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β-Cyclodextrin hydrogels for the ocular release of antibacterial thiosemicarbazones

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ABSTRACT

Two types of hydrophilic networks with conjugated beta-cyclodextrin (β-CD) were developed with the aim of engineering useful platforms for the localized release of an antimicrobial 5,6-dimethoxy-1-indanone N4-allyl thiosemicarbazone (TSC) in the eye and its potential application in ophthalmic diseases. Poly(2-hydroxyethyl methacrylate) soft contact lenses (SCLs) displaying β-CD, namely pHEMA-co-β-CD, and super-hydrophilic hydrogels (SHHs) of directly cross-linked hydroxypropyl-β-CD were synthesized and characterized regarding their structure (ATR/FT-IR), drug loading capacity, swelling and *in vitro* release in artificial lacrimal fluid. Incorporation of TSC to the networks was carried out both during polymerization (DP method) and after synthesis (PP method). The first method led to similar drug loads in all the hydrogels, with minor drug loss during the washing steps to remove unreacted monomers, while the second method evidenced the influence of structural parameters on the loading efficiency (proportion of CD units, mesh size, swelling degree). Both systems provided a controlled TSC release for at least two weeks, TSC concentrations (up to 4000 μg/g dry hydrogel) being within an optimal therapeutic window for the antimicrobial ocular treatment. Microbiological tests against *P. aeruginosa* and *S. aureus* confirmed the ability of TSC-loaded pHEMA-co-β-CD network to inhibit bacterial growth.

1. Introduction

Aqueous solutions and oily suspensions to be instilled into the conjunctival fornix represent approximately 90% of all formulations aimed to treat ocular diseases (Hu et al., 2011; Lang, 1995). Drugs applied topically to the eye penetrate through the cornea and biodistribute to ocular tissues (Alvarez-Lorenzo, Hiratani, & Concheiro, 2006; Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez, Concheiro, & Torres-Labandeira, 2007; Urtti, 2006). However, the ocular drug bioavailability is limited due to a fast clearance from the ocular surface, mediated by lacrimation, and systemic absorption; the latter usually resulting in systemic side effects (Alvarez-Lorenzo, Hiratani, & Concheiro, 2006; Rodriguez-Tenreiro et al., 2007; Urtti, 2006). In fact, topical application of a drug to the eye normally entails a loss of approximately 95% of the administered dose (Alvarez-Lorenzo, Yañez, & Concheiro, 2010; Tieppo et al., 2012). The primary goals of the development of ophthalmic drug delivery systems (ODDS) are to increase the amount of the drug that penetrates through the cornea, to prolong its permanence in the ocular structures, and to reduce the required dose and the administration frequency, thus increasing patient compliance (Alvarez-Lorenzo, Hiratani, et al., 2006; Alvarez-Lorenzo et al., 2010; Rodriguez-Tenreiro et al., 2007; Tieppo et al., 2012).

Soft contact lenses (SCLs) loaded with a drug payload have been evaluated during the last decades as a way to combine the correction of vision defects and the treatment of different ocular conditions such as glaucoma, infections, allergies and chronic dry eye (Alvarez-Lorenzo et al., 2002; Alvarez-Lorenzo, Yañez, et al., 2006; Alvarez-Lorenzo et al., 2010; Andrade-Vivero, Fernandez-Gabriel, Alvarez-Lorenzo, & Concheiro, 2007; Malaekeh-Nikouei, Ghaeni, Motamedshariaty, & Mohajeri, 2012). Compared to conventional ophthalmic formulations that stay on the eye surface for a few minutes, medicated SCLs can maintain the drug in the pre-corneal area for prolonged time, increasing the likelihood of

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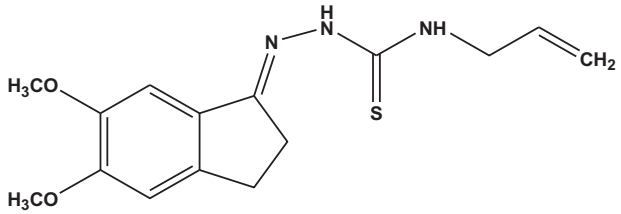


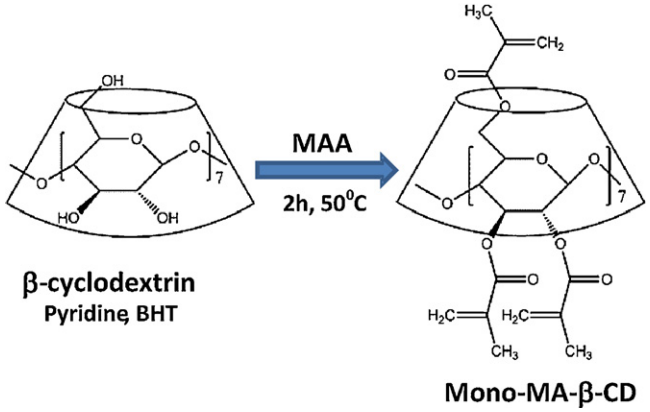
Scheme 1. Chemical structure of 5,6-dimethoxy-1-indanone N4-allyl thiosemicarbazone.

drug penetration (Alvarez-Lorenzo, Hiratani, et al., 2006; Alvarez-Lorenzo et al., 2002). However, the high hydrophilicity of most monomers of SCLs limits the uptake of hydrophobic drugs and the control of the release of the hydrophilic ones (Alvarez-Lorenzo, Hiratani, et al., 2006; Alvarez-Lorenzo et al., 2002; Alvarez-Lorenzo, Yañez, et al., 2006; Andrade-Vivero et al., 2007; Malaekeh-Nikouei et al., 2012; Peng, Burke, Carbia, Plummer, & Chauhan, 2012; Tieppo et al., 2012). For example, poly(2-hydroxyethyl methacrylate) (pHEMA) is a highly hydrophilic component of SCLs and intraocular lenses, vascular grafts, soft tissue substitutes and hydrogels for drug delivery (González-Chomón, Concheiro, & Alvarez-Lorenzo, 2011; Lin & Metters, 2006; Peng et al., 2012; Xinming et al., 2008).

