Hydrogels for diabetic eyes: Naltrexone loading, release profiles and cornea penetration

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1. Introduction

Cornea, sclera, conjunctiva and tear film are functionally linked as one system, including tear glands, to protect the eye against external adverse events and pathogens [1]. In addition, lachrymal fluid and cornea are the first refracting elements. Thus, cornea integrity and an adequate tear production are essential to preserve a healthy vision [2]. Metabolic changes suffered by persons with diabetes lead to important ocular surface complications such as decreased tear production, diminished corneal sensitivity, and delayed wound healing [3]. Disruption of tear film barrier function and poor tear quality production can also result in irritation, inflammation of the stroma and impaired vision.

Enkephaline is a natural opioid growth factor (OGF) that, when interacts with its receptor (OGFR), negatively regulates cell proliferation and tissue growth. Enkephaline has been found to be at higher levels in persons with diabetes compared to healthy people [4,5]. Elevated concentrations of this peptide, which have also been confirmed in diabetes animal models, are responsible for complications such as ulceration and increased susceptibility to infection due to delayed epithelialization during wound healing. It should be noted that the OGF-OGFr axis plays a key role in the homeostasis of cornea and retina [1], and thus antagonists of OGFr, such as naltrexone (NTX), may revert effects of enkephaline [4,6]. NTX is approved in oral formulations for treatment of addiction to opioids or alcohol, although it undergoes a relevant hepatic first pass effect. Preclinical studies have demonstrated that NTX, either systemically or topically applied, can notably improve corneal wound healing and reverse severe dry eye [6,7]; for example, studies on type I diabetic rats and normal rats with episodic dry eye have shown that one drop of
NTX restore tear production in < 1 h and the effect persists for 72 h [6]. Importantly, repeated doses of NTX eye drops (50 μM; 4 drops) over a 24 h period did not cause adverse events on healthy human volunteers [2]. However, NTX tends to autoxidation when it is formulated as eye drops [8]. Furthermore, conventional ocular formulations provide low ocular bioavailability because of short precorneal residence time and limited cornea permeability. Thus, to achieve a therapeutic effect, frequent instillations are needed. Alternatively, mucoadhesive in situ ocular gelling formulations [9] and niosomes [10] have been explored.

Contact lenses (CLs) are safe devices widely used to correct refractive errors and are attracting an increasing interest as drug delivery platforms [11–14]. Several approaches have been implemented to endow CLs with ability to host drugs such as timolol, acetazolamide, olopatadine, amphotericin B, ciprofloxacin or epalrestat, among others [15–20]. CLs can act as a reservoir that provides sustained levels of drug in the precorneal area (post lens lachrymal fluid) while minimizes drug loss due to blinking, reflex lachrymation or nasolacrimal drainage [21]. One useful approach to enhance the capability of CLs to load therapeutic amounts of drug is the molecular imprinting [11,22]. This technique relies on the use of the target drug molecules as templates during polymerization in order to induce monomers arrangement as a function of their affinity. After polymerization, the template molecules are removed and specific cavities for the target drug, named imprinted pockets, are revealed. Physical stability of the imprinted cavities is quite limited in the case of loosely crosslinked networks, as occurs for soft CLs. Therefore, a precise selection of functional monomers that can endow the cavities with sufficient affinity for the target molecule even after the swelling of the network is required for the success of the recognition. Bioinspired strategies that rely on mimicking the pharmacological target of the drug in the hydrogel network have been shown useful for the rational selection of functional monomers [17,23,24].

The aim of the present work was to design NTX-imprinted poly(2-hydroxyethyl methacrylate) (HEMA) hydrogels suitable as soft CLs that can load therapeutic amounts of NTX and provide sustained release on the ocular surface. The hydrogels were designed taking into account the information available about the interactions of NTX with the μ-opioid receptor (MOR) [25,26], which are mainly driven by binding to amino acid residues Asp147 (bearing a carboxylic acid group) and Tyr148 (bearing a phenyl group) and polar interactions with Lys233 [27,28]. Taking into account this information, functional monomers were chosen among those bearing carboxylic acid groups (acrylic acid, AAc) and aromatic groups (benzyl methacrylate, BzMA). It can be hypothesized that the addition of NTX before polymerization may drive the adequate spatial arrangement of the monomers for a more efficient formation of ad hoc artificial receptors (imprinted hydrogels). Both imprinted and non-imprinted hydrogels were carefully washed and then loaded with NTX by soaking in aqueous solutions. Feasibility of carrying out loading and sterilization processes in one step was investigated. Since there is not a standardized release test for ocular solid or semisolid formulations, the diversity of setups and conditions reported in literature (some far from mimicking cornea environment) make comparisons and predictions difficult [29]. Thus, to gain an insight into the effect of the tests conditions on the release profiles, NTX release from the hydrogels was recorded using both a conventional test in bulk medium and also a microfluidic device mimicking lachrymal fluid turn over [30] and the information obtained compared to elucidate whether correlations among both methods could be established. Finally, after confirming biocompatibility, most promising formulations were also evaluated regarding corneal accumulation and permeability.

