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Research paper

Triclosan-loaded poloxamine micelles for enhanced topical antibacterial activity against biofilm

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Abstract

Our research group is interested in the study of different technological approaches to treat hospital biofilm as a means to constrain nosocomial-acquired infections. The present work investigated the effect of the incorporation of the antibacterial agent triclosan (TS) into polymeric micelles of poloxamine T1107 ($M_W = 15$ kDa, 70 wt% PEO). The aggregation phenomenon was primarily investigated by means of Critical Micellar Concentration in a broad range of pH. Then, the effect of the polymer concentration on the micellar size was evaluated by Dynamic Light Scattering. Solubility levels increased up to 4 orders of magnitude. The drug inclusion affected the micellization, resulting in size increase and micellar fusion. This phenomenon was only apparent in TS-saturated systems. TS-loaded aggregates proved to be active *in vitro* against a broad spectrum of bacteria but more importantly, also against two representative clinical pathogens: methicilin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VREF). While the former was sensitive to even very low TS levels attainable in poloxamine-free aqueous media, the later was inhibited only when exposed to higher drug levels affordable exclusively using an inclusion system. These findings indicated the release of the drug from the reservoir. Finally, the activity of a TS-containing 5% poloxamine combination of pH 7.4 was assessed on biofilms of *Staphylococcus epidermidis*. Results showed a significant decrease ($p < 0.001$) in the number of Colony-Formation Units when the biofilm was exposed to the TS/poloxamine as compared to the limited activity of the polymer-free TS control.

Keywords: Poloxamine polymeric micelles; Tetronic 1107; Triclosan water-solubilization; Methicillin-resistant *Staphyloccoccus aureus*; Vancomycin-resistant *Enterococcus faecalis*; Bacterial biofilm

1. Introduction

Bacteria adhere to the surface of medical devices, tissues and working areas of different industries, secreting a hydrated extracellular matrix of polysaccharides and proteins that leads to the formation of a slimy layer known as biofilm [1]. This process comprises two stages: (1) phase one, involving a physical and reversible interaction between the surface and the microorganism and (2) phase two, an irreversible time-dependent adhesion based on chemical and cellular mechanisms [2]. Encased bacteria usually withstand antimicrobial chemotherapy; the minimal inhibitory concentration (MIC) of pathogens embedded in biofilms raises. Biofilms are also associated with common diseases like periodontitis and infective endocarditis [3,4]. The type of bacteria involved in this phenomenon depends on the site of colonization. In the case of biomaterials centered infections (BCI) like infected hip prostheses [5], *Staphylococcus epidermidis* was reported to be a highly recurrent one [6]. In hospitals, the formation of biofilm is associated

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with the spreading of intra-hospital secondary infections. Pathogens involved are, often, antibiotic resistant, affecting most importantly immune-compromised or immune-suppressed patients. Furthermore, the extension of hospitalization periods up to 15 days usually has a strong economical impact on healthcare systems worldwide. Among multiresistant pathogens responsible for nosocomial infections, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VREF) present increasing noteworthy incidence [7]. Bottone et al. described the ineffectiveness of handwashing soaps in the removal of these microorganisms from contaminated fingerprints [8]. On the other hand, they also stressed hand hygiene as the most important infection control measure.

Triclosan (TS) is a synthetic broad spectrum antibacterial agent displaying high chemical stability and persistent activity [9,10]. The drug is used in topical applications, and it was approved in oral care products in 1997 [11]. Friedman and collaborators developed buccal patches for the sustained release of TS that affected the viability of *Streptococcus mutans* in periodontal biofilms [12]. The main hurdle in the development of effective topical formulations is, as in the case of more than 50% of the drugs approved for use, the low solubility of TS in aqueous media [13]. In order to guarantee a suitable drug solubilization in water, nanotechnological strategies like nanoparticle engineering were pursued [14–17]. Another strategy investigated in order to enhance the solubility of TS was the design of complexes with β-cyclodextrins [18–20]. More recently, the group of Alonso developed chitosan-hydropropylcyclodextrin nanocarriers and investigated the hydrosolubilization of TS [21]. Solubility values increased almost 20 times.

Inclusion of hydrophobic molecules within polymeric micelles is one of the most important approaches intended to enhance water-solubilization and bioavailability of poorly water soluble drugs. Polymeric micelles are nanoscopic structures formed by the self assembly of amphiphilic block copolymers in water, above the critical micelle concentration [22]. Micelles comprise an inner hydrophobic domain called core and an outer hydrophilic corona. Due to the hydrophobic nature of the core, these entities particularly suited for the solubilization of water insoluble molecules [23]. The most broadly studied amphiphiles belong to the group of poly(ethylene oxide)–poly(propylene oxide) block copolymers [24,25]. These derivatives display an incomplete micellization that could affect stability upon dilution [26]. However, the fact that they are commercially available in a wide variety of compositions, the proven biocompatibility shown and, more crucially, the approval of several PEO–PPO block copolymers by FDA and EPA as additives in pharmaceutical and cosmetic industries still constitute, from the technological perspective, a very attractive feature [27–29]. Two families are commercially available: (1) the linear PEO–PPO–PEO poloxamers (Pluronic®) and the branched counterparts named poloxamine (Tetronic®). The most extensive work was carried out on micellar systems of poloxamers [25,30]. However, poloxamines display a unique feature worth to be investigated: the presence of two tertiary amine groups in the center of the molecule. This structure confers the molecule a dual behavior: temperature and pH sensitiveness [31]. In addition, amine groups enabled further modification of the molecule [32]. Thus, in the last years the interest for the application of poloxamine has gradually risen.

Modification of surfaces to prevent bacteria adhesion and biofilm formation is the mostly pursued alternative [33]. However, in the case of for example prostheses, these procedures would require additional biomaterial evaluations to assure that the material maintained the original properties and remained totally biocompatible.

