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Ocular injectable formulation assessment for oxidized dextran-based hydrogels

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Abstract

Initiator-free injectable hydrogels are very interesting for drug and/or cell delivery applications, since they can be administered in a minimally invasive way, and avoid the use of potentially harmful chemical initiators. In the current work, oxidized dextran crosslinked with adipic acid dihydrazide hydrogels were further characterized and tuned to produce formulations, with the aim of producing an injectable formulation for the possible treatment of posterior eye diseases. The gelation rate and the hydrogel dissolution profile were shown to be dependent on the balance between the degree of dextran oxidation, and the concentration of both components. For the in vitro studies, rabbit corneal endothelial cells were seeded on the hydrogels to assess cytotoxicity. Hydrogels prepared with low oxidized dextrans were able to promote cell adhesion and proliferation to confluence in just 24 h, while more highly oxidized samples promoted cell adhesion and proliferation, but without achieving confluence. Cell viability studies were performed using MTS assays to verify the non-cytotoxicity of hydrogels and their degradation byproducts, rendering these formulations attractive for further in vivo studies.

Keywords: Injectable; Hydrogel; Oxidized dextran; In vitro; Ocular delivery

1. Introduction

Ocular diseases of the posterior eye are the most common cause of visual disorders in industrialized countries [1]. These illnesses include, for example, cataracts, diabetic retinopathy, macular degeneration associated with aging, and retinitis pigmentosa [1,2]. These visual disorders cause discomfort, anxiety and fear of vision loss in patients.

The ocular drug market is dominated by drugs that were conceived to treat illnesses that affect the anterior eye; such medicines include antibiotics, anti-inflammatory agents or anti-glaucoma drugs, usually as eye-drop formulations [1,3]. These topical applications, in the form of eye-drops, are ineffective in many cases, mainly due to the drainage system of the eye, which leads to poor ocular bioavailability [4]. New drugs have been developed to reach the posterior eye, although most of these are administered through repeated intravitreal injections. This method is associated with complications such as pain, increased intraocular pressure, retinal detachment and endophthalmitis (which may lead to blindness). New methods of drug delivery that are more secure, efficient, comfortable and with prolonged activity are needed in order to minimize the number of injections. Currently, there are a number of systems for controlled drug delivery on the market or being tested, such as implants, hydrogels and colloids [1].

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Hydrogels are hydrophilic polymer networks, which may absorb thousands of times their dry weight in water. Hydrogels may be chemically stable or they may degrade and eventually disintegrate and dissolve. They are called "reversible" or "physical" gels when the networks are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces. Hydrogels are called "permanent" or "chemical" gels when they contain covalently crosslinked networks [5].

Recently, Van Tomme and Hennink [6] reviewed the different strategies used in producing dextran-based hydrogels, and demonstrated that dextran is a very versatile starting polymer for hydrogel synthesis. Furthermore, pharmaceuticals and various potential therapeutic agents can easily be incorporated and their release profile controlled. Recently, human embryonic stem-cell encapsulation was also successfully achieved in bioactive hydrogels of dextran-acrylate [7].

Dextran is biocompatible and can be degraded through the action of dextranases in various organs in the human body, including liver, spleen, kidney and colon [8].

Dextran oxidation by sodium periodate is an easy and well-known way to functionalize dextran with aldehyde moieties [9]. This chemical functionality has been widely tested to conjugate N-nucleophiles, due to their fast and almost complete reaction [10]. This approach has been tested on the synthesis of pro-drugs [11], as a spacer in enzyme immobilization [12] or for growth factor controlled release [13]. For the preparation of hydrogels, different aminated crosslinkers, such as chitosan [14,15], gelatin [16], 8-arm polyethylene glycol (PEG) amine [17] or polyhydrazides [18] have been used to yield chemical initiator-free formulations.

Recently, we described an injectable formulation composed of oxidized dextran (dexOx) crosslinked with adipic acid dihydrazide (AAD) [19]. The dexOx with 15% oxidation degree (OD) was crosslinked with AAD, forming a gel within 2–4 min, depending on the AAD concentration used. The obtained hydrogels were characterized by their mechanical properties (7–32 kPa), swelling and degradation (9–23 days) behavior under physiological conditions.