Cyclodextrins (CDs) are extensively used to solubilize, physicochemically stabilize and increase the bioavailability of hydrophobic drugs (Brewster & Loftsson, 2007; Loftsson & Duchêne, 2007; Loftsson & Jarvinen, 1999; Loftsson & Stefánsson, 2002). The number of ophthalmic formulations containing CDs has shown a marked increase over the last years (Loftsson & Jarvinen, 1999; Loftsson & Stefánsson, 2002). SCLs and other types of covalently crosslinked hydrogels that contain conjugated CD units emerged as versatile platforms for the localized delivery of high drug cargos to the eye or other mucosal surfaces (Blanco-Fernandez, Lopez-Viota, Concheiro, & Alvarez-Lorenzo, 2011; Moya-Ortega, Alvarez-Lorenzo, Concheiro, & Loftsson, 2012; Ribeiro et al., 2012; Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez, Concheiro, & Torres-Labandeira, 2006; Rosa dos Santos et al., 2009; Rosa dos Santos et al., 2010; Xu, Li, & Sun, 2010).

1-Indanone thiosemicarbazones (TSCs) are a group of novel low molecular-weight drugs that have shown promising antibacterial (Brousse et al., 2004), antifungal (Brousse et al., 2004), antiviral (Castro et al., 2011; Finkielsztein et al., 2008, 2010; Garcia et al., 2003; Glisoni, Cuestas, et al., 2012), antiprotozoal (Caputto et al., 2011) and antileukemic (Gómez et al., 2011) activities. TSCs are very hydrophobic molecules (aqueous solubility 1.5–13.0 μg/mL; $\log P_{experimental}$ = 1.79–2.83) and they display a relatively high tendency to self-aggregate in water (Glisoni, Chiappetta, Finkielstein, Moglioni, & Sosnik, 2010). This behavior hampered the reproducible and reliable evaluation of the antiviral activity *in vitro* (Finkielsztein et al., 2008). To circumvent this critical biopharmaceutic drawback, Glisoni et al. thoroughly investigated the development of complexes of different TSCs with a variety of native and chemically modified CDs (Glisoni, Chiappetta, Moglioni, & Sosnik, 2012). Then, the activity against the hepatitis C virus was confirmed in a robust *in vitro* model of the disease (Glisoni, Cuestas, et al., 2012).

Aiming to extend the application of the TSC/CD platform to the treatment of ocular infections, the present work explored the engineering of two types of CD-conjugated ODDS for the localized release of an antibacterial allyl N4-substituted TSC (Scheme 1): (i) networks of pHEMA-co-β-CD prepared through conventional free radical polymerization using a polymerizable β-CD derivative, namely 2,3-di-O-methacrylated-6-O-methacrylated-β-CD monomer (mono-MA-β-CD); and (ii) networks prepared by direct cross-linking of hydroxypropyl-β-CD (HPβ-CD) and



Scheme 2. Synthesis of the methacrylated monomer of β-cyclodextrin.

HPβ-CD/hydroxypropylmethyl cellulose (HPMC) (1.0%, w/v) with ethylene glycol diglycidyl ether (EGDE) (Rodriguez-Tenreiro et al., 2007). To the best of our knowledge, a synthetic pathway that incorporated the drug cargo during the copolymerization of HEMA and mono-MA-β-CD is reported herein for the first time. *In vitro* release assays in artificial lacrimal fluid provided a sustained release of TSC for at least two weeks. Finally, the evaluation of the antibacterial activity against two pathogens commonly associated with ocular infections demonstrated the potential of these ODDS to serve as platform for the localized release of this novel therapeutic agent to the eye.

2. Materials and methods

2.1. Materials

HEMA ophthalmic grade was from Merck (Darmstadt, Germany). Methacrylic acid anhydride (MAA), 2,6-di-tert-butyl-4-methylphenol (BHT), 2,2′-azo-bis(isobutyronitrile) (AIBN), ethylene glycol dimethacrylate (EGDMA, technical grade 98%), EGDE (technical grade 50%), hydroxypropylmethyl cellulose K4M (HPMC K4M) and dichloromethylsilane (DCMS) were from Sigma–Aldrich Chemicals (Madrid, Spain). β-CD (M_w = 1135 g/mol) and HPβ-CD (M_w = 1400 g/mol) were supplied by Roquette-Laisa (Valencia, Spain). 5,6-Dimethoxy-1-indanone N4-allyl thiosemicarbazone (TSC, M_w = 305 g/mol) (Scheme 1) was synthesized and purified as described elsewhere (Glisoni et al., 2010). Ultrapure water (resistivity > 18.2 MΩ cm) was obtained by reverse osmosis (MilliQ®, Millipore Ibérica SA, Madrid, Spain). All other reagents and solvents were of analytical grade and used without further purification.

2.2. Synthesis of methacrylated-β-CD monomer (mono-MA-β-CD)

Mono-MA-β-CD (M_w = 2311 g/mol) was primarily synthesized employing the methodology previously described (Rosa dos Santos, Couceiro, Torres-Labandeira, & Alvarez-Lorenzo, 2008; Saito & Yamaguchi, 2003) (Scheme 2). In brief, pristine β-CD (8.0 g) was dried in an analytical balance thermostated at 105 °C (24 h) and solubilized in dry pyridine (36 mL) together with BHT (40.0 mg). MAA (19.8 g) was carefully added and the mixture was stirred at room temperature for 2 h and then at 50 °C (5 h). The reaction crude was poured into cold distilled water (300 mL) and kept at 4 °C (12 h) to promote the precipitation of mono-MA-β-CD. The precipitate was isolated by filtration (0.22 μm Nylon membranes, Teknokroma, Sant Cugat del Vallés, Spain) and purified by dissolution in MeOH (20 mL) and subsequent re-precipitation in cold distilled water

(300 mL); this procedure was repeated three times. The white solid obtained was dried in a vacuum desiccator containing silica gel (5 days) and characterized by ^{1}H NMR spectroscopy (Bruker AMX500 spectrometer, Bruker Daltonic GmbH, Tübingen, Germany; room temperature, deuterated dimethyl sulfoxide (DMSO-d_6) solution) at 500 MHz and Attenuated Total Reflectance/Fourier-transformed Infrared Spectroscopy (ATR/FT-IR; see below) (Rosa dos Santos et al., 2008).