Aiming at constraining the spreading of hospital-acquired infections, our group is working on the development of formulations for topical application with higher bactericide activity against biofilm. In this first study, we present the water-solubilization behavior of TS in polymeric micelles of poloxamine T1107, a relatively hydrophilic derivative. Different parameters affecting the aggregation of the amphiphile and, consequently, the solubilization ability were evaluated. The antibacterial activity of TS-containing micelles was investigated *in vitro* primarily on a wide variety of bacteria collections. Afterwards, the activity of TS–poloxamine complexes was tested against two representative clinical pathogens: methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VREF), isolated in local hospitals. Finally, the effect on pre-formed *Staphylococcus epidermidis* biofilms was investigated with promising results.

2. Materials and methods

2.1. Materials

Tetronic T1107 ($M_W = 15{,}000$ Da, 70 wt% PEO, HLB 18–23, a kind gift from BASF, USA), triclosan (TS, Pharmed Medicare PVT. Ltd., India), KH_2PO_4 (Carlo Erba, Italy), KCl (Merck, Germany), NaOH (Merck, Germany) and concentrated HCl (Anedra, Argentina) were used as received. Buffers of pH 2.0, 5.8 and 7.4 were prepared in distilled water according to the USP XXIV edition. Buffer pH 12.0 was prepared using Na_2HPO_4 (Merck, Germany) and the pH adjusted with NaOH 0.2 M.

2.2. Preparation of poloxamine micelles

Poloxamine micellar systems (0.1–10%) were prepared by dissolving the required amount of polymer in different media at 4 °C and equilibrating the system at 23 °C, 24 h prior to use in order to allow the formation of the micelles. Concentrations are expressed in % w/v.

2.3. Measurement of the critical micellar concentration

The critical micellar concentration (CMC) of poloxamine T1107 in the pH range between 2.0 and 12.0 at

23 °C ± 0.5 °C was determined by means of surface tension using the du Nöuy ring method (Fernández Berlusconi y Rocca SRL, Argentina).

2.4. Preparation of TS-containing micelles

Triclosan (in excess, ~50 mg/mL poloxamine solution) was added to T1107 micellar systems (3 mL, 0.1–10% w/v) in caramel glass vials (10 mL) and sealed appropriately with parafilm. Specimens were vigorously shaken (48 h) in a temperature-controlled horizontal shaker at 23 °C (Minitherm-Shaker; Adolf Kuhner AG, Switzerland). Suspensions were filtered through clarifying filters (0.45 μm, cellulose nitrate membrane, Microclar, Argentina) and dried in a vacuum oven at room temperature. Dry samples were re-dissolved in ethanol and the concentration adjusted as required. Drug concentrations were determined by measuring the absorbance in a UV spectrophotometer (282 nm, CARY [1E] UV–Visible Spectrophotometer Varian, USA) at 23 °C using a calibration curve of TS solutions in ethanol covering the range between 0.01 and 0.06 mg/mL (0.001–0.006%, correlation factor was 0.9998–1.0000). Concentrations are expressed in μg/mL or mg/mL. Ethanol was used as blank. Solubility factors (f_s) were calculated according to the equation

$$f_s = S_a/S_{water}$$

where S_a and S_{water} are the apparent solubility of TS in poloxamine micellar systems and the intrinsic solubility in a free-poloxamine aqueous medium, at certain pH. Molar solubilization ratios (MSR) were calculated by ratioing the molar concentration of the drug by the molar concentration of the polymer.

2.5. Measurement of the micellar size by dynamic light scattering (DLS)

The average hydrodynamic radius of drug-free poloxamine aggregates formed in aqueous medium of pH 7.4 was measured in a Zetasizer Nano Series (Malvern Instruments, UK). Drug-containing samples were assayed in a NICOMP 380 ZLS (Particle Sizing Systems Inc., CA, US). Measurements were performed in PMMA disposable cuvettes at 23°C. Samples were filtered by clarifying filters.

2.6. Visualization of drug-containing micelles

TS-loaded poloxamine micelles were studied by Transmission Electron Microscopy (TEM, EM 109T Zeiss Transmission Electron Microscope, Karl Zeiss, Berlin, Germany). Samples were prepared following a previously described technique [34]. Briefly, TS-containing poloxamine systems were placed on grids covered with Formvar film and stained with 2% w/v phosphotungstic acid solution in water. Then, samples were dried in a closed container with silica gel and observed under the microscope.

2.7. Physical stability of drug-containing micelles

In order to study the stability of the TS-containing micelles along the time, specimens previously prepared were stored at RT for different periods of time (at 1, 2 and 3 months) and the TS concentration determined by UV (see above). Samples were worked up as previously described. Results of % of remaining TS (% TS) are expressed as means ± SD ($n = 3$).

2.8. Antibacterial activity of TS/poloxamine complexes

Antibacterial evaluation of the different TS-containing micelles (pH 5.8 and 7.4) was performed on diverse bacteria collections (American Type Cell Collection, ATCC except for a *Salmonella cholerasuiss* obtained from an enterobacteria collection of the Malbrán Hospital, Buenos Aires) and hospital isolated clinical strains. Different bacteria were cultured on Tryptone Soy Agar (TSA, Britannia, Argentina) plates and were incubated at 35 °C for 24 h. Then, microorganisms were removed from the isolation medium, suspended in 0.9% NaCl to a final concentration equivalent to an optical density of 0.6 (at 600 nm) and diluted 1/10 (0.9% NaCl). Suspensions (0.1 mL) were diluted in cold molten TSA (10 mL) containing 2,3,5-triphenyltetrazolium hydrochloride (TTC, 0.007% final concentration, Sigma, USA), plated in sterile Petri dishes and allowed to solidify. Paper discs (6 mm diameter) were embedded in the corresponding preparations, namely, poloxamine-free saturated TS solution in buffer (pH 5.8 or 7.4, control), TS-free poloxamine micellar systems (blank) and TS/poloxamine systems (pH 5.8 or 7.4, samples), incubated (35 °C, 24 h) and the inhibition zone in disc diffusion tests measured. Results of inhibition are expressed in diameter. Inhibition zones of <6 mm indicate no antibacterial activity.

