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Imprinted contact lenses for sustained release of polymyxin B and related antimicrobial peptides

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Abstract

The aim of this work was to develop drug-soft contact lens combination products suitable for controlled release of antimicrobial peptides on the ocular surface. Incorporation of functional monomers and the application of molecular imprinting techniques were explored to endow 2-hydroxyethyl methacrylate (HEMA) hydrogels with the ability to load and to sustain the release of polymyxin B and vancomycin. Various HEMA:drug:functional monomer:crosslinker molar ratios were evaluated to prepare polymyxin B imprinted and non-imprinted hydrogels. Acrylic acid-functionalized and imprinted hydrogels loaded greater amounts of polymyxin B and led to more sustained release profiles, in comparison to non-functionalized and non-imprinted networks. Polymyxin B-loaded hydrogels showed good biocompatibility in HET-CAM tests. Functionalized hydrogels also loaded vancomycin and sustained its release, but the imprinting effect was only exhibited with polymyxin B, as demonstrated in rebinding tests. Microbiological assays carried out with *Pseudomonas aeruginosa* allowed identification of the most suitable hydrogel composition for efficient bacteria eradication; some hydrogels being able to stand several continued challenges against this important bacterial pathogen.

Keywords:hydrogels; ophthalmic drug delivery; contact lens; peptide delivery; drug-device combination product; molecular imprinting; polymyxin B; vancomycin; *Pseudomonas aeruginosa*.

INTRODUCTION

Efficient topical ocular drug delivery is a still challenging task due to the numerous barriers that prevent drug penetration through the cornea.[1] Maintenance of therapeutic levels for a long time, as required for antimicrobial treatments, is not fully achieved using traditional eye drops. In-situ forming gels, suspensions, muco-adhesive polymers, nanoparticles and implants have been shown to enhance, to a certain extent, ocular drug bioavailability, as long as they can prolong drug residence on the ocular surface or promote its penetration.[2-4] Nevertheless, discomfort symptoms caused by the formulation itself, like sticking and blurring effects, do not facilitate patient compliance. Using soft contact lenses as drug delivery platforms can minimize these drawbacks, while significantly increase ocular bioavailability by entrapping the drug in the lachrymal fluid between the lens and the cornea.[5-7] It should be noticed that hydrogel contact lenses can be used not only for vision correction, but also as neutral corneal bandages for improving healing processes.[8]

In general, commercially available contact lenses are quite hydrophilic networks and exhibit limited affinity for most ophthalmic drugs. As a consequence, the amount of drug loaded cannot reach therapeutic levels in the cornea or it is released too fast and prolonged effects cannot be attained. By contrast, a few lens types bear components that strongly bind certain drugs, which hinders complete release when in contact with the lachrymal fluid.[9] Therefore, the design of drug-contact lens combination products requires an adequate match of the chemical functionalities of the contact lens network with those of the drug to be delivered, while preserving the unique light transparency and oxygen permeability of the contact lenses.[10,11]. In recent years, several new strategies have been explored to endow contact lenses with tunable affinity for drugs.[10] Amongst these strategies, the use of small proportions of monomers, which bear chemical groups complementary to those of the drug of interest (named functional monomers), is a promising advance, as these can be easily copolymerized with the main backbone monomers routinely used to prepare contact lenses. The functional monomers must be chosen such that they do not significantly alter the physical properties and the biocompatibility of the lenses. This approach can benefit from the application of the molecular imprinting technology, which aims to optimize the affinity for the drug by means of the appropriate arrangement of those functional monomers.[12,13] To 'imprint' a polymer, the target drug molecules are used as templates around which the monomers organize as a