In this work, oxidized dextran, with various ODs, was studied in order to improve the control of the system properties. The influence of dextran OD and concentration in the solution viscosity was monitored. We observed that the hydrogel swelling and dissolution could also be controlled by the dextran OD and that the dissolution profile could be extended for more than 2 months, improving the previous studied formulation. Cell toxicity assays were carried out in 96-well plates with different dexOx hydrogels. Cell viability studies were performed using rabbit corneal endothelial cells, which were seeded on top of the dexOx hydrogels. The cell adhesion and growth was visualized by optical microscopy and dehydrogenase activity of cells was evaluated by reduction of the MTS reagent.

2. Materials and methods

2.1. Materials

Dextran (from *Leuconostoc mesenteroides*; M_w 60,000 Da, according to Fluka's specification), sodium periodate, adipic acid dihydrazide, *tert*-butyl carbazate (tBC), ethyl carbazate (EtC), phosphate-buffered saline (PBS), dialysis membranes (MWCO ~12,000 Da), amphotericin B, L-glutamine, Eagle's Minimum Essential Medium (MEM), penicillin G, streptomycin and trypsin were purchased from Sigma (Sintra, Portugal). Fetal bovine serum was purchased from Biochrom AG (Berlin, Germany). The 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and electron coupling reagent (phenazine methosulfate; PMS) were purchased from Promega. T-flasks and 96-well plates were purchased from Nunc (Denmark).

2.2. Dextran oxidation

An aqueous solution of dextran (1 g; 0.125% w/v) was oxidized with 2 ml of sodium periodate solution with different concentrations (33–264 mg ml^{-1}) to yield theoretical oxidations from 5 to 40%, at room temperature. The reaction was stopped after 4 h. The resulting solution was dialyzed for 3 days against water, using a dialysis membrane with a MWCO 12–14,000 Da, and then lyophilized (Snijders Scientific type 2040, Tillburg, Holland). The scale-up of the reaction was done using the same procedure though using 30 g of dextran and a calculated amount of periodate to yield a theoretical oxidation of 5, 10, 25 and 40%.

2.3. Nuclear magnetic resonance (NMR) and size exclusion chromatography (SEC)

The OD of dexOx is defined as the number of oxidized residues per 100 glucose residues (OD refers to the theoretical value unless otherwise stated) and quantified by using tBC [20,21] and EtC. The carbazates react with aldehyde groups to form carbazones in the same way that hydrazones are formed in the presence of hydrazides.

^{1}H spectra were acquired on a Varian 600 NMR spectrometer (Palo Alto, CA) using a 3 mm broadband NMR probe. ^{1}H NMR spectra were recorded in D_2O (20–25 mg in 0.2 ml; pD of ~5.0) using a 90° pulse and a relaxation delay of 30 s. The water signal, used as reference line, was set at δ 4.75 ppm and was partially suppressed by irradiation during the relaxation delay. A total of 32 scans were acquired for each ^{1}H NMR spectra. The spectra were analyzed with iNMR software, version 2.6.4 (www.inmr.net).

SEC was performed in a HPLC system composed of a degasser and a WellChrom Maxi-Star k-1000 pump (Knauer), coupled to an LS detector (evaporative light scattering PL-EMD 960) and a single column (PL aquagel-OH Mixed 8 μm) from Polymer Laboratories. The

whole system was kept at room temperature and the eluent used was KNO_3 (0.001 M, pH 3.9) at a flow rate of 0.4 ml min^{-1}. Samples and standards were dissolved in the eluent at 4–6 mg ml^{-1} (Fluka Chemie AG, dextran standards from 12 to 80 kDa).

2.4. DexOx solution viscosity

Solutions of dextran and dexOx, with concentrations ranging from 10 to 30% (w/w) in PBS, were prepared and analyzed in a Brookfield Programmable D-II+ Viscometer with a S18 spindle, assisted by DVLoader v1.0 software. The chamber temperature was controlled by an external bath to 25 °C, and the chamber was loaded with 8 ml of solution.

2.5. Hydrogel preparation and characterization

The several oxidized dextrans were dissolved in different concentrations; dexOx 5% (D5) and 10% (D10) solutions were used at 30% (w/w) and dexOx 25% (D25) at 20% (w/w), in aqueous solvent (PBS) at 37 °C until a liquid solution was obtained and then kept at 5–8 °C, until further use. Then, 250 μl of a given dexOx solution was mixed with 250 μl of a given AAD solution on homemade Teflon® molds and allowed to crosslink for at least 2 h (except for the D5 hydrogels, which were left to cure overnight). The AAD concentrations used are calculated based on a given molar percentage of dextran residues. The hydrogel nomenclature is as follows: dexOx 5% + AAD 5% = D5A5, etc.