The technique for the evaluation of the antibacterial activity of drug-loaded micelles on *Staphylococcus epidermidis* (SE) biofilms was adapted from a previously described methodology [35]. Sterile glass slides (1 cm^2) were immersed in Tryptein Soy Broth (20 mL, TSB, Britannia, Argentina) and a loopful of a 1 day-culture of the microorganism was inoculated. The system was incubated under static conditions (35 °C, 48 h). Then, the slides were removed from the culture medium and were washed individually (NaCl 0.9%, 3 × 30 mL) in order to remove Planktonic cells. Biofilm formation was confirmed qualitatively using Crystal Violet staining [35]. Briefly, biofilm-containing slides were incubated (15 min) in Crystal Violet solution (0.1%) and washed with distilled water (3 × 30 mL). The stain was dissolved in ethanol and the absorbance measured at 570 nm. Results were compared to slides without biofilm. Viable microorganisms in the biofilm were estimated at every stage from the number of Colony-Formation Units (CFU) by immersing 3 slides in an antagonist diluent solution (10 mL, 0.9% NaCl, 3% Tween 80, 1% gelatin bacteriological grade, 0.1% sodium thiosulfate)

and vortexing the mixture (2 × 1 min.) to release the microorganisms from the biofilm. Bacterial suspensions were appropriately diluted, the dilution (1 mL) was plated in TSA by duplicate and incubated (35 °C, 48 h). In order to investigate the antibacterial effect on the biofilm, TS-saturated 5% poloxamine systems (pH 7.4, 20 mL) were poured into sterile Petri dishes, 6 biofilm-coated slides were immersed in each preparation and incubated at 20 °C for different periods of time (3 slides were counted after 30 min and 3 after 90 min, following the previous procedure). For comparison, a similar procedure was carried out with TS in buffer pH 7.4 (control) and drug-free poloxamine micelles (pH 7.4, blank). Values were compared to the initial number of microorganisms (time zero) and are expressed as Log_{10} of means ± SD ($n = 3$, determined by duplicate). Statistical differences ($p < 0.001$) between biofilms exposed to the test sample, the control and the blank were analyzed using a Tuckey–Turner multiple comparisons test.

3. Results and discussion

3.1. Drug-free poloxamine systems in aqueous media

The present work aimed at exploring the inclusion of TS into polymeric micelles formed by poloxamine T1107 in aqueous medium as a means for the enhanced water-solubility and the antibacterial activity of the drug against MRSA and VREF, two highly prevalent microorganisms in hospital-acquired infections. The preliminary stage comprised the investigation of the polymer aggregation phenomenon. Some solubilization improvement can be attained below the CMC. However, maximization of the solubilizing effect demands the formation of micelles [25]. As described before, poloxamines are pH-sensitive molecules and consequently the aggregation phenomenon is affected by the pH of the medium. In order to characterize the system and define the polymer concentration for the solubilization experiments, the CMC was determined at four different pHs (in the 2.0–12.0 range, Table 1). As expected, findings showed a decrease in CMC values as pH increased: from 0.61% at pH 2.0 to 0.16% at pH 12. Poloxamine pK_a values generally are 3.8–4.0 for the first tertiary amine group and around 7.9–8.0 for the second [31]. Modifications in the size of the blocks of PPO and PEO bounded to the ethylenediamine central molecule do not change these values in a very significant manner [31]. At pH ~2, poloxamine displays a diprotonated form. At pH ~5–7, only one positive charge remains and the percentage of aggregation increases, being even higher at pH >8. When pH < pK_a (in the range 2.0–7.4) repulsion of diprotonated or monoprotonated central blocks resulted in incomplete micellization [36] and higher CMC values (0.49–0.61%). Once the molecule displayed an unprotonated form (pH = 12.0 > pK_a), the CMC decreased pronouncedly to a value of 0.16%.

Table 1
Poloxamine 1107 CMC values at four different pHs

pH	CMC (% w/v)
2.0	0.61
5.8	0.51
7.4	0.49
12.0	0.16

The size of the aggregates and the size distribution at pH 7.4 was performed by means of DLS. Findings of a 0.1% poloxamine system showed the presence small structures of about 6 nm. These small entities could be associated to the presence of unimers (single polymer molecules) [37]. An increase in the polymer concentration from 0.1% (below the CMC) to 3% (above the CMC) led to an increase in their size to about 8 nm. Further polymer concentration increase to 10% resulted in values of ~12 nm, being consistent with the presence of micelles.

3.2. Water-solubilization of triclosan

The evaluation of the ability of poloxamine micelles to water-solubilize TS and the extent of this phenomenon were among the main goals of the study. Two parameters affecting the micellization process and consequently solubilization were investigated: (A) the concentration of the polymer and (B) the pH. A third parameter, the temperature, was kept constant at 23 °C. Accordingly, TS-saturated poloxamine systems (0.1–10%) in 4 different media (pH range 2.0–12.0) were prepared and the apparent solubility (S_a) of TS measured as poloxamine concentration increased (Fig. 1). Since several points overlapped, results for different pH were presented in separate graphs in order to allow a clearer observation. According to the range of poloxamine concentration, three well-differentiated zones were observed: (1) 0.1–0.5%, (2) 0.5–3% and (3) >3%. At very low T1107 concentrations (0.1%, pH 2.0 to 7.4) solubility values were similarly low to those obtained in poloxamine-free medium (data not shown), indicating negligible aggregation levels due to polymer concentrations below the CMC. An increase in poloxamine concentration to 0.5% resulted in an increase in the solubility: S_a values increased from ~2 μg/mL in poloxamine-free media to 0.62, 0.73 and 0.75 mg/mL for pHs 2.0, 5.8 and 7.4, respectively. At pH 12 (pH > pK_a), a different behavior was expected. TS contains a phenol functional group in its structure. In alkaline medium (pH >8), this moiety ionizes, constituting an additional parameter of noteworthy consideration. This fact was supported by the relatively high solubility showed by TS in a poloxamine-free solution of pH 12.0 (1.4 mg/mL) that contrasted with the very low extent in lower pH systems (2 μg/mL). Thus, even though a final value around 1.8 mg/mL was attained in a 0.5% poloxamine solution of pH 12.0, this solubility level indicated a moderated increase of only 0.4 mg/mL from the basal level (poloxamine-free system). This improvement represented a lower extent than the observed in a more acidic media. The