2.3. Synthesis of β-CD-conjugated pHEMA SCLs (pHEMA-co-β-CD)

Mono-MA-β-CD (0.0, 0.6 and 1.2 g) was solubilized in HEMA (constant volume of 6.0 mL; final concentration of mono-MA-β-CD in HEMA 0%, 10% and 20%, w/v, respectively) at 25 °C (30 min). Two methods were employed to load TSC into pHEMA-co-β-CD networks: (i) during-copolymerization loading (DP) and (ii) post-copolymerization loading (PP). In the case of DP systems, TSC (6.0 mg) was firstly solubilized in a mixture of HEMA (6.0 mL) and mono-MA-β-CD (corresponding amount; see above), yielding a final TSC concentration of 1.0 mg/mL of reaction mixture. Then, EGDMA (crosslinker agent, 8.0 mM, 10.0 μL) and AIBN (initiator, 10.0 mM, 9.8 mg) were added, the mixture stirred until complete homogenization and carefully injected into a mold constituted by two glass-made plates and a silicon frame (thickness of 0.4 mm) (Scheme 3). To prevent the adhesion of the networks to the plates, their internal surface was coated with a layer of DCMS (2 h), and subsequently washed with distilled water and acetone. The molds were heated at 50 °C for 12 h and then at 70 °C for 24 h to complete the copolymerization. In the case of PP systems, the same procedure was followed, though without the addition of TSC. The reagent feeding ratios are summarized in Table 1. After heating at 70 °C, pHEMA sheets were dismounted from the molds, weighed and immediately immersed in boiling water (1 L) for 15 min to remove unreacted monomers and to facilitate the cutting of disks (10 mm in diameter) with a punch. The disks were washed until no monomers were detected in the washing solution (three days with medium replacements every 12 h), as determined by UV–vis at λ between 190 and 600 nm (Urtti, 2006) (Agilent 8453, Agilent Technologies, Böblingen, Germany). In addition, the amount of TSC released during the washings was monitored by UV–vis at λ_{max} of 331 nm (Glisoni, Cuestas, et al., 2012). Finally, the disks were dried at 50 °C and weighed.

2.4. Synthesis of super-hydrophilic hydrogels (SHHs) of HPβ-CD and HPβ-CD/HPMC

HPβ-CD (4.8 g), NaOH aq. solution (0.2 M, 16 mL) and EGDE (crosslinker agent, 8 mL) were thoroughly mixed. The final reaction mixture (24 mL) was divided into aliquots A and B (12 mL each), and HPMC K4M (0.12 g) was added exclusively to aliquot B (12 mL), yielding a final HPMC concentration of 1% (w/v). Then, aliquots A and B were stirred (15 min) until homogeneity, poured into thin-glass tubes (diameter of 5 mm) and heated at 50 °C (12 h) to complete the reaction. HPβ-CD (20%, w/v) and HPβ-CD/HPMC (20%/1%, w/v) SHHs were obtained by breaking down the tubes and washing the crosslinked networks successively with water (12 h), 10 mM HCl (12 h) and water (24 h). Finally, SHHs were cut into 5 mm diameter disks with a scalpel and dried in an oven at 40 °C (24 h).

2.5. Loading of TSC into HEMA (PP method) and SHH networks

A fine TSC suspension in water (250 μg/mL) was prepared by sonication (30 min, Bath Branson 3510, Danbury, CT, USA) at 45 °C and magnetic stirring at room temperature (24 h). Dry disks of (i) pHEMA-co-β-CD containing 0%, 10% and 20% (w/v) of mono-MA-β-CD (average weight 40.0 ± 9.3 mg) and (ii) SHHs of HPβ-CD (20%, w/v) and HPβ-CD/HPMC (20%/1%, w/v) (average weight 167.3 ± 27.8 mg) were immersed into TSC aqueous suspensions (10 mL, see above) and stirred at 25 °C (1 day) protected from light. Additionally, pHEMA-co-β-CD disks were autoclaved in a TSC suspension (20 min, 121 °C) to promote TSC loading (Rosa dos Santos et al., 2008, 2010). Finally, TSC-loaded disks were rinsed with water to remove the drug adsorbed on the surface of the networks.

The TSC loading efficiency was calculated as follows (Natu, Sardinha, Correia, & Gil, 2007):

$$\mathrm{LE}\,(\%) = \frac{\mathrm{TSC_{LH}}}{\mathrm{TSC_{SS}}} \times 100$$

where $\mathrm{TSC_{LH}}$ is the total amount of TSC loaded in the sample as determined during the release assays and $\mathrm{TSC_{SS}}$ is the amount of TSC in the loading medium. All the assays were performed in triplicate and expressed as mean ± SD.

2.6. Hydrogel characterization

2.6.1. ATR/FT-IR

Dry TSC-loaded systems were characterized by ATR/FT-IR over the range 400–4000 cm^{-1} employing a Varian-670 FTIR spectrometer (Varian Inc., Santa Clara, CA, USA) equipped with a GladiATR® diamond crystal (PIKE Instruments, Madison, WI, USA). Spectra analysis was conducted with Agilent Resolutions Pro-software (Agilent Technologies, Santa Clara, CA, USA). The respective TSC-free networks were used as controls.

2.6.2. Swelling

The water uptake capacity of TSC-free pHEMA-co-β-CD networks and HPβ-CD and HPβ-CD/HPMC SHHs was assessed in artificial lacrimal fluid (6.78 g/L NaCl, 2.18 g/L $NaHCO_3$, 1.38 g/L KCl and 0.084 g/L $CaCl_2{\cdot}2H_2O$; pH 8.0) (Ali et al., 2007) at 25 °C. TSC-loaded DP pHEMA-co-β-CD networks with 0%, 10% and 20% (w/v) of mono-MA-β-CD were also analyzed for comparison. Individual samples were immersed into artificial lacrimal fluid (10 mL) and weighed at different time points after a careful wiping of the surface with tissue paper to remove the excess of water. The degree of swelling, Q_t (%), was determined as follows (Blanco-Fernandez et al., 2011; Ribeiro et al., 2011; Rosa dos Santos et al., 2010):

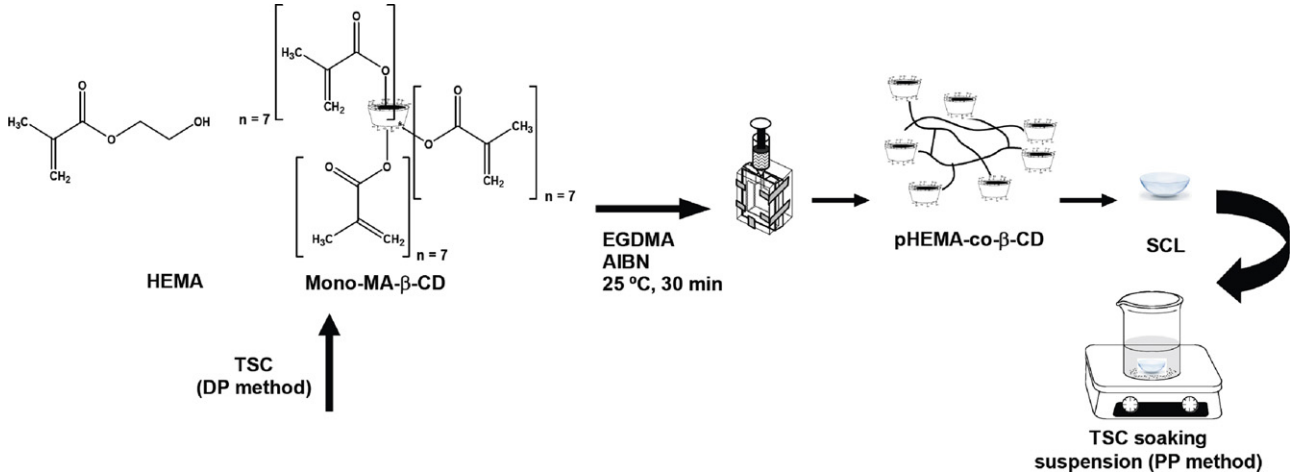
$$Q_t\,(\%) = \frac{W_t - W_0}{W_0} \times 100$$