2.6. Dynamic swelling experiments

DexOx hydrogels, after being prepared and weighed (W_i), were immersed in PBS (~5 ml) in 6-well cell culture plates at 37 °C. At regular intervals, they were removed from the aqueous solution, blotted on filter paper, weighed (W_t) and returned to the original well while PBS was replaced.

Swelling index:

$$SI = \frac{W_t}{W_i} \quad (1)$$

2.7. Rheological analysis

Rheological experiments were carried out using the parallel plate geometry (20 mm diameter, steel) of a Haake Rheostress RS 1. To calculate the gelation period, both solutions (190 μl each) were mixed on the bottom plate, and the upper plate was positioned at a gap of 1 mm (after gap optimization). This procedure took around 20 s, after which the experiment was started at low frequency (0.5 Hz) and stress (0.1 Pa), to avoid interference with the network formation. The gelation rate was followed and the gelation period was considered to be the crossover point between G' and G'' ($G' = G''$).

2.8. Cell source and growth

Rabbit corneal endothelial cells were obtained as previously described [22]. Subsequently, cells were plated in 25 cm^3 T-flasks with MEM with heat-inactivated fetal bovine serum (FBS, 10% v/v) and growth factors to achieve primary culture in agreement with the procedures previously described in the literature [23]. T-flasks with cells were incubated in a 5% CO_2 humidified atmosphere at 37 °C. On day 3, the medium was changed and every 3 days thereafter. Six days later, cells attained confluence.

After confluence was obtained, cells were subcultivated using 5 min incubation in 0.18% trypsin (1:250) and 5 mM EDTA. The free cells were added to an equal volume of culture medium. Following centrifugation, cells were resuspended in sufficient culture medium and seeded in 96-well plates containing the biomaterials.

2.9. Cell culture and in vitro cytotoxicity studies

Two crosslinking degrees of AAD for each dexOx were selected and used for cytocompatibility tests. The dexOx samples and AAD were either dissolved in PBS or in the appropriate cell culture medium, i.e. MEM.

Each formulation in the form of hydrogel was introduced ($n = 6$) into the wells of 96-well cell culture plates, in amounts that never exceeded 60 μl. The plates were irradiated for 30 min with UV, before being seeded with cells.

Fourth-passage endothelial corneal cells were seeded, at a density of 90,000 cells per well, into a 96-well plate containing the hydrogels. The plate was incubated at 37 °C, under a 5% CO_2 humidified atmosphere. After 1, 3 and 7 days, cell viability was assessed through MTS assay. A CellTiter 96® AQueous Assay composed of MTS and PMS (Promega) were used. Twenty microliter of MTS/PMS were added to each sample and incubated for 4 h at 37 °C, under a 5% CO_2 atmosphere. The absorbance of the samples was determined at 492 nm using a Biorad Microplate Reader Benchmark.

Wells containing cells in culture medium without biomaterials were used as negative control (K−). Ethanol (96%) was added to wells containing both types of cells as a positive control (K+). The samples were analyzed using an Olympus CX41 optical microscope equipped with an Olympus SP-500 UZ digital camera.

3. Results and discussion

3.1. DexOx characterization

Following previous work [19], we characterized hydrogels made from dexOx with a wider range of ODs, crosslinked with different amounts of AAD. Dextran was oxidized by using sodium periodate at different percentages and characterized by ^1H NMR. The OD was not estimated by the TNBS assay, due to the good correlation of the NMR titration with the colorimetric assay, as shown in previous work [19]. The

Table 1
Oxidation degree of several oxidized dextrans calculated by ^{1}H NMR analysis after titration with different carbazates and molecular weight evolution.

Sample	Oxidation degree			Mn[c]	PDI[d]
	$NaIO_4$[a]	tBC[b]	EtC[b]		
Dextran	–	–	–	40.9	1.47
D5	5	2.5 ± 0.3	3.6 ± 0.1	40.8	1.59
D10	10	7.4 ± 0.9	8.6 ± 0.2	37.9	1.61
D25	25	18.9 ± 0.4	22.2 ± 0.7	29.7	2.03
D40	40	–[e]	33.0 ± 0.8	8.3	3.05

[a] Theoretical OD, calculated as the molar ratio of sodium periodate per initial glucose unit in dextran.
[b] Calculated by ^{1}H NMR after titration with tBC/EtC, taking into account the ratio between the integral of the peak at δ 7.3 ppm and the integral of the anomeric proton at δ 4.9 ppm. Average and standard deviation of five independent integrations.
[c] Number-average molecular weight estimated by SEC.
[d] Polydispersity index corresponding to M_w/M_n.
[e] Precipitation occurred.