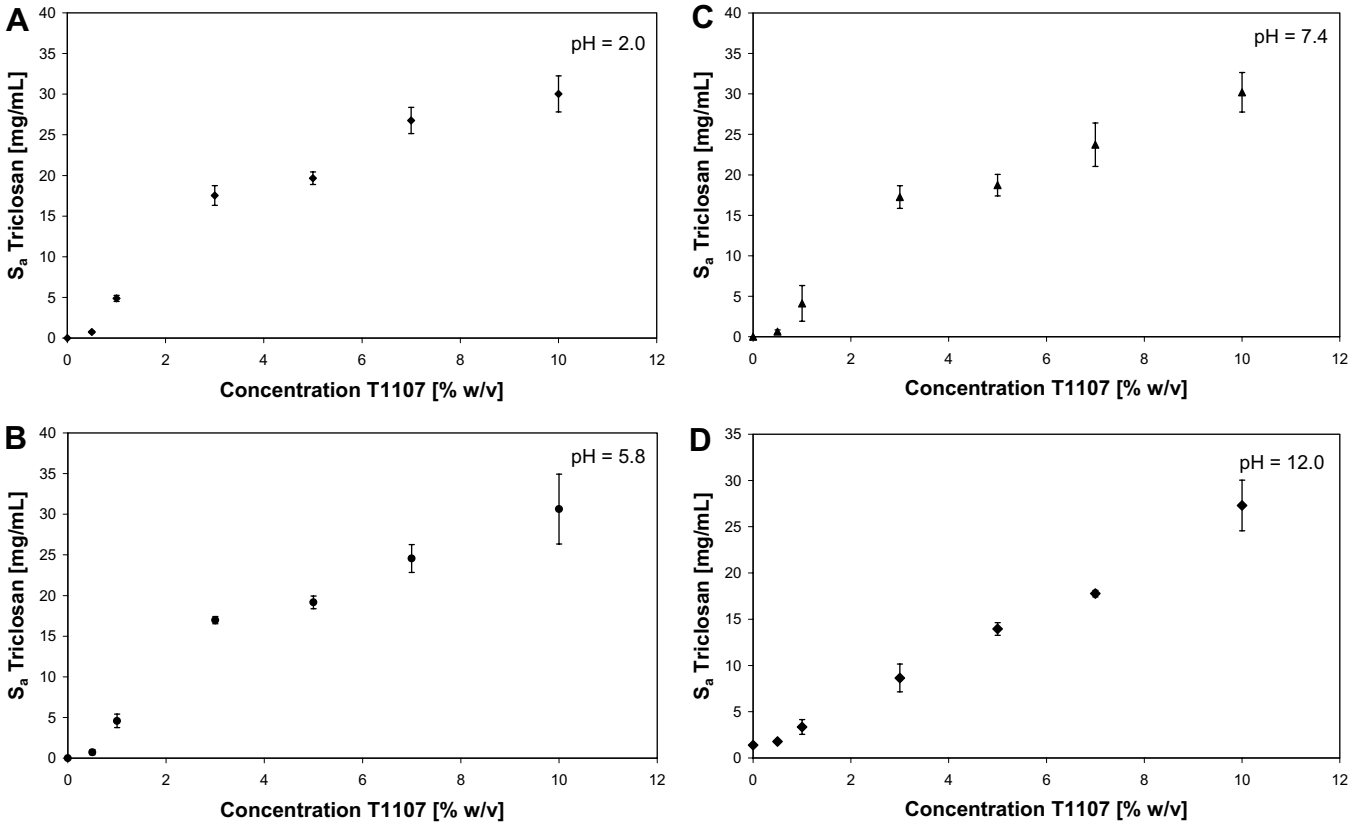

Fig. 1. S_a of TS versus poloxamine concentration at four different pHs. (A) pH 2, (B) pH 5.8, (C) pH 7.4 and (D) pH 12.

increase in solubility for 0.5% poloxamine systems for pHs between 2.0 and 7.4 was in agreement with an incipient aggregation around that concentration, as indicated by the CMC values presented before (Table 1). The solubility profile observed in this range (0.1–0.5%) and the slight slope supported the fact that solubilization improvement fundamentally relied on the solubilizing effect of the unimers available below the CMC rather than on micellization [38]. Doubling the poloxamine concentration to 1% resulted in a solubility increase from 0.62 to 4.1 mg/mL, at pH 7.4. These findings were in accordance with the presence of micelles (poloxamine concentration > CMC ~0.5%) and molecules were able to accommodate within the hydrophobic micellar core. Then, an additional increase in the concentration of the polymer to 3% led to apparent solubility values around 17 mg/mL (pH 7.4). Finally, for T1107 concentrations >3%, the solubilizing effect became less pronounced (i.e., a 10% poloxamine system rendered a S_a ~30 mg/mL) as expressed by the lower slope in this concentration range. The more pronounced linear curve between 1 and 3% poloxamine concentration would suggest that an increase in poloxamine concentration probably resulted in the formation of additional micelles (that solubilized more TS) accompanied by no relevant changes in the micellar size [39]. Then, further increases in the concentration of the amphiphile (>3%) only resulted in the incorporation of more polymer molecules to the existing aggregates with the consequent increase in the micellar size and resulting in a more limited solubility improvement [41]. A similar trend was found for the other pHs. This was supported by data presented separately (see below *Effect of TS on the aggregation of poloxamine*) where an increase in poloxamine concentration between 3 and 10% in TS-saturated systems resulted in larger aggregates. As previously described, poloxamine displays a pH-dependent aggregation behavior that will expectedly influence the water-solubilization of TS [31]. Accordingly, the analysis of the water-solubility performance of the drug under a broad range of pH was of interest. Fig. 2 represents S_a data for systems in the 1–10% poloxamine range, though, in this case versus the pH. Findings showed similar S_a levels in the 2.0–7.4 pH range, though the trend indicated slightly higher extents at a lower pH. For example, S_a values for 7% poloxamine systems were 26.8 and 23.7 mg/mL at pH 2.0 and 7.4, respectively. A relatively limited number of works reporting on drug solubilization properties of poloxamine derivatives at different pHs were previously published [34,36,40,41]. These works agreed that the higher the pH, the higher the solubilization extent (due to a more complete micellization) [31,34,37,42]. However, it should be stressed that as opposed to the present work, drugs used in these preceding studies were pH-insensitive. The present work investigated a more complex system where both polymer and drug were ionizable. Our findings indicated a higher drug/core affinity at lower pH, as expressed by the slightly higher solubility levels attained

