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Synergistic performance of cyclodextrin–agar hydrogels for ciprofloxacin delivery and antimicrobial effect

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ABSTRACT

Combination of cyclodextrins (CDs) and agar may render hydrogels that interact with drugs by forming inclusion complexes and/or ionic bonds. Hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin (20%) in alkaline solutions containing or not agar (up to 1.5%) were cross-linked with ethyleneglycol diglycidylether at 50 °C. Agar increases hardness, compressibility and modulus of deformability, but does not interfere in the loading and release of a probe (3-methyl benzoic acid) with high affinity for CD. Agar notably promoted the loading of the zwitterion ciprofloxacin and endowed the hydrogels with ability to retain the drug in water medium. The release can be triggered by ions or changes in pH, leading to a 12-h sustained delivery. Loading and release profiles were compared to those of maltodextrin-based hydrogels, and the contribution of CDs and agar elucidated. Microbiological tests with Gram-negative and Gram-positive bacteria confirmed the suitability of the "green" CD–agar hydrogels for the development of drug delivery systems with tunable physical and drug loading/release properties.

1. Introduction

Synthetic hydrogels have found a relevant niche in the biomedical field and are being increasingly used in the last decades as components of medical devices and drug-device combination products (Hoffman, 2002; Deligkaris, Tadele, Olthuis, & van den Berg, 2010; Jagur-Grodzinski, 2010). Versatile and reproducible synthesis procedures and finely tunable mechanical features have greatly facilitated their use as biomaterials (Kopecek, 2009; Alvarez-Lorenzo & Concheiro, 2008; Johnson, Turro, Koberstein, & Mark, 2010). Nevertheless, the inherently high content in water, essential for the biocompatibility, limits the success of hydrogels as drug delivery systems. Only polar drugs can be effectively loaded in the aqueous phase of the hydrogels, and once administered to the body, the release is usually too fast for therapeutic purposes (Santos, Couceiro, Concheiro, Torres-Labandeira, & Alvarez-Lorenzo, 2008). This fact is explained by the negligible hydrodynamic hindrance to the movement of small hydrophilic drugs, due to the low microviscosity of the network (Alvarez-Lorenzo, Gomez-Amoza, Martinez-Pacheco, Souto, & Concheiro, 1999). On the other hand, concerns about environmental contamination caused by residues of synthetic hydrogels have arised (Stahl, Cameron, Haselbach, & Aust, 2000; Maia, Majcherczyk, Schormann, & Hüttermann, 2002). Differently from the monomers, synthetic polymers are in general non-toxic for the soil, but cannot be easily degraded by the microorganisms (Wen, Chen, Zhao, Zhang, & Feng, 2010). This leads to a dramatically increasing accumulation of plastic waste in the ecosystems (Sasek et al., 2006). Therefore, current design of advanced drug carriers attempts to endow the hydrogels with optimized drug loading and control of release and to make them more greenish. Since there is nothing greener than components from Nature, the use of natural components is raising increasing attention (Scott, 2000).

Polymers obtained from renewable sources are gaining interest as a way of addressing ecological concerns and also of adding value to natural products that are secondary outcomes of agriculture- or sea-related factories. The availability of techniques for a deeper characterization of these natural polymers and the improvements in the knowledge about sources of variability in their structure may pave the way for a broader use in the pharmaceutical field (Mouradi-Givernaud, Givernaud, Morvan, & Cosson, 1992; Lahaye, 2001). Marine-derived polysaccharides are attracting much attention as components of oral and topical drug dosage forms or wound dressings owing to their biodegradability, resemblance to human tissues components, and abundance (Coviello, Matricardi, Marianecci, & Alhaique, 2007; Bao, Yang, Mao, Mou, & Tang, 2008; Gomez d'Ayala, Malinconico, & Laurienzo, 2008; Laurienzo, 2010). In particular, agar or agar–agar is obtained from the cell walls of some species of red algae or seaweed and widely employed as ingredient of foods and microbial cultures due to its performance

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as thickener and stabilizer. Agar is a heterogeneous mixture of two unbranched polysaccharides: agaropectin and agarose, which share the same galactose-based backbone. Agaropectin is heavily modified with acidic side-groups, such as sulphate and pyruvate, while agarose has neutral charge and possesses longer chains (Freile-Pelegrín & Murano, 2005). Despite agar has been barely used as component of drug delivery systems, physical blends of agar and other polymers have shown suitable performance for such a purpose. For example, the blending of agar or agarose with κ-carrageenan or gelatin enables the tuning of mechanical properties of physical gels and slows down to a certain extent drug release rate (Sjöberg, Persson, & Caram-Lelham, 1999; Liu, Lin, Li, & Liu, 2005) probably due to multiple interactions in the interpenetrated networks and to competition between gelling and demixing, which can result in suspensions of gelled droplets of one component in a gelled matrix of the other or in more complex phase behaviour (Amici, Clark, Normand, & Johnson, 2002; Frith, 2010). Grafting to agar of synthetic polymers, such as poly(vinyl pyrrolidone), poly(acrylic acid) or poly(vinyl alcohol) is being used to render hydrogels for wound dressing containing antimicrobials and for agrochemical delivery (Prasad, Mehta, Meena, & Siddhanta, 2006; Varshney, 2007; Meena, Chhatbar, Prasad, & Siddhanta, 2008; Meena, Prasad, & Siddhanta, 2009; Pourjavadi, Farhadpour, & Seidi, 2009).

Since hydrogels made of natural components may be even more hydrophilic than the synthetic ones, optimization of the drug loading/release performance requires a detailed knowledge of how mesh size and drug-network interactions can be modulated. Chemical cross-linking or grafting to a substrate of polysaccharides bearing ionizable groups (Rodríguez, Alvarez-Lorenzo, & Concheiro, 2003a,b; Wang & Zhang, 2010) or of the oligosaccharide-based cyclodextrins (CDs) (Demir, Kahraman, Bora, Apohan, & Ogan, 2008; Zhang, Xue, Gao, Huang, & Zhuo, 2008; Mocanu, Mihai, LeCerf, Picton, & Moscovici, 2009; Santos et al., 2009) has been shown able to improve the affinity of natural and synthetic hydrogels for specific drugs. CDs are known to form inclusion complexes with a large number of drugs by hosting the molecules totally or partially in the apolar cavities (Loftsson & Duchene, 2007). The affinity of the drug for the CD cavities endows the hydrogels with an unique mechanism to control drug loading and delivery (Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez, Concheiro, & Torres-Labandeira, 2007; Rodriguez-Tenreiro, Diez-Bueno, Concheiro, Torres-Labandeira, & Alvarez-Lorenzo, 2007; Vyas, Saraf, & Saraf, 2008; Otero-Espinar, Torres-Labandeira, Alvarez-Lorenzo, & Blanco-Mendez, 2010). To do that, the CDs have to be fixed to the network and they should still be able to form inclusion complexes with the drug of interest. Among the many procedures reported to prepare CD-based hydrogels, condensation with ethyleneglycol diglycidylether (EGDE) enables the obtaining of CD networks in one step without previous derivatization of the CD unities with acrylic moieties (Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez, Concheiro, & Torres-Labandeira, 2006). EGDE has two epoxy groups that react with the hydroxyl groups of CDs and of certain polysaccharides (Rodríguez, Alvarez-Lorenzo, & Concheiro, 2003a). This cross-linking method does not require any modification in the CD structure and takes places in aqueous medium under mild conditions, being environmentally friendly (Rodriguez-Tenreiro, Alvarez-Lorenzo, et al., 2007; Moya-Ortega, Alvarez-Lorenzo, Sigurdsson, Concheiro, & Loftsson, 2010).

