affected the two systems differently. At lower DES aqueous concentrations (10% w/w), the viscosity of both DES aqueous solutions with PF-127 was similar. Nevertheless, while the increase in Bet:gly concentration (up to 30% w/w) led to an increase in the viscosity, the increase in Bet:xyl concentration (up to 30% w/w) led to a decrease in the viscosity of the system (as observed in Figure S4a, Supporting Information). On the other hand, the viscosity of both DES aqueous solutions with PF-127 becomes comparable when chloramphenicol is solubilized in

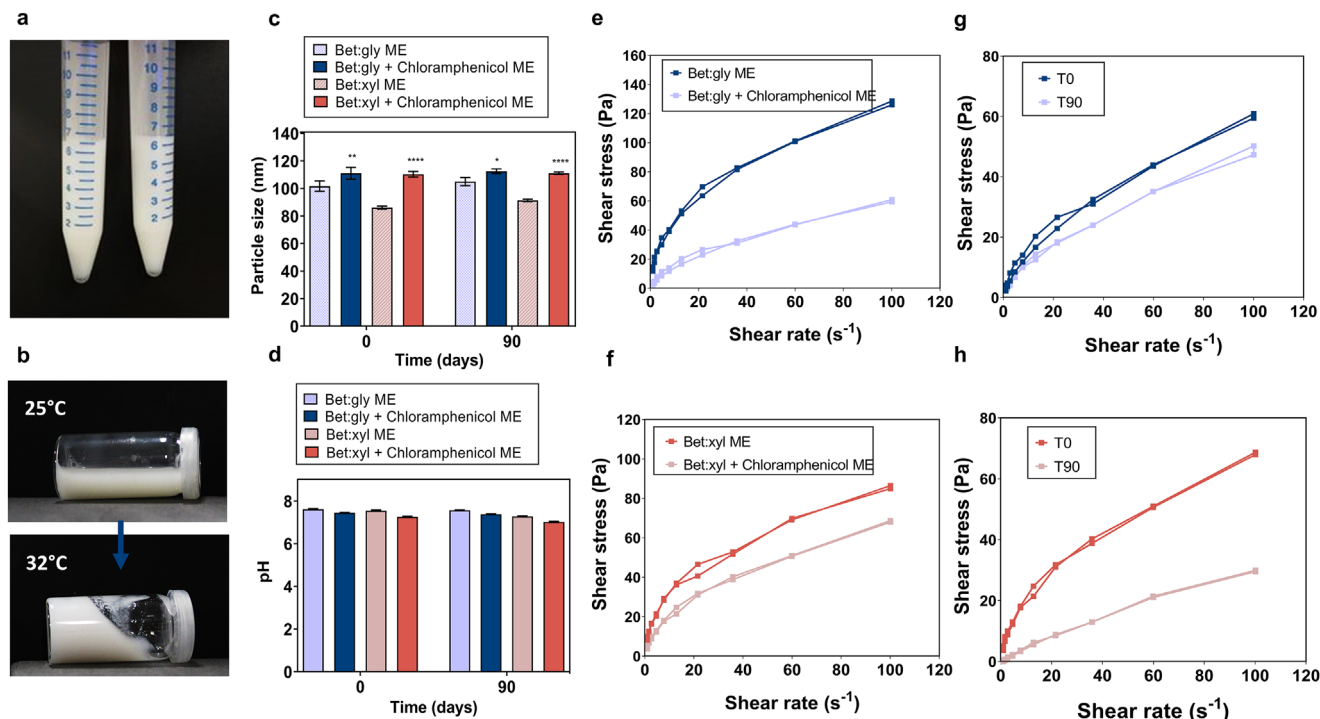


Figure 2. a) Photographs of the Bet:gly and Bet:xyl based microemulsions comprising chloramphenicol at the day of their preparation, b) effect of temperature in the viscosity (visual appearance) of a microemulsion, c) droplet size, and d) pH of DES-based microemulsions (ME) with and without chloramphenicol upon 90 days of storage at 4 °C. * $p < 0.0115$, ** $p < 0.0020$, and **** $p < 0.0001$ particle size of DES-based ME comprising the antibiotic in comparison to the respective DES-based MEs without the drug. Data points represent mean \pm standard deviation ($n = 3$). Rheograms of the DES-based microemulsions before and after the incorporation of chloramphenicol e) Bet:gly-based systems and f) Bet:xyl-based systems and g,h) their respective stability when comprising the antibiotic after 90 days of storage at 4 °C.

the media at similar concentrations to those used in eye drops, namely at 4% w/w, independently of the DES concentration (Figure S4b, Supporting Information).

Based on the described results, the 10% w/w of DES aqueous solution was selected for further studies and used in each water phase of w/o/w microemulsions. The viscosity of these DES-based microemulsions with PF-127, displayed similar tendencies to those observed for the respective DES aqueous solutions with PF-127, as illustrated in Figure 2e,f. This was particularly assessed by determination of an ascending and descending curve of the variation on the shear rate to conclude about the behavior of these fluids. For both DES-based microemulsions, viscosity decreases with the increase of the shear rate (consistent with the pseudoplastic or shear thinning behavior), which is in accordance with the behavior of natural tears, also categorized as a non-Newtonian, shear thinning fluid.^[35] Interestingly, these microemulsion formulations enhance the viscosity, even at lower concentrations of PF-127 (5% w/w), which is comparable to the results reported for other microemulsions with higher polymer concentration.^[36]

Similarly to the DES aqueous solutions with PF-127 (Figure S4, Supporting Information), the corresponding Bet:gly-based microemulsions have also higher viscosity values than the ones with Bet:xyl (Figure 2e,f, respectively). This can be attributed to the effect of the co-solvents on the gelation ability of PF-127. Glycerol has shown to promote the formation of strong hydrogen bonds with poloxamers such as PF-127, causing a slight decrease

in the gelation temperature; and therefore, stronger gels.^[37] Conversely, the use of alcohols, such as ethanol or propylene glycol, has proven to increase the sol–gel transition temperature, decreasing the viscosity of the resulting systems, which might be similar to the behavior observed for xylitol.^[38]

After the incorporation of the antibiotic in the DES-based microemulsions, the viscosity decreases for both formulations. In fact, the incorporation of drugs in PF-127 formulations or the inclusion of several additives has been stated to greatly modify the sol–gel transition boundaries of this polymer^[39,40] because it might increase the gelation temperature and decrease the adhesive forces. Other active ingredients, such as diclofenac, have shown the ability to reduce the gel strength of PF-127 formulations.^[41]

The stability of the DES-based microemulsions comprising chloramphenicol over 90 days stored at 4 °C was also studied (Figure 2g,h). After 90 days, the Bet:gly-based microemulsions comprising the antibiotic presented only a slight decrease in the shear stress, and thereby, in the systems' viscosity ($\approx 10\%$) (Figure 2g), maintaining the characteristics suitable for ocular application. Bet:xyl-based microemulsions comprising chloramphenicol present about a twofold reduction in their overall viscosity (Figure 2h). However, no visual changes or evidence of flocculation in both microemulsions were observed during the storage period.

