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Free and copolymerized γ-cyclodextrins regulate performance of dexamethasone-loaded dextran microspheres for bone regeneration

A. C. Lima, A. M. Puga, J. F. Mano, A. Concheiro and C. Alvarez-Lorenzo

Polymeric particles acting as source of biological cues to promote tissue regeneration are currently an interesting topic in bone tissue engineering research. In this study, microspheres of dextran-methacylated (dextran-MA) and γ-cyclodextrins (γ-CD) for the delivery of osteogenic agents were prepared by means of photopolymerization on biomimetic superhydrophobic surfaces. The effects of the incorporation of the γ-CD units as free entities or as structural monomers (acylamidomethyl-γ-cyclodextrin, γ-CD-NMA) on dexamethasone loading and release performance were evaluated in detail in order to achieve osteogenic differentiation of human stem cells. The copolymerization of dextran-MA with γ-CD-NMA improved the loading capacity of the particles and also provided a sustained release of dexamethasone for several days. The biological studies revealed that such microspheres were cytocompatible and capable to induce the differentiation of human adipose-derived stem cells (hASCs) to osteoblasts, as determined from an increase of alkaline phosphatase (ALP) activity between days 3 and 7. Such results were also confirmed using ALP staining. Therefore, immobilization of γ-CDs onto dextran-MA network may be particularly useful for the development of cytocompatible implantable spherical biomaterials for bone tissue engineering purposes.

Introduction

Cyclodextrins (CDs) are appealing biocompatible tools for the design of drug delivery systems based on hydrophilic hydrogels. The capability of CDs to form inclusion complexes with a variety of active substances usually does not diminish, but even enhances when the CDs are trapped in a polymer network. Thus, poorly soluble drugs can be homogeneously loaded up to great extent in hydrophilic networks forming complexes with CDs. Regarding control of the release kinetics, the inclusion complex capability of CDs can be exploited in two different ways: a) as free entities, i.e., not chemically bound to the network, CDs can accelerate or retard drug diffusion through the network depending on drug solubility and interactions with the polymer component of the hydrogel; and b) as chemically bound moieties, CDs act as binding points for the drug, regulating the release by an affinity-dependent mechanism. Differently to other additives, such as micelle-forming surfactants or liposome-forming lipids that require self-assembly for hosting drugs and regulating the diffusion from polymer networks, CDs offer relevant advantages as highly stable unimolecular guest agents. Several approaches can be followed to immobilize CDs in polymer networks through covalent bonds under mild conditions. Recently, γ-CDs nanogels obtained via direct cross-linking in an emulsified system were shown able to load dexamethasone for application as eye drops, being highly biocompatible in vivo studies performed in rabbits. The aim of this work was to use the superhydrophobic surface technology to produce spherically shaped microgels combining dextran-MA and γ-CD that can deliver dexamethasone and induce the differentiation of stem cells into osteoblasts, for tissue engineering or regenerative medicine purposes. The influence of free γ-CDs and copolymerized γ-CD-NMA (acylamidomethyl-γ-cyclodextrin) on the capability of hydrogel particles to load and to control the release of the poorly water soluble corticoid drug was evaluated in detail. Dextran is a highly hydrophilic and biocompatible natural polysaccharide consisting mainly of linear chains of α-1,6-linked glucopyranose monomers, widely used as carrier for hydrophilic bioactive agents and as component of scaffolds. On the other hand, γ-CDs are highly soluble in water (18.5-39 g/100 mL at 20-35°C) allowing the production of hydrogels with high content in freely dispersed γ-CDs. Moreover, monomers of γ-CD have been previously shown useful to enhance the uptake of hydrophobic drugs by acrylic networks. Despite the potential advantages of combining dextran and CDs, very few
binary systems have been reported yet. Fernández et al. synthesized a copolymer of dextran and amino derivatives of β-CD to be applied as thermoprotectant of trypsin. Later, Ramirez et al. reported a covalently bonded β-CD-dextran as macromolecular carrier for naproxen, a poorly soluble non-steroidal anti-inflammatory drug. Using click chemistry, Nielsen and co-workers synthesized an open-chain polymer resultant of the combination of alkyne-modified dextran and β-CDs functionalized with azide groups. Using two formulations proposed in such work, Fulop et al. studied their complex forming properties with a model drug and their aggregation ability. Stable nanoassemblies spontaneously formed by mixing dextran-β-CD polymers and dextran-adamantyl have been explored for drug delivery due to their low toxicity. However, to the best of our knowledge, dextran-CD networks have not been explored yet for sustained release in tissue engineering purposes. In this context, particulate drug delivery systems are especially attractive due to its potential to be implanted via minimal invasive maneuvers.