The aim of this work was to combine the features of CDs and agar to render chemically cross-linked hydrogels that can interact with drugs by forming inclusion complexes and/or ionic bonds with the polysaccharide and that may be suitable for medicated wound dressings or oral drug delivery systems. This may result in an enhanced loading ability and dually controlled delivery; the release profile being regulated by the affinity of the drug for the CD and tunable by external stimuli, such as the pH or the ionic strength of the physiological environment. Ciprofloxacin was chosen as a model of antimicrobial agent that can form inclusion complexes with βCD derivatives in solution or after being immobilized on medical devices (Chao, Meng, Li, Xu, & Huang, 2004; El Ghoul, Blanchemain, Laurent, Campagne, El Achari, Roudesli, Morcellet, Martel, & Hildebrand, 2008; Blanchemain, Laurent, Chai, Neut, Haulon, Krump-konvalinkova, Morcellet, Martel, Kirkpatrick, & Hildebrand, 2008). This wide-spectra fluoroquinolone behaves as zwitterion (Hernández-Borrel & Montero, 1997) and, consequently, its ability to interact ionically may be greatly dependent on the pH of the medium. Hydrogels with a fix content in hydroxypropyl-β-cyclodextrin (HPβCD) or methyl-β-cyclodextrin (MβCD) and various proportions of agar were prepared and the influence of agar on the degree of swelling and the mechanical properties was firstly evaluated. Loading and release behaviour of ciprofloxacin was compared to that of a probe molecule that only interacts with the hydrogel forming inclusion complexes. Furthermore, the replacement of the βCD derivatives by a maltodextrin was tested to gain insight into the contribution of CD–drug complex formation to the uptake and the control of release. Finally, the antimicrobial performance of the hydrogels was tested in vitro against common pathogens of skin and mucosa.

2. Materials and methods

2.1. Materials

Agar–agar (10,000 cPs) was from Guinama (Spain). Hydroxypropyl-β-cyclodextrin (HPβCD, KLEPTOSE® HPB, M.S. 0.65), methyl-β-cyclodextrin (MβCD; KLEPTOSE® CRYSMEB; 4 methyl groups per native cyclodextrin molecule, M.S. 0.57) and maltodextrin (MX, Glucidex® 12D) were from Roquette (France). Ethyleneglycol diglycidylether (EGDE, 50% in water) was from Fluka (Germany), ciprofloxacin·HCl from Fagron (Spain) and 3-methylbenzoic acid (3-MBA) from Merck (Germany). Purified water with resistivity above 18.2 MΩ cm^{-1} was obtained by reverse osmosis (MilliQ®, Millipore Spain). All other reagents were of analytical grade.

2.2. Agar elemental analysis and intrinsic viscosity

The elemental composition of agar was determined using a Thermo Finnigan Elementary Analyzer Flash EA 1112 (Waltham MA, USA). The viscosity of 0.05, 0.075, 0.10, 0.15, 0.20 and 0.30 g/dl agar dispersions in 0.21 M NaOH was measured in triplicate at 37 °C in a Cannon-Fenske capillary viscometer (Afora, Barcelona, Spain). Intrinsic viscosity ([η]) was estimated by fitting Huggins equation to the results thus obtained (Singh, Bohidar, & Bandyopadhyay, 2007). The critical overlapping and the entanglement concentration were calculated as the reciprocal of the intrinsic viscosity multiplied by 1 or 10, respectively (Morris, Cutler, Ross-Murphy, & Rees, 1981).

2.3. Hydrogel synthesis

HPβCD or MβCD (2 g) were dissolved in freshly prepared 0.21 M NaOH solutions (10 ml) without agar or containing 1% or 1.5% agar. Dispersions with MX were similarly prepared, replacing HPβCD or MβCD by MX. After homogenization, EGDE (5 ml) was added to each dispersion and stirred for 5 min at 20 °C. The dispersions were transferred to test tubes (8.6 mm internal diameter), which were hermetically closed and kept at 50 °C for 24 h. Then, the CD, MX, CD–agar and MX–agar hydrogels were carefully removed from the moulds, and placed in 10 mM HCl (250 ml) for 12 h and latter in water (250 ml), replacing the medium several times for 1 week. Finally, cylindrical pieces of each gel (4–5 mm thick) were

Table 1
Composition upon synthesis of the hydrogels prepared, swelling percentage and textural parameters, including modulus of deformability (ED), once swollen in water. Mean values and, in parenthesis, standard deviations.

Hydrogel	CD or MX (%w/w)	Agar (%w/w)	EGDE (%w/w)	Swelling (%)	Hardness (N)	Compressibility (N mm)	ED (kPa)
HPβCD	11.26	0	16.20	905 (68)	4.5 (0.1)	5.0 (0.2)	0.065 (0.001)
MβCD	11.26	0	16.20	866 (5)	3.1 (0.1)	4.0 (0.1)	0.037 (0.001)
MX	11.26	0	16.20	268 (7)	27.4 (0.6)	21.5 (2.4)	2.46 (0.24)
HPβCD + agar 1%	11.26	0.56	16.20	554 (17)	6.3 (0.1)	5.9 (0.9)	0.113 (0.012)
MβCD + agar 1%	11.26	0.56	16.20	698 (37)	10.0 (0.3)	12.1 (0.3)	0.118 (0.002)
MX + agar 1%	11.26	0.56	16.20	249 (6)	19.0 (0.3)	21.1 (3.3)	1.72 (0.19)
HPβCD + agar 1.5%	11.26	0.84	16.20	582 (25)	7.6 (0.3)	7.2 (0.5)	0.117 (0.005)
MβCD + agar 1.5%	11.26	0.84	16.20	685 (46)	10.3 (1.1)	12.8 (1.4)	0.124 (0.012)
MX + agar 1.5%	11.26	0.84	16.20	246 (9)	31.6 (0.3)	33.4 (3.2)	2.46 (0.29)

cut and maintained in water. Elemental analysis of dried pieces of the hydrogel was carried out as described above.