function of their binding interactions. Subsequent polymerization fixes these binding arrangements in place and, when the templates are removed, receptors (imprinted cavities) that are chemically and spatially complementary to the template molecule are revealed. Imprinting of small molecules in rigid polymer networks is now well developed and also incipiently proved in swellable hydrogels.[12,14,15] However, preparation of hydrogels imprinted for peptide drugs is still challenging for two main reasons: (i) the peptide has to be soluble in the monomer solution and be able to diffuse through the network during removal and rebinding (which is difficult due to steric hindrance of the network mesh size); and (ii) the relatively low cross-linking density of the contact lenses and their swelling in aqueous media (including lachrymal fluid) after polymerization may reduce the stability of the imprinted cavities.[16] Therefore, the imprinting procedure should be designed to yield the optimum network stability and maximum interactions between the drug and the network.[17] Among other applications, antimicrobial peptides are being used for the treatment of severe ocular infections, when formulated as solutions or suspensions that incorporate thickening agents in order to enhance the residence time on the cornea.[18] Polymyxin B, a lipopeptide antibiotic that can electrostatically interact with the negatively charged lipopolysaccharide (LPS), exhibits a rapid activity against multidrug-resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa*.[19] Ophthalmic eye drops containing polymyxin B solely or in combination with trimethoprim are commercially available and intended for treatment of multidrug-resistant pathogens.[20,21] To the best of our knowledge, delivery of polymyxin B using contact lenses has not been evaluated yet. Moreover, no previous attempts to prepare imprinted networks for polymyxin B have been carried out, although a polymerizable derivative of polymyxin B has been used as functional monomer to develop a sensor for detection of LPS in sepsis, which relies on the strong affinity of this antimicrobial peptide for LPS.[22] Thus, the aim of our work was to develop hydrogel contact lenses suitable for loading and release of therapeutic amounts of polymyxin B and related antimicrobial peptide drugs, such as vancomycin. To do that, 2-hydroxyethyl methacrylate (HEMA) (a common component of soft contact lenses) was copolymerized with low proportions of acrylic acid (AAc), acting as functional monomer, at various monomer:template:crosslinker ratios. Imprinted and non-imprinted hydrogels series were prepared, and each set of hydrogels was characterized paying special attention to the drug loading/release capability and also to the

maintenance of optical properties, oxygen permeability and other features critical for the performance as soft contact lenses. Memorization of the imprinting effect and effectiveness against *Pseudomonas aeruginosa* were also investigated.

MATERIALS AND METHODS

Materials

Ophthalmic grade 2-hydroxyethyl methacrylate (HEMA) and acrylic acid (AAc) were from Merck (Germany); 2,2′-azo-bis-isobutyronitrile (AIBN) from Acros (New Jersey, USA); ethyleneglycol dimethacrylate (EGDMA) and dichlorodimethylsilane from Sigma-Aldrich (Germany); vancomycin HCl from Fagron (Spain); and polymyxin B sulfate from Alfa Aesar (Germany). Ultrapure water was obtained by reverse osmosis (resistivity>18.2 MΩ cm; Milli-Q®, Millipore, Spain).

Hydrogel preparation

HEMA (4.5 mL) was transferred to vials containing different amounts of polymyxin B, and then AAc, water and EGDMA were added in the proportions indicated in Table 1. AIBN (10 mg) was incorporated to the vials and the systems were stirred until complete dissolution. The solutions were then injected into moulds made of two glass plates previously treated with dichlorodimethylsilane and separated by a silicon frame (0.4 mm thickness). The moulds were kept at 50˚C for 12 h and then at 70 ˚C for 24 h to complete the polymerization of the hydrogel films. Each hydrogel was boiled in water for 30 min to remove un-reacted monomers. A cork borer was used to take discs out of the hydrogels (10 mm in diameter). The discs were immersed in water and the absorbance of the supernatant was monitored by UV spectrophotometry (Agilent 8453, Germany) to detect removal of polymyxin B used as template. Water was replaced until the discs were clean. All discs were then immersed in phosphate buffer pH 7.4 for 24 hours, rinsed with water and dried at 37 ˚C for 12 h.

Hydrogels physical characterization

Differential scanning calorimetry (DSC) runs of polymyxin B and hydrogel discs (2-4 mg) were recorded in a DSC Q-100 (TA instruments, UK) heating from room temperature to 100°C, cooling from 100°C to 0°C, and finally heating from 0°C to 300°C at 10°C/min. Transmittance of hydrogel pieces was recorded in the 200 nm to 700 nm range using a UV spectrophotometer (Agilent 8453, Germany). Oxygen permeability (Dk) and transmissibility of hydrogels previously swollen in 0.9% NaCl solution were measured in triplicate using a Createch permeometer (model 210T, Rehder Development Company, Castro Valley, USA) fitted with a flat polarographic cell and in a chamber at 100 % relative humidity. Degree of swelling was evaluated in water and in 0.9% NaCl solution. Dry discs were weighed (W_d) and placed in the aqueous medium at room temperature. At various time points they were removed from the medium, the surface was wiped with a piece of paper and the weight recorded again (W_s). The degree of swelling was calculated as follows:

$$Swelling\ (\%) = \frac{(W_s - W_d)}{W_s} \cdot 100 \qquad (1)$$

Polymyxin B loading and release

Three discs of each hydrogel type (weights ranging from 0.033g to 0.042g) were immersed independently in 2 mL of polymyxin B solution (4 mg/mL) prepared in previously autoclaved water (121ºC, 30 min). The systems were kept protected from light at room temperature, and the absorbance of loading solutions was monitored at 258 nm (UV spectrophotometer, Agilent 8453, Germany). The amount of polymyxin B loaded was calculated from the decay in absorbance and referred to the weight of each disc. After 3 days in the loading solution, the discs were removed, wiped with a piece of paper, and immediately transferred to vials containing 2 mL of 0.9% NaCl solution also prepared using autoclaved water. Samples of release media were frequently withdrawn to measure drug concentration using UV-Vis spectrophotometry (258 nm); the samples were then returned to the vials. The amount of drug (mg) released per gram of disc was calculated. After 174 hours the discs were weighed and transferred to fresh release media. In all cases, the release tests were carried out under sink conditions.

Vancomycin loading and release

Three discs of each hydrogel type were immersed independently in 2 mL of vancomycin aqueous solution (4 mg/mL) at room temperature. The systems were kept protected from light at room temperature, and the absorbance of loading solutions was monitored at 281 nm (UV spectrophotometer, Agilent 8453, Germany). The amount of vancomycin loaded was calculated from the decay in absorbance and referred to the weight of each disc. For the release test, vancomycin-loaded discs were removed from the loading solution, wiped with a piece of paper and placed in water for one day and then moved to 2 mL of 0.9% NaCl solution. Samples of release media were frequently withdrawn to measure drug concentration using UV-Vis spectrophotometry (281 nm); the samples were then returned to the vials. The amount of drug (mg) released per gram of disc was calculated. After 47 hours the discs were weighed and transferred to fresh media, and the release study continued as described above.

Polymyxin B loading and release after vancomycin release

The discs were thoroughly washed with water after the vancomycin release study in order to ensure a complete removal of the drug. Then, they were immersed in phosphate buffer pH 7.4 for 24 hours, rinsed with water and dried at 37 ˚C for 12 h. Polymyxin B loading and release tests were carried out as described above.

Microbiological tests

Non-loaded and polymyxin B-loaded hydrogel discs were placed on plates containing Müller-Hinton agar, previously seeded with *Pseudomonas aeruginosa CECT 110* (Spanish Type Culture Collection) grown in tryptic soy agar (TSA1). The plates were then kept at 38 °C for 24 hours and after measuring the inhibition zones, the hydogels were transferred to new plates seeded as described above and incubated in the same way. The inhibition zones were measured again. In parallel, the antimicrobial effects of samples of release medium from polymyxin B-loaded contact lenses, which were collected after 4 and 8 hours of the beginning of the release test, were measured by placing 20 µL onto paper discs (6 mm in diameter; Whatman, Sigma-Aldrich, St. Louis MO, USA) placed on similar plates also seeded with *Pseudomonas aeruginosa*. After 24 h of incubation, the inhibition zones were measured.

HET-CAM Tests

The ICCVAM-recommended hen's egg test-chorioallantoic membrane test (HET-CAM) method protocol[23] was followed, as previously described.[24] Briefly, fertilized broiler chicken eggs (Coren, Spain) were incubated at 37 ± 0.3 ºC and 60 ± 2.6% relative humidity until day 10. Then, the upper part of the eggshell was removed using a rotary saw (Dremel 300, Breda, The Netherlands) and the intact inner membrane of the eggs was moistened with 0.9% (w/v) NaCl solution for 30 min and then detached with a forceps. Polymyxin B-loaded hydrogel discs were placed on the chorioallantoic membrane and the irritation potential was monitored for 5 min. The experiments were carried out in triplicate. Negative (0.9% NaCl solution) and positive (0.1 N NaOH) controls were performed under the same conditions. Irritation scores (IS) were calculated as follows:

$$\mathrm{IS} = \left[\left(\frac{301-H_{time}}{300}\right) \mathrm{x}\ 5 + \left(\frac{301-L_{time}}{300}\right) \mathrm{x}\ 7 + \left(\frac{301-C_{time}}{300}\right) \mathrm{x}\ 9\right] \quad (2)$$

In this equation, H_{time}, L_{time} and C_{time} represent the time (in seconds) at which hemorrhage, lysis and/or coagulation started.