Based on all the discussed results, generally, both DES-based microemulsions present a long-term shelf-life stability for the

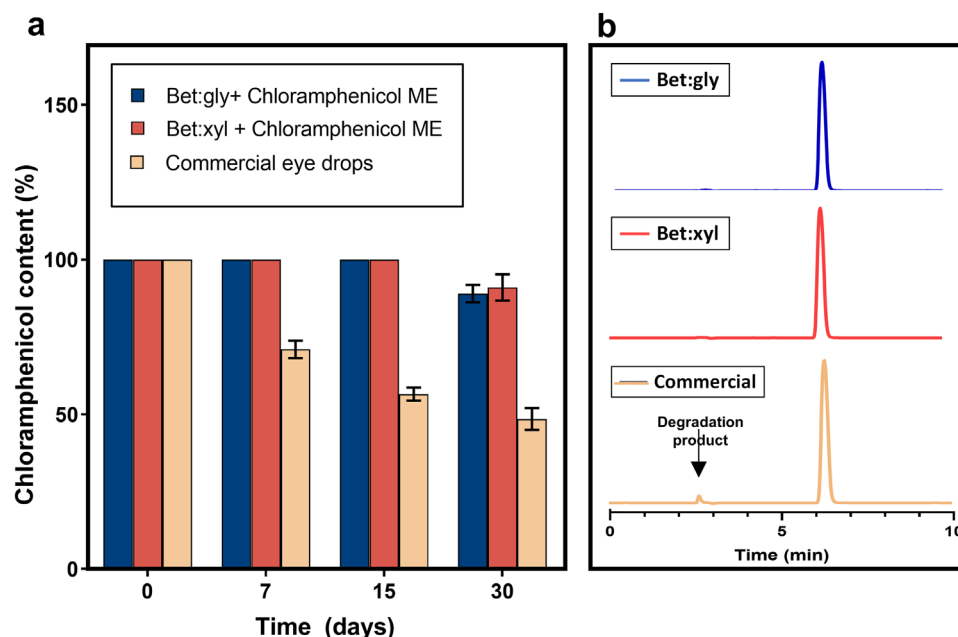


Figure 3. a) Chloramphenicol content of each DES-based microemulsion (ME) and in commercial eye drops, quantified regularly over 1 month period. Each value is the respective mean \pm standard deviation. b) HPLC chromatograms of chloramphenicol in each formulation 30 days (T_{30}) after preparation.

evaluated parameters. Furthermore, the advantageous use of the selected DES should be highlighted for this purpose because it avoids the addition of further excipients to control changes in these parameters in pharmaceutical formulations.

2.3. Drug Stability in DES-Based Microemulsions

The stability of chloramphenicol in the DES-based microemulsions was assessed by measuring the drug concentration by HPLC-DAD. This parameter was evaluated over a month, to allow its comparison with a commercial formulation of eye drops containing chloramphenicol, whose shelf-life was up to 28 days after opening. For this purpose, the commercial eye drops were stored following the manufacturer's recommendations ($T \leq 25$ °C in the dark) and the DES-based microemulsions followed the storage conditions previously screened (4 °C in the dark). **Figure 3a** shows the chloramphenicol content at each time point relative to the initial drug concentration. For both DES-based microemulsions, for at least 15 days of storage, the amount of the drug was kept constant. At the end of 1 month, the drug content was found to be 89% of the initial value for the Bet:gly-based microemulsion and 91% for the Bet:xyl-based one. For the commercial formulation, the decrease in the drug concentration was more evident. Actually, in the commercial formulation, after 7 days, only 71% of chloramphenicol was detected, and, after 1 month, the drug concentration decreased to 48%, highlighting the low stability of the antibiotic in the commercial aqueous eye drops.^[42,9] In fact, a peak of a degradation product was observed in the HPLC chromatogram of the commercial eye drops after 30 days of storage (**Figure 3b**). This has been already described for other commercial eye drops^[42] and happens due to the common degradation of chloramphenicol by hydrolysis

of the amide group in aqueous media.^[43] Remarkably, this is not verified in the DES-based microemulsions developed in this work, which not only allows us to significantly enhance the drug stability but also prevents the formation of hydrolysis products after storage, which is certainly due to the entrapping of chloramphenicol in the microemulsion droplet interface. The DES-based microemulsions have also shown the possibility to increase the stability over other microemulsions reported in the literature to this purpose,^[42] while offering a higher shelf-life for the drug over commercial formulations.

2.4. Cytotoxicity of the DES-Based Formulations

The *in vitro* cytotoxicity of chloramphenicol was studied in aqueous solution and in the DES-based microemulsions towards human adult retinal pigment epithelial cells (ARPE-19) by exposure for 24 h. Each formulation comprising chloramphenicol was evaluated in concentrations ranging from 12.5 to 100.0 $\mu\text{g mL}^{-1}$. For comparison purposes, the commercial eye drops were also tested in the same range of concentrations. These concentrations were selected considering the limited amount of drug that crosses the protective mechanisms of the eye and that becomes in contact with the cells.^[44] Initially, the effect of chloramphenicol in aqueous solution in ARPE-19 cells viability was appraised. This antibiotic seems to present low cytotoxicity in the studied range of concentrations because all cell viability was above 90% (**Figure 4a**). When formulated as eye drops, the effect of chloramphenicol and the respective excipients demonstrate dosage-dependent toxicity, which decreases in the studied range of concentrations up to 80% for a drug dosage of 100.0 $\mu\text{g mL}^{-1}$ (**Figure 4b**). Similar values have been found for the cell viability of human keratinocytes cells, when exposed to diluted