where W_t is the weight of the swollen sample at time t and W_0 is the weight of the dry sample. All assays were performed in triplicate and expressed as mean ± SD.

2.7. TSC release in artificial lacrimal fluid

Hydrogel disks were immersed into artificial lacrimal fluid (10 mL) at 25 °C, and the release of TSC was monitored by UV–vis spectrophotometry (λ_{max} = 331 nm) (Glisoni, Cuestas, et al., 2012). For quantification, a calibration curve of TSC in artificial lacrimal fluid (1.0–7.0 μg/mL; $R^2 > 0.9987$) was prepared. The release medium volume (10 mL) was not great enough to ensure sink conditions. Thus, part of the artificial lacrimal fluid (2–3 mL) was exchanged by fresh pre-heated medium at different time intervals to prevent saturation. All assays were performed in triplicate and expressed as mean ± SD.

First-order, Higuchi and Korsmeyer–Peppas models (Costa & Sousa Lobo, 2001; Gibaldi & Feldman, 1967; Higuchi, 1963; Korsmeyer, Gurny, Doelker, Pierre, & Peppas, 1983) were fitted



Scheme 3. Synthetic pathway of TSC-loaded β-CD-conjugated pHEMA SCLs produced by the DP and the PP method.

to the release profiles. In the Korsmeyer–Peppas kinetic model (Korsmeyer et al., 1983; Seremeta, Chiappetta, & Sosnik, 2013).

$$\frac{M_t}{M_\infty} = kt^n$$

M_t and M_∞ are the absolute cumulative amounts of drug released at time t and at infinite time, respectively; k is a constant incorporating the structural and geometric properties of the release system, and n provides information about the release mechanism. Only M_t/M_∞ data in the 0.1–0.6 range was used. The fitting of first-order and Higuchi models was conducted with Microsoft® Excel 2003 and of Korsmeyer–Peppas model with SigmaPlot® 2001 software.

2.8. Antibacterial activity of TSC-loaded hydrogels

To determine the minimal inhibitory concentration (MIC) of TSC, the broth microdilution testing against two species with high incidence in ocular infections, namely *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 9027) and *Staphylococcus aureus* (*S. aureus*, ATCC 6538P), was conducted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2011a). Final concentrations of TSC ranged from 1 to 1024 μg/mL. After 20 h of incubation at 35 °C, the MICs were visually evaluated and defined as the lowest drug concentration that showed complete growth inhibition.

The agar diffusion method of Mueller–Hinton (under the experimental conditions suggested by the CLSI) (CLSI, 2011b) was also used to evaluate the antimicrobial activity of TSC against both pathogens. TSC solution (20 mg/mL in DMSO) was seeded (10 μL) onto 6-mm paper disks (200 μg of TSC per disk). TSC-loaded (test samples, $n = 3$) and TSC-free (negative control, $n = 3$) paper disks were dried in a laminar flow cabinet at room temperature and then at 37 °C (15 min) protected from light to remove any DMSO trace. Then, paper disks were placed onto agar plates inoculated with the corresponding microorganisms and incubated at 35 °C for 24 h. Commercial disks impregnated with reference antibiotics (Britannia Laboratories, Buenos Aires, Argentina) were used as controls, as well as solvent-alone impregnated disks as negative control.

To evaluate the antibacterial activity of TSC-loaded systems of pHEMA-co-βCD, dry DP and PP pHEMA-co-βCD hydrogel disks (10%, w/v of mono-MA-β-CD, average weight of disks 40.0 ± 3.0 mg) were placed on agar previously seeded with the microorganism of interest and incubated at 35 °C for 24 h. Finally, the inhibition zone was measured. Drug-free and microorganism-free controls were included. All experiments were done in triplicate and expressed as mean ± SD.

2.9. Statistical analysis

One-way ANOVA combined with the Dunnett Multiple Comparision Test or t-test (significance level $P > 0.05$) was performed using the GraphPad Prism software version 5.00 for Windows (GraphPad Software Inc., USA).

3. Results and discussion

3.1. Synthesis of pHEMA-co-β-CD and SHH hydrogels

Two different synthetic routes were followed to produce the hydrogels: (i) the SCL-type networks of pHEMA-co-β-CD were prepared through conventional free radical polymerization of HEMA and a polymerizable β-CD derivative, namely mono-MA-β-CD (Rosa dos Santos et al., 2008; Saito & Yamaguchi, 2003). ^{1}H NMR

Table 1
Reagent and TSC feeding ratios used during the copolymerization of HEMA and mono-MA-β-CD to produce pHEMA-co-β-CD matrices. In the case of PP systems, TSC was incorporated only after the production of the matrix.

pHEMA network	TSC loading method	HEMA (mL)	TSC (mg)	Mono-MA-β-CD (g)	EGDMA (mM)	AIBN (mM)	Mono-MA-β-CD content (%, w/v)
pHEMA		6.0	6.0	0.0	8.0	10.0	0
pHEMA-co-β-CD	DP[a]	6.0	6.0	0.6	8.0	10.0	10
pHEMA-co-β-CD		6.0	6.0	1.2	8.0	10.0	20
pHEMA		6.0	-	0.0	8.0	10.0	0
pHEMA-co-β-CD	PP[b]	6.0	-	0.6	8.0	10.0	10
pHEMA-co-β-CD		6.0	-	1.2	8.0	10.0	20

[a] TSC was loaded during the copolymerization.
[b] TSC was loaded after the copolymerization.