2.4. FTIR spectra

FTIR-ATR spectra of agar, HPβCD, MβCD, MX, and dried hydrogels were recorded using a Varian 670IR (Varian, Inc., Santa Clara, CA, USA) equipment fitted with universal ATR sampling accessory (PIKE MIRacle crystal, which is composed of a diamond ATR with a zinc selenide focusing element in direct contact with the diamond).

2.5. Degree of swelling

Hydrogel disks were weighed (W_t) and dried in an oven along 3 days, raising the temperature from 40 to 70 °C. Then they were weighed again (W_0). The degree of swelling was estimated as:

$$Q(\%) = \frac{(W_t - W_0)}{W_0} \times 100 \quad (1)$$

CD and CD–agar hydrogel disks dried under the above described conditions were immersed in 10 ml of water at 4 °C or 37 °C. The weight was determined at pre-established time intervals. The degree of swelling was estimated again using Eq. (1).

2.6. Hardness and compressibility

Texture properties were determined (in duplicate) at room temperature using a TA-TX Plus Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK) with a cylindrical aluminum probe (Ref. P/20). The experiments were carried out by compressing disks of 5 mm thickness with the probe at a rate of 1 mm/s and to a depth of 2 mm. The stress and the displacement were recorded. Then, the probe was removed and the recovery of the sample was also monitored. The hardness was estimated as the maximum resistance to compression (i.e., the peak value in the force–distance plot), and the compressibility was quantified as the work carried out in the compression (i.e., the area under the force–distance plot) (Andrews & Jones, 2006). The modulus of deformability, ED, was estimated as the slope of the initial linear portion of the stress–strain plot, converting the force to a true stress:

$$\sigma_T = \frac{F(t)[h_0 - \Delta h]}{A_0 h_0} \quad (2)$$

and the distance to Hencky's strain:

$$\varepsilon_T = \ln\left(\frac{h_0}{h_0 - \Delta h}\right) \quad (3)$$

In these equations h_0 is the original height of sample, Δh is the change in height, $F(t)$ the compressive force at time t, and A_0 the original cross-sectional area (Konstance, 1993).

2.7. 3-MBA loading and release

Hydrogels were immersed in 10 ml of 3-MBA aqueous solution (0.125 mg/ml) at 25 °C protected from light. The absorbance of the medium was periodically recorded at 281 nm (Agilent 8453, Boeblingen, Germany) and the total amount of 3-MBA taken up by the hydrogels was calculated as the difference between the initial and the final amounts of 3-MBA in the solution. The experiments were carried out in triplicate. The amount loaded into the aqueous phase of the network, due to equilibrium between the solute concentration in the loading solution and that in the aqueous phase of the hydrogel, can be estimated using the following equation (Kim, Bae, & Okano, 1992):

$$\text{Loading}_{(\text{aq.phase})} = \frac{V_s}{W_p} \cdot C_0 \quad (4)$$

where V_s is the volume of water sorbed by the hydrogel, W_p the dried hydrogel weight, and C_0 the solute concentration in the loading solution. The 3-MBA-loaded disks were carefully wiped with a paper and immersed in water. The absorbance at 281 nm was recorded again for 48 h and then the disks were transferred to pH 7.4 phosphate buffer at 37 °C and the absorbance monitored at 275 nm for 5 days.

2.8. Ciprofloxacin loading and release

Hydrogel disks (4–5 mm thick, 8.6 mm in diameter) were individually placed in 10 ml of ciprofloxacin aqueous solution at 25 °C. Two drug concentrations were tested, i.e., 0.010 mg/ml and 0.050 mg/ml. The absorbance of the solution was periodically recorded at 276 nm (Agilent 8453, Boeblingen, Germany) for 5 days. The total amount of drug loaded was estimated from the difference between the initial amount of drug in the solution and that remaining 5 days latter. All experiments were performed in triplicate and repeated twice. The amount of drug hosted in the aqueous phase of the hydrogels was estimated applying Eq. (4). Release experiments were carried out in triplicate at 37 °C under three different conditions. Those hydrogels loaded in 0.010 mg/ml ciprofloxacin solution were immersed in 5 ml of water for 48 h and then the medium was replaced by 5 ml of pH 7.4 phosphate buffer. Those hydrogels loaded in 0.050 mg/ml ciprofloxacin solution were directly immersed in HCl 0.1 N or pH 7.4 phosphate buffer; the volume of release medium being 20 ml for CD and MX hydrogels and 40 ml for CD–agar and MX–agar hydrogels. 1-ml samples were periodically taken, the absorbance measured, and immediately returned to the corresponding vial.

2.9. In vitro microbiological tests

Dried CD and CD–agar hydrogel disks (five replicates) were immersed in 0.050 mg/ml ciprofloxacin solution for 48 h at 25 °C. Then, they were carefully centered on Petri plates containing

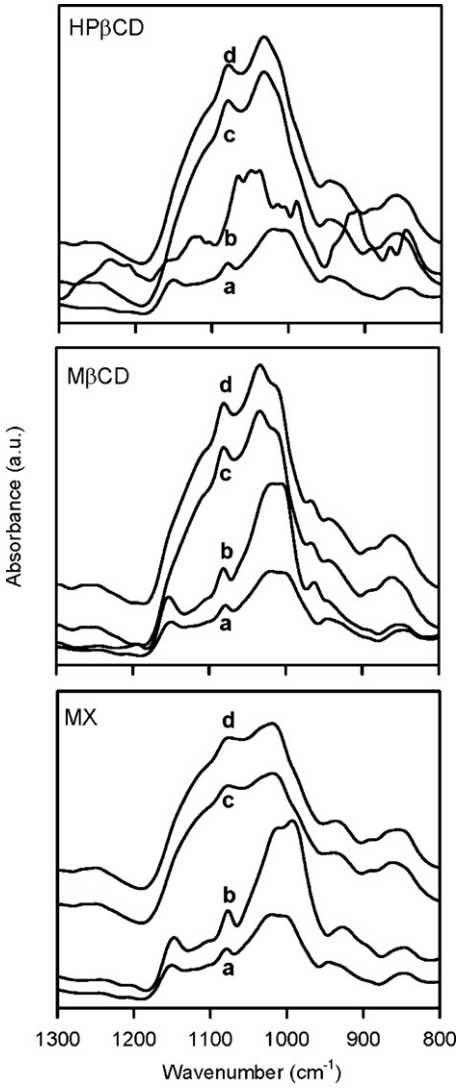

Fig. 1. FTIR spectra of agar (a, in all plots), HPβCD, MβCD, and MX pure components (b), and HPβCD, MβCD, and MX hydrogels without agar (c) or with agar 1.5% (d).

Müeller Hinton-agar medium previously seeded with *Pseudomonas aeruginosa* (CECT 110), *Edwardsiella tarda* (NCIMB 2034), *Escherichia coli* (FV9180), *Staphyloccocus aureus* (ATCC 25923) or *Staphylococcus epidermidis* (CECT 4184) suspensions (10^9 CFU/ml). The Petri plates were incubated for 48 h at 37 °C and the inhibition zones measured after 24 and 48 h.