2. Experimental

2.1. Materials

Naltrexone hydrochloride (NTX), ethylene glycol dimethacrylate (EGDMA) and dichlorodimethylsilane were from Sigma-Aldrich (Steinheim, Germany); 2-hydroxyethyl methacrylate (HEMA) and acrylic acid (AAc) were from Merk (Dramstad, Germany); benzyl methacrylate (BzMA) from Polysciences Inc. (Warrington, UK) and 2,2′-azobis(2-methylisopropionitrile) (AIBN) from Across (New Jersey, USA). WST-1 cell proliferation reagent was from Roche (Mannheim, Germany); phosphate buffer saline (PBS), MEM Alpha and fetal bovine serum (FBS) were from Sigma-Aldrich (Saint Louis, USA); penicillin/streptomycin (10,000 U/mL and 10,000 μg/mL), L-glutamine (200 mM) and 0.25% trypsin-EDTA were from Gibco (Paisley, UK). Water was purified using reverse osmosis (resistivity > 18 MΩ cm, MilliQ, Millipore® Spain). Simulated lachrymal fluid (SLF) was prepared with the following composition: 6.78 g/L NaCl from Scharlau (Barcelona, Spain), 2.18 g/L NaHCO3 from Panreac (Barcelona, Spain), 1.38 g/L KCl, and 0.084 g/L CaCl2·2H2O from Merck (Dramstad, Germany) with pH 7.8. Carbonate buffer pH 7.2 was prepared mixing buffer solution A (100 mL): 1.24 g NaCl from Scharlau (Barcelona, Spain), 0.071 g KCl from Merck (Dramstad, Germany), 0.02 g NaH2PO4·H2O from Merck (Dramstad, Germany) and 0.49 g NaHCO3 from Panreac (Barcelona, Spain); and buffer solution B (100 mL): 0.023 g CaCl2 from Merck (Dramstad, Germany) and 0.031 g MgCl2·6H2O from Scharlau (Barcelona, Spain).

2.2. Hydrogel synthesis

Various sets of monomer mixtures were prepared with the composition shown in Table 1. The components were placed into vials and mixed at room temperature under magnetic stirring (400 rpm). After dissolution of BzMA, AAc and EGDMA in HEMA, NTX and AIBN were added and stirred for 30 min. Final concentrations of BzMA: AAc: NTX: EGDMA: AIBN were 50:50:10:80:10 mM, respectively when all components were present. Monomer mixtures were injected into molds constituted by two glass plates (10 × 10 cm2) pretreated with dichlorodimethylsilane and separated by a silicone frame of 0.5 mm thickness. Polymerization was carried out at 50 °C for 12 h and then at 70 °C for 24 h more.

Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>HEMA</th>
<th>NTX</th>
<th>BzMA</th>
<th>AAc</th>
<th>EGDMA</th>
<th>AIBN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>45.20</td>
<td>4.93</td>
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<tr>
<td>A2</td>
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<td>10</td>
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<td>0</td>
<td>45.20</td>
<td>4.93</td>
</tr>
<tr>
<td>B1</td>
<td>3</td>
<td>0</td>
<td>25.41</td>
<td>0</td>
<td>45.20</td>
<td>4.93</td>
</tr>
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<td>25.41</td>
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</tr>
<tr>
<td>C1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>10.30</td>
<td>45.20</td>
<td>4.93</td>
</tr>
<tr>
<td>C2</td>
<td>3</td>
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<td>45.20</td>
<td>4.93</td>
</tr>
<tr>
<td>D1</td>
<td>3</td>
<td>0</td>
<td>25.41</td>
<td>10.30</td>
<td>45.20</td>
<td>4.93</td>
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<tr>
<td>D2</td>
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<td>10</td>
<td>25.41</td>
<td>10.30</td>
<td>45.20</td>
<td>4.93</td>
</tr>
</tbody>
</table>
magnetic stirring (200 rpm) at room temperature and dried at 70 °C for 3 h.