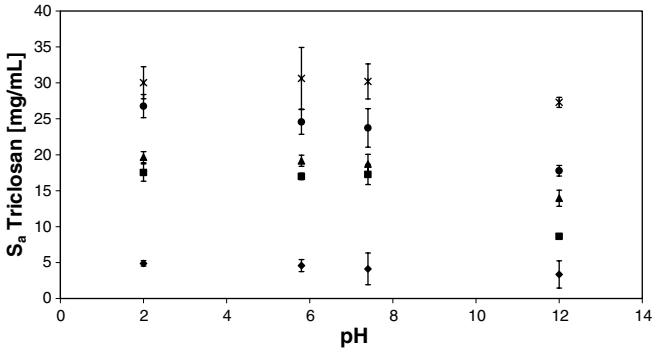

Fig. 2. S_a values of four different poloxamine solutions represented versus the pH. 1% (diamonds), 3% (squares), 5% (triangles), 7% (circles) and 10% (crosses).

at pH 2 and they contrasted with previous investigations. At pH 12, a moderated decrease in S_a was observed. In the TS–poloxamine system depicted herein, two extreme situations could be predicted. At very low pH, TS displays an aromatic –OH moiety that would favor the formation of H bonds with the –O– group of the polyether chains. Contrary to this, at higher pH TS becomes ionized through the phenol group (phenoxy), curtailing the formation of such bonds and constraining solubilization due to a lower affinity. These results were in agreement with a previous work by Mahugo-Santana et al. that investigated the extraction of chlorophenol derivatives using surfactant solutions [43]. In those investigations the ionization of the aromatic –OH group at higher pH led to a lower affinity between phenol derivatives and the micellar core (the extractive phase) and decreased the extraction levels attained. The substantial improvement in the aqueous solubility of TS can be further appreciated in Table 2, where f_s values are presented. For example, a 10% poloxamine solution of pH 2.0, increased the solubility more than 15,000 times. As indicated above, the lower the pH, the higher the solubility enhancement. Slight deviations from this trend were observed in some cases and could be assigned to experimental error. Due to ionization of the phenol group, triclosan solubility in poloxamine-free PBS of pH 12 was dramatically higher than at lower pH (about 700 times). Since f_s values are ratios of S_a and S_{water}, and the basal solubility in polymer-free medium was relatively high (1.8 mg/mL) at the highest pH, a pronounced decrease (750 times) in f_s was apparent: from about 15,000 to about 20. However, the absolute values of S_a remained very close in the whole range of pH investigated. It should be remarked that previous reports indicated that micellization of poloxamine at pH 2.0 is negligible [42]. However, solubilization could be also enhanced by a polymer in a non-micellar state [40]. Accordingly, a priori, the fact that different mechanisms were involved in the solubilization process cannot be underestimated. In our specific drug-polymer system, the very similar solubility extents for identical poloxamine concentrations in the broad pH range investigated suggested that the same mechanism was involved. CMC measurements indicated that micelles were present even at such low pHs. Since we are dedicating efforts to the development of formulations for topical application with potential activity in the treatment of biofilm, further investigations at extreme pHs (2.0 and 12.0) were out of the scope of the work. In advance, studies focused on systems with a more biocompatible pH (in the 5.8–7.4 range).

Table 2
Triclosan solubility factor (f_s) in poloxamine systems between 0.5 and 10% at four different pHs

pH	Triclosan solubility factor (f_s)					
	0.5%	1.0%	3.0%	5.0%	7.0%	10.0%
2.0	374	2443	8765	9830	13,380	15,010
5.8	367	2292	8490	9580	12,280	15,315
7.4	311	2060	8625	9360	11,865	15,100
12.0	1.3	2.4	6.2	10.0	12.8	19.6

3.3. Effect of TS on the aggregation of poloxamine

The size of the drug-loaded aggregates is a critical parameter in their eventual interaction with cells [44]. Thus, investigating the effect of the solubilized drug on the aggregation pattern of the polymer was a fundamental task. The size of the TS-loaded aggregates in saturated specimens at pH 7.4 was measured by DLS. A priori, an increase in the micellar size due to both incorporation of solute molecules and the increase of the aggregation number was expected [45,46]. Table 3 shows the size of the aggregates formed in 3, 5 and 10% TS-saturated poloxamine systems and the corresponding population distribution. A clear trend was apparent in TS-saturated systems: a gradual increase in concentration of T1107 (and TS) led to the formation of larger aggregates that became also more predominant as concentration rose. Smaller sizes (14 and 11 nm for 5 and 10%) were consistent with the presence of a limited fraction of regular micelles containing low TS concentrations (see below). This group decreased as polymer concentration increased. Fractions with an intermediate size (~54–60 nm) would indicate the presence of enlarged micelles due to the incorporation of TS into the core. In the case of a 10% poloxamine solution, a sharp growth in size of the intermediate aggregates to 137 nm was observed. Finally, the size of the largest aggregates went from 190 to about 500 nm for 5 and 10% systems, respectively. These large aggregates would fit the association of several structures of intermediate size to form a larger one. This phenomenon would rely on the stronger interaction between more densely packed aggregates that resulted in micellar fusion in order to stabilize the system. Another evidence of a secondary association taking place upon saturation was the increasing opalescence of saturated systems between 1 and 10% [47]. Aiming at obtaining further insight, an additional experiment was performed. 10% poloxamine specimens (pH 7.4) that were unsaturated in TS were prepared; they contained amounts of TS corresponding to saturated systems of 3, 5 and 7% poloxamine. None

Table 3
Micellar size and size distribution (% volume) of TS-loaded 3, 5 and 10% T1107 systems