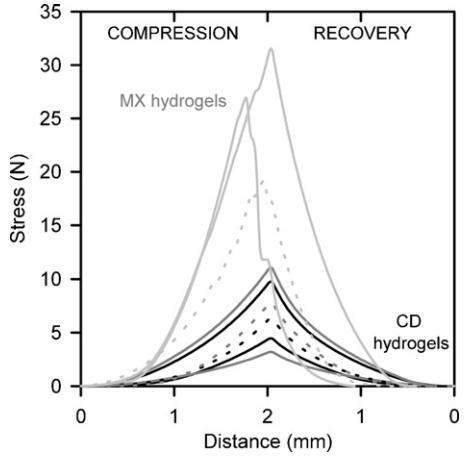

Fig. 2. Force–displacement curves obtained for swollen HPβCD (black lines), MβCD (dark grey lines), and MX (clear grey lines) hydrogels without agar (lower continuous line), with agar 1% (dotted line) or with agar 1.5% (upper continuous line).

3. Results and discussion

3.1. Preparation of CD–agar and MX–agar hydrogels

The composition of the hydrogels upon synthesis is summarized in Table 1. Agar solely solution did not lead to hydrogels at the concentrations evaluated (1 and 1.5%) probably because the proportions were too low for entanglement and the repulsion between acidic side-groups of agaropectine may make the reaction with EGDE difficult. In fact, the intrinsic viscosity of agar in the alkaline medium used to prepare the hydrogels was 3.58 (0.02) dl/g, which means that the critical overlapping concentration is about 0.28% and the entanglement concentration ca. 2.8%. Higher proportions were tested, but the high viscosity of the agar solutions hindered the homogeneous distribution of the cross-linker and no hydrogels were obtained either. On the other hand, anionic repulsions have been found to hinder the cross-linking of sulfobutyl-β-cyclodextrin solely with EGDE, even at so high concentration as 20% (Rodriguez-Tenreiro, Alvarez-Lorenzo, et al., 2007). Agar is a poly-anionic polysaccharide and the batch used to prepare the hydrogels had a content in sulphur of 5.4 (0.2)%, which indicates that it is heavily substitute with sulphate groups that can make difficult the

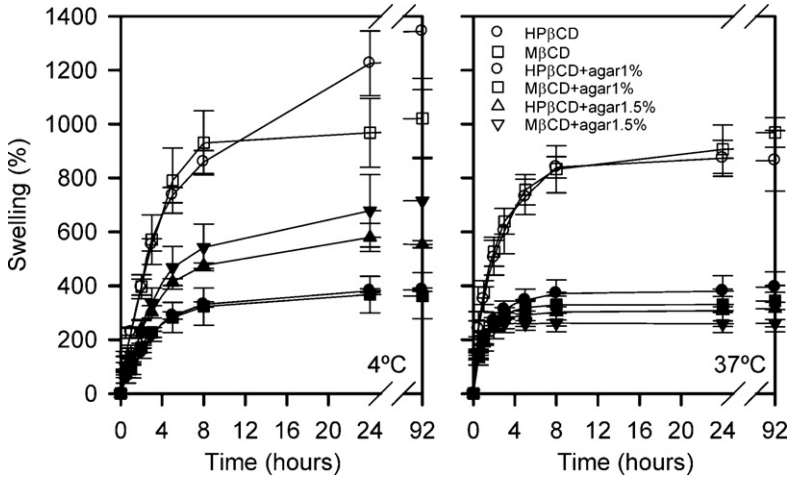

Fig. 3. Swelling kinetics of CD and CD–agar hydrogels in water at 4 and 37 °C.

Table 2
Total amount of 3-MBA loaded by the hydrogels, loading in the aqueous phase and the network/water partition coefficient ($K_{N/W}$) at 72 h. Mean values and, in parenthesis, standard deviations.

Hydrogel	3-MBA total loading		Loading in the aqueous phase (mg/g)	$K_{N/W}$
	mg/g	μmol/g		
HPβCD	5.9 (0.3)	43.4 (1.9)	1.05 (0.13)	41.2 (1.2)
MβCD	6.5 (0.1)	48.0 (1.0)	1.00 (0.03)	46.9 (1.1)
MX	1.6 (0.1)	11.7 (0.9)	0.31 (0.00)	10.9 (1.0)
HPβCD + agar 1%	4.5 (0.6)	32.8 (4.2)	0.61 (0.13)	32.6 (3.7)
MβCD + agar 1%	5.5 (0.4)	40.6 (2.7)	0.80 (0.02)	40.2 (3.2)
MX + agar 1%	1.0 (0.1)	7.0 (0.4)	0.29 (0.00)	5.7 (0.5)
HPβCD + agar 1.5%	4.8 (0.2)	35.0 (1.1)	0.70 (0.01)	34.6 (1.3)
MβCD + agar 1.5%	5.9 (0.1)	43.3 (0.4)	0.80 (0.02)	43.3 (0.3)
MX + agar 1.5%	0.9 (0.1)	6.5 (0.7)	0.29 (0.00)	5.1 (0.8)

cross-linking. Differently, 20% HPβCD and MβCD solutions with or without agar did render viscoelastic hydrogels that resist handling without breaking. The proportion of EGDE was chosen to be sufficient to react with all hydroxyl groups present in the βCD structure. This CD/EGDE ratio enabled to complete the cross-linking process in a few hours under the synthesis conditions applied (Rodriguez-Tenreiro et al., 2006). Thus, several sets of hydrogels were prepared with a fix content in CD and EGDE but various agar proportions (0, 1, and 1.5%). CD solely hydrogels were totally transparent to the visible light, while the presence of agar made the hydrogels slightly opalescent. When the CDs were replaced by maltodextrin (MX), brownish and more brittle hydrogels were obtained. Differently from the cyclic structure of CDs, MX Glucidex® 12D is formed by linear polysaccharides with a dextrose equivalent of 11–14. The molecular weight of MX Glucidex® 12D is roughly 16,000 Da, i.e., 12–16 times larger than that of HPβCD and MβCD. The relatively long linear chains of MX may find it easier to entangle each other and with agar and to react with EGDE, giving harder networks.