2.4. Swelling degree, light transmittance and oxygen permeability

Swelling in water and artificial lachrymal fluid was monitored at room temperature in triplicate, recording the increase in weight of the discs at pre-established times, as follows:

\[
\text{Swelling degree (\%) } = \frac{W_f - W_0}{W_0} \times 100
\]  

where \(W_0\) and \(W_f\) represent the weight of each dried and swollen hydrogel disc, respectively.

Swollen discs were fitted to the internal wall of a quartz cell filled with water and the transmittance was recorded, in duplicate, from 190 to 700 nm in a UV-Vis spectrophotometer (Agilent 8453, Germany). The oxygen permeability of the hydrogels swollen in 0.9% NaCl was measured (in duplicate) using a Creactive permeometer model 210T (Rehder Development Company, Castro Valley, CA USA) fitted with a flat cell in a 100% RH chamber.

2.5. Naltrexone loading

Imprinted and non-imprinted discs previously washed and dried (as explained above) were weighed and immersed in a NTX solution (0.3 mg/mL, 3 mL) in water at room temperature under magnetic stirring (200 rpm). Aliquots of medium (2 mL) were taken at pre-established time points for 24 h. Each sample was immediately measured spectrophotometrically (Agilent 8534, Germany) at 281 nm and returned to the corresponding vial.

In parallel, some imprinted and non-imprinted discs were weighed and placed in vials containing NTX aqueous solution (0.3 mg/mL). Then, the vials were sealed and sterilized in autoclave (121 °C, 20 min). After autoclaving, the vials were stored at room temperature during 2 days and the excess of liquid blotted with filter paper. The discs were placed in vials containing NTX aqueous solution (0.3 mg/mL, 3 mL) and autoclaved (121 °C, 20 min). After autoclaving, the vials were stored at room temperature during 2 days and fluctuations in NTX concentration were measured as explained above.

2.6. Effect of autoclaving on NTX-loaded discs

Discs loaded with the drug at room temperature were rinsed with water and then the excess of liquid blotted with filter paper. The discs were placed in vials containing NTX aqueous solution (0.3 mg/mL, 3 mL) and autoclaved (121 °C, 20 min). After autoclaving, the vials were stored at room temperature during 2 days and fluctuations in NTX concentration were measured as explained above.

2.7. Naltrexone release

2.7.1. Release under agitated sink conditions

Drug-loaded discs (at room temperature) were rinsed with water and then the excess of liquid blotted with filter paper. The discs were placed in vials (3 mL) containing 0.9% NaCl at 36 °C under magnetic stirring (50 rpm). Aliquots of medium were taken at pre-established periods of time, measured spectrophotometrically at 281 nm and returned to the vials.

To infer about the drug transport mechanism through the polymeric matrix, the obtained NTX release profiles were fitted to the Korsmeyer-Peppas equation [32]:

\[
\frac{M_t}{M_infinity} = k t^n
\]

where \(M_t/M_{\infty}\) is the fraction of drug released at time \(t\), with respect to the total amount of drug released (\(M_{\infty}\)), \(k\) is the release rate constant which accounts for the characteristics of the macromolecular network/drug system and the dissolution medium, and \(n\) is the diffusion exponent that determines the dependence of the release rate on time. According to the value of \(n\), different transport mechanisms may be identified: \(n = 0.5\) corresponds to Fickian diffusion, \(0.5 < n < 1.0\) to non-Fickian or anomalous transport, \(n = 1.0\) to Case II transport and \(n > 1.0\) to Super Case II transport. Eq. (4) was applied for the first 60% of the amount released [32].

For the systems with \(n = 0.5\), where Fickian diffusion occurs, the effective diffusion coefficients of NTX, \(D\), were determined by applying the equation:

\[
\frac{\partial C(x,t)}{\partial t} = D \frac{\partial^2 C(x,t)}{\partial x^2}
\]

This equation describes the mass transfer from the polymeric matrix with a certain concentration of drug (\(C(x,t)\), being \(x\) the space coordinate in the transverse direction and \(t\) the time). The boundary conditions for the drug release experiments are:

\[
\frac{\partial C(x,t)}{\partial t} (x = 0) = 0
\]

\[
C(x,t) = C_i, t = h
\]

Eq. (6) assumes symmetry at the centre of the disc, while Eq. (7) is based on the perfect sink assumption [33]. In the last equation, \(h\) corresponds to half of the thickness of the disc. The initial condition is:

\[
C_t (0,x) = C_i
\]

The concentration profile in the disc was calculated through the following equation [33]:

\[
C = \sum_{n=0}^{\infty} \frac{(-1)^n 4C_i}{(2n + 1)\pi} \cos \left(\frac{(2n + 1)\pi}{2h}x\right) e^{-(2n + 1)\pi^2 D t/4h^2}
\]

The value of \(D\) can be obtained fitting the experimental data to this equation in Matlab program, using the ‘fminsearch’ function to minimize the error.