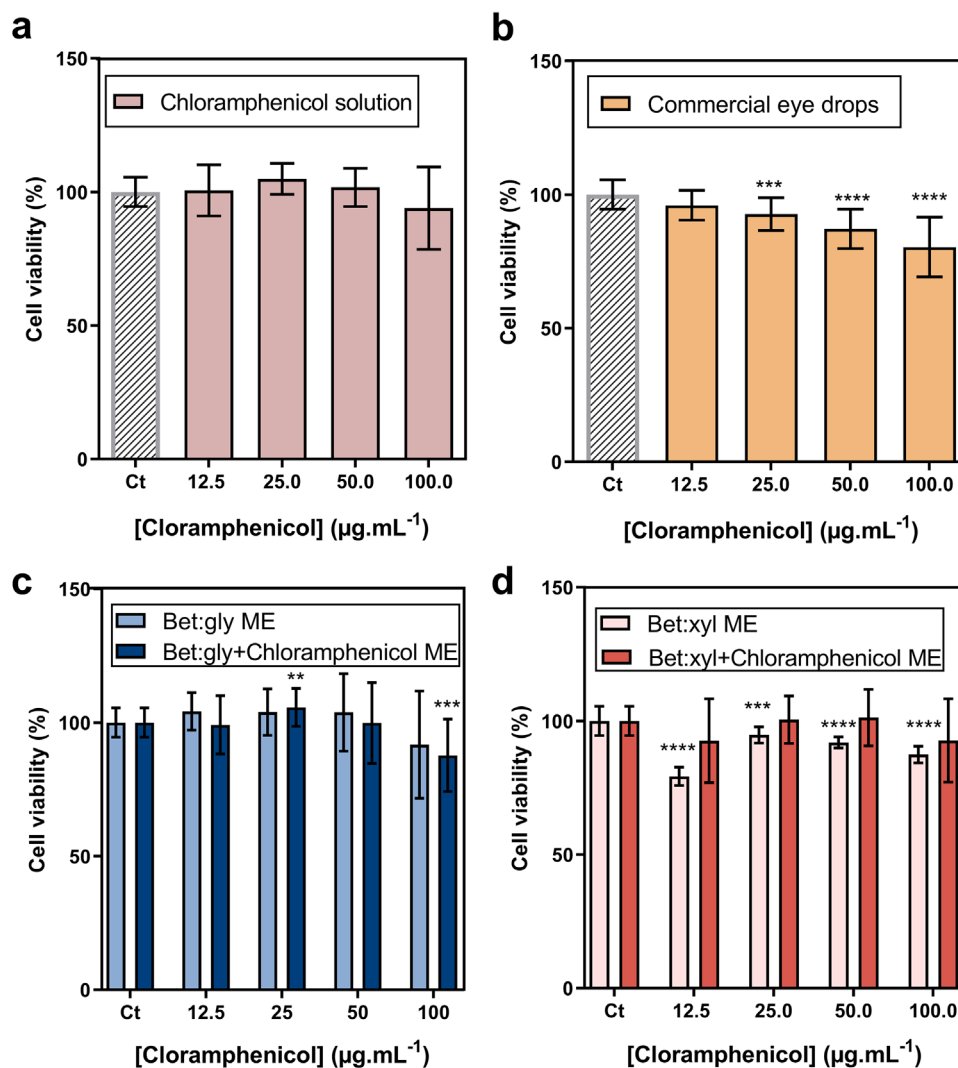


Figure 4. Cytotoxicity values after 24 h of exposure versus control ARPE-19 cells (Ct) determined for a) chloramphenicol in aqueous solution, b) commercial eye drops, c) Bet:gly microemulsions (ME) with and without chloramphenicol, and d) Bet:xyl MEs with and without chloramphenicol incorporated. Results are expressed as mean \pm standard deviation of four independent experiments. ** $p < 0.0043$, *** $p < 0.0003$, and **** $p < 0.0001$ cell viability in comparison to the control cells.

commercial eye drops in the same range of drug concentrations here explored.^[45] Therefore, the possible decrease in cell viability might be attributed to the formulation excipients commonly found in commercialized eye drops.

Following this, the cytotoxicity of both microemulsions, with and without the drug, was investigated. The DES-based microemulsions without the antibiotic exhibit non-cytotoxicity toward ARPE-19 cells with cell viabilities always above 85% (Figure 4c,d). The incorporation of the antibiotic in both DES-based microemulsions does not significantly impact the cytotoxicity of the resultant formulations (Figure 4c,d). These exhibit similar profiles with comparable cell viability values within the studied range of concentrations. At higher concentrations, namely 100 $\mu\text{g mL}^{-1}$, the cell viability decreases to 88% for the Bet:gly-based microemulsion when containing chloramphenicol (Figure 4c) and to 91% for the Bet:xyl-based containing the same drug (Figure 4d). Such values are comparable to those observed for aqueous

solution containing the same chloramphenicol concentration (100 $\mu\text{g mL}^{-1}$). Such findings highlight that safe chloramphenicol concentrations were used in the present work and that the incorporation of this drug in the DES-based microemulsions does not create a more toxic ophthalmic formulation. Overall, the two developed DES-based microemulsions present comparable cytotoxicity values to already commercialized eye drops. Therefore, for the next experiments, the highest chloramphenicol concentration tested (100 $\mu\text{g mL}^{-1}$) was considered.

2.5. In Vitro and Ex Vivo Drug Permeation Studies

The in vitro release profile of chloramphenicol from both DES-based microemulsions was investigated in PBS (pH 7.4) at 32 $^{\circ}\text{C}$ for 3 h, to simulate ocular physiological conditions (Figure 5a). Contrary to commercial eye drops, in which the drug release into