RESULTS AND DISCUSSION

Hydrogels preparation and physical properties

Polymyxin B, as well as other cationic polypeptides, performs as an antimicrobial agent due to its capability to bind into the cell membrane of Gram-negative bacteria and, as a consequence, to alter its permeability. Solely or combined with trimethoprim, polymyxin B is used in ophthalmic drops at 1 mg (equivalent to 10,000 units) per mL, and one drop should be instilled every three hours (up to 6 doses per day) for 7 to 10 days.[25] Polymyxin B consists of a mixture of five structurally-related components (B1, B1-I, B2, B3 and B6), which posses an identical polypeptide head and a slightly different fatty acid tail (Figure 1) and, thus, show very similar MIC values.[26] As a related antimicrobial agent, vancomycin is a bulkier glycosylated peptide (Figure 1) that cannot penetrate Gram-negative bacteria membranes (except some *Neisseria spp.*) but binds to the terminal D-alanyl-D-alanine moieties of the wall of Gram-positive bacteria, hindering their cross-linking. Detailed studies on the interaction of vancomycin with acrylic acid (AAc), a suitable monomer for contact lenses which can also perform as functional monomer for imprinted networks, revealed that in an

aqueous environment AAc strongly binds to the primary amino and amido groups, but also at the secondary amino groups up to a drug:AAc stoichiometry of ca. 1:10.[27] Polymyxin B has five primary amino and eleven secondary amino groups, as well as a large number of carbonyl groups that can establish hydrogen bonds with AAc (Figure 1). Thus, to prepare the hydrogels, AAc amount was fixed in 0.2 mL in order to have an AAc:HEMA ratio of approx. 1:12.5 mol/mol (i.e., 8 mol% in AAc), which is a common proportion when preparing ionic, hydrophilic contact lenses.[10] In addition to one control non-imprinted hydrogel (A in Table 1), three different amounts of polymyxin B (12.5, 25, and 50 mg) were tested to obtain imprinted networks, which led to AAc:drug ratios of 288, 144, and 72 mol/mol. To avoid solubility problems, HEMA was first added to the drug powder in vials and then AAc was added solely or with a small volume of water, and afterwards the other components were incorporated. Addition of water was only tested for one of the hydrogels (F) prepared with the largest content in template drug, and its effect was compared to that of a hydrogel with a similar composition without water (G). Such a high proportion of polymyxin B took several hours to be totally solubilized; water shortening the process. The cross-linker EGDMA was incorporated at 4.4 mol% with respect to HEMA (as it is common when preparing soft contact lenses) and, in hydrogel D, at 2.2 mol% in order to elucidate if a less cross-linked network may allow a faster and more complete diffusion of polymyxin B. In total, seven different compositions were tested to prepare the hydrogels as indicated in Table 1.

The melting point of polymyxin B sulfate, ca. 235 ºC, was not seen after hydrogel synthesis which indicated complete dissolution of the drug in the monomers solution. Moreover DSC scans of the hydrogels (Figure S1 in Supporting Information) showed the typical glass transition of HEMA networks close to 110 ºC.[28] Nevertheless, hydrogels prepared with the greatest content in polymyxin B were translucent and showed a limited light transmittance; namely, ca. 45%, 25% and 15% transmittance for hydrogel E, F and G, respectively, in the 300 to 600 nm range. This finding suggests a phase separation process (probably due to ionic interactions of polymyxin B with AAc) that may limit the use of these hydrogels as contact lenses, although it should be noticed that the thickness of the discs (0.4 mm) is greater than the central thickness of common contact lenses (0.07 mm to 0.35 mm depending on power). Therefore for thinner lenses, the detrimental effect on light transmittance may be attenuated.

Regarding oxygen permeability, all hydrogels exhibited typical values of soft contact lenses;[29] ranking in the order hydrogel C $[65\cdot 10^{-11}\ cm^3\cdot cm^2/(cm^3\cdot s\cdot mmHg)] < E \approx F \approx G \approx B \approx A\ [70\cdot 10^{-11}\ cm^3\cdot cm^2/(cm^3\cdot s\cdot mmHg)] < D\ [80\cdot 10^{-11}\ cm^3\cdot cm^2/(cm^3\cdot s\cdot mmHg)]$. This order correlates well with the degree of swelling of the hydrogels (Table 2).