2.7.2. Release in microfluidic flow chamber

NTX release was also carried out using a microfluidic cell described elsewhere [30]. The measurements were carried out in triplicate at 36 °C with a continuous flow of 3 μL/min of 0.9% NaCl solution. The exit solution was collected at scheduled times and analyzed spectrophotometrically at 281 nm to determine the NTX concentration.

2.8. HET-CAM test

The Hen’s Egg Test on Chorio-Allantoic Membrane (HET-CAM) assay is a validated alternative to animal testing of ocular irritancy [34]. The HET-CAM test was carried out using fertilized hen's eggs.
(50–60 g; Coren, Spain) incubated at 37 °C and 60% RH during 7 days [34]. A rotator saw (Dremel 300, Breda, The Netherlands) was used to make a circular cut of 1 cm in diameter on the wider extreme (where the air chamber is placed), on the eighth day, to remove the eggshell. The inner membrane was wet with 0.9% NaCl for 30 min and then carefully removed to expose the chorioallantoic membrane (CAM). NTX aqueous solution (0.3 mg/mL, 300 μL) and hydrated drug-loaded discs (imprinted and non-imprinted in duplicate) were placed on the CAM. 0.9% NaCl and 0.1 N NaOH solutions (300 μL) were used in triplicate as negative and positive controls, respectively. The vessels of CAM were observed during 5 min, under white light, for haemorrhage, vascular lysis or coagulation. The irritation score (IS) was calculated as previously reported [20,35].

2.9. Cytocompatibility assay

Human mesenchymal stem cells derived from bone marrow (hMSC, ATCC-PCS-500-012™) were cultured in 175 cm² culture flask with MEM Alpha supplemented with fetal bovine serum heat inactivated (PBS, 10%), and antibiotics (penicillin/streptomycin 10,000 Units/mL and 10,000 μg/mL respectively, 1%). hMSCs were harvested, at approximately 90% of confluence, with 0.25% trypsin-EDTA. Cells were seeded (10,000 cells/well) in 24 wells plate and kept at 37 °C, 5% CO₂ and 95% HR for 12 h allowing cell adhesion.

Pieces of NTX-loaded and non-loaded imprinted hydrogels bearing AAc and BzMA (19.6 m^3/m²) were placed in the 24 wells plate (10,000 cells/well) the plate was kept at 37 °C, 5% CO₂ and 95% HR. Solution of NTX (50 μM) in MEM Alpha was used as control. After 72 h, WST-1 cell proliferation assay was carried out following manufacturer instructions. Briefly, the medium was replaced by MEM Alpha (0.5 mL, with no supplements), and WST-1 cell proliferation reagent (50 μL; Roche, Mannheim, Germany) was added. Plates were incubated at 37 °C, 5% CO₂ and 95% HR for 1 h and measured spectrophotometrically at 450 nm in a Bio-Rad plate reader 680 (California, USA).

2.10. Bovine corneal permeability test

Fresh bovine eyeballs were collected from the local slaughterhouse and transported following BCOP protocol alternative to in vivo testing [36]. Eyes were carried completely immersed in PBS with antibiotics 1% (100 IU/mL penicillin, 100 μg/mL streptomycin) in an ice bath. Once arrived, corneas were excised with 2–3 mm of surrounding sclera, rinsed with PBS and mounted in vertical diffusion (Franz) cells. Carbonate buffer pH 7.2 (6 mL) was used to fill receptor chamber and a small stir bar was also incorporated. The cornea was placed on the receptor chamber (maintained at 37 °C) and the donor chamber was fixed and filled also with carbonate buffer pH 7.2 (0.785 cm² area available for permeation). After 1 h equilibration, the buffer in the donor chamber was removed and the corneas were exposed to NTX-loaded discs (C2 and D2 discs with 1 mL of 0.9% NaCl) or to control drug solution (350 μg/mL NTX, 2 mL). All experiments were carried out in triplicate. The donor chamber was covered with parafilm to prevent evaporation. Samples (1 mL) were taken from the receptor chamber at 1, 2, 3, 4, 5 and 6 h, replacing with the same volume of bicarbonate buffer each time and taking care of removing air bubbles from the diffusion cell. NTX in the receptor medium was quantified by means of an HPLC equipment (Waters 717 Autosampler, Waters 600 Controller, 996 Photodiode Array Detector) fitted with a C18 column (Waters Symmetry C18 5 μm; 3.9 × 150 mm) and Empower2 software. Mobile phase consisted of acetonitrile: 10 mM ammonium acetate buffer (40:60 v/v, pH adjusted to 5.6 with acetic acid) at 0.5 mL/min and 30 °C [37]. The injection volume was 50 μL, and naltrexone was quantified at 220 nm (retention time 3.08 min). Calibration was carried out with NTX standard solutions (1–10 μg/mL) in water (filtered through 0.2 μm, 13 mm GMP Minispine filters).