The degree of cross-linking of the hydrogels was estimated by comparison of the FTIR absorbance of secondary to the primary hydroxyl groups ratio of the CDs and MXs before and after reaction with EGDE. FTIR spectra of HPβCD and MβCD raw materials (Fig. 1) showed 1078/1030 cm^{-1} absorbance ratios of 0.63 and 0.53, respectively, while for MX the 1076/1018 cm^{-1} absorbance ratio was 0.53. The HPβCD and MβCD hydrogels exhibited an increase in the absorbance ratio up to 0.88 and 0.84, respectively, which indicates that the etherification with EGDE notably reduces the number of primary hydroxyl groups (Rodriguez-Tenreiro et al., 2006). In the presence of agar (disregarding its concentration) the ratio slightly increased (0.90 and 0.85, respectively). This means that most available hydroxyl groups in the CD and probably some in the agar structure are efficiently linked to others through the EGDE spacer. FTIR spectra of agar and CDs almost overlap, preventing the identification of specific signal of agar in the hydrogels spectra. MX solely hydrogels exhibited an absorbance ratio of 0.91, which increased up to 0.95 in the presence of agar. Therefore, the relative number of hydroxyl groups that reacted with EGDE was greater in the case of MX hydrogels, suggesting a larger degree of cross-linking. No peak at 1250 cm^{-1} was recorded indicating that no free EGDE remains in the network. Elemental analysis of the hydrogels did not enable to quantify the content in sulphur because of the low proportion of agar in the network. If all agar were effectively linked into the network, the content in sulphur of those hydrogels made with the highest agar concentration would be 0.16%, which is too low for an accurate quantification by elemental analysis (detection limit is around 0.3%). Therefore, the presence of agar can be indirectly perceived by the effect of agar on the physical features of the hydrogels and their drug loading ability.

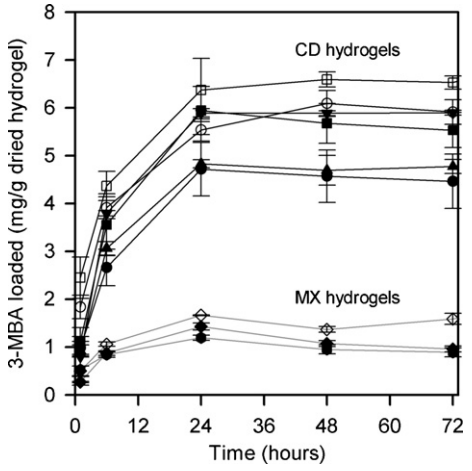

Fig. 4. 3-MBA loading profile obtained during immersion in 0.125 mg/ml 3-MBA solution of HPβCD (open circles), MβCD (open squares), MX (open diamond), HPβCD + agar 1% (solid circles), MβCD + agar 1% (solid squares), MX + agar 1% (solid diamond), HPβCD + agar 1.5% (solid up triangles), MβCD + agar 1.5% (solid down triangles) and MX + agar 1.5% (solid hexagon) hydrogels. Black lines correspond to CD-based hydrogels and grey lines to MX-based hydrogels.

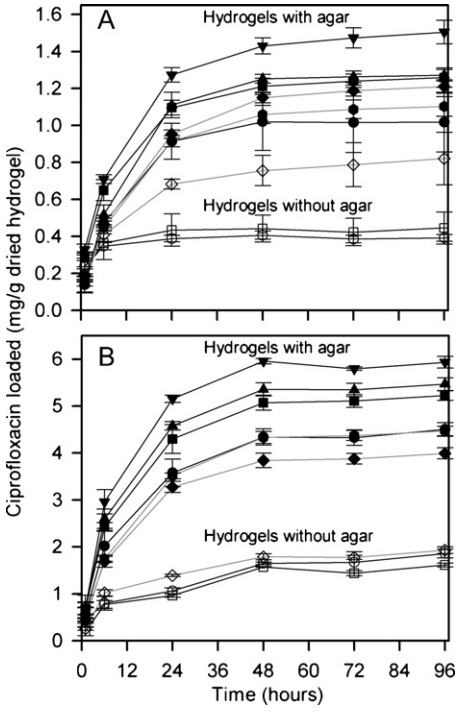

Fig. 5. Ciprofloxacin loading profiles of hydrogels immersed in 0.01 mg/ml (A) and 0.05 mg/ml (B) ciprofloxacin solution. Symbols and lines as in Fig. 4.

Table 3
Total amount ciprofloxacin loaded by the hydrogels, loading in the aqueous phase and the network/water partition coefficient. Mean values and, in parenthesis, standard deviations.

Hydrogel	Ciprofloxacin total loading in 0.01 and 0.05 mg/ml		Loading in the aqueous phase in 0.01 and 0.05 mg/ml		$K_{N/W}$	
	μmol/g	μmol/g	mg/g	mg/g		
HPβCD	1.1 (0.1)	5.1 (0.2)	0.087 (0.005)	0.46 (0.03)	33 (4)	33 (4)
MβCD	1.1 (0.2)	4.4 (0.3)	0.084 (0.005)	0.44 (0.02)	35 (6)	29 (4)
MX	2.2 (0.4)	5.1 (0.1)	0.027 (0.001)	0.13 (0.00)	82 (15)	38 (2)
HPβCD + agar 1%	2.6 (0.4)	12.5 (0.6)	0.052 (0.011)	0.22 (0.04)	96 (16)	88 (11)
MβCD + agar 1%	3.3 (0.2)	14.6 (0.4)	0.067 (0.002)	0.36 (0.02)	121 (6)	103 (6)
MX + agar 1%	3.1 (0.1)	11.0 (0.4)	0.024 (0.001)	0.13 (0.04)	120 (4)	83 (2)
HPβCD + agar 1.5%	2.2 (0.1)	15.2 (0.4)	0.056 (0.003)	0.29 (0.02)	121 (3)	111 (4)
MβCD + agar 1.5%	3.9 (0.2)	15.6 (0.5)	0.066 (0.002)	0.35 (0.03)	145 (7)	114 (12)
MX + agar 1.5%	2.8 (0.1)	11.7 (0.2)	0.024 (0.001)	0.12 (0.00)	108 (5)	92 (4)

3.2. Hardness and compressibility

A compression test was carried out to characterize the hardness and compressibility of disks of hydrogels of 8.6 mm in diameter, i.e., the surface in contact with the probe was 58.09 mm^2 (Fig. 2). HPβCD and MβCD hydrogels deformed quite easily under the pressure of the probe (compression step in Fig. 2), but were able to recover their initial shape when the probe was removed (recovery step in Fig. 2). Incorporation of agar made the hydrogels to remarkably increase the hardness and the compressibility; the higher the agar proportion, the greater the strength of the network (Table 1). This means that agar is indeed involved in the cross-linking, reinforcing the mechanical features of the network. MX hydrogels showed notably higher hardness and compressibility, which is in agreement with their greater degree of cross-linking. MX solely networks did not stand well the pressure and cracks occurred, which explains the drop in the force–distance profile. Agar provided certain elasticity to the hydrogels, and full recovery was observed for MX–agar 1.5% hydrogels.