Under osteogenic stimulus, some stem cells have the ability to differentiate into osteoblasts, expressing characteristic markers such as bone-specific alkaline phosphatase (ALP). This ability of the stem cells can be favorable in the development of bone tissue engineering strategies. For example, human adipose-derived stem cells (hASCs) have been shown to exhibit potential to differentiate into mesodermal phenotypes including osteoblasts, chondrocytes, adipocytes, endothelial, neural and muscle cells. An advantage of adipose tissue as a source of stem cells is its abundance and easy accessibility. The osteogenic differentiation of hASCs in vitro has been reported by supplementing the cell culture medium with ascorbic acid, β-glycerophosphate (β-GP) and a glucocorticoid such as dexamethasone. The osteogenic differentiation varies with the dexamethasone dose; the optimal concentration being between 10 and 100 nM. Toxic effects have been found at 1000 nM dexamethasone. In fact, the prolonged duration of exposure in vitro may cause severe osteoporoses. To prepare the proposed spherical microgels (dextran + CDs) a technique consisting in the photopolymerization of aqueous dispersion droplets onto superhydrophobic surfaces was implemented. Compared to other techniques, this bioinspired methodology is simpler, cheaper and faster, does not require the use of organic solvents or complex apparatus and allows obtaining particles with narrow size distribution and 100% encapsulation yield of freely diffusible species. This technology permits also the production of multilayered particles for protein or cell encapsulation under mild conditions. The loading capability, the ability to sustain the release of dexamethasone, the cytocompatibility of the final formulated systems and their potential to be applied in bone regeneration were evaluated in detail.

Experimental

Materials

Polystyrene (PS) sheets from square petri dishes (Bdbioscience, Enzifarma, Portugal) and polystyrene granules from a grade for injection molding (Styrolution PS 158k; UL Ides, Portugal) were used in the preparation of the superhydrophobic substrates. Tetrahydrofuran (THF, 99.9%) was from Riedel de Haen (Germany). 1H,1H,2H-2H-perflurodecylethoxyxylylamine (PFDTs, 97%), dextran from Leucosostoc mesenteroides (MW 100-200 kDa), 4-(N,N-dimethylamido)pyridine (DMAP, 99%), hydrochloric acid (HCl, 37%), acetic acid, dexamethasone for cell culture, penicillin/streptomycin solution, L-glutamine, β-glycerophosphate disodium salt hydrate (β-GP), ascorbic acid, ALP substrate, p-nitrophenyl phosphate liquid substrate, naphthol AS-BI phosphate disodium salt, fast red violet, 2-amino-2-methyl-1,3-propanediol, and Dulbecco’s Modified Eagle medium (DMEM) nutrient mixture F-12 Ham were from Sigma-Aldrich (USA). BCA kit was purchased from Pierce (USA), Cell Proliferation Kit (MTT) and Cytotoxicity Detection Kit (LDH) were supplied by Roche (Switzerland). Paraformaldehyde tablets were from Panreac (Spain). Mesenchymal stem cells StemPro® human adipose-derived stem cells (hASCs), MesenPro RS basal Medium and MesenPro RS Growth supplement were from Gibco (Invitrogen, USA). Phosphate buffer solution 10x and dimethyl sulfoxide (DMSO) were from Fisher Chemicals (UK). Glycidyl methacrylate (GMA) was from Fluka (Sigma-Aldrich, USA), Irgacure 2959 from Ciba (BASF, Germany) and dexamethasone was from Fagron (Spain). γ-Cyclodextrin (γ-CD) Cavamax W8 was from ISP and N-(hydroxymethyl)acrylamide (NMA) from Merck (Germany). Purified water (resistivity >18 MΩ cm; MilliQ®, Millipore, Spain) was obtained by means of reverse osmosis. All the other chemicals were used as received.