After 6 h of test, the formulations were removed from the donor chambers and the corneas were rinsed at least three times with PBS. Then, the corneas were placed in tubes with 2 mL acetonitrile for 24 h. The amounts of NTX extracted from the cornea were measured by HPLC as explained above.

2.11. Statistical analysis

The effects of sterilization process on NTX loading, and of hydrogels with and without NTX on NTX diffusion coefficients, hMSCs viability and cornea permeability were analyzed using ANOVA and multiple range test (Statgraphics Centurion XVI 1.15, StatPoint Technologies Inc., Warrenton VA).

3. Results and discussion

3.1. Hydrogel synthesis

Naltrexone hydrochloride (NTX) is a white powder soluble in water (up to 100 mg/mL) with a peak of absorbance at 281 nm (Fig. S1, Supporting information). The UV/Vis quantification method was validated regarding linearity, accuracy and precision in water, SLF and 0.9% NaCl medium. NTX is a weak acid that at 32 °C (~ocular surface temperature) has a pKa of 8.20 due to the dissociation of the proton on aliphatic nitrogen and a pKa of 9.63 associated to the dissociation of the phenolic proton [38]. Thus, it is expected that NTX can establish ion interactions through its positively charged aliphatic nitrogen with the negatively charged AAc. Also, hydrogen bonding may occur between AAc and the hydroxyl and carbonyl groups of NTX. It is known that in the pharmacological receptor (MOR) hydrophobic interactions contribute to stabilize ligands binding [28], and hence BzMA was chosen as an additional functional monomer (Fig. 1).

NTX easily dissolved in the monomers mixture up to 10 mM. Higher concentrations were not tested since that amount of NTX should already provide therapeutic amounts to hydrogel pieces of dimensions in the range typical of CLs. After polymerization, hydrogel sheets were immersed in boiling water (500 mL) and the amount of NTX removed was quantified spectrophotometrically at 281 nm. Boiling is typically used to clean soft CLs after fabrication, and thus both imprinted and non-imprinted hydrogels underwent the same cleaning process. Imprinted hydrogel sheets were polymerized in the presence of 10 mg of NTX (~3.3 mg/g) and nearly the whole amount of template drug was removed during boiling (Fig. 2). Differences in the UV–Vis spectra of washing medium of imprinted and non-imprinted discs were clearly observed despite the large volume of water used for boiling. Interference of residual monomers in the peak absorbance of the drug was minor (Fig. S2, Supporting information). Subsequently, the discs were
immersed in SLF pH 7.8 to complete NTX removal before loading assay (Fig. 2). Salts in the SLF medium participated in ionic competition for AAc monomers and thus triggered the release of residual NTX from the polymer network. All hydrogels were prepared in triplicate, and reproducible behavior was observed.

3.2. Hydrogel characterisation

Swelling degrees in water and SLF of both imprinted and non-imprinted discs were in the 46–57% range (Fig. S3, Supporting information), which is typical of HEMA hydrogels. Thus, functionalization with small proportions of AAc and BzMA did not alter water uptake. Regarding light transparency, all swollen discs showed transmittance above 85% in the visible range. A decrease in transmittance below 300 nm was recorded for NTX-loaded hydrogels (Fig. 3). This means that NTX loading is not expected to disturb a clear vision but even could protect the eye against UV radiation.

Fig. 2. Accumulated amounts of NTX removed from imprinted hydrogel sheets during boiling in water (blue bars) and after soaking in SLF (purple portion). Hydrogel codes as in Table 1. The straight line indicates the amount of NTX added to each monomer mixture used to prepare one hydrogel sheet. The slightly higher values recorded are due to a small interference of monomers traces. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Light transmittance profiles of swollen C1 and C2 after boiling in water, and NTX-loaded C2.

(a) 

![Graph A](image1)

(b) 

![Graph B](image2)

(a) Loading profiles of naltrexone in water (0.3 mg/mL, 3 mL) at room temperature, and (b) amounts of naltrexone loaded after autoclaving and storage at room temperature for 2 or 24 h.