Since the cross-sectional area and length of the hydrogel disks do change substantially when force is applied, the Young's modulus cannot be accurately calculated from the slope of the force–distance plot (Moya-Ortega, Alvarez-Lorenzo, Sigurdsson, Concheiro, & Loftsson, 2010). Thus, a modulus of deformability, ED, was estimated using the Hencky's model, in which the true stress is obtained by correcting the engineering stress ($F(t)/A_0$) to account for cross-sectional area expansion of the deformed specimen (Konstance, 1993). ED is an index of the stiffness and has been widely used for characterizing hydrogels and soft materials of varied nature (Konstance, 1993). The ED values (Table 1) of HPβCD and MβCD hydrogels increased 2-fold when agar was incorporated to the networks. As expected, MX hydrogels both without or with agar exhibited notably higher values.

3.3. Degree of swelling

All hydrogels behaved as superabsorbent being able to uptake various times their weight in water (Table 1), although the presence of agar in the HPβCD and MβCD hydrogels reduced considerably the water incorporating capability. Replacement of CD with MX led to a lower degree of swelling at equilibrium and no significant effect of agar was recorded. This finding confirms the higher degree of cross-linking and consequently lower mesh size of MX networks.

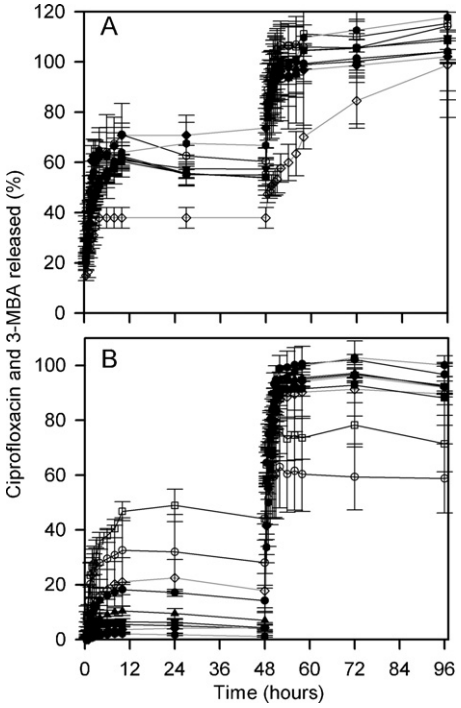

Fig. 6. 3-MBA (A) and ciprofloxacin (B) release profiles in water (first 48 h) and in pH 7.4 phosphate buffer from hydrogels. Symbols and lines as in Fig. 4.

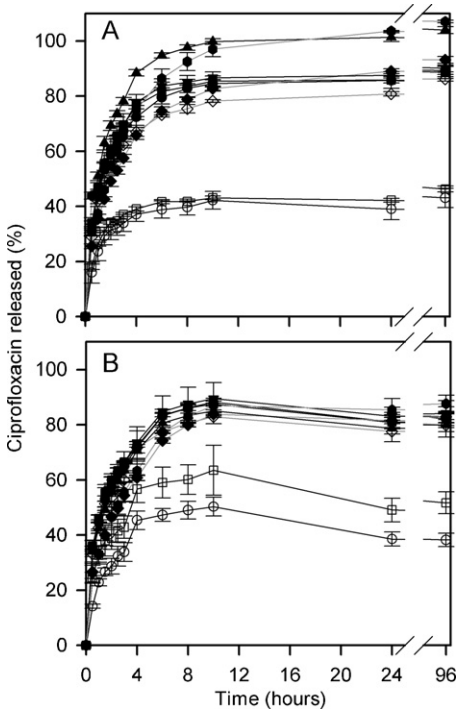

Fig. 7. Ciprofloxacin release profiles in HCl 0.1 N (A) and pH 7.4 phosphate buffer (B) from hydrogels. Symbols and lines as in Fig. 4.

Table 4
Inhibition zone diameter (mm) caused by the ciprofloxacin-loaded hydrogels.

Microorganism	HPβCD		MβCD		HPβCD + agar 1%		MβCD + agar 1%		HPβCD + agar 1.5%		MβCD + agar 1.5%	
Hours	24	48	24	48	24	48	24	48	24	48	24	48
P. aeruginosa	25	28	33	35	34	35	32	32	32	30	32	35
E. tarda	35	39	40	45	45	47	44	47	44	47	44	48
E. coli	30	33	34	34	35	38	35	36	36	37	36	37
S. aureus	–	25	–	27	–	34	–	34	–	28	–	31
S. epidermidis	28	35	35	38	37	43	40	41	38	40	39	42

Regarding the swelling kinetics, MX hydrogels did not stand well successive drying and swelling cycles, breaking apart into small pieces. Therefore, swelling kinetics could only be precisely recorded for CD and CD–agar hydrogels. HPβCD and MβCD hydrogels showed a fast swelling during the first 4 h, and the equilibrium was attained after approximately 8 h (Fig. 3). The relatively slow water uptake can be attributed to the collapse of the network during drying at air, which causes the porosity to decrease and consequently makes the diffusion of water into the network difficult. A small influence of temperature on the degree of swelling was noticed; the uptake of water being faster although slightly lower at 37 °C than at 4 °C. As temperature raises, water diffusion coefficient increases, but the ability of polysaccharides to interact with water molecules forming hydrogen bonds diminishes (Savage, 1971). An increase in temperature from 4 °C to 37 °C may also make the chains to rearrange establishing hydrophobic interactions (Sandolo, Matricardi, Alhaique, & Coviello, 2007) and, consequently, the degree of swelling decreases.

3.4. Loading of 3-MBA and ciprofloxacin

3-MBA has a high affinity for βCD (stability constant of the complex $1.3 \times 10^7\ M^{-1}$) and, thus, its uptake can be used as an index of the amount of CDs in the hydrogel that keep their ability to form inclusion complexes (Fundueanu et al., 2003). HPβCD and MβCD hydrogels incorporated larger amounts of 3-MBA than MX ones (Fig. 4 and Table 2). The presence of agar caused minor changes in the loading ability of MβCD hydrogels, but a more evident decrease in that of HPβCD networks. This finding can be related to the decrease in swelling caused by agar, which makes the movement of the probe towards the inner HPβCD units more difficult.

Although MX is commonly used as a control to elucidate if a drug is loaded by inclusion complex formation with CDs or by unspecific hydrophobic interactions with the sugar rings (Tabary et al., 2007), the differences in saccharide composition and molecular weight between MX and CD have to be considered when conclusions are stated. Particularly, a lower loading could be directly related to a smaller degree of swelling, i.e., less volume of aqueous phase, and not to a lower affinity of MX network for 3-MBA. Thus, to elucidate the relative contribution of swelling and drug interactions of CD-based and MX-based hydrogels on the distribution of the 3-MBA molecules (i.e., free in the aqueous phase or interacting with the network), we determined for each hydrogel the amount of 3-MBA in the aqueous phase applying Eq. (4), and the partition coefficient, $K_{N/W}$, between the polymer network and the loading solution using the following equation (Kim, Bae, & Okano, 1992):

$$\text{Loading}_{(\text{total})} = \frac{V_s + K_{N/W} \cdot V_p}{W_p} \cdot C_0 \quad (5)$$

where V_p is the volume of dried polymer and the other symbols maintain the same meaning as in Eq. (4).