Poloxamine concentration (%)	Peak 1		Peak 2		Peak 3	
	Size (nm)	%	Size (nm)	%	Size (nm)	%
3	–	–	54	100	–	–
5	14	12.8	60	35.2	194	52.0
10	11	1.4	137	6.7	492	91.9

of them presented opalescence, suggesting the absence of secondary association in this case [47]. Moreover, DLS measurements of these unsaturated dispersions revealed the exclusive presence of small structures with sizes around 10–14 nm and confirmed that secondary association was not taking place in the unsaturated systems. Calculations of the MSR of the different systems showed a clear decrease at poloxamine concentrations >3% (Table 4). For example, at pH 2, the ratio increased sharply from 7.83 to 30.28 for 0.5 and 3% poloxamine solutions, respectively. Then, values gradually decayed to about 15. In the case of unsaturated 10% samples, these ratios were substantially lower (8.89, 9.65 and 12.23 for TS-contents of 3, 5 and 7% poloxamine saturated specimens) than the values observed for similar TS-saturated systems. These findings constituted additional support that the association phenomenon took place as the amount of drug within the micelles increased (and approximated to the upper capacity limit). TS-saturated specimens were also investigated by TEM. Thus, in 10% samples the micellar association was clearly observed (Fig. 3A and B), confirming that increasing concentrations of the drug induced the formation of larger aggregates through the fusion of smaller ones.

Table 4
Triclosan/poloxamine molar ratios (MSR) in poloxamine systems between 0.5 and 10% at four different pHs

pH	Triclosan/poloxamine molar ratio (MSR)					
	0.5%	1.0%	3.0%	5.0%	7.0%	10.0%
2.0	7.83	25.18	30.28	20.58	19.67	15.48
5.8	7.67	23.63	29.33	20.06	18.05	15.79
7.4	6.51	21.24	29.79	19.59	17.44	15.57
12.0	15.48	17.27	14.94	14.60	13.06	14.07

3.4. Physical stability of the drug-containing micelles

The solubilization process comprises the incorporation of hydrophobic TS molecules into the core of poloxamine aggregates to render the inclusion complexes. However, under regular storing conditions temperature fluctuations could modify the CMC of the polymer and consequently alter the solubilization ability of the systems, resulting, for example, in drug precipitation and titer loss. In this context and from a technological perspective, the study of the physical stability of the formulations intended for topical application was of interest. Samples of drug-loaded poloxamine systems between 1 and 10% of pH 5.8 and 7.4 were stored at RT (~23 °C) and the TS concentration assayed at different time points. Findings indicated that in 1% systems of pHs 5.8 and 7.4 S_a values sharply decrease to about 30 and 20% of the initial level, respectively, during the first month (Fig. 4). This effect was more pronounced at the higher pH, where values dropped below the 10% level after 2 months. This behavior indicated that the proximity of the poloxamine concentration to the CMC (~0.5%) rendered relatively unstable systems that unassembled, releasing the drug to the medium. The lower drug–core affinity at a higher pH due to the absence of H bonding probably made these systems less stable. In contrast, more concentrated systems displayed much higher stability extents with values from about 70% for 3% samples and up to 80–90% for more concentrated ones, after 3 months of study. Also, no relevant difference among pHs was found. Another noteworthy technological aspect was to assay the ability to re-dissolve triclosan/poloxamine mixtures that were previously freeze-dried under controlled conditions. Findings showed that all the samples re-dissolved completely in aqueous media upon reconstitution.

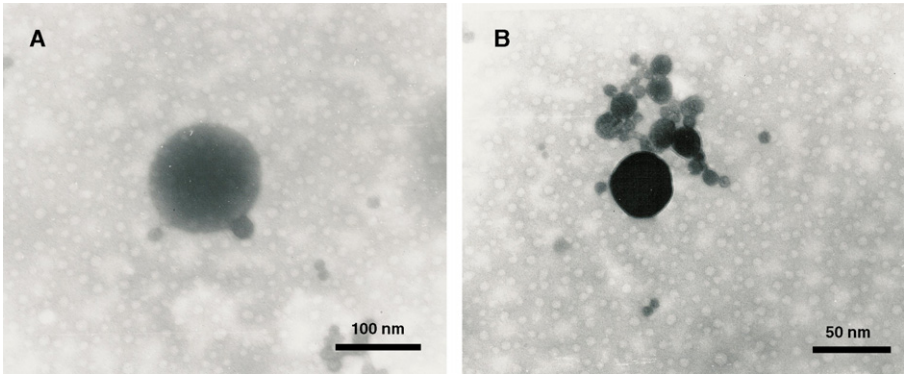

Fig. 3. TEM micrographs of TS-saturated 10% poloxamine T1107 micelles negatively stained with phosphotungstic acid at pH 7.4. (A) Scale bar = 100 nm (microscope mag. = 50,000×) and (B) Scale bar = 50 nm (microscope mag. = 100,000×).

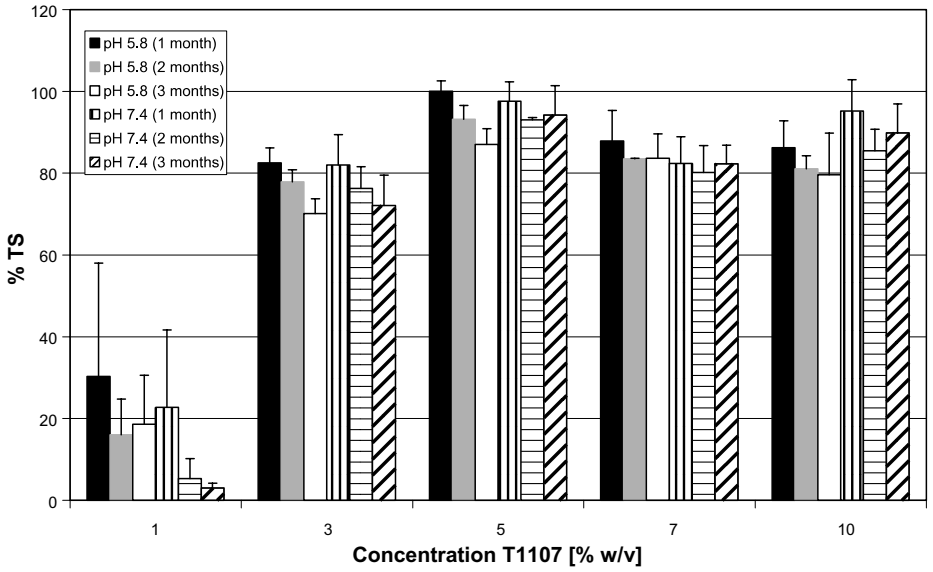

Fig. 4. % TS along the time of different TS-loaded micellar systems of different poloxamine concentrations stored at 23 °C.