tBC titration, however, causes sample precipitation when reacted with high OD samples, thereby precluding acquisition of the NMR spectra (D40, Table 1). We suggest that this effect is caused by the large *tert*-butyl moiety and we hypothesized that a less bulky molecule, such as EtC, would not cause sample precipitation. In fact, both spectra are similar except for the ethyl proton peak which is sharper and slightly shifted upfield in comparison to the *tert*-butyl peak (spectra not shown). The real OD was calculated by taking into account the ratio between the integral of the peak at δ 7.3 ppm (arising from the carbazone group formed either with tBC or EtC) and the integral of the anomeric proton at δ 4.9 ppm. The ODs obtained with EtC were slightly higher (Table 1) than that obtained with tBC titration, which may suggest that tBC as some difficulty in reacting further due to its bulkier moiety.

The dexOx number-average molecular weight (Mn), estimated by a SEC analysis, showed a clear decrease with increasing OD, but also an increasing polydispersity index (PDI), reflecting the higher range of dexOx molecular weights (Table 1).

3.2. DexOx solution viscosity

In the design of injectable hydrogel formulations, details such as viscosity become increasingly important, in order to choose the best syringe geometries and/or needle gauges. In general, the hydrogel characteristics can be tailored by the feed concentration [14], degree of polymer modification and, in other cases, chemical initiator concentration [24]. The system reported in this paper involves the mixing of two equal-volume solutions, each of which has its own reactive species: DexOx residues and AAD. Thus, the formulation has to take into account the halving of the feed concentration of both solutions after mixing.

Every dexOx sample, at any of the concentrations tested, showed a linear shear stress increase regarding the different shear rates, and hence a Newtonian behavior. For every concentration tested, across the different ODs, the obtained viscosities for each dexOx series show a natural trend directly related to the concentration, as can be observed on Fig. 2A for dextran and D40. The influence

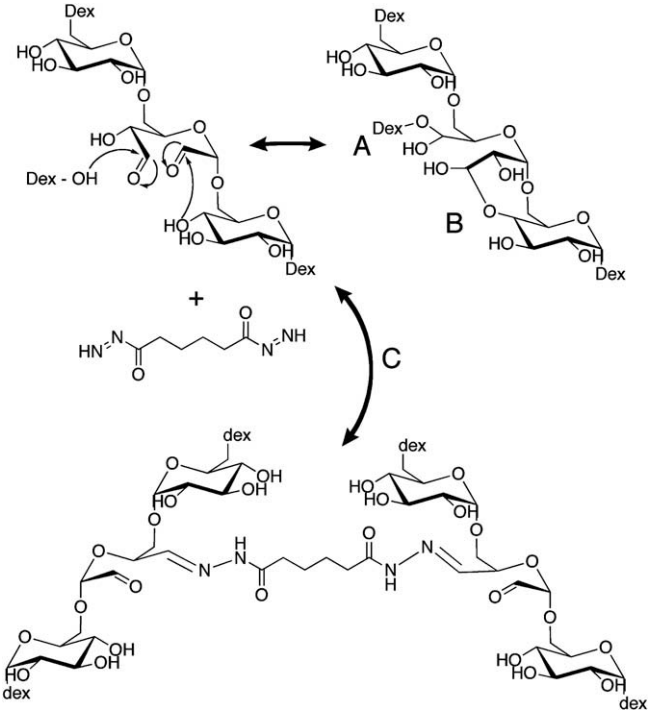

Fig. 1. The reactive aldehyde groups formed upon periodate oxidation are prone to establish inter (A) or intra (B) hemiacetals, when reacting with hydroxyl groups from nearby residues. The addition of AAD (C) promotes the reversible crosslinking with the formation of hydrazones.