Polystyrene superhydrophobic surfaces

Polystyrene (PS) superhydrophobic surfaces were prepared using a phase separation method as described elsewhere. Briefly, a PS solution (70 mg/mL) in THF was mixed with ethanol (2:1.3 v/v). The mixture was dispensed onto smooth PS commercial substrates which were then immersed in ethanol for 1 min and dried under nitrogen flow. In order to increase the superhydrophobicity of obtained surfaces, the rough PS surfaces were modified with PFDTs (1% v/v in ethanol) after argon plasma treatment for 40 s at 30 W (Plasma Prep5, Gala Instruments, Germany).

Synthesis of dextran-methacrylated (dextran-MA)

Dextran-MA was synthesized as previously described. Briefly, dextran (25 g) was dissolved in DMSO (225 mL) under nitrogen atmosphere. Then, 4-(N,N-dimethylamido)pyridine (5 g) was added and, when the dissolution was complete, glycidyl methacrylate (20.5 mL) was incorporated. The solution was stirred at room temperature for 48 h, and then the reaction was stopped by adding an equimolar amount of concentrated HCl solution (3.3 mL of HCl, 37%) to neutralize 4-(N,N-dimethylamido)pyridine. The reaction mixture was transferred to a dialysis tube (MWCO 12,400 Da) and dialyzed during 1 month against demineralised water. Finally, dextran-MA was lyophilized (Manifold freeze-drier, Telstar cryodos, Spain). 1H NMR spectra of unmodified and modified dextrans (0.3 g in 0.7 mL of deuterated water) were recorded (Mercury 300, 300 MHz, Varian, Australia) and then analyzed using MestReNova software (MestreLab, Spain).
Synthesis of acrylamidomethyl-\(\gamma\)-cyclodextrin (\(\gamma\)-CD-NMA)

\(\gamma\)-CD (17.12 g) and NMA (13.36 g) (NMA-\(\gamma\)-CD 10:1 molar ratio) were added to 50 mL of HCl solution (1\% v/v). The solution was heated at 80°C and stirred continuously for 30 min. Then, 300 mL of acetone were added to precipitate \(\gamma\)-CD-NMA. The flask was kept at 4°C overnight. The precipitate was filtered (Albet® 145, Spain), repeatedly washed with acetone (200 mL) and filtered again (2-3 cycles). Finally, the product was dried under vacuum for 2 days at room temperature and stored at 4°C. IR spectrum of \(\gamma\)-CD-NMA monomer was recorded over the range 400-4000 cm\(^{-1}\) (Bruker IFS 66V FT-IR, Germany) using the potassium bromide (KBr) pellet technique, and \(^1\)H NMR spectra of unmodified and modified \(\gamma\)-CD (30 mg/mL in deuterated water) were recorded (Mercury 300, 300 MHz, Varian, Australia) and then analyzed using MestReNova software (MestreLab, Spain).

Microgels preparation

Dextran-MA solutions (5\% or 10\% w/v; 1 mL) were prepared in water or in an aqueous medium containing 200 mg of \(\gamma\)-CD or \(\gamma\)-CD-NMA. Irgacure 2959 (60\µL or 100\µL of 0.5\% w/v in water) was added to the solutions (Table 1). 5\µL of each solution were dispensed onto the PS superhydrophobic surfaces, and crosslinked by UV-Vis detector (275 nm, SpectraSystem UV8000 - Thermo Scientific) and acetonitrile:water (90:10, 0.5 mL/min flow) as isocratic mobile phase.

Table 1 – Composition of the dispersions used to prepare the microgel particles. Amounts refer to 1 mL water.

<table>
<thead>
<tr>
<th>Code</th>
<th>Dextran-MA</th>
<th>(\gamma)-CD</th>
<th>(\gamma)-CD-NMA</th>
<th>Irgacure 2959</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextran-MA</td>
<td>50 mg</td>
<td>-</td>
<td>-</td>
<td>60 \µL</td>
</tr>
<tr>
<td>5% Dextran-MA + 20% (\gamma)-CD</td>
<td>50 mg</td>
<td>200 mg</td>
<td>-</td>
<td>60 \µL</td>
</tr>
<tr>
<td>5% Dextran-MA + 20% (\gamma)-CD-NMA</td>
<td>50 mg</td>
<td>-</td>
<td>200 mg</td>
<td>60 \µL</td>
</tr>
<tr>
<td>10% Dextran-MA</td>
<td>100 mg</td>
<td>-</td>
<td>-</td>
<td>100 \µL</td>
</tr>
<tr>
<td>10% Dextran-MA + 20% (\gamma)-CD</td>
<td>100 mg</td>
<td>200 mg</td>
<td>-</td>
<td>100 \µL</td>
</tr>
<tr>
<td>10% Dextran-MA + 20% (\gamma)-CD-NMA</td>
<td>100 mg</td>
<td>-</td>
<td>200 mg</td>
<td>100 \µL</td>
</tr>
</tbody>
</table>

Microgels characterization

Quantification of unreacted photoinitiator. Three particles of each formulation were immersed in 1 mL of acetonitrile and kept under magnetic stirring for at least 6 h at room temperature. The amount of Irgacure 2959 released was quantified by HPLC using an Accucore C18 (3 mm x 150 mm, 2.6 \µm) column kept at 37°C, a UV-Vis detector (275 nm, SpectraSystem UV80000 - Thermo Scientific) and acetonitrile:water (90:10, 0.5 mL/min flow) as isocratic mobile phase.

Double bonds (C=C) detection (bromine test). 5\% (w/v) dextran-MA, 10\% (w/v) dextran-MA, 20\% (w/v) \(\gamma\)-CD and 20\% (w/v) \(\gamma\)-CD-NMA precursor solutions (40 \µL, equivalent volume to prepare 8 particles) and the different formulated cross-linked particles (8 units) were placed in contact with 200 \µL of bromine in water at room temperature. After 5 min, the color of the solutions was observed and compared with the original bromine in water solution. The bromine in water was prepared mixing 32 mL of water, 7.6 mL of NaClO solution (33 mg/mL) and 10.7 mL of a solution of NaBr (1.1 g) in 1M HCl.

Degree of swelling. The degree of swelling was evaluated in sextuplicate as the difference between the weight of 10 particles swollen in water for 6 days (\(m_s\)) and the initial weight of the dried particles (\(m_0\)), as follows:

\[
% \text{Swelling} = \frac{m_s - m_0}{m_0} \times 100
\]

Particle size. The size of dried and swollen particles was evaluated using an Olympus SZ-CTV optical stereomicroscope (Tokyo, Japan) connected to a JCV TK-S350 video camera (Tokyo, Japan). Eight particles of each formulation were sized using an image analysis software (Soft Imaging System®, Münster, Germany).

Scanning Electron Microscopy (SEM). The particles were allowed to swell in water at room temperature. Then, the images of the particles, after gold coating, were obtained using an EVO LS15 microscope (Zeiss, Oberkochen, Germany).

Dexamethasone solubilization

A large excess of dexamethasone (8 mg) was dispersed into 50 mL of water and maintained under magnetic stirring during 48 h. Then, the dispersions were filtered through 0.22 \µm nylon membrane to remove the non-dissolved drug. The concentration of the dissolved dexamethasone was spectrophotometrically measured at 241 nm (Agilent 8453, Waldbornn, Germany).

Dexamethasone loading

Ten particles of each formulation were dried at air overnight and then immersed in 10 mL of aqueous solution of dexamethasone (30 \µg/mL) for 6 days at room temperature. The absorbance of each solution was measured at 241 nm over time. The experiments were repeated six times for each formulation. The amount of drug loaded via equilibrium between the aqueous phase of the microgels and the loading solution was estimated as follows:

\[
\text{Loading (aqueous phase)} = (V_s/W_p)xC_o
\]

where \(V_s\) is the volume of water sorbed by each particle, \(W_p\) the dried weight of the particles, and \(C_o\) the concentration of drug in the loading solution.

Dexamethasone release

Dexamethasone-loaded particles (10 units) were dried overnight and then immersed directly in 2 mL of water and placed in an incubating mini shaker (VWR) at 37°C and 200 rpm. At 24, 120, 264 and 432 h, 1 mL of water was added to the initial release medium. Samples of the release medium were taken periodically and returned to the container.
after measuring the absorbance at 241 nm. The experiments were repeated four times for each formulation.

**Cytocompatibility studies**

Two particles of each formulation (4 replicates) were placed in 48 well plates containing SaOs-2 cells (ATCC HTB-85; 50,000 cells/well, 1 mL) in DMEM-F12 with 10% FBS and 1% penicillin/streptomycin, and kept in humidified incubator at 5% CO₂ and 37°C. After 24 h and 72 h incubation, aliquots of 100 µL of the culture medium were taken and mixed with 100 µL of the reaction medium contained in the Cytoxicity Detection Kit (LDH). Blank (100 µL of cell culture medium), negative (aliquot collected from a well containing just cells) and positive (aliquot collected from a well to which 20 µL lysis factor was added) controls were also performed. The plates were incubated 10 min at room temperature protected from the light. 50 µL of stop solution was added to each well and the absorbance measured at 490 nm using a microplate reader (BIORAD Model 680 Microplate Reader). The cytotoxicity was estimated as follows:

\[
\text{% Cytotoxicity} = \frac{\text{abs}_{\text{experiment}} - \text{abs}_{\text{negative control}}}{\text{abs}_{\text{positive control}} - \text{abs}_{\text{negative control}}} \times 100
\]  

**Osteogenic differentiation**

hASCs were cultured in MesenPro® RS medium supplemented with 2% (v/v) growth supplement, 1% (v/v) L-glutamine (200 mM) and 1% (v/v) penicillin/streptomycin (Medium 1). Then, they were seeded (passage 5, 25,000 cells/well, 1 mL) in 6 well plates and after 24 h the medium was replaced by 5 mL of fresh medium. Three particles of each formulation (placebo or loaded plates) were placed into direct contact with the cells and the Medium 1 was supplemented with 10 mM β-glycerophosphate and 50 µM ascorbic acid. Four controls were carried out in this experiment:

(i) **negative control 1**: cells cultured in MesenPro® supplemented with 2% (v/v) growth supplement, 1% (v/v) L-glutamine (200mM) and 1% (v/v) penicillin/streptomycin (Medium 1);
(ii) **positive control 1**: cells cultured in standard osteogenic differentiation medium, i.e., Medium 1 supplemented with 10 mM β-GP, 100 nM dexamethasone for cell culture and 50 µM ascorbic acid (Medium 2);
(iii) **negative control 2**: cells cultured in Medium 1 supplemented with 10 mM β-GP and 50 µM ascorbic acid (Medium 3);
(iv) **positive control 2**: cells cultured in Medium 1 supplemented with 10 mM β-GP, 50 µM ascorbic acid and 400 ng/mL of dexamethasone as used to load the formulations under study (Medium 4).

The plates were incubated at 37°C in a humidified atmosphere with 5% CO₂ and 2 mL of medium were replaced twice a week. The cells were routinely observed using inverted microscopy (x10) in order to discard any contamination. Experiment were carried out in triplicate. Cell proliferation was evaluated using the MTT assay. At 3, 7 and 12 days of incubation 4 mL of culture medium were withdrawn and 100 µL of MTT solution (5 mg/mL in PBS) were added to each well. These plates were incubated at 37°C for 4 h and then 1 mL of MTT solvent (10% SDS in 0.01 M HCl) was added to each well and the plates incubated again at 37°C overnight. Aliquots of 200 µL were transferred to 96 well plates and the absorbance read at 550 nm (BIORAD Model 680 Microplate Reader). The respective calibration curve was constructed using cultures with known number of cells in order to estimate the cell number in the experiment.

For ALP quantification, the cells were lysed at 3, 7 and 12 days by addition of 300 µL 10 mM Tris-HCl buffer (pH 7.5) with 0.1% Triton X-100. Samples were exposed to three freezing (-80°C for 45 min)/thawing cycles. Lysates were centrifuged at 14,000 rpm for 15 min at 4°C. 50 µL of the supernatant were incubated with 150 µL of ALP substrate in 37°C for 30 min. The absorbance at 405 nm was read at 5, 10, 15, 20 and 30 min using an ELISA plate reader (BIORAD Model 680 Microplate Reader). The ALP activity was quantified using a calibration curve prepared with p-nitrophenylphosphate solutions. The obtained values were normalized by the protein concentration measured using a BCA protein detection kit in order to express the results as nmoles of ALP per min per mg of protein.

ALP staining was carried out as follows. After each culture period, the medium was removed, and the cells were fixed with 4% paraformaldehyde in PBS for 5 min at room temperature and then washed twice with PBS. The cells were then soaked in a staining solution prepared by mixing 0.2% naphthol AS-BI phosphate in 112 mM 2-amino-2-methylpropanediol (AMPD) and 0.2% fast red violet in 1:1 (v:v) proportion, incubated in darkness for 10 min at room temperature and visualized in