3.5. Antibacterial activity of triclosan complexes

Sequestration of hydrophobic molecules by the core of polymeric micelles could be defined as the first stage in the process to attain improved solubility. However, in order to display pharmacological activity, the drug must be released from the reservoir to the medium. At glance, a very strong drug–core interaction could curtail the antibacterial activity as the drug remains sequestered in the core. Accordingly, studies of the antibacterial activity of the complex were of interest. Primarily, a broad spectrum of collection pathogens was assayed (Table 5). Findings showed a clear increase in the antibacterial activity (expressed by a larger inhibition area) of the complexes when compared to controls on most of the cases. For example, while TS aqueous solutions did not display any antibacterial activity against cultures of *Salmonella ssp*, a 1% poloxamine TS-loaded system already resulted in a clear inhibition at both pHs. In other cases where strains were sensitive even to low TS concentrations present in poloxamine-free solutions, a clear increase in the inhibition area was observed. It should be stressed, though, that two tested bacteria (*Burkholderia cepacia* and *Pseudomona aeruginosa*) were insensitive to the drug, regardless the concentration attained in the complex, indicating an intrinsic resistance to TS. Afterwards, two clinical strains isolated in public hospitals that present high incidence in nosocomial-acquired infections were tested. MRSA was inhibited

Table 5
Inhibition zone diameters of TS-loaded 1, 3 and 5% T1107 micelles at pHs 5.8 and 7.4

Microorganism	Source	Diameter of inhibition zone (mm)									
		pH 5.8					pH 7.4				
		Control[a]	1%	3%	5%	Blank[b]	Control[a]	1%	3%	5%	Blank[b]
Staphylococcus aureus	ATCC-6538	<6[c]	>44	>44	>44	<6	27	44	44	>44	<6
Escherichia coli	ATCC-8739	<6	21	22	25	<6	14	21	24	27	<6
Streptococcus *faecium*	ATCC-10541	10	24	26	25	<6	15	23	25	27	<6
Burkholderia cepacia	ATCC-25416	<6	<6	<6	<6	<6	<6	<6	<6	<6	<6
Pseudomonas aeruginosa	ATCC-9027	<6	<6	<6	<6	<6	<6	<6	<6	<6	<6
Salmonella spp.	ATCC-13076	<6	22	23	23	<6	<6	23	23	24	<6
Klebsiella pneumonie	ATCC-10031	17	27	30	31	<6	22	32	32	33	<6
Salmonella cholerasuiss	Enterobacteria collection of the Malbrán Hospital, Buenos Aires	14	29	30	32	<6	14	30	31	32	<6
Staphylococcus epidermidis	ATCC-1228	30	>44	>44	>44	<6	40	>44	>44	>44	<6
Methicillin-resistant *Staphylococcus aureus*	Hospital strain	34	38	44	>44	<6	34	40	41	44	<6
Vancomycin-resistant *Enterococcus faecalis*	Hospital strain	<6	20	22	22	<6	<6	20	20	25	<6

[a] Control denotes a poloxamine-free saturated aqueous solution of TS.
[b] Blank denotes a drug-free poloxamine micellar system.
[c] A diameter <6 denotes no inhibition.

even by polymer-free TS. However, inclusion of TS into polymeric micelles led to a more effective inhibition due to the higher drug levels attained: the inhibition zone increased from 34 mm in the control to about 44 mm in a 5% T1107 systems. In the case of VREF, more remarkable results were found. Whereas the pathogen was not affected by a TS control solution, a 1% poloxamine-based formulation clearly killed it (Fig. 5). Findings suggested a clear improvement in the bactericidal activity of TS by inclusion in poloxamine polymeric micelles due to higher concentrations attained. However, assays on plates could not lead to any conclusive statement regarding the activity against the same pathogen in a biofilm conformation. Bacteria embedded in this unique matrix usually resist chemotherapy and constitute a major challenge in medicine and a main cause of intra-hospital infections. Regardless the antibacterial activity displayed by TS-loaded micellar formulations on plate assays, the effectiveness against bacteria in a biofilm was a more difficult challenge. In order to investigate this aspect, *Staphylococcus epidermidis* biofilms were generated on glass slides and exposed to a TS-loaded 5% poloxamine complex at pH 7.4. *Staphylococcus epidermidis* constitutes a highly frequent pathogen related to BCIs [6] and primary experiments on plate showed sensitivity to TS even in poloxamine-free systems. Thus, this strain was a good candidate to assess a potential enhanced activity due to inclusion into polymeric micelles and to compare the performance against the same pathogen in plate and embedded in biofilm. Coated slides were qualitatively assessed with Violet Crystal in order to confirm the formation of the matrix. Findings showed a sharp increase in the absorbance, indicative of the presence of this network of polysaccharides and proteins. Then, specimens of biofilm-coated slides were exposed to aqueous TS (control), TS–poloxamine (sample) and poloxamine (blank) and the number of bacteria determined by counting the CFUs after 30 and 90 min (Fig. 6). Initial bacterial levels in biofilms were $\sim 6.1 \times 10^6$ cells/slide. Exposure to controls showed an initial slight decrease in the count to a value $\sim 1.9 \times 10^6$ cells/slide (at 30 min), stressing some antibacte-

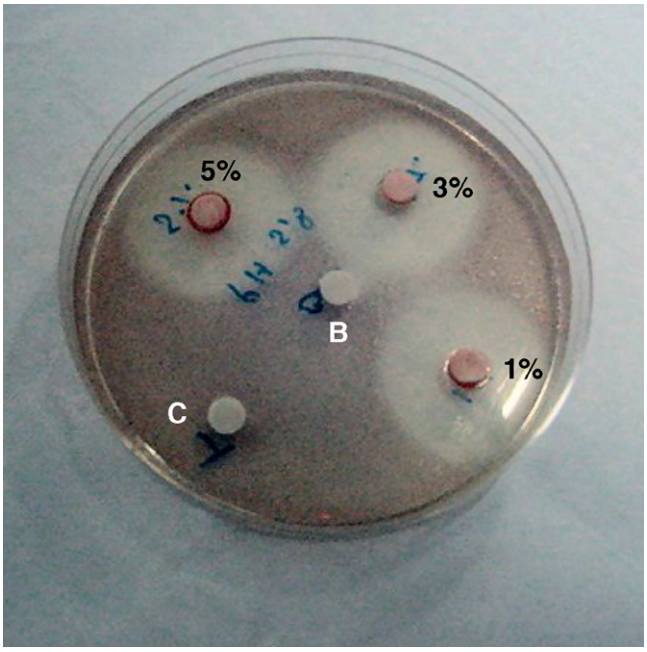

Fig. 5. Photograph of the antibacterial activity of TS-loaded samples with poloxamine concentrations between 1 and 5% at pH 5.8. The blank (5% drug-free poloxamine) and the control (TS aqueous solution) are labeled as B and C, respectively.