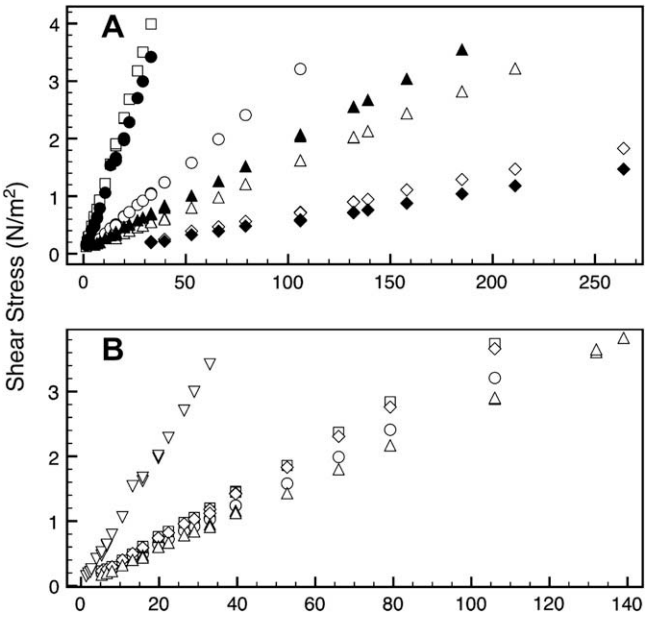

Fig. 2. Shear stress vs. shear rate. (A) Dextran with concentrations (w/w) of: □ – 30%, ○ – 20%, △ – 15% and ◇ – 10%; and D40 with concentrations (w/w) of: ● – 20%, ▲ – 15% and ◆ – 10%. (B) All solutions at 20% (w/w): ○ – dextran, □ – D5, ◇ – D10, △ – D25 and ▽ – D40.

of OD on viscosity seems to be greater when the polymer concentration is high, but as the polymer solution becomes less concentrated, the viscosity tends to decrease and eventually becomes lower than that of the reference solution (dextran).

Within the same concentration range, only the D25 viscosity falls below the dextran viscosity, but on the other hand, D40 has an extremely high viscosity, reflecting the influence of the high reactivity, due to the high OD (Fig. 2B).

We suggest that this effect is due to the decrease in molecular weight with oxidation, which, below a certain concentration, counterbalances the crosslinking via hemiacetals (Fig. 1A), decreasing the solution viscosity (Fig. 2A). This effect could possibly be further enhanced by a dexOx chain coil due to the intrahemiacetal formation (Fig. 1B), demonstrating how both parameters (OD and M_w) can affect the viscosity of a given dexOx solution.

3.3. Hydrogel characterization

The hydrogel preparation took into consideration three factors: the dexOx concentration, the OD of the dexOx and the AAD concentration. The first two are closely related due to the viscosity issues discussed above. Therefore, the concentration used was as high as the viscosity would allow. Solutions of D5 and D10 with 30% concentration allow a good homogenization, despite the high viscosity, but for D25, we had to use a 20% concentration. The D40 macromonomer was not studied in as much detail as the others, due to its high reactivity in the desired concentration range. The D40 with a 20% concentration yields a very viscous solution that reacts promptly with AAD, impairing good homogenization. Lower concentrations produce better hydrogels, though with less attractive and more unpredictable swelling profiles. Nevertheless, the D40 gelation rate data is presented (15% feed concentration) to emphasize the effect that the balance between OD and AAD feed concentration has on the gelation rate (discussed below).

The balance between the available crosslinking points (OD) and the amount of crosslinker used (AAD feed concentration) clearly affects the gelation rate, as shown on Table 2, but not always in the same direction. The gelation periods of D25 and D40 hydrogels decrease with increasing AAD, while for the D5 and D10 hydrogels, the gelation periods increase directly with AAD concentration. For low ODs, the excess AAD increases the number of non-valid crosslinks, caused by the reaction of a single hydrazide, leaving a dangling end [25], and retarding hydrogel formation. The polymer feed concentration also affects the gelation periods, as can be observed with the D40 hydrogels, which should be faster than the D25 for the same AAD feed concentrations.

Table 2
Gelation periods estimated for each dexOx with different AAD concentrations.

dexOx	Feed conc. % (w/w)	Gelation period (min)[a]		
		5% AAD	10% AAD	20% AAD
D5	30	66.7 ± 1.4	77.8 ± 5.2	–[b]
D10	30	14.9 ± 1.7	16.8 ± 0.5	18.8 ± 1.3
D25	20	2.8 ± 0.3	1.6 ± 0.9	1.3 ± 0.2
D40	15	3.7 ± 0.4	2.6 ± 0.1	2.3 ± 0.1

[a] The gelation period was considered when $G' = G''$; values are means ± SD ($n = 4$).
[b] Hydrogel not formed.