Polymyxin B loading

Polymyxin B loading profiles (Figure 2) clearly indicated that the functional monomer AAc is required for the uptake of the drug by the hydrogels. Hydrogel C, the only one synthesized without AAc but polymerized in the presence of the drug, did not show any affinity for polymyxin B. This means that the template drug molecules by themselves did not improve the subsequent loading; hydrogel C swelled in the loading medium without perturbing drug concentration, which means that polymyxin B concentration in the aqueous phase inside the hydrogel is the same as in the loading solution and no drug adsorption to the polymer backbone occurs.[30] In contrast, hydrogel D which was imprinted with the smallest amount of drug (12.5 mg) and cross-linked with the lowest proportion of EGDMA, exhibited a very rapid and remarkably high drug loading. As mentioned above, except for hydrogel C, all networks contained a fixed proportion of AAc. Therefore, the rapid uptake observed in the case of hydrogel D can be attributed to its greater mesh size (because of the lower cross-linking density), which in turn makes diffusion of the peptide drug into the network easier and facilitates the interaction with the available acrylic acid groups. In fact, hydrogel D was the one that swelled more both in water and in 0.9% NaCl medium (Table 2). If compared with the AAc-functionalized non-imprinted hydrogel A, the hydrogels B, E and G imprinted with 12.5, 25 or 50 mg drug, respectively (Table 1), loaded somehow less drug, which is not uncommon for imprinted networks.[31] Interestingly, just adding a small volume of water to the monomers before polymerization (hydrogel F) remarkably facilitated the loading (compared to hydrogel G). The small volume of water added favored drug dissolution and interaction with AAc before polymerization, and it may also increase hydrogel mesh size without leading to pores formation.[32] In summary, proportions of AAc, cross linker, drug and water play all an important role in the polymyxin B loading performance of the hydrogels.

The loading study was limited to 3 days in order to avoid drug instability problems. The maximum loading, recorded for hydrogels D and F, was around 90 mg drug per gram of disc,

which corresponds to an AAc:drug 10:1 mol ratio. For a disc with weight (around 15 mg) and dimensions similar to those of a soft contact lens, this loading may correspond to 1.35 mg polymyxin B per lens, which is roughly equivalent to the amount provided by 27 drops of 50 µL commercial eye drops.

To quantify the increase in affinity provided by the functional monomer and the molecular imprinting approach, the partition coefficient of polymyxin B between the hydrogel network and water, $K_{n/w}$, was calculated as follows:[30]

$$K_{n/w} = \frac{Total\ amount\ \left(\frac{mg}{g}\right) - Amount\ in\ the\ aqueous\ phase\ \left(\frac{mg}{g}\right)}{C_0\left(\frac{mg}{g}\right)} \quad (3)$$

In this equation, "total amount" represents the amount of drug loaded by the hydrogel (mg drug/g), "amount in the aqueous phase" is the amount of drug in the aqueous phase of the hydrogel estimated as the product of the water volume absorbed by the hydrogel (mL water/g) and the concentration of the drug in the soaking solution (mg drug/mL water). This later concentration is C_0 assuming that water density is 1 mg/mL.

Compared to hydrogel C (which did not contain AAc), all other hydrogels showed approx. 10-times greater $K_{n/w}$ values (Table 2), which means that the functional monomer increased 10-fold the affinity of the network for the drug in spite of being incorporated at a low proportion. No significant differences were recorded between hydrogels A and B, which means that addition of a low amount of polymyxin B as template during polymerization has minimal repercussion on the total rebinding of the drug. The highest $K_{n/w}$ values were recorded for hydrogels D and F, which respectively indicate that an increase in mesh size (low cross-linker proportion in hydrogel D) and incorporation of more template molecules (and also a small amount of water to facilitate dissolution and interaction with functional monomers, in hydrogel F) favor the access to, and the interaction with, the network of the antimicrobial peptide drug. In fact, regarding $K_{n/w}$ values hydrogels with a fixed EGDMA proportion ranked in the order: $B < E \cong G < F$; namely, the greater the proportion of template molecules, the highest the $K_{n/w}$ value.