analysis of mono-MA-β-CD confirmed that all the 21 hydroxyl groups of pristine β-CD were substituted with MAA (data not shown) (Rosa dos Santos et al., 2008). The synthetic pathway of β-CD-conjugated pHEMA SCLs is depicted in Scheme 3. Three mono-MA-β-CD concentrations (0%, 10% and 20%, w/v, in HEMA) were used to generate networks with different capacity of TSC incorporation (Table 1). The drug was added to some hydrogels before polymerization (DP method), while other pieces were loaded after polymerization (PP method). The amount of EGDMA was relatively low to ensure an appropriate degree of crosslinking that would enable the effective diffusion of the drug payload (see below). The SHHs were obtained by direct cross-linking of HPβ-CD (20%, w/v) and HPβ-CD (20%, w/v)/HPMC (1.0%, w/v) with EGDE. The alkaline conditions required for the cross-linking of SHHs and the subsequent extensive washing before use did not allow the loading of TSC during the preparation of the hydrogel. Both pHEMA-co-β-CD and SHH hydrogels were transparent with a transmittance above 90% at 600 nm. These results suggested a correct copolymerization (Rosa dos Santos et al., 2008). Conversely, PP hydrogels became slightly opalescent after incorporation of TSC from a drug suspension.

3.2. Loading of TSC into DP and PP networks

The drug loading method can determine the mechanisms by which the drug is released from the ODDS (Lin & Metters, 2006). In this framework, two methods of TSC loading were explored: (i) addition to the monomer soup (DP method) and (ii) soaking of the hydrogels in a drug suspension (PP method). The TSC cargo was indirectly determined during the *in vitro* release assays. The amount of drug that can be loaded when a matrix is immersed into a drug solution or suspension (PP) depends on the drug concentration, the crosslinking density (mesh size), and the affinity of the drug for the network (Lin & Metters, 2006; Rosa dos Santos et al., 2008). The ratio between the amount of TSC incorporated by the SCLs and SHHs and the amount of TSC in the soaking suspension resulted in a LE of 5% at 24 h. Since autoclaving has been previously demonstrated as a useful way to enhance the loading of hydrophobic drugs in CD networks (Rodriguez-Tenreiro et al., 2007), this technique was also tested for pHEMA-co-β-CD networks soaked in the TSC suspension. However, the conditions were too drastic and TSC underwent hydrolysis. The resulting degradation products 5,6-dimethoxy 1-indanone (λ_{max} = 314 and 269 nm) and N4-allyl thiosemicarbazide (λ_{max} = 240 nm) were detected by UV–vis (Glisoni et al., 2010). Thus, the DP method appeared as a more straightforward and efficient one. Although one common disadvantage of DP method is the partial loss of the drug cargo during the post-production washing, such effect was not seen with the present networks; the elution of TSC to the washing solution being negligible.

3.3. Hydrogels characterization

3.3.1. ATR/FT-IR

TSC has typical bands at 1643.1, 955.3 and 921.6 cm^{-1} (CH_2—CH═CH_2 st) (Glisoni et al., 2010; Glisoni, Chiappetta et al., 2012). pHEMA SCLs prepared with and without mono-MA-β-CD and loaded with TSC by the DP and the PP methods showed the strong bands of the vibration of —OH and the stretching of ester groups of pHEMA at 3386.3–3432.2 cm^{-1} and 1714.5–1716.3 cm^{-1}, respectively (Rosa dos Santos et al., 2008; Saito & Yamaguchi, 2003; Xu et al., 2010). In DP samples, the characteristic signals of TSC completely disappeared and spectra were identical to those of TSC-free hydrogels. These results were consistent with a very efficient incorporation of the drug into the matrix bulk and with the absence of drug adsorbed non-specifically to the surface of the hydrogels. Conversely, hydrogels loaded applying the PP method showed the characteristic signals of TSC, probably caused by fine drug particles dispersed in the networks or adsorbed onto their surface. Even though ATR/FT-IR strongly suggested that TSC is at a different dispersion state in the DP and the PP networks, analyses were not conclusive about the drug cargo in the hydrogels. The *in vitro* release studies in artificial lacrimal fluid were crucial to quantify the TSC in each system and to study the effect of different parameters on the release pattern.

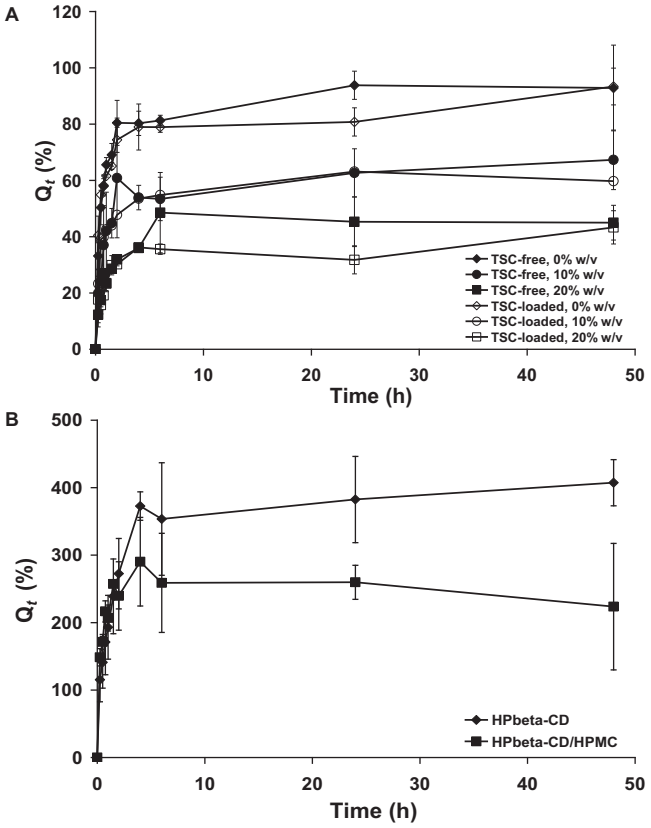


Fig. 1. Swelling of pHEMA-based SCLs in artificial lacrimal fluid, at 25 °C. (A) TSC-free and TSC-loaded pHEMA-co-β-CD containing 0%, 10% and 20% (w/v) of mono-MA-β-CD and obtained by the DP method and (B) TSC-free SHHs of HPβ-CD and HPβ-CD/HPMC. All data are expressed as mean ± SD of at least 3 independent experiments.