The dexOx/AAD hydrogels were prepared in Teflon molds and allowed to cure for at least 2 h, except for the D5 hydrogels, which were left overnight due to their slow curing rate. Equal volumes of dexOx and AAD solutions were added and mixed vigorously with the pipette tip, to achieve a good homogenization of both solutions.

The hydrazide groups in AAD react with the aldehyde groups in dexOx, forming hydrazone bonds, which are hydrolyzable (Fig. 1C), therefore making these hydrogels soluble in different timeframes. The swelling index (Eq. (1)) of the different sets of hydrogels show how the dissolution profiles could be controlled, not only by the amount of AAD but also by the dextran OD. By increasing the OD, we can significantly increase the dissolution time of the hydrogels. This oxidation increase enables more AAD to be used as a crosslinking agent, resulting in longer-lasting hydrogels. By balancing these two variables it is possible to control the dissolution profile of the hydrogels.

As shown, low ODs do not allow long dissolution times. However, it is perfectly possible to control the dissolution profile with the AAD concentration, within a range of 5 days (box in Fig. 3). By increasing the OD, hydrogels more resistant to dissolution are obtained. The D25 hydrogels have very interesting profiles (Fig. 3). The dissolution time could be extended up to 70 days. Furthermore, one can observe an interesting plateau, with 20% AAD, resembling a saddle, approximately from the second until the 50th day

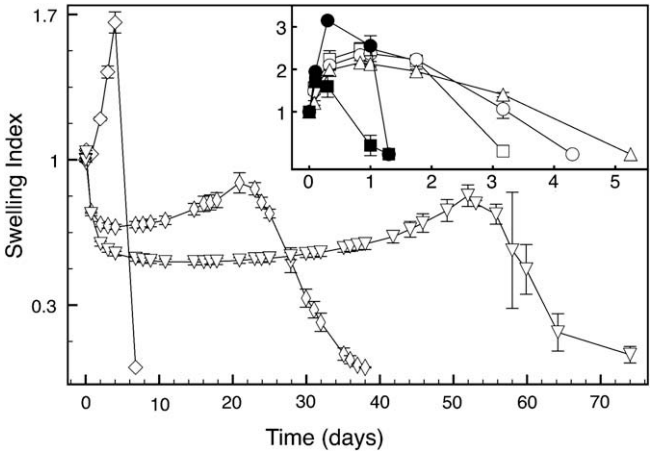

Fig. 3. Swelling index profile of dexOx + AAD hydrogels. D25 with feed concentration of 20% and D10 and D5 with feed concentration of 30%. ◇ – D25A5; ◇ – D25A10; ▽ – D25A20; inset: □ – D10A5; ○ – D10A10; △ – D10A20; ■ – D5A5; ● – D5A10.

of immersion in phosphate buffer. Within this period the hydrogels maintained their size and shape without abrupt variations in swelling. This profile seems to be interesting for the proposed application, as it will not swell above the injected volume and takes a few weeks to dissolve. Just before the hydrogel collapses and dissolves, a maximum swelling peak is observed. We hypothesize this peak to be a fracture point, reached when the osmotic force equals the polymeric matrix force, which weakens through time due to diffusion of AAD out of the hydrogel.

The water content of the vitreous humor is around 98%—mainly composed of collagen, hyaluronic acid [26] and hyalocytes of Balazs, which take care of removing cellular debris and reprocessing the hyaluronic acid [27]. Since the dexOx hydrogels are soluble in water and electrolyte solutions, we think that the vitreous humor will naturally contribute to the dissolution of the hydrogel and help the elimination of hydrogel. The by-products of the designed hydrogel are its initial components: dexOx and AAD. Both by-products should be excreted into the aqueous humor via the anterior route. This elimination is probably faster than through the blood–retina barrier to the systemic circulation [1], even though dextrans up to 150 kDa can diffuse through the sclera [28].

3.4. Assessment of cytotoxic potential

The characteristics of these formulations—their injectability, controlled gelation rate and dissolution profile—render them suitable as drug carriers to the posterior part of the eye. Formulations composed partially by dexOx have been tested for their biocompatibility. Recently, Bhatia et al. [17] seeded 3T3 fibroblast cells on top of oxidized dextran (20–50% OD) crosslinked with eight-arm PEG amine and showed that their formulation was non-toxic and could lead to cell confluence after 24 h of growth. This suggests that the crosslinked dexOx can promote better cell adhesion than dextran itself [29], despite showing some toxicity when present, alone, in cell culture with mesothelial cells [30].