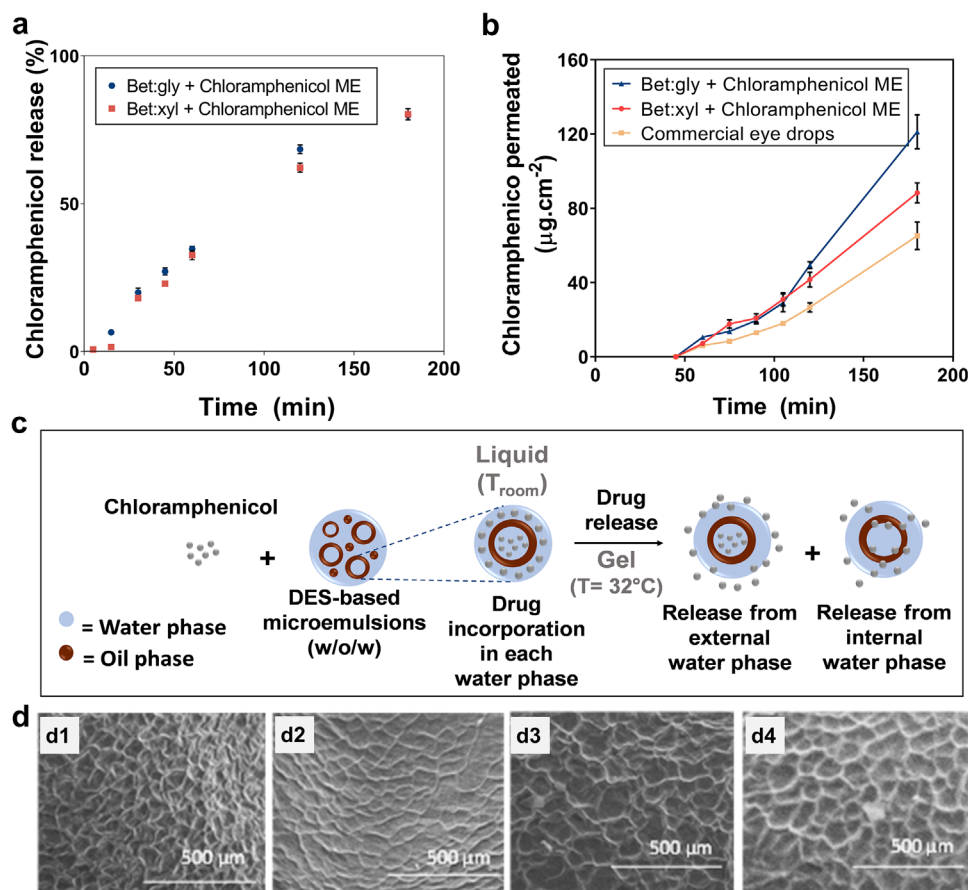


Figure 5. a) In vitro release profile of chloramphenicol from Bet:gly and Bet:xyl-based microemulsions (ME) over 3 h. b) Cumulative amount of chloramphenicol permeated across corneal tissue during 3 h for chloramphenicol from Bet:gly and Bet:xyl-based MEs and for commercial eye drops. All profile data represented as mean \pm standard deviation of three independent experiments. $*p < 0.03$, $***p < 0.003$, and $****p < 0.0001$ amount of chloramphenicol permeated from each ME in comparison to the commercial eye drops. c) Schematic representation of chloramphenicol loading into the water phases of both DES-based microemulsions and the two-phase drug release at ocular temperature. d) SEM micrographs of the corneal tissue after 3 h of exposure to d1) Bet:gly and d2) Bet:xyl-based MEs comprising chloramphenicol, d3) commercial eye drops, and of d4) control.

the ocular media is instantaneous, and for that reason is not here presented, the results of this study revealed a sustained release of the antibiotic from DES-based microemulsions. Furthermore, these microemulsions present similar release profiles over the assay period. After 1 h, 34.7% and 32.6% of chloramphenicol were released from Bet:gly and Bet:xyl-based microemulsions, respectively (Figure 5a). At the end of the total assay period (3 h), 79.8% of the drug was released from the Bet:gly-based microemulsion and 80.2% from the Bet:xyl-based one. These results are quite relevant because due to the low solubility of chloramphenicol, its release into aqueous media can be as low as 37%, even after 7 days, without the application of any solubilization strategy.^[46]

Studies have reported the use of bi-layered polymer-based films,^[47] nanoparticles,^[46] and other microemulsions^[48] to offer an improvement in drug delivery over commercial eye drops. Although these can achieve high amounts of drug released, most still present a slow-release rate, which delays the therapeutic onset of the drug. The DES-based microemulsions herein prepared allow a sustained release of the drug, achieving a high drug content release within only 3 h. In fact, after only 5 min, both DES-

based microemulsions could deliver chloramphenicol concentrations far above the MIC for *Staphylococcus* species (>30% the MIC value for *S. aureus*^[49]).

To infer the impact of the formulations on the permeation of chloramphenicol, ex vivo studies through corneal tissue were conducted over 3 h at 32°C , using Franz diffusion cells. Figure 5b presents the results obtained for each DES-based microemulsion and for the commercial formulation. As observed in the in vitro release assays, the permeation of chloramphenicol across the corneal tissue follows a sustained permeation pattern. This sustained drug delivery is attributed to the fact that DES-based microemulsions were designed to comprise chloramphenicol in both external and internal DES:water phases, providing an immediate therapeutic effect from the external phase of the microemulsion and a more sustained release of the drug from the internal phase, as depicted in Figure 5c. These release abilities associated with an increase in the viscosity under temperatures closer to the ocular environment result in a successful continuous delivery of chloramphenicol and, thereby, a sustained release of the drug.

Both DES-based microemulsions present similar permeation profiles within the first 120 min, with permeated amounts

of chloramphenicol up to $\approx 49.4 \mu\text{g}\cdot\text{cm}^{-2}$. After this time, the Bet:gly-based microemulsion enabled a higher permeation of the antibiotic across the corneal tissue than the Bet:xyl-based one. Such an effect can be expected because glycerol has been used as a permeation enhancer to improve the penetration of active ingredients across biological membranes.^[50] The two DES-based microemulsions promote the permeation of higher amounts of the drug across the corneal membrane than commercial eye drops, during the assay period. This can also be anticipated as DES are known to facilitate the permeation of solubilized active ingredients across membranes without negatively affecting the cells.^[51,52] These values reflect an amount of $44.0\text{--}46.1 \mu\text{g}\cdot\text{mL}^{-1}$ of chloramphenicol permeated 120 min after application, which is in accordance with previous values reported for chloramphenicol permeation from ointment formulations.^[53] As these formulations allow reducing the initial drug concentration while distributing similar amounts to commercial eye drops through the corneal tissue with comparable safety, it is possible to model the amount of drug to be incorporated into DES-based microemulsions.

We have further extended the assay period for 180 min to understand the influence of the developed formulations on the corneal tissue during longer periods of exposure. Interestingly, the microemulsions, and more particularly the Bet:xyl-based microemulsion (Figure 5d2), did not induce higher structural changes than the eye drops (Figure 5d3) for the same period of exposure (180 min), demonstrating that their effect on the morphology of the corneal tissue is comparable to the commercial formulation under similar conditions. The most sustained drug release and permeation in the DES-based microemulsions are, in this sense, advantageous as these formulations allow a higher drug accumulation in contact with the cornea, which can locally prolong the therapeutic effect comparatively to the commercialized eye drops formulations.

2.6. Antimicrobial Efficacy

Based on the chloramphenicol's action and the sustained release ability of the DES-based microemulsions, their antimicrobial activity in the treatment of multidrug-resistant infections was finally evaluated. For this purpose, the efficacy of these formulations was studied in the eradication of Gram-positive bacteria, namely the MRSA. The commercial chloramphenicol eye drops were also used in the same drug concentration for comparison purposes.