Fig. 4. (a) Loading profiles of naltrexone in water (0.3 mg/mL, 3 mL) at room temperature, and (b) amounts of naltrexone loaded after autoclaving and storage at room temperature for 2 or 24 h.

Fig. 5. Release profiles of naltrexone-loaded discs in 0.9% NaCl at 36 °C under magnetic stirring (50 rpm).
Table 2. Parameters \( n \) and \( k \) from Korsmeyer-Peppas equation and respective correlation coefficients, \( R^2 \), and estimated effective diffusivity, \( D \).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Diffusion exponent ( n )</th>
<th>Pseudokinetic constant ( k ) (h)</th>
<th>Correlation coefficient ( R^2 )</th>
<th>Effective diffusivity ( D ) (( \times 10^{-13} ) m²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>2.21</td>
<td>0.003</td>
<td>0.98</td>
<td>–</td>
</tr>
<tr>
<td>A2</td>
<td>0.86</td>
<td>0.037</td>
<td>0.94</td>
<td>–</td>
</tr>
<tr>
<td>B1</td>
<td>1.13</td>
<td>0.016</td>
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<td>B2</td>
<td>1.12</td>
<td>0.016</td>
<td>0.94</td>
<td>–</td>
</tr>
<tr>
<td>C1</td>
<td>0.50</td>
<td>0.220</td>
<td>0.99</td>
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<tr>
<td>C2</td>
<td>0.58</td>
<td>0.213</td>
<td>0.99</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>D1</td>
<td>0.43</td>
<td>0.220</td>
<td>0.93</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>D2</td>
<td>0.61</td>
<td>0.190</td>
<td>0.99</td>
<td>2.4 ± 0.3</td>
</tr>
</tbody>
</table>

Fig. 6. NTX release profiles under dynamic microfluidic conditions. a) NTX accumulated released mass; and b) NTX concentration in the exit solution of the microfluidic cell. The graphic inside shows NTX concentration (g/mL) in the exit point within the first 10 h. The green line indicates maximum NTX concentration in lachrymal fluid provided by eye drops (formulated as \( 10^{-5} \) M drug concentration) at the moment of administration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Naltrexone loading

After the drug removal step (applied to both imprinted and non-imprinted hydrogels), dried discs were immersed in a NTX aq. solution (0.3 mg/mL) to quantify the drug loading ability (Fig. 4a). Non-imprinted hydrogels without functional monomers or with only BzMA (A1 and B1) did not show any affinity for NTX. Differently, non-imprinted hydrogels with AAc could load 8.06 ± 0.27 mg/g and 7.28 ± 0.41 mg/g for C1 and D1 (codes as in Table 1) respectively. The FMF values indicated that the functional monomer AAc increases the amount of NTX loaded (\( \text{FMF} = 41.18 \)) while BzMA has no influence in NTX uptake (\( \text{FMF} = 0.26 \)). The combination of AAc and BzMA led to a FMF similar to that of hydrogels with AAc (\( \text{FMF} = 38.14 \)) confirming the minor role of BzMA. Besides, imprinted hydrogels were able to increase the amount of NTX loaded (up to 10.41 ± 0.40 mg/g and 10.83 ± 0.38 mg/g for C2 and D2 respectively), with \( K_{\text{NW}} \) of 34.4 for C2 and 35.9 for D2. Calculated imprinting factors (\( K_{\text{IF}} = 1.31 \) and \( D_{\text{IF}} = 1.46 \)) indicated that hydrogels synthesized with functional monomers in presence of the drug can uptake more amount of drug due to specific cavities formation inside the network. Interestingly D2 hydrogels bearing both functional monomers, although loaded NTX in a similar amount as C2 hydrogels, had a few larger imprinting factor, which means that BzMA when correctly oriented in the imprinted cavity plays a role in the loading, increasing drug affinity for the binding regions.

From a manufacturing point of view, drug loading may involve an additional, time-consuming step in the processing of drug-eluting CLs. To minimize the impact of this step, we then evaluated the feasibility of carrying out both drug loading and final sterilization in one step. First, we verified that NTX aqueous solution can withstand autoclaving by recording the UV–Vis spectrum and the drug concentration before and after sterilization (Fig. S1, Supporting information) and no significant differences were observed. Hydrogels that underwent autoclaving in the NTX solution showed similar loading (Fig. 4b) than those loaded by soaking at room temperature (Fig. 4a) although a slight increase in the amounts loaded was recorded. For comparative purposes the hydrogels autoclaved in the NTX solution were allowed to stabilize at room temperature for 24 h (i.e., under the same conditions as the non-autoclaved hydrogels). After this time, the amount of NTX loaded by autoclaved hydrogels was 8.46 ± 0.72 mg/g for C1, 8.96 ± 1.1 mg/g for D1, 11.03 ± 0.76 mg/g for C2 and 9.25 ± 0.12 mg/g for D2.