In the present work, endothelial cells from rabbit cornea were chosen based on the need to assess the cytotoxicity of the dexOx–AAD crosslinked hydrogel for the proposed application.

To assess the cytotoxicity of these materials, several combinations of hydrogels involving the different concentrations of dexOx and AAD were prepared. The 96-well cell culture plates were covered with 60 μl of hydrogels or just controls and the cells were seeded on top, after an overnight curing period. The materials used were not only dissolved in PBS but also in the appropriate cell culture media. Occasionally, the initial dexOx feed concentration had to be slightly decreased, due to the high viscosity, probably due to proteins in the medium crosslinking with the dexOx through existing amine groups.

After cell seeding on top of the hydrogels, each well was visualized by optical microscopy, to observe whether there was any cell adhesion and/or proliferation. Cells grew in

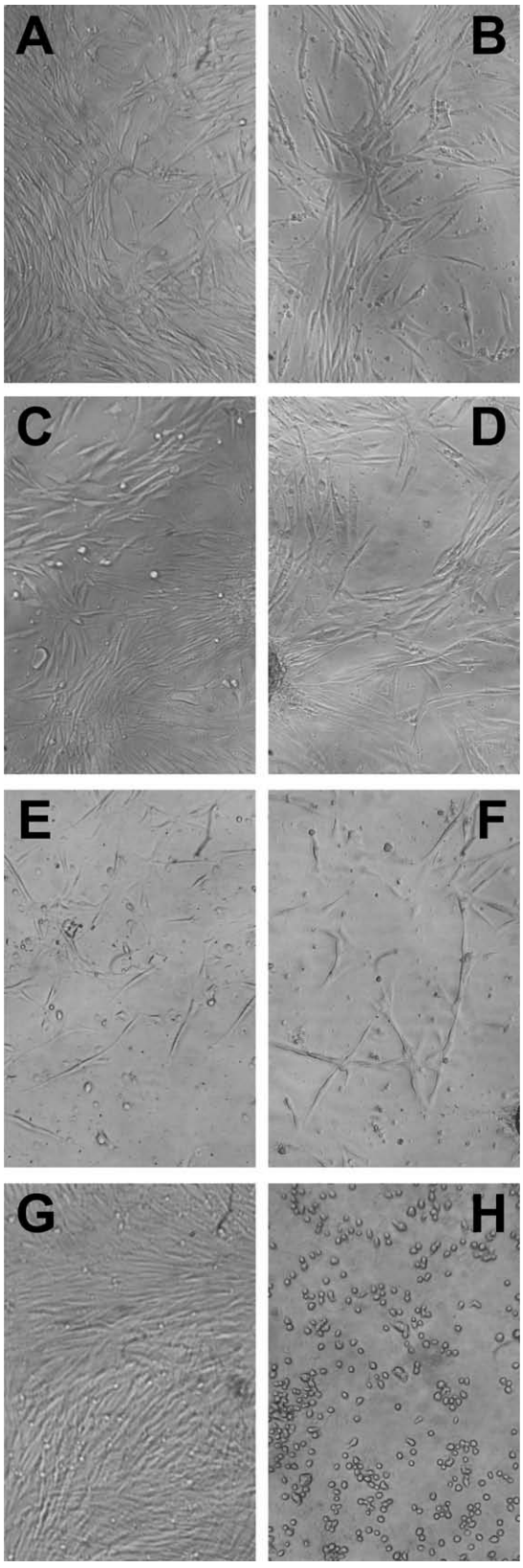

Fig. 4. Endothelial cells isolated from rabbit cornea seeded in dexOx + AAD hydrogels, dissolved in MEM or PBS, after 72 h. (A) D5A5, PBS; (B) D5A5, MEM; (C) D10A10, PBS; (D) D10A10, MEM; (E) D25A10, PBS; (F) D25A10, MEM; (G) negative control; (H) positive control. Original magnification 100×.

the presence of all hydrogels tested, although cell growth, in the presence of D25 hydrogels, did not achieve confluence during the period of study (Fig. 4).

Dextran is neutrally charged and we expect dexOx to maintain this neutrality as the periodate oxidation reaction does not yield any charged chemical groups. However, the crosslinking of dexOx with AAD (pK_a 2.5) yields hydrazone bonds which were reported to have a pK_a of ~3–4 [31]. Hence, due to the nature of the crosslinking bond, we suggest that the hydrogel zeta potential shifts negatively, allowing cell growth and proliferation.