First, the determination of the antimicrobial susceptibility to the DES-based microemulsions, with and without chloramphenicol and eye drops, was performed (Figure 6a). It is possible to verify that the DES-based microemulsions without the drug do not present antimicrobial activity. The composition of the DES-based microemulsions differs only in the hydrogen-bond donor. Glycerol does not present relevant antimicrobial activity but is used in commercial antibiotic formulations and xylitol presents only a slight ability to interfere in biofilm formation by inhibiting the bacterial adherence of *S. aureus*.^[54] Overall, the bacteria seem to be susceptible to chloramphenicol in all the tested formulations comprising the drug, according to EUCAST classifications.^[49] The Bet:gly and Bet:xyl-based microemulsions containing chlo-

ramphenicol exhibited growth inhibition zones of 28 ± 2 mm and 27.5 ± 2 mm, respectively, whereas eye drops commercialized formulation showed one with 25 ± 2 mm (Figure 6a).

When considering the treatment of infections caused by resistant bacteria to systemic antibiotics, topical treatment can be a more effective alternative due to the higher local concentrations. Generally, two drops of a chloramphenicol formulation are prescribed every 2 to 3 h in the first 48 h, reducing afterward to 4 to 6 h. The drug should be administered for further 48 h after the eye appears to be normal. Based on the clinical prescription of this antibiotic, we have simulated a severe eye infection caused by a resistant bacterium (MRSA), treating it with specific drug dosages ($100 \mu\text{g}\cdot\text{mL}^{-1}$) initially administered at each 2 h for 48 h, and then each 4 h up to 5 days to guarantee complete bacterial eradication (results depicted in Figure 6b). We monitored the drug action of both DES-based microemulsions comprising chloramphenicol and the respective commercial eye drops in this strain. A bacterium positive control (Ct) containing only the bacterial inoculum in PBS was also carried out, being cultured at the same time points but in the absence of the antibiotic or the DES-based microemulsions.

In the absence of the antibiotic and DES-based microemulsions, the bacterium growth increases in the first 48 h ($1.9 \log_{10}$, $p < 0.0001$), remaining stable during the rest of the assay period at high bacterial concentrations. When the bacterium was exposed to the antibiotic formulations, the decrease in the bacterium growth seemed to be not only time-dependent but also reliant on the number of drug applications, reflecting the growth inhibition capability of the formulations studied. The DES-based microemulsions containing chloramphenicol present a similar profile of eradication of MRSA infection without statistical differences between both microemulsions, being more effective in the eradication of MRSA infections than the commercial eye drops, as presented in Figure 6b. The growth inhibition of the drug is comparable between all the studied formulations up to 24 h after the treatments' start, presenting a $2.4 \log_{10}$ reduction of the bacterium growth ($p < 0.0001$).

However, at the end of the second day of dosage applications, the differences between the DES-based microemulsions and the commercial eye drops start to become obvious. While 4.5 and $4.1 \log_{10}$ reductions in the bacterium growth ($p < 0.0001$) were verified for Bet:gly-based and Bet:xyl-based microemulsions, the eye drops enabled only a $3.4 \log_{10}$ decrease ($p < 0.0001$). After 48 h, the commercial eye drops only allowed a $3.8 \log_{10}$ reduction in the bacterium growth ($p < 0.0001$) (Figure 6b,1), while both DES-based microemulsions were capable of fully eradicating the MRSA bacteria (Figure 6b,c2,c3). In fact, the commercial eye drops took 72 h to eradicate the MRSA bacteria till the detection limit of the methodology, that is, more 24 h than the DES-based microemulsions. Considering that low drug concentrations were tested, it is expected that with the application of one drop of each DES-based microemulsion (4.0 mg mL^{-1}), the drug efficacy would be even better than the eye drops with similar drug concentration, requiring fewer applications to fully eradicate the MRSA infection.

In parallel, we have also evaluated the toxicity of the DES-based microemulsions toward the bacterial cells, performing a cell viability test after exposure to a single chloramphenicol dosage of $100 \mu\text{g mL}^{-1}$ (data shown in Figure S5, Supporting Information).