Polymyxin B release

Polymyxin B-loaded discs were removed from the soaking solution, wiped with a piece of paper to remove excess loading medium, and immediately transferred either to release medium (0.9% NaCl) or to agar plates seeded with *Ps. aeruginosa* (as discussed below). Moreover, samples of release medium were taken at 4 and 8 hours in order to check their antimicrobial activity. It should be noticed that this bacteria is the most common pathogen in contact lens-related ocular infections.[33]

Release profiles (depicted in Figure 3) were in all cases recorded under sink conditions and the release medium was not exchanged in the first days of the test in order to investigate the effects of the affinity of the lens for the drug on the release process. Thus, differences in release rate can attributed to differences in affinity and not to solubility restrictions. As expected, hydrogel C only released a minimal amount of drug, which corresponded to that hosted in the aqueous phase of the network, and the delivery occurred in few hours. This hydrogel exhibited the fastest release rate, also in agreement with an unspecific loading. Interestingly, when placed on *Ps. aeruginosa* plates a small inhibition zone was recorded (Figure 4; Table 2) which corresponded to the rapid release of its small payload. Hydrogel C did not stand a second challenge against the bacteria.

The other hydrogels sustained the release for more than two weeks, even though the 0.9% NaCl medium weakened the drug-polymer interactions. Compared to the non-imprinted hydrogel A, release profiles from hydrogels G and F (prepared with AAc:drug ratios of 72 mol/mol and, in the last case, incorporating water) were quite similar, but hydrogels B and E showed more sustained release (Figure 3). This means that adding small amounts of polymyxin B during synthesis (AAc:drug ratios of 288 and 144 mol/mol, respectively), enables the arrangement of AAc mers in a more favorable conformation for drug retention. In fact, hydrogel B exhibited the slowest release. It seems that for hydrogels G and F, the amount of template is in excess for creating well-defined imprinted cavities and thus no differences with the non-imprinted polymers in release rate can be detected.

The behavior of hydrogel D (homologous to B but with cross-linker reduced by 50%) was again different to the others, and led to the second faster release profile; at day 7, hydrogel D released 71% drug loaded compared to hydrogel B that only released 41%. The lower crosslinking density should not only facilitate drug diffusion, but also compromise the physical stability of the imprinted cavities. Hydrogel D swelled more than the other hydrogels

both in the loading and the release media, and thus the imprinted cavities could be distorted more greatly than in hydrogel B. These results are in agreement with previous reports that indicate that if the cross-linker proportion is below the proportion in functional monomers, imprinted cavities fail in maintaining the conformational memory engraved upon synthesis.[34]

With the exception of hydrogel C, all other hydrogels avoided the growth of *Ps. aeruginosa* on their surface and led to remarkable inhibition zones on agar plates (Figure 4). In agreement with the loading and release profiles, hydrogel D caused the greatest inhibition of bacteria growth. The differences with respect to the other hydrogels were even larger when the same hydrogel discs were exposed to a second challenge with the bacteria (Table 2). Its counterpart formulation, hydrogel B, led to smaller inhibition diameters, as expected from its slower release of the drug. Thus, this microbiological test appears as a suitable tool for discriminating hydrogels regarding loading and release of polymyxin B. Overall, the large inhibition zones recorded for all AAc-functionalized hydrogels even after the second challenge clearly indicates that this monomer, at proportions suitable for preparing soft contact lenses, endows the hydrogels with affinity for polymyxin B sufficient to provide drug levels well above the minimum inhibitory concentration (MIC) of *Ps. aeruginosa*. Although the differences among the imprinted hydrogels were small, once again the inhibition zones recorded in the two challenges were greater for those hydrogels prepared with more template molecules, which can be related to their greater loading and relatively faster release of polymyxin B.

Since testing the hydrogels on agar plates allows release of the drug for the full 24 h of incubation of each challenge, an additional test was carried out to analyze the capability of polymers which released the drug over shorter times (4 and 8 h) to inhibit bacterial growth. Thus, samples of the release medium were taken at 4 and 8 h and placed on sterile paper disk on the bacteria culture. Samples taken at 4 h did not show distinguishable inhibition zones around the paper disc with the exception of those coming from release medium of hydrogel D (Figure 4). After 8 h of release, samples from hydrogel C release medium still did not cause any inhibition and in fact the bacteria grew well all around the paper disc. Samples from hydrogels A, B, E, F, and G showed a small inhibition zone, similar to that observed

for hydrogel D at 4 h. Once again, samples of release medium from hydrogel D at 8 h were the ones that provided the highest bacteria inhibition.