It is well known that the surface chemistry of hydrogels can affect cell adhesion, proliferation and other phenomena. Chen et al. [32] showed how the nature of the polymer and the crosslinker concentration can dictate the surface charge density of the gels and strongly influence the cell behavior. They have identified a threshold for the surface zeta potential (−20 mV), below which cells adhere and proliferate.

Schneider et al. [33] also reported that the charge density of the hydrogel can regulate cell attachment either directly in the absence of extracellular matrix components, or indirectly through the association of extracellular matrix proteins found within the serum due to charges on the hydrogel surface.

The MTS assay is a quick and effective method for testing mitochondrial impairment and correlates quite well with cell proliferation. In recent years, it has been frequently used as a preliminary screen for the evaluation of *in vitro* cytotoxicity of polymeric components.

The MTS assays were performed at 1, 3 and 7 days after cells being seeded on top of the hydrogels. Cellular activity did not seem to be affected by the medium used to prepare the hydrogels (MEM or PBS). The results (Fig. 5) emphasize that every formulation promoted dehydrogenase activity. In the first 24 h, all hydrogels promoted higher enzyme activity than the negative control. By the 3rd day, the activity had doubled for most hydrogels, with a few exceptions, and by the 7th day the activity reached a constant level. These results demonstrate that the tested formulations are non-cytotoxic.

4. Conclusions

In order to estimate the degree of oxidation of dextran, we have identified EtC as a better and more accurate titrant than tBC, especially for highly oxidized samples. The bulkier tBC moiety causes sample precipitation and also seems to impair good access to the oxidized residues, lowering the measured OD.

The viscosity studies revealed how the dextran OD can limit the concentration used. However, for diluted dexOx solutions, the lower molecular weight counterbalances the oxidation effect, decreasing the viscosity.

The characterized hydrogels have a dual crosslinking control method, which depends on the dextran OD and the amount of AAD used. The OD allows a certain proportion of residues to serve as reactive points for molecules such as AAD. The amount of AAD used is crucial in defining the gelation period, mechanical properties and dissolution profile of the hydrogels. It should be within the same range of the amount of oxidized residues present in dextran in order to avoid forming dangling ends rather than valid mechanical crosslinks.

In the cytotoxicity assay, the different dextran ODs tested were successful in promoting cell adhesion and growth. However, with the increasing oxidation, the cells took longer to proliferate in the hydrogels. Oddly, the metabolic activity, measured by the MTS assay, showed very high levels of activity for the more highly oxidized

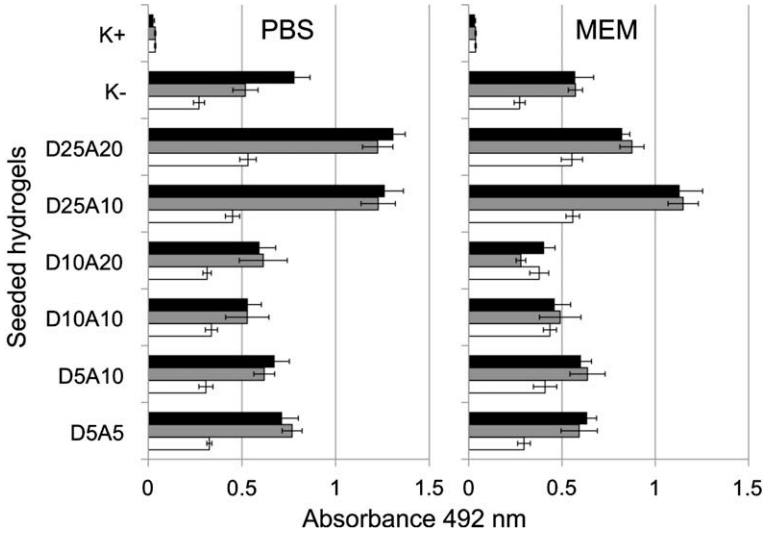

Fig. 5. Cellular activities measured by the MTS assay after 1 day (open bars), 3 days (gray bars) and 7 days (black bars). Endothelial cells from rabbit cornea, seeded onto dexOx + AAD hydrogels. The materials were either dissolved in PBS or MEM. K+, positive control; K−, negative control. All error bars represent one standard deviation from six experiments.

hydrogels, which does not correspond to the cellular proliferation observed.

The system investigated here aims to provide a reliable drug delivery device for the posterior part of the eye that uses a single injection, but does not require further surgery for the removal of the device. The designed formulation is expected to dissolve and be eliminated naturally by the organism.

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