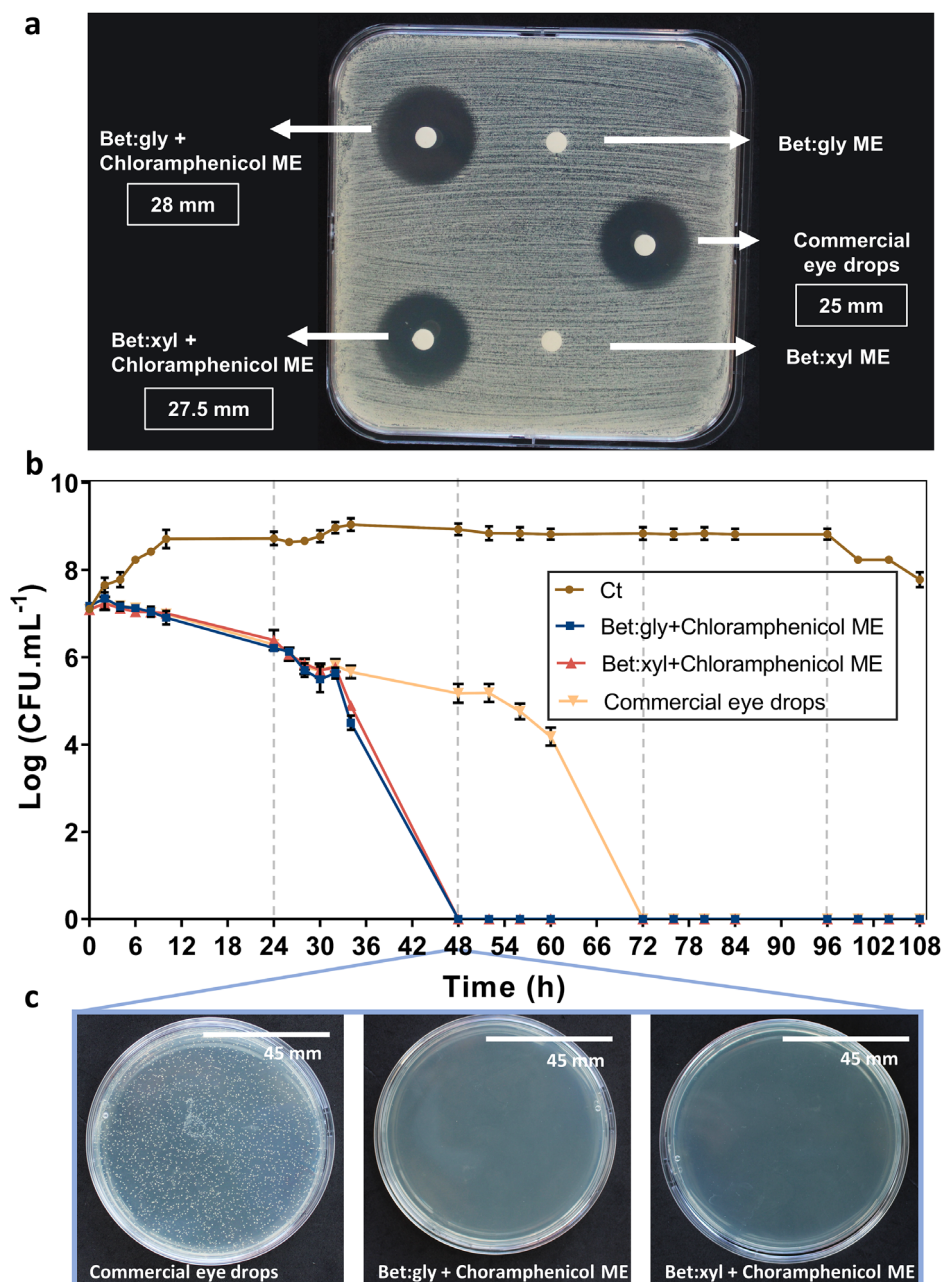


Figure 6. a) Antimicrobial susceptibility of *S. aureus* (MRSA) DSM 25693 to Bet:gly-based ME and Bet:xyl-based ME with and without chloramphenicol and to the commercial eye drops (disks with $100 \mu\text{g}\cdot\text{mL}^{-1}$ of antibiotic). b) Growth inhibition profiles of Bet:gly-based ME and Bet:xyl-based ME and commercial eye drops determined based on the Colony Forming Units (CFU $\cdot\text{mL}^{-1}$) from the samples collected over each time point after administration of an antibiotic dose of $100 \mu\text{g}\cdot\text{mL}^{-1}$ and of the bacterium without being submitted to any formulation (Ct). Dashed lines represent the different days of drug administration. Data are presented as mean \pm standard deviation values of three independent studies for each sample. c) Plate photographs of the colonies formed in agar plates after 48 h of treatment with each formulation.

Such study indicated that the two DES-based microemulsions and the commercial eye drops do not present toxicity towards MRSA, and that the effective bacterium eradication by chloramphenicol in the microemulsions is due to an enhanced bactericidal action. Therefore, the behavior observed can be possibly attributed to a synergetic effect of both the DES aqueous solutions comprising chloramphenicol and the microemulsion formulation. The thermo-responsive microemulsions might act

as more effective carriers to deliver the drug into the bacterial cells. In fact, both DES and microemulsions have been reported to enhance the cellular permeability of bacteria, enabling an increase in intracellular concentrations of certain compounds.^[56–58] This effect might explain the improved activity of the DES-based microemulsions because chloramphenicol is more readily available inside the bacterial cell to exert its action, to bind to the bacterial ribosome structure and inhibit protein synthesis.^[7]

3. Conclusion

Bet:gly and Bet:xyl-based microemulsions with thermo-responsive character were developed and characterized aiming to improve the therapeutic action of the antibiotic chloramphenicol typically used to treat ocular infections. The use of aqueous solutions of DES proved to be a promising strategy to improve the drug water solubility of the antibiotic up to 140-fold, while avoiding the use of common organic solvents. Furthermore, their incorporation as water-phases in the development of w/o/w microemulsions enabled the design of thermo-responsive microemulsions with a final drug concentration of 4 mg·mL⁻¹. The investigated DES-based microemulsions present pH, droplet size, and viscosity values adequate for ophthalmic administration. The use of DES in these formulations allowed to overcome the use of further preserving excipients, resulting in formulations that are stable over, at least, 3 months. Furthermore, the use of DES improved the gelling properties of the system requiring the use of a lower percentage of the in situ gelling polymer (PF-127) to achieve a higher viscosity at ocular temperature. In addition, when containing chloramphenicol, the DES-based microemulsions improved the preservation of the drug stability comparing to the commercial eye drops.

The microemulsions developed herein are non-cytotoxic to ARPE-19 cells (cell viability >88%), presenting similar cytotoxic values to those achieved for commercial formulations. The incorporation of chloramphenicol in the outer and inner phase of the w/o/w microemulsions and the thermo-responsive character of these systems allowed to obtain a sustained-release of the antibiotic from the Bet:gly- and Bet:xyl-based microemulsions, reaching a drug release of 79.8% and 80.2% within 3 h, respectively. These profiles translate into an ex vivo sustained permeation of the drug through the corneal tissue with higher amounts of permeated drug.

The use of the DES-based microemulsions shortened the treatment period from 72 to 48 h, when compared to a commercial eye drops formulation. These results might translate into a decrease in the chloramphenicol concentration needed for the development of future ophthalmic formulations containing this antibiotic.

In summary, the developed DES-based microemulsions can enhance the efficacy of chloramphenicol ophthalmic application by improving its retention in the ocular mucosa, allowing a higher drug contact with the affected area. This results in an enhancement in the treatment of ocular infections caused by resistant bacteria, such as MRSA. The results here reported pave the way to the use of DES in the development of drug delivery systems with improved performance, deserving to be further investigated toward their recurrent application.

4. Experimental Section

Materials: The DES studied in this work were prepared using betaine anhydrous (98%, Alfa Aesar, Germany), xylitol (≥99%, Acros Organics, Thermo Scientific, New Jersey, USA) and glycerol (≥99%, Sigma-Aldrich, St. Louis, MO, USA). Chloramphenicol (≥98%) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The preparation of the microemulsions required the use of Tween 80 and Pluronic F-127 (≥ 98%), both provided by Sigma-Aldrich (St. Louis, MO, USA), while Span 80 and isopropyl myristate were purchased from Acofarma (Madrid, Spain).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and fluorescein from Thermo Scientific (New Jersey, USA). Phosphate buffered saline solution (PBS, pH 7.4) was acquired from Sigma-Aldrich (St. Louis, MO, USA) in the form of tablets. Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) from Liofilchem (Italy) were used in the antimicrobial studies. All other solvents and reagents were from analytical or high-performance liquid chromatography (HPLC) grades.

DES Composition and Preparation: The DES were prepared by mixing the respective precursors (betaine and xylitol or glycerol) in sealed glass vials with constant heating and stirring, until a homogeneous transparent liquid was formed (at a maximum temperature of 85 °C). DES were prepared at 1:2 and 1:1 molar ratios for betaine:glycerol (Bet:gly) and betaine:xylitol (Bet:xyl), respectively. The mixtures were then kept for 1 h at this maximum temperature and then allowed to return to room temperature.

The DES composition was confirmed by NMR spectroscopy. The ¹H NMR and ¹³C NMR spectra were recorded using a Bruker Avance 300 at 300.13 and 75.47 MHz, respectively. The DES were analyzed in deuterated water and using trimethylsilyl propanoic acid (TMSP) as an internal reference.