As expected from the swelling data, MX hydrogels cannot host so many molecules of 3-MBA in the aqueous phase as CD hydrogels do (Table 2). Nevertheless, the differences in the total amount loaded cannot be attributed only to the degree of swelling, but to the larger $K_{N/W}$ values exhibited by the CD hydrogels. In the absence of agar, $K_{N/W}$ values of HPβCD and MβCD hydrogels were 4-fold those of MX ones (Table 2). The differences became even larger as the content in agar increased. This means that the high affinity of 3-MBA for the CD cavities prompts the effective hosting in the hydrogels through inclusion complex formation. Such a strong interaction (particularly intense in the case of MβCD hydrogels) is not disturbed by the presence of agar. Oppositely, 3-MBA molecules interact with the MX chains more weakly than with the CD rings, and the hydrophobic interaction is altered by the presence of the hydrophilic agar chains. Unspecific hydrophobic interactions between polysaccharide derivatives (cellulose ethers) and hydrophobic drugs (ibuprofen, diclofenac) have been previously reported (Rodríguez, Alvarez-Lorenzo, & Concheiro, 2003b; Rodriguez-Tenreiro et al., 2006).

Loading tests of ciprofloxacin were carried out similarly by immersion of the hydrogels in 0.01 and 0.05 mg/ml drug solutions, in order to evaluate the influence of the drug concentration. The amounts of ciprofloxacin loaded by HPβCD and MβCD hydrogels were notably lower than the values achieved for 3-MBA (Fig. 5A and B, see total loading as μmol/g in Table 3), probably because the lower affinity constant of the complexes (stability constant values of 2.78×10^2 and $3.43 \times 10^2\ M^{-1}$ for βCD and HPβCD, respectively; Chao, Meng, Li, Xu, & Huang, 2004; Jianbin, Liang, Hao, & Dongpin, 2002) and the lower concentration of the drug solution compared to that of 3-MBA (0.125 mg/ml). This indicates that most CDs in the hydrogel remain vacant (at least temporally, as a dynamic equilibrium of the drug with adjacent CDs can be established) once immersed in the ciprofloxacin solution, although the loading was greater as the drug concentration became higher. When drug concentration increased 5-fold, the amount loaded raised between 4- and 5-fold, as expected from Eq. (4). However, differently from 3-MBA, the presence of agar (despite its low proportion) enhanced 2- and 3-fold the ciprofloxacin loading. This finding suggests that the ionic interactions between ciprofloxacin and the acidic groups of agar promoted drug uptake. At the pH of loading solution (slightly acid), the acidic agar groups are negatively charged and ciprofloxacin appears like a zwitterion. Thus, ionic interactions can be established between agar and the protonized amine of ciprofloxacin, increasing the amount of drug loaded compared to CD solely hydrogels. According to the loading data summarized in Table 3, agar increased roughly 10 μmol/g the amount of ciprofloxacin loaded. If the content in sulphur of those hydrogels was 0.16%, there would be 5×10^{-5} moles of sulphate groups per gram of dry network (i.e., 50 μmol/g). Thus, the proportion of agar present in the hydrogels can explain by itself the increase in loading and also suggest that the hydrogels have not reached the saturation value.

MX hydrogels without agar behaved apparently as the CD ones, loading similar amounts of ciprofloxacin. All hydrogels containing agar showed $K_{N/W}$ values 3–4 times higher than those made solely of CD or MX (Table 3); the partition coefficient being also much larger than that recorded for 3-MBA (Table 2). These results indicate that the presence of agar notably enhances the affinity of ciprofloxacin for the network (due to ionic interactions), being