3.3.2. Swelling

Dried TSC-loaded pHEMA-co-β-CD and TSC-free SHHs networks took-up large amounts of artificial lacrimal fluid at 25 °C during the first 2–4 h of the assay, reaching the swelling equilibrium after 24 h (Fig. 1). For pHEMA SCLs, Q_t values were between 43% and 93%, indicating a moderate affinity for water (Fig. 1A). The incorporation of growing mono-MA-β-CD amounts resulted in more crosslinked, stiffer and less hydrophilic networks (Rosa dos Santos et al., 2008; Saito & Yamaguchi, 2003). Also, it is noteworthy that TSC-loaded networks produced by the DP method showed only a slight decrease in the swelling capacity, probably due to the hydrophobicity of the drug (Fig. 1A). For example, the Q_t of mono-MA-β-CD-free networks was 93% at 48 h. The addition of 10% (w/v) of mono-MA-β-CD resulted in Q_t values of 67% and 59% for TSC-free and TSC-loaded networks, respectively (Fig. 1A). A further increase of the CD content reduced the water uptake to less than 50%. The swelling values found for the TSC-free and TSC-loaded pHEMA-co-β-CD networks are in the range of the typical ones for hydrophilic contact lenses (Alvarez-Lorenzo, Yañez, et al., 2006). It should be noticed that the networks are intended to be applied onto the eye only after the swelling, as occurs with the conventional

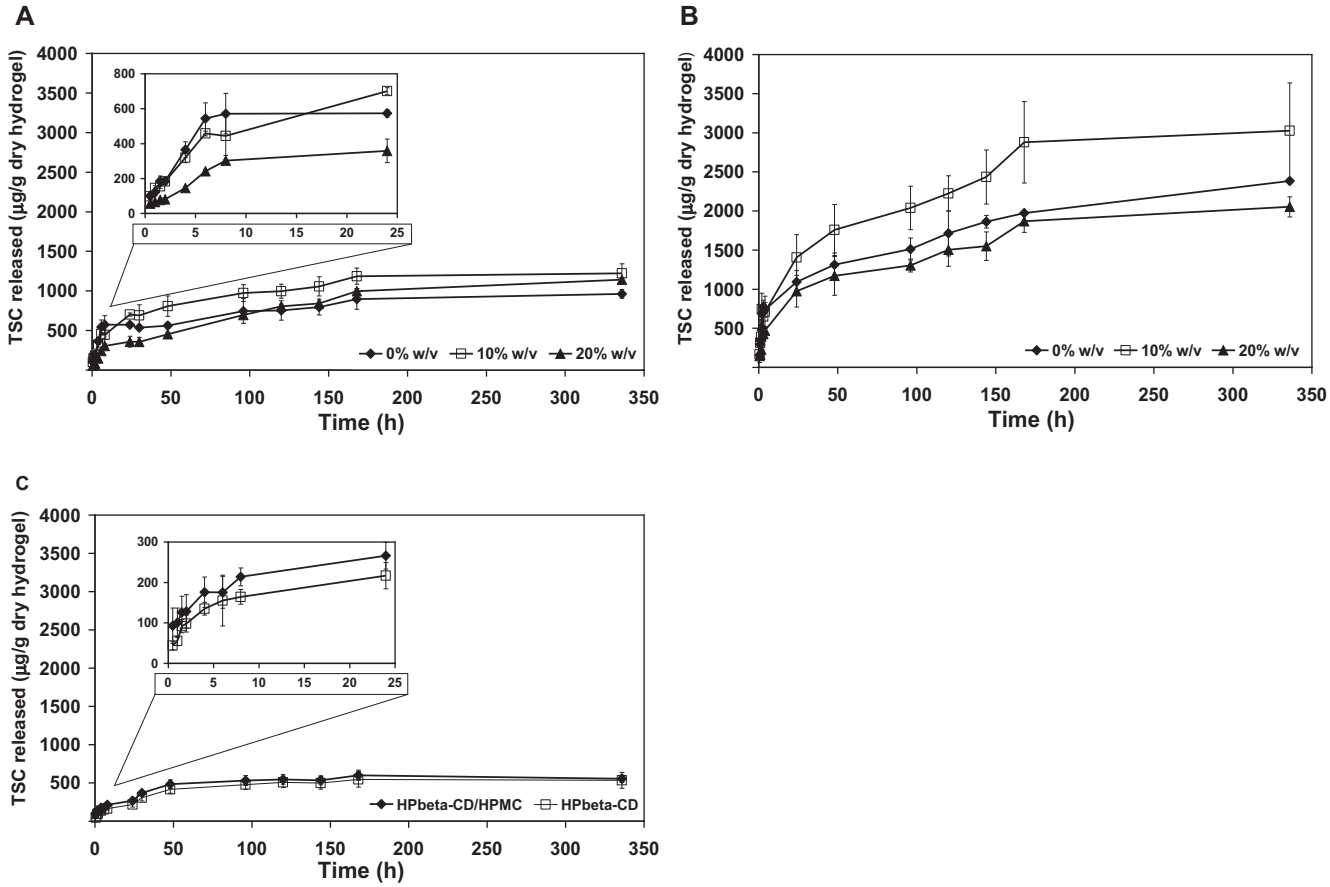


Fig. 2. TSC release kinetics from different hydrogels over two weeks, at 25 °C. (A) pHEMA-co-β-CD produced by the DP method, (B) pHEMA-co-β-CD produced by the PP method and (C) HPβ-CD and HPβ-CD/HPMC SHHs. The artificial lacrimal fluid medium was replaced to maintain sink conditions every (A) 30 h, (B) 4 h and (C) 8 h. Figure insets show the release during the first 24 h. All data were expressed as mean ± SD of at least 3 independent experiments.

contact lenses. Therefore, uncomfortable feelings due to swelling phenomena are not expected during use.

SHHs based on HPβ-CD and HPβ-CD/HPMC showed greater water uptake and reached 2–4 times the dry weight in 4 h (Fig. 1B). The greatest Q_t value of 407% corresponded to HPβ-CD networks (Fig. 1B). The addition of HPMC probably reduced the porosity and increased the hydrophobicity of the network, resulting in a decrease of the water uptake capacity (Lin & Metters, 2006; Lopez-Montero, Rosa dos Santos, Torres-Labandeira, Concheiro, & Alvarez-Lorenzo, 2009). The TSC cargo did not affect much the swelling capacity of the SHHs.

3.4. In vitro TSC release

The *in vitro* release profiles of the different TSC-loaded hydrogels are shown in Fig. 2. Regardless of the method of the drug loading (DP or PP) and in spite of their relatively fast swelling in artificial lacrimal fluid, pHEMA-co-β-CD SCLs sustained the release for several days (Fig. 2A and B). CD-containing SCLs prepared applying the DP method released the whole amount of TSC that was initially solubilized in the reaction mixture (1000 µg/mL) after one week (Fig. 2A). In the DP method, drugs or drug-polymer conjugates are mixed with a precursor solution and the copolymerization

Table 2
Release kinetics of TSC from different SCLs and SHHs.