Solubility Assays of Chloramphenicol: Chloramphenicol solubility in water and in aqueous solutions of DES was determined by adding the drug in excess to water and to 2.0 g of each DES aqueous solution (0–90% w/w of DES). These mixtures were placed in sealed glass vials with a stirring bar and allowed to equilibrate in a specific aluminum disk with a stirring plate at 900 rpm and at constant temperature (25 °C and 32 °C) during 72 h. After achieving saturation, the samples were removed and then centrifuged. An aliquot of the supernatant was taken and diluted in water. After this, the samples were carefully filtered with a 0.20 μm syringe filter to remove any solid from the liquid phase and subsequently quantified by high-performance liquid chromatography with diode-array detection (HPLC-DAD), on a PROMINENCE model (Shimadzu, Kyoto, Japan), equipped with an analytical Kinetex 5 μm C18 100 Å reversed-phase column (250 × 4.60 mm), from Phenomenex, using similar conditions to those described in the method previously reported.^[57] The wavelength was set at 277 nm.

Preparation of Thermo-Responsive Microemulsions: Two DES-based microemulsions comprising a final concentration of 4 mg·mL⁻¹ of chloramphenicol were prepared by emulsification and ultrasonication technique via a three-step approach. Initially, water phase I of each microemulsion was prepared by solubilization of chloramphenicol in the aqueous solutions of each DES (70% [w/w]). Then, these DES aqueous solutions were dispersed into an oil phase based on isopropyl myristate (75.5% [w/w]) stabilized with Span 80 (12% [w/w]). To guarantee homogeneity and to generate the water-in-oil pre-emulsion, the samples were stirred at high-speed using Ultra-Turrax T25 equipment (Janke & Kunkel IKA Labortechnik, Staufen, Germany) at 7000 rpm during 5 min. Second, 40% of this water-in-oil pre-emulsion was added to the water phase II, an aqueous solution composed of a 2:9 mixture of the aqueous solutions of DES (70% w/w) comprising chloramphenicol and an aqueous solution of Tween 80 (5% (w/w)). For better homogenization of the resulting water-in-oil-in-water (w/o/w) emulsions, stirring using an Ultra-Turrax under the previous conditions was carried out, followed by sonication at 70% amplitude for 10 min using a probe sonicator (Sonics & Materials Inc. Vibra Cell VCX 130 Model CV 18, Newtown, CT). The resultant microemulsions were allowed to equilibrate at room temperature and were then stored at 4 °C. After cooling, the last step consisted in adding PF-127 to each DES-based microemulsion using the cold method and under constant stirring. Upon complete dispersion of the polymer, DES-based microemulsions with PF-127 (5% [w/w]) were obtained and stored at 4 °C. **Table 1** presents the full composition of the DES-based thermo-responsive microemulsions investigated.

pH and Droplet Size: The pH of the different DES-based microemulsions was determined at room temperature using a HI 2550 multiparameter (Hanna Instruments, Woonsocket, Rhode Island, USA). The droplet size of the oil phase of the w/o/w microemulsions was assessed by dynamic light scattering using a Mastersizer 3000 (Malvern Instruments,

Table 1. Composition of each DES-based microemulsion (ME) prepared for a final mass of 25 g of emulsion.

Sample	Water phase I		Oil phase		Water phase II			
	DES aqueous solution [%]	Drug [%]	Span 80 [%]	Isopropyl myristate [%]	Tween 80 [%]	DES aqueous solution [%]	Drug [%]	Water [%]
Bet:gly ME	10.5	—	12.0	77.5	5.0	10.0	—	45.0
Bet:gly + chloramphenicol ME	10.0	0.5	12.0	77.5	5.0	10.0	0.5	45.0
Bet:xyl ME	10.5	—	12.0	77.5	5.0	10.0	—	45.0
Bet:xyl + chloramphenicol ME	10.0	0.5	12.0	77.5	5.0	10.0	0.5	45.0

Malvern, UK). To avoid multiple light scatterings due to high droplet concentration, the microemulsions were diluted with ultrapure water (1:100). Aiming to evaluate the stability of the prepared microemulsions, both parameters were analyzed immediately after preparation and at day 90. All measurements were reported as mean values \pm standard deviations of triplicates for each microemulsion.

Rheological Measurements: Initially, PF-127 was dispersed at 15% w/w in both Bet:gly-based and Bet:xyl-based aqueous solutions (10% and 30% w/w of DES in water). This was carried out at low temperature (4 °C) to facilitate the polymer dispersion. Posteriorly, the viscosity of these DES-based solutions with PF-127 was evaluated at ocular temperature (32 \pm 0.5 °C) using a Thermo Haake VT-550 (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) rotational viscometer equipped with a SV-DIN coaxial cylinder sensor. The rheological analysis was performed with a stabilization time of 900 s and with a variation on the shear rate from 0.1 to 500 s⁻¹ (ascending curve) and from 500 to 0.1 s⁻¹ (descending curve). After selection of the best DES concentration and preparation of each DES-based microemulsion, the viscosity of these formulations was also appraised in the day of preparation and after 90 days using the same conditions described.

Drug Stability: To evaluate the stability of the drug in the novel formulations upon storage, thermo-responsive DES-based microemulsions with 4.0 mg·mL⁻¹ of chloramphenicol were prepared as previously described and kept in the dark at 4 °C for 30 days. A sample of a commercial formulation of the same drug with the same concentration was also stored according to the manufacturer instructions during 30 days. As the shelf-life of the commercial formulation after opening is 28 days, the experiments were performed during the same period to allow the comparison between formulations. An aliquot of each formulation was collected and analyzed by HPLC-DAD according to the previously described protocol, at given time points (7, 15, and 30 days).

In Vitro Cytotoxicity Assay: The cytotoxic effect of the DES-based microemulsions containing chloramphenicol was assessed on human adult retinal pigment epithelial cells (ARPE-19) by the colorimetric MTT assay. The cells were seeded in 96-well plates at a density of 30 000 cells per well in 200 μ L Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) medium (Gibco) supplemented with 10% v/v of fetal bovine serum (FBS; Life Technologies, Carlsbad, California, USA) and antibiotic/antimycotic containing 100 units mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, and 0.25 μ g mL⁻¹ amphotericin B (Sigma). 24 h after plating, cells were exposed to a range of four concentrations, 12.5–100.0 μ g mL⁻¹ of chloramphenicol diluted in sterile PBS. These were then incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. After this, the wells were washed with PBS, and 50 μ L of fresh medium and 10 μ L of MTT solution of 3 mg mL⁻¹ were added to each well. After 4 h of incubation, 150 μ L of isopropanol (with HCl 0.04 M) was added to dissolve the formazan crystals. Cell viability was measured at 570 nm using a microplate reader (Synergy HT from BioTeK Instruments Inc., Winooski, Vermont, EUA) and the percentage of viable cells was calculated as the ratio between the absorbance of treated versus control cells.