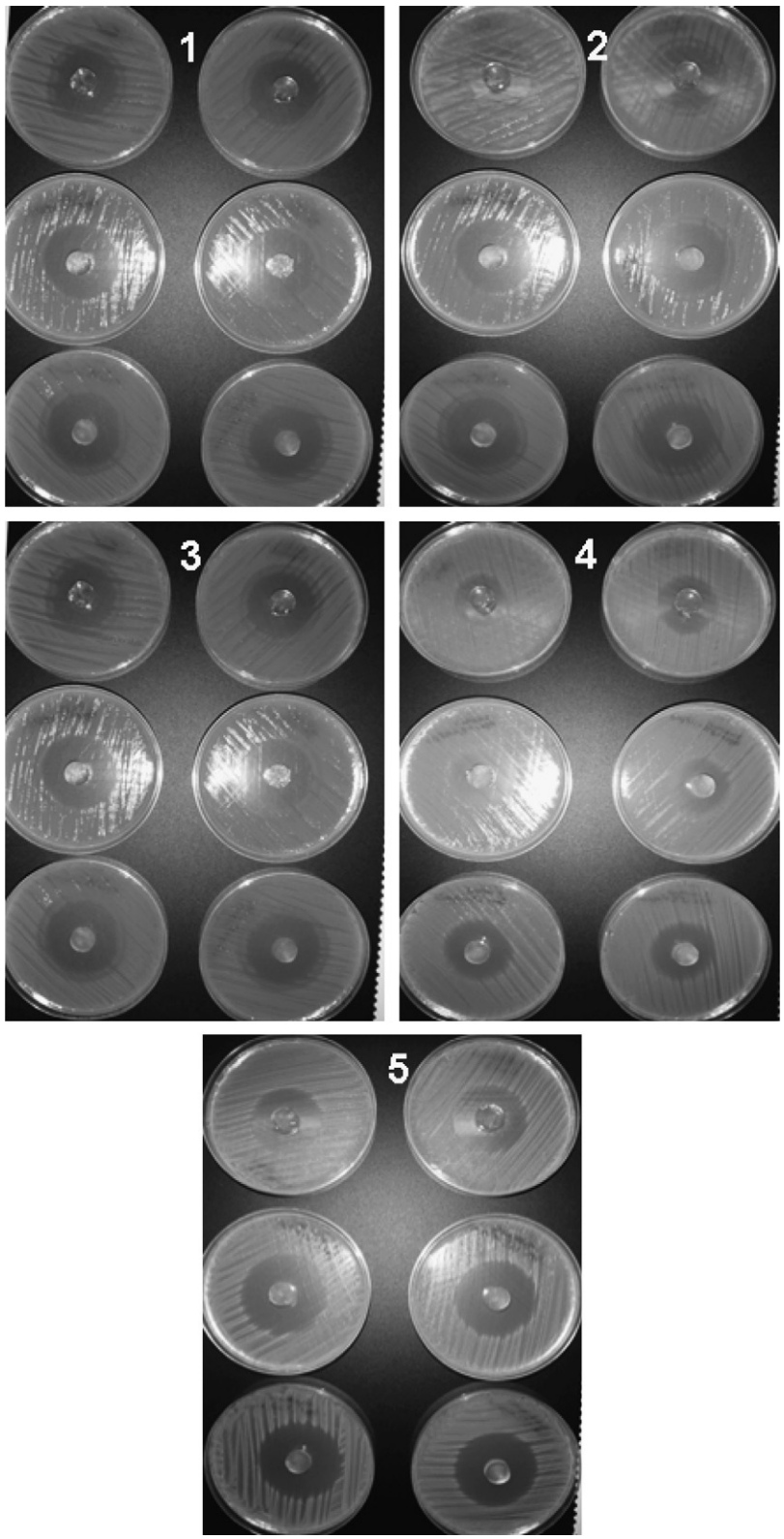

Fig. 8. Zones of inhibition against *Pseudomonas aeruginosa* (1), *Edwardsiella tarda* (2), *Escherichia coli* (3), *Staphylococcus aureus* (4), and *Staphylococcus epidermidis* (5) after 48 h on Müller–Hinton agar plates with ciprofloxacin-loaded hydrogels. From up left to bottom right: HPβCD, MβCD, HPβCD + agar 1%, MβCD + agar 1%, HPβCD + agar 1.5%, and MβCD + agar 1.5% hydrogels.

larger than that exhibited by CD and MX hydrogels (which can only establish inclusion complexes or weak hydrophobic interactions, respectively). Thus the combination of the ability of CD cavities to host the drug and the feasibility of agar to ionically interact remarkably enhances the affinity of the networks for ciprofloxacin, compared to that observed for solutes such as 3-MBA that can only interact through inclusion complex formation.

3.5. Release of 3-MBA and ciprofloxacin

Hydrogels loaded with 3-MBA exhibited sustained delivery in water, releasing 50–60% after 10 h (Fig. 6). Then the release stopped indicating that equilibrium between 3-MBA concentration in the release medium and the residual 3-MBA in the hydrogels had been achieved. When the medium was replaced by fresh water, the release was reactivated. All hydrogels rendered very similar 3-MBA release pattern, suggesting that the inclusion complex formation and the hydrophobic interactions with the network govern the release process.

Ciprofloxacin release profile in water (Fig. 6) from HPβCD, MβCD and MX hydrogels without agar was similar to that found for 3-MBA; i.e., pulsate profile in which the release stops when inner and outer drug concentrations reach equilibrium. By contrast, the presence of agar made the hydrogels to effectively retain the drug without releasing it to water. Only when the medium was replaced by phosphate buffer pH 7.4, the release was triggered. This finding indicates that, in the absence of competitive ions, the electrostatic interactions between ciprofloxacin and agar in water are responsible for the retaining of the drug inside the hydrogel, despite the network is highly swollen. The buffer ions can shield the drug/network interactions and promote the release, which was complete in a 10-h time period.

The release from ciprofloxacin-loaded hydrogels was also tested by direct immersion in HCl 0.1 N and phosphate buffer pH 7.4 (Fig. 7). In these media, the hydrogels with agar completed the release in 10 h. By contrast, CD hydrogels without agar reached an equilibrium when the release was about 40%. This indicates that affinity of the drug for the CD cavities leads to stop the release when an equilibrium between the free drug and that forming complexes in the hydrogel is attained. Namely, the complex formation constant controls the amount of drug released and the release rate. Thus, the release could be completed when the equilibrium is disturbed by replacing the medium for fresh one. This means that the CD hydrogels can stop the release when a certain concentration in the medium is reached, which could be useful for avoiding toxic levels in surrounding tissues. Such an ability was not exhibited by MX hydrogels, which means that although MX possesses monosaccharide units suitable for hydrophobic interactions, the peculiar structure of the CD cavity is responsible for more efficient hosting of ciprofloxacin and for regulation of the release process.

The fact that the hydrogels that incorporate agar released the whole amount of drug, although sustainedly, in HCl 0.1 N or phosphate buffer pH 7.4 is explained again by the zwitterionic character of ciprofloxacin (Hernández-Borrel & Montero, 1997). At acidic pH, the amino and the carboxylic acid groups of the drug are protonized and thus the repulsion with the also protonized groups of agar–agar makes the drug–CD complex unstable and promotes the release. At pH 7.4, the carboxylic acid groups of ciprofloxacin are negatively charged, as the acid groups of agar–agar do. Therefore electrostatic repulsions between anionic groups prompt the release.

3.6. In vitro microbiological tests

Microbiological tests were carried out with the purpose of elucidating if the amount of ciprofloxacin loaded was enough and the release rate adequate to inhibit the growth of ciprofloxacin-sensible microorganisms. Three Gram-negative (*Pseudomonas aeruginosa*, *Edwardsiella tarda* and *Escherichia coli*) and two Gram-positive (*Staphyloccocus aureus* and *Staphylococcus epidermidis*) bacteria were tested (Table 4). All hydrogels effectively inhibited the growth of the microorganisms tested, however, some differences in the inhibition zone diameter were observed (Fig. 8). CD–agar hydrogels displayed greater inhibition zones than CD solely hydrogels (Table 4), which may be due to the greater loading capability and that they release all the ciprofloxacin incorporated in few hours.

4. Conclusions

Cross-linking of CDs and agar mixtures in aqueous medium under mild conditions renders hydrogels endowed with the ability of CDs to form inclusion complexes and the capability of agar to ionically interact with amphiphilic cationic drugs. Replacement of CDs for MXs also leads to hydrogels, although with a higher degree of cross-linking and lower swelling. Agar may play three different roles in the loading: (i) almost no interference in the loading of a hydrophobic molecule with so high affinity for the CD cavities as 3-MBA has; (ii) promotion of the loading of drugs (such as ciprofloxacin) with medium affinity for the network (either through CD complex or MX unspecific interaction) but that possess chemical groups available for interacting with the ionizable groups of agar; (iii) hindrance in the uptake of a hydrophobic molecule (namely 3-MBA) that unspecifically interact with the MX networks, as a consequence of an increase in the hydrophilicity of the networks. Regarding the release process, agar plays a minor role in the control of the release of molecules that do not interact directly with it (namely 3-MBA). By contrast, agar can effectively retain ciprofloxacin into the hydrogels when immersed in aqueous medium without ions, while enables the triggering of the release when ion concentration raises or a change in pH (from slightly acid to pH 1 or pH 7.4) diminishes the intensity of the ionic interactions. Electrostatic repulsions between ciprofloxacin and agar may destabilize the inclusion complexes with CD and prompts the release of the whole dose of drug, although still sustainedly for several hours. The achieved ciprofloxacin loading and release patterns were shown to be suitable for the *in vitro* inhibition of the growth of Gram-negative and Gram-positive bacteria. Therefore, both the components and the process can be considered environmentally friendly and the CD–agar hydrogels may be useful for the development of oral/topical antimicrobial drug delivery systems with tunable physical and drug loading/release features.

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