Preliminary evaluation of the biocompatibility of polymyxin B-loaded hydrogels was carried out using the HET-CAM assay, which has been proposed as an alternative method of Draize eye irritation test by the ICCVAM.[23] It is important to note that none polymyxin B-loaded hydrogels caused hemorrhage, lysis or coagulation in the chorioallantoic membranes, which suggests adequate biocompatibility in spite of their potent antibacterial effect.

Vancomycin loading and release

Hydrogels were also tested regarding their affinity for vancomycin in order to elucidate whether the loading and release behavior exhibited with polymyxin B could be extrapolated to other related antimicrobial peptide drugs. Structural similarities/differences among related drugs may favor/hinder the loading especially in the imprinted cavities. Moreover, selectivity and memorization of the imprinting effect attained for polymyxin B can be investigated by first loading and release of vancomycin and, subsequently, monitoring the loading and release of polymyxin B with the same hydrogel discs.

In contrast to polymyxin B, vancomycin exhibits a broad spectrum against Gram-positive bacteria and is the first-choice antimicrobial agent against methicillin-resistant *Staphylococcus aureus* (MRSA). Both polymyxin B and vancomycin have a similar molecular size, but vancomycin is a stronger base (pKa 8.89)[35] and bears more aromatic rings (Figure 1). Therefore, each of these molecules should fit differently into the imprinted cavities. When monitoring the loading of vancomycin (Figure 5), it was again observed that hydrogel C did not uptake drug while hydrogel D was the one that loaded the drug faster, as happened with polymyxin B. As indicated above, this means that the functional monomer AAc is mandatory to endow the networks with affinity for the peptide drugs, and that a low cross-linking density facilitates the diffusion of the drug. However, two remarkable differences can be observed: (i) hydrogel D was the one that loaded more; and (ii) all other hydrogels (except C) either imprinted or non-imprinted loaded the same. These findings indicate that, for a fixed cross-linker proportion (hydrogels A, B, E, F and G), vancomycin loading is only driven by the interaction with AAc (which is quite strong as previously

reported)[27] and vancomycin molecules interact the same with the AAc in the non-imprinted orientation and in the cavities imprinted for polymyxin B. Thus, vancomycin did not adjust well into the polymyxin B-imprinted regions. In rigid, highly-crosslinked networks the lack of affinity for the imprinted cavities is commonly shown as a decrease in the amount loaded because the movement of the functional monomers is strongly restricted.[36] In the case of loosely cross-linked networks, as it is the case of swellable soft contact lenses, the arrangement of the functional monomers upon synthesis can be distorted in the presence of a strong interacting molecule. As a consequence, imprinted and non-imprinted networks behave the same against a non-imprint drug (vancomycin in the present case).

Performance of the hydrogels during the release confirmed the loss of the imprinting effect in the presence of vancomycin. The vancomycin-loaded discs were first immersed in water and no release occurred for 1 h, which confirmed the strength of the drug-network interactions. When transferred to 0.9% NaCl, the release was triggered due to competitive displacement of the drug by ions of the medium, but almost stopped 24 h latter, which indicated that an equilibrium was attained between the drug molecules free in the medium and those interacting with the network (as previously observed for networks of diverse composition).[37,38] It should be noticed that all release experiments were carried out under *sink* conditions. Therefore, the discontinuation in the release confirms the strength of vancomycin-AAc interaction. Exchange of the release medium with fresh 0.9% NaCl allowed the release to commence again.

Regarding hydrogel composition, no appreciable differences were observed among the various imprinted and non-imprinted hydrogels prepared with fixed cross-linker proportion (hydrogels A, B, E, F and G). That is to say, the imprinting effect observed for polymyxin B disappeared in the case of the non-imprint vancomycin. Interestingly and oppositely to what had been observed for polymyxin B, hydrogel D was the one that released the drug more slowly. This hydrogel showed a higher loading of vancomycin than of polymyxin B, which again confirms that vancomycin did not accommodate in the imprinted cavities, but directly interacted with AAc functionalities. From consideration of the greater accessibility of AAc units in hydrogel D because of its lower cross-linking density, it is likely that during the release experiments, the drug molecules were able to find more AAc binding points and be

temporally retained before reaching the surface of the hydrogel. This mechanism is typical of an affinity-controlled release process and has been previously described for cyclodextrin networks.[37,38]