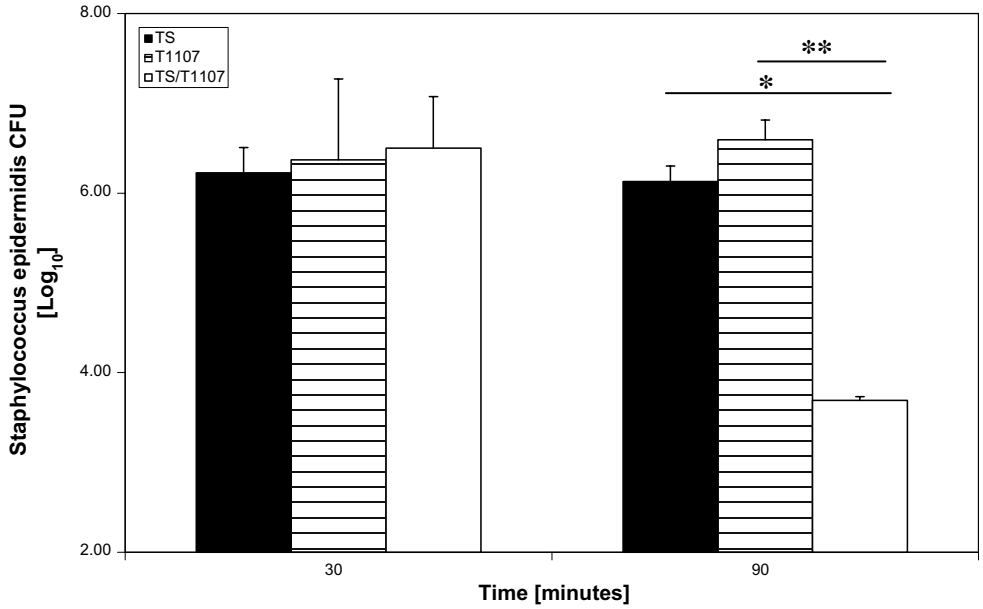

Fig. 6. *Staphylococcus epidermidis* CFUs (Log_{10}) in a biofilm assay after 30 and 90 min. The Log_{10}CFU value at time = 0 was 6.65 ± 0.44. * and ** denote significantly different ($p < 0.001$).

rial effect, even at such low TS concentrations (in agreement with plate assays). However, a longer treatment (90 min) rendered only a minor additional decrease in viability to about 1.4×10^6 cells/slide and was indicative of the limited activity of the drug against the pathogen in biofilm. Before the study of the complex, the compatibility of the polymer needed to be demonstrated. Biofilms were incubated in contact with blank specimens (poloxamine micelles) and the number of viable microorganisms assayed. Cell numbers primarily grew up to a value of 8.6×10^6 cells/slide, though a later decrease to about 4.2×10^6 cells/slide was apparent. The fact that cell numbers were of the same order of magnitude agreed with previously reported results that indicated the high cytocompatibility of this polymer in contact with eukaryotic cells [48,49]. Finally, the effect of TS-loaded micelles was investigated. After 30 min, a limited bactericidal effect was observed as cell counts stayed high, around 4.8×10^6 cells/slide. In contrast, one hour later, a dramatic decrease in 3 orders of magnitude to levels around 5×10^3 cells/slide was observed. Values of CFU for the TS/poloxamine combination were significantly lower than the levels shown by both the control and the blank ($p < 0.001$) and confirmed the substantial enhancement attained by the proposed strategy. These findings stressed the fact that the drug needs to be released from the inclusion complex to the medium in order to display any pharmacological activity. This process could be initially slow, leading to an early limited effect (0.5 h). Afterwards, the higher levels attained led to a strong antibacterial effect on the biofilm under investigation. Further studies will comprise specific studies to elucidate these fundamental aspects.

4. Conclusions

We capitalized on the improved aqueous solubility of TS (up to 4 orders of magnitude) by incorporation into polymeric micelles of poloxamine T1107. To the best of our knowledge, this is the first report investigating the solubilizating effect of the temperature and pH-responsive poloxamines on a pH-sensitive molecule, like triclosan. In contrary to previous works where higher solubilization extents were found at higher pHs, in this case a slight decrease in solubility was found at higher pHs. This phenomenon could be explained by the pH-dependent ionization of the aromatic –OH group. At low pHs, unionized phenol groups can interact with the –O– of the polyether chain, forming H bonds. Oppositely, once ionized, a detrimental effect was observed and a lower solubility attained. In this framework, additional studies replacing TS by analogs without phenol groups are being conducted. It is worth stressing that higher TS levels could have a detrimental effect on the compatibility of the formulation in contact with skin. Since the formulation is intended for topical application or treatment of infected surfaces of implants and working areas, cytocomaptibility studies were out of the scope of the work. However, for applications where a direct contact with internal tissues or organs could be involved, further studies will be mandatory. Finally, the effectiveness of the formulation was demonstrated on collection *Staphylococcus epidermidis* biofilms with promising results. Future studies will demand the exploration of the activity of TS/poloxamine systems against biofilms formed by clinical MRSA and VREF strains, characteristic of intra-hospital infections. Also, the extension to other pathogens associated with biofilm in the pharmaceutical industry will be conveniently evaluated. These investigations are underway and findings will be reported separately.

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