In Vitro Drug Release: For the drug release performance, dialysis bags with 5 mL of each microemulsion containing 4 mg mL⁻¹ of chloramphenicol were used. Each bag was completely immersed in 200 mL of PBS as

dissolution medium (pH 7.4) and was maintained under continuous stirring at 150 rpm and a temperature of 32.0 °C \pm 1 °C. An aliquot (1 mL) from each container was collected at specific time points (5, 15, 30, 45, 60, 120, and 180 min). Each sample was diluted 1:1 in running buffer, filtered, and analyzed by HPLC-DAD following the previously described method.^[57] For each release profile, three different samples were tested and, for each time point, the aliquots were measured two times at 277 nm.

Ex Vivo Corneal Permeation Studies: The permeation of chloramphenicol through corneal tissue was performed using static Franz diffusion cells (PermeGear, Inc., Hellertown, Pennsylvania, USA) with a diffusion area of 0.636 cm² and a receptor compartment of 5 mL. To this purpose, fresh porcine corneal epithelium was provided by a local slaughterhouse. Briefly, on the experimental day, corneal tissue was carefully harvested from porcine eye and immersed in PBS. After that, the tissue was cut to appropriate size and clamped between the donor and receptor compartments faced up. A PBS solution was used as receptor media stirred at 600 rpm. The receptor solution was maintained at 37 °C \pm 0.5 °C by a thermostatic water pump; thus, the human eye conditions were mimicked because the temperature at the ocular surface (32 °C) was assured. An aliquot of 500 μ L (with \approx 100 mg mL⁻¹ of chloramphenicol) for each formulation was placed in the donor compartment. Then, 300 μ L of the receptor medium was removed at designated time points (15, 30, 45, 60, 90, 120, 240, 360, and 480 min) and immediately replaced with the same volume of fresh solution. Each collected sample was diluted to 1:3 in acetonitrile, filtered, and analyzed by HPLC-DAD following the former method described.^[57] The release studies were conducted in three independent studies and expressed as average of permeated drug \pm standard error of mean. Permeation profiles were obtained by plotting the cumulative amount of chloramphenicol permeated per surface area against time.

Corneal Morphology and Integrity: The apical surface of the corneas treated with the different formulations was observed by scanning electron microscopy (SEM) to study their morphology and topography. Before the analysis, the samples were dried under vacuum and properly spread on a double-sided carbon tape mounted onto an aluminum stud. SEM micrographs were registered using a tungsten cathode scanning electron microscope JSM 6010LV/6010LA, (Jeol, Tokyo, Japan) Secondary electron mode, an acceleration voltage of 1 kV, a spot size of 30, and a working distance of 10 mm, were selected as the operational conditions.

Bacterial Culture Conditions: The strain of methicillin-resistance *S. aureus* (MRSA) DSM 25693, positive for SE A, C, H, G, and I enterotoxins, was grown on solid medium, Trypticase Soy Agar (TSA), at 37 °C for 24 h and was posteriorly stored at 4 °C. Prior to each assay, the bacterium strain was inoculated in liquid medium, Trypticase Soy Broth (TSB), and grown aerobically at 37 °C under stirring (up to 100 rpm) for 24 h. For each assay, a 300 μ L aliquot of the referred culture was transferred into a new fresh TSB medium (subcultured in 30 mL twice) and grown under constant stirring overnight at 37 °C.

Antimicrobial Efficacy of Chloramphenicol in the DES-Based Microemulsions: The drug efficacy was first evaluated by testing the antimicrobial susceptibility by a modified Kirby–Bauer disk diffusion method. The bacterial suspension in PBS was set for a turbidity of 0.5 on the McFarland scale, prepared by peaking up 1–2 colonies from the pure culture. The

suspension was spread plated using a swab on Mueller–Hinton Agar plate. Disks containing 100 µg mL⁻¹ of the drug from each formulation were used for MRSA evaluation. The DES-based microemulsions without the drug were also assessed as the respective controls to determine its impact on the antimicrobial susceptibility. The agar plates with all samples were incubated at 37 °C for 18–24 h. Following this, the susceptibility of each formulation was determined by measuring the diameter of the inhibition zones and comparing it to the breakpoint established by the EUCAST European Committee on Antimicrobial Susceptibility Testing and according to the Clinical and Laboratory Standards Institute [CLS].^[49,58]

The antimicrobial efficacy was determined based on the continuous exposure of the bacterium to the DES-based microemulsions with chloramphenicol and the respective commercial formulation containing the same drug for comparison purposes. The bacterial culture was grown overnight. After dilution and adjustment to 0.5 MacFarland scale using liquid medium TSB, the bacterial suspensions were distributed equally in 5 mL tubes. Subsequently, a drug dosage of 100 µg mL⁻¹ of each DES-based microemulsion comprising chloramphenicol and the tested commercial eye drops was added to the bacterial suspensions at specific time points. A positive bacterium control (Ct) containing only the bacterial inoculum in TSB and the DES-based microemulsions without the drug were additionally carried out. Time points were selected according to the clinical prescription of the drug for severe skin infections: first applications every 2 h for 48 h, and then every 4 h up to 5 days. Aliquots of each bacterial suspension were taken after each dosage application. Each aliquot was serially diluted in PBS and each sample dilution was pour-plated TSA being posteriorly incubated at 37 °C for 24 h. The growth inhibition ability of chloramphenicol in both DES-based microemulsions and in the commercial eye drops was evaluated by quantifying the number of colonies forming units (CFU) per milliliter (CFU mL⁻¹). Experiments were carried out in duplicate with three replicates for each sample.

Statistical Analysis: The results obtained were expressed as mean ± standard deviation of independent experiments. In the case of cell viabilities, at least four independent studies were conducted, analyzing six different replicas for each measurement. For the permeation profiles, three independent studies were performed using a total of six different corneal samples for each formulation. The statistical analysis of all data was done using a two-way ANOVA, with multiple comparisons. The levels of significance were set at probabilities of ***p* < 0.0043, ****p* < 0.0003, *****p* < 0.0001 cell viability for cell viability and of **p* < 0.03, ***p* < 0.003, ****p* < 0.0001 for the amount of chloramphenicol permeated from each ME, all analyzed with Graphpad Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

antibiotics, chloramphenicol, deep eutectic solvents, drug delivery, microemulsions, thermo-responsive systems

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