Hydrogel	Mono-MA-β-CD concentration (%, w/v)	TSC loading method	First order[c]		Higuchi[d]		Korsmeyer–Peppas[e]		
			k_1 (% h^{-1})	R^2_{adj}	k_H (% $h^{-1/2}$)	R^2_{adj}	k_{KP} (h^{-n})	n	R^2_{adj}
pHEMA-co-β-CD	0		−0.0120	0.9486	4.8890	0.8092	0.2192	0.3245	0.9011
	10	DP[a]	−0.0120	0.9365	5.7330	0.8978	0.1369	0.4382	0.9602
	20		−0.0120	0.9664	5.8270	0.9757	0.0802	0.4190	0.9793
	0		−0.0110	0.9503	5.0000	0.9458	0.1774	0.3015	0.8542
	10		−0.0130	0.9541	5.6091	0.9476	0.1335	0.3851	0.9520
	20	PP[b]	−0.0120	0.9622	5.4720	0.9479	0.1304	0.3900	0.9298
HPβ-CD	–		−0.0140	0.9152	6.2860	0.8871	0.1336	0.4008	0.9420
HPβ-CD/HPMC (20/1)	–		−0.0150	0.8618	6.0035	0.8471	0.2003	0.2826	0.9713

[a] TSC was loaded during the copolymerization.
[b] TSC was loaded after the copolymerization over 24 h.
[c] First order equation ($\ln M_t - \ln M_0 = k_1 t$); M_t: absolute cumulative amount of drug released at time point t, M_0: initial amount of drug in the solution, and k_1: first order release kinetic constant (Gibaldi & Feldman, 1967).
[d] Higuchi equation ($M_t = k_H t^{1/2}$); M_t: absolute cumulative amount of drug released at time point t, k_H: Higuchi release kinetic constant (Higuchi, 1963).
[e] Korsmeyer–Peppas equation ($M_t/M_\infty = k_{KP} t^n$); M_t/M_∞: fraction of the drug released at time point t, k_{KP}: Korsmeyer–Peppas release kinetic constant (Korsmeyer et al., 1983).

and the drug incorporation are both accomplished simultaneously (Lin & Metters, 2006). Thus, in these networks, the drug release could be governed by diffusion, polymer swelling and rupture of the labile drug-polymer bonds and/or hydrophobic interactions between the drug and the polymer network (Lin & Metters, 2006). The slower release rate observed for networks containing 20% of mono-MA-β-CD would rely on their higher crosslinking density and their greater ability to form inclusion complexes. Pure pHEMA hydrogels loaded by the PP method (soaking in TSC suspension for 24 h) hosted 2380 μg TSC/g dry hydrogel (Fig. 2B). The incorporation of 10% mono-MA-β-CD resulted in a pronounced increase of the loading capacity to 3025 μg TSC/g dry hydrogel. These data suggest that the formation of TSC/CD complexes favors the incorporation and retention of additional 700 μg of TSC within the network. Further increase of the CD concentration to 20% (w/v) had a detrimental effect and the loading capacity decreased to 2050 μg TSC/g dry hydrogel, a value that was smaller than the one observed for CD-free networks. This phenomenon probably stemmed from the generation of a denser network with smaller mesh size that makes drug diffusion into the hydrogel more difficult and hampers the interactions of TSC with the network. As for the hydrogels prepared by the DP method, the amount of TSC released from pHEMA-co-β-CD depended on the proportion of mono-MA-β-CD in the order 10% > 0% > 20% of mono-MA-β-CD. SHHs hosted smaller amounts of TSC; the loading capacity being approximately 530 μg TSC/g dry hydrogel (Fig. 2C). This finding can be related to