Polymyxin B re-loading and release

From a theoretical point of view, evaluation of the ability of hydrogels that were exposed to vancomycin to recover the same affinity for polymyxin B as they initially had after synthesis, may provide useful information regarding the "memorization" of the imprinting conformation in loosely cross-linked networks. From a practical point of view, it could help to foresee the possibility of reuse of the same hydrogel discs for successive loading and release of peptidic drugs. Polymyxin B loading profiles of re-used discs (Figure 6) followed the same ordering as that shown by freshly prepared discs (Figure 2). Although the total amount loaded by the imprinted networks was slightly lower, it again ranked in the order B < E ≅ G < F. This suggests that the imprinted cavities recovered their conformation upon synthesis, which can occur through an induced fit mechanism when polymyxin B molecules interact with the imprinted regions, as previously suggested for other imprinted hydrogel systems.[39] Polymyxin B release profiles (Figure 6) also resembled those recorded for freshly prepared discs (Figure 3), although the re-used hydrogels exhibited slightly higher release rate, which may indicate that the reconstituted imprinted cavities are less spatially-defined than the pristine ones. Thus, they cannot retain the drug so strongly. Nevertheless, the re-used hydrogels still sustained the release of drugs for two weeks.

CONCLUSIONS

Acrylic acid (AAc) is suggested as a valuable monomer for preparing soft contact lenses able to load and release therapeutically useful doses of peptidic antimicrobial agents. The proportion of AAc is in the range commonly used to prepare hydrogel lenses and therefore it does not alter physical properties required for the primary mechanism of action of the contact lenses (if intended as vision correctors). Addition of low amounts of polymyxin B during synthesis to prepare imprinted networks enables modulation of the loading and release

profiles, but if the amount is above a certain threshold the contact lenses become translucent, which in turn may compromise their application as ophthalmic devices. Nevertheless, those translucent hydrogels might be useful for other applications that require prolonged release of antimicrobial agents (e.g., as constituents of topic or implantable formulations or of ocular inserts). In vitro microbiological tests evidenced the role that the monomer:template:crosslinker ratios play on having hydrogels that can effectively stand several challenges against common bacteria on the eye surface. Moreover, the hydrogels could be used for the hosting of other related antimicrobial peptides useful against common Gram-negative and Gram-positive bacteria responsible of ocular infections and biofilm formation onto contact lenses. Memorization of the imprinting may be exploited for rebinding and release of polymyxin B.

Acknowledgements

Work supported by MICINN (SAF2011-22771) Spain and FEDER. N. Malakooti thanks to NanoFar program "European Doctorate in Nanomedicine and Pharmaceutical Innovation" (funded by EACEA Erasmus Mundus Joint Doctorate) for a European PhD grant. The authors acknowledge B. Magariños for help with the microbiological tests and A. Concheiro for valuable comments during discussion of the results.

The authors have no conflict of interest.

Supporting information: DSC scans of polymyxin B and hydrogels.

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Figure captions

Figure 1. Structure of polymyxin B1 (1301.56 g/mol),vancomycin (1449.30 g/mol) and acrylic acid monomer (AAc, 72.06 g/mol). AAc can ionically interact with amine groups (indicated with green arrows) and through hydrogen bonding with the atoms of peptide bond (purple arrows), as depicted (in red color) for two of the groups of polymyxin B.

Figure 2. Polymyxin B loading profiles for different hydrogel formulations. Codes as in Table 1.

Figure 3. Polymyxin B release profiles in 0.9% NaCl from different hydrogel formulations. The plot on the right shows the first 24-h time period. Codes as in Table 1.

Figure 4. Inhibition zones in *Pseudomonas aeruginosa* cultures in agar plates recorded after 24 hours of incubation with: first row, 0.9% NaCl solution, polymyxin B solution (4 mg/mL), and polymyxin B-loaded discs of hydrogels A, B, C and D (in duplicate); and second row, polymyxin B-loaded discs of hydrogels E, F and G (in duplicate) and samples of release medium from hydrogel D taken at 4 and 8 h since the beginning of the release test (which showed inhibition zone diameters of 1.0 and 1.6 cm, respectively).

Figure 5. Vancomycin loading in water (up) and release profiles in water and in 0.9% NaCl (down) from different hydrogel formulations. Codes as in Table 1. The release medium was initially water and after 24 h it was replaced with 0.9% NaCl. Five days later, the medium was replaced with fresh 0.9% NaCl.

Figure 6. Polymyxin B loading in water (up) and release profiles in 0.9% NaCl (down) from hydrogel discs that were firstly exposed to vancomycin. Codes as in Table 1.