3.4. Effect of autoclaving on NTX-loaded discs

Batches of imprinted and non-imprinted discs were loaded at room temperature (as explained above) and then transferred to vials containing fresh NTX storage solution (0.3 mg/mL, 3 mL) and autoclaved (121 °C, 20 min). The purpose of this step was to elucidate whether a subsequent sterilization process may alter the amounts loaded, either by promoting further uptake or by triggering discharge. After autoclaving, samples were stored at room temperature during 2 days. Non-imprinted BzMA/AAc discs (D1) showed a minor decrease in the amount of NTX loaded after autoclaving, but it was reloaded during storage (Fig. S4a, Supporting information). Imprinted discs prepared with BzMA/AAc (D2) were able to uptake a little more amount of NTX (1.71 mg/g; Fig. S4b, Supporting information) in addition to that previously loaded. Overall, the discs retained the initial amount of NTX with minor changes during sterilization and storage.

3.5. Naltrexone release

The lack of standardized methods to evaluate drug release from ocular solid formulations, and particularly CLs, makes comparison among published data difficult. In this regard, two main setups have been identified as the most suitable ones in terms of comparison of the ability of the CLs to provide sustained release and to predict in vivo behavior. These two setups consist in either agitated sink conditions or microfluidic flow devices [11,29,30,39]. For comparison purposes, release from NTX-loaded discs was investigated using both setups. Discs that had been loaded with NTX at room temperature were rinsed with water and the excess of solution on their surface carefully wiped. Infinite sink conditions were achieved using 3 mL of 0.9% NaCl under magnetic stirring (Fig. 5). NTX was sustainably released for 24–48 h; the discs providing almost 100% drug released without showing
plateaus of pseudo-equilibria [29]. Discs functionalized with AAc released higher amounts of drug to the medium than those contained in experimental eye drops (10^{-5} M) [6] already from the first half an hour. Imprinted discs C2 and D2 released 12.11 ± 0.33 and 13.16 ± 0.11 mg/g, respectively, and non-imprinted discs C1 and D1 released 9.90 ± 0.14 and 7.17 ± 0.05 mg/g, at day 8, in good agreement with the total amounts loaded. The well agitated conditions (200 rpm) prevented that a boundary layer was formed between the disc and the surrounding solvent, which could lead to false slow release. Thus, even under agitated sink conditions, which are prone to trigger a rapid discharge of the CLs, the NTX-loaded functionalized discs could cover one-whole day treatment.

Fitting of Korsmeyer–Peppas equation (Eq. (4)) to the experimental release data is shown in Table 2. In all cases, high correlation coefficients were achieved ($R^2 \geq 0.93$). The $n$ parameter values indicated that only the discs functionalized with AAc provide release profiles compatible with Fickian diffusion ($n \approx 0.5$). Thus, the calculation of the effective diffusivity was carried out for these hydrogels. Although all values of $D$ were of the same order of magnitude, a lower diffusivity was found for the non-imprinted hydrogels (especially for D1) compared to the imprinted ones ($p < 0.05$).

Release experiments were also performed under physiological-mimicking dynamic conditions using a microfluidic flow cell (Fig. S5, Supporting information). The cell used [30] simulates the hydrodynamic conditions of the eye (lachrymal fluid volume and renovation rate), which allows estimating the drug concentration in the volume of liquid in direct contact with the CLs. The knowledge of this concentration is critical to predict the in vivo efficacy of the drug released. Fig. 6a shows that, at each time, the amount of NTX released was lower than in the bulk test, and that the release occurred in a sustained way. The imprinted discs (C2 and D2) along with the non-imprinted disc C1 released the highest amounts of drug, being the obtained values similar. A similar tendency had been observed in the bulk test. All the other systems without AAc released the drug at a nearly constant rate. An overall analysis of the results indicated that the amount of NTX released under dynamic microfluidic conditions is well below the tolerance value reported for NTX (50 μM, i.e. 17 μg/mL [2]), which corresponds
to 73.7 μg in the lachrymal fluid per day, assuming an average
novation rate of the fluid of 3 μL/min. Moreover, the analyses of
the concentration curves (Fig. 6b) pointed out hydrogels C2 and D2 may
exert a therapeutic action during at least 3 and 3.5 days, respectively,
since the NTX concentrations remained above those provided by eye
drops (10^{-8} M) [6], i.e. 3.4 μg/mL, during these periods. It should be
noted that eye drops rapidly dilute in the lachrymal fluid and therefore
the effective concentration may be lower. Indeed, the IC50 for the
inhibition of the opioid receptor is around 5 nM, i.e. 0.0017 μg/mL [40].
In any case, renovation rate of lachrymal fluid in the post-lens region,
i.e. between a CL and the cornea, is slower than when an eye drop is
applied [21] and, therefore, relevant advantages of using CLs as NTX
release platforms compared to eye drops in terms of prolonged ther-
apeutic levels can be envisioned.