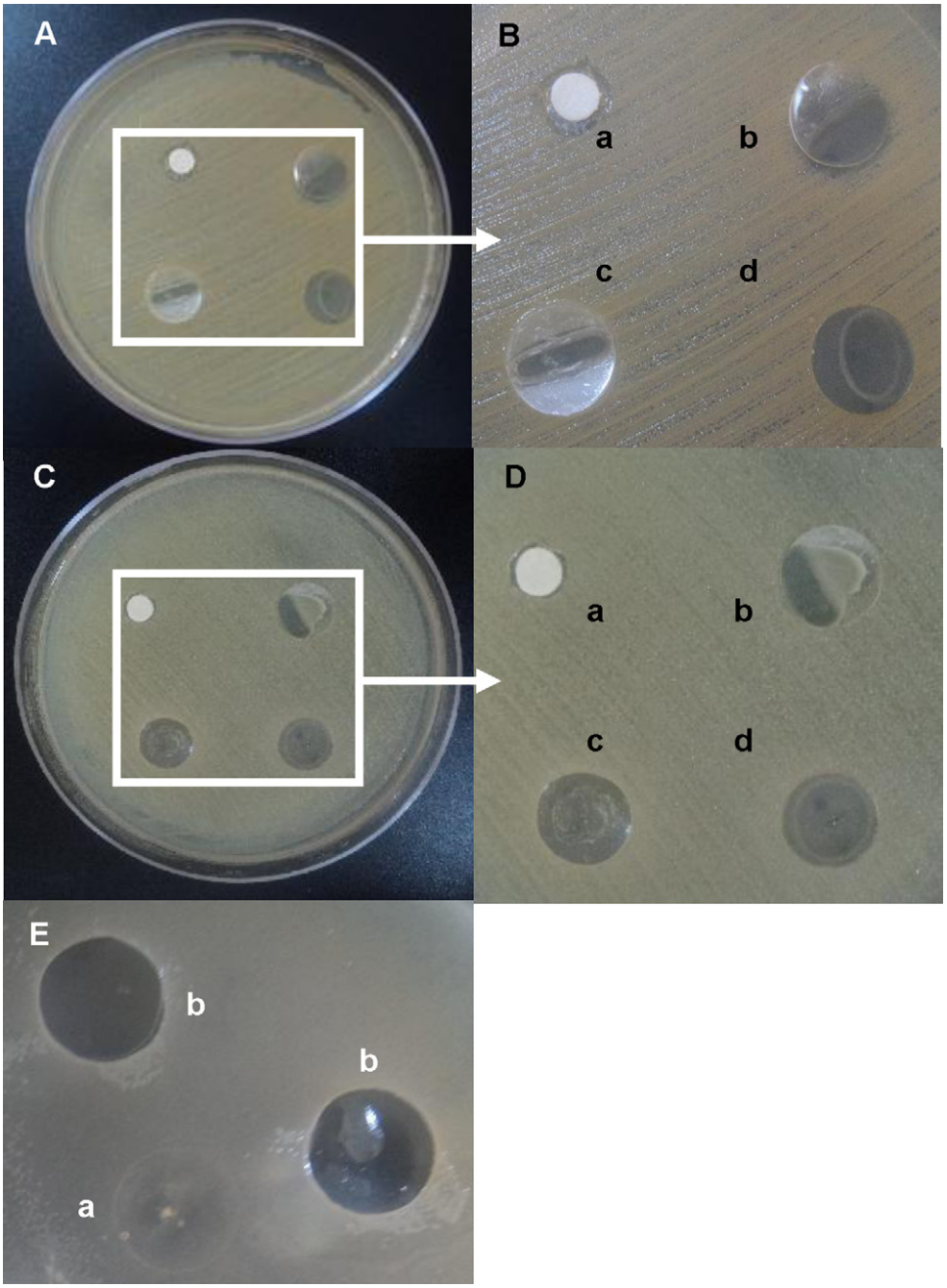


Fig. 3. Antibacterial activity in (A, B and E) *S. aureus* ATCC 6538 and (C and D) *P. aeruginosa* ATCC 9027 cultures after 24 h. (a) Paper disk loaded with 200 μg of TSC (positive control), (b) SCL without TSC (negative control), (c) TSC-loaded SCL obtained by the PP method with stirring of 24 h and (d) TSC-loaded SCL obtained by the DP method. All the SCLs are pHEMA-co-β-CD with 10% (w/v) of mono-MA-β-CD. (B and D) The magnification of (A) and (C), respectively. (E) Bacterial growth beneath the surface of (a) TSC-free and (b) TSC-loaded SCLs.

the greater hydrophilicity of the SHHs compared to the pHEMA-co-β-CD networks, which determines that this hydrophobic drug can be hosted in the network only through the formation of an inclusion complex with the CD units, and not through unspecific hydrophobic interactions. Regardless of the difference in composition, crosslinking density and swelling, HPβ-CD and HPβ-CD/HPMC showed identical loading capacities and release profiles, which confirm the role of the CD units in both processes mainly through an affinity controlled mechanism (Fig. 2C). Interestingly, the mesh size is much larger than the size of the TSC molecules and differences in porosity and swelling had only a negligible impact on the release. On the other hand, it is important to stress that pHEMA-co-β-CD and SHHs networks sustained the release of TSC for at least two weeks (Fig. 2) and maintained concentrations within the antimicrobial therapeutic range (see below). Further studies should comprise the adjustment of the TSC release amounts to ensure therapeutic efficacy and, at the same time, safety.

To gain a deeper insight into the mechanisms that govern the release of TSC from the different hydrogels, various release models were fitted to the release data (Bastakoti, Guragain, Yokoyama, Yusa, & Nakashima, 2011). In general, TSC release from pHEMA-co-β-CD SCLs followed first order release kinetics ($R^2_{adj} > 0.9365$) (Table 2). The initially rapid release could be related to the drug that is not effectively enclosed within the crosslinked matrix, but adsorbed on the surface. The slower release observed later could be attributed to the drug molecules effectively hosted into the polymeric network, through hydrophobic interactions or forming inclusion complexes with pendant CDs.

The Korsmeyer–Peppas model fitted better the release from SHHs, with R^2_{adj} of 0.9420 and 0.9713. The release exponent n was smaller than 0.5, indicating a purely diffusive release (Table 2). This finding is compatible with the fact that SHHs did not erode during the release assays and their swelling led to mesh sizes that were, most probably, remarkably larger than the size of TSC molecules to alter the release rate. Considering that pHEMA-co-β-CD SCLs showed a greater TSC loading capacity, a less pronounced burst effect and a more sustained release, the microbiological evaluation *in vitro* focused on these ODDSs.

3.5. Antibacterial activity of TSC-loaded systems

Microbiological assays were primarily performed in liquid medium to elucidate whether TSC displays antibacterial activity against two representative bacteria that are commonly associated with ocular infections. TSC displayed MICs of 128 and 256 μg/mL against *P. aeruginosa* and *S. aureus*, respectively. It is noteworthy that these values could be overestimated because of the tendency of these molecules to self-aggregate in aqueous media that leads to smaller effective concentrations (Glisoni et al., 2010; Glisoni, Chiappetta, et al., 2012; Glisoni, Cuestas, et al., 2012). Then, the activity of free TSC and the drug loaded in the SCLs was assessed in agar. pHEMA-co-β-CD networks produced with 10% w/v of monoMA-β-CD by DP and PP methods combined the greatest loading capacity of all the investigated hydrogels with optimal release kinetics that were in the range of the MICs. Thus, the study focused on these specific networks. The results showed the inhibition of bacterial growth, indicating that TSC displays antibacterial activity against both pathogens also under these more complex experimental conditions (Fig. 3A–D). On the other hand, *in vitro* release studies from the hydrogels were conducted under liquid conditions, as opposed to semi-solid agar medium where the slow release and a more limited diffusion led to a relatively small inhibition area. *In vitro* release studies in artificial lacrimal fluid indicated that approximately 150 μg of TSC per lens was released after 168 h, this amount being equivalent to 3700 μg/g of dry hydrogel (Fig. 2B). Noteworthy, the release of TSC from pHEMA-co-β-CD networks in agar was probably substantially slower than from TSC/CD complexes directly solubilized in culture medium, as recently shown during the evaluation of the activity against HCV (Glisoni, Cuestas, et al., 2012). Additional evidence that pointed out the diffusion of TSC from the hydrogel as the most critical parameter in this type of assay was related to the fact that, in TSC-free SCLs, bacteria could grow beneath the surface of the matrix that was in direct contact with the agar (Fig. 3E), while the opposite was true for TSC-loaded counterparts. These data also confirmed that the network does not display any antibacterial activity by itself. In contrast, TSC-loaded SCLs prevented bacterial proliferation.

4. Conclusion

The present work investigated the development of SCLs functionalized with CDs and loaded with a novel thiosemicarbazone that displays a broad antibiotic spectrum. The systems engineered enabled the loading of high TSC cargos and they provided sustained release for at least two weeks. Moreover, they inhibited the growth of two prominent bacteria associated with ocular infections. Nevertheless, a more comprehensive study of the antibacterial activity against specific pathogens in liquid culture medium would be required to unequivocally define the potential therapeutic boundaries of these devices.

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