3.6. HET-CAM and cytocompatibility tests

Potential ocular irritation effects of NTX-loaded discs and NTX were
evaluated on the chorioallantoic membrane (CAM) of fertilized hen
eggs. Neither the loaded discs nor NTX aqueous solution (300 μL,
0.3 mg/mL) directly placed on the CAM induced haemorrhage, lysis or
coaugulation, and behaved as the negative control (0.9% NaCl) (Fig. 7),
which is in good agreement with the good ocular tolerance reported for
NTX eye drops [2]. Cytocompatibility tests with hMSCs confirmed the
high biocompatibility of hydrogels bearing AAc and BzMA after 72 h
direct contact (Fig. S6, Supporting information); all hydrogels were as
cytocompatible as the D2 group.

3.7. Bovine corneal permeability

Bovine corneal permeability test was carried out by monitoring the
amount of NTX that diffused from the CLs (C2 discs loaded with
835.97 ± 33.02 μg, and D2 discs loaded with 866.92 ± 38.21 μg)
towards the cornea and the receptor medium mimicking the aqueous
humour. As a control, a concentrated solution of NTX (350 μg/mL,
2 mL) in 0.9% NaCl was used; the total amount of drug supplied to the
donor compartment (700 μg) being slightly lower to the total amount of
drug contained in the CLs.

After 6-h test, NTX concentration in the 0.9% NaCl solution (1 mL)
of the donor chamber was 293.82 μg/mL for C2 and 295.93 μg/mL for
D2 discs. In the case of the control, the corneas were exposed to a high
NTX concentration since the very first minute; after 6 h test the NTX
decreased to 306.56 μg/mL. Nevertheless, the amount of NTX in the
receptor chamber was quite low and only quantifiable after 3 h (Fig. S7,
Supporting information). This means that NTX mainly accumulated into
the cornea and only a very small portion of the drug molecules (< 1%) reached the receptor compartment that mimicked the aqueous humour.

Amounts accumulated in cornea were 41.23 ± 5.77 μg/cm² for C2 and
37.66 ± 6.63 μg/cm² for D2 discs (Fig. 8). These values were not
statistically different from those recorded for the NTX solution
(32.37 ± 3.91 μg/cm²) in spite that the hydrogel discs sustainedly
released the drug.

4. Conclusion

Incorporation of AAc to HEMA network increases affinity for NTX
through weak interactions with the aliphatic nitrogen, hydroxyl and
carbonyl groups of NTX. The presence of the drug during polymeriza-
tion facilitates monomers arrangement creating specific cavities that
contribute to increase even more NTX affinity. Although OGF includes
hydrophobic interaction with its ligands, BzMA only plays a minor role
in the loading of imprinted networks. Swelling degree, oxygen perme-
ability and light transmission of functionalized hydrogels are in the
common range for CLs. Besides, no potential ocular irritation is
observed on the CAM neither cytotoxicity in hMSCs monolayer.
Relevantly from the fabrication point of view, loading and sterilization
can take place either simultaneously or in separate steps with minor
changes in total amount loaded. Imprinted hydrogels are able to control
NTX release for at least 2 days in well-agitated bulk medium and for a
more prolonged period in physiological-mimicking dynamic conditions,
maintaining therapeutic concentrations in the lachrymal fluid until day
3. Despite the sustained release, the NTX-loaded hydrogels allow re-
levant amounts of drug accumulate in the cornea in the first 6 h of
application. Thus, hydrogels containing AAc as functional monomer
may be suitable for preparing NTX-eluting CLs containing therapeutic
amounts of drug. The effects of a small change in thickness and cur-
vature of CLs on NTX release rate are expected to be minor but would
require further investigation.

Acknowledgements

This work was supported by MINECO [SAF2017-83118-R], Agencia
Estatal de Investigación (AEI) Spain, Xunta de Galicia [ED431C 2016/
008] and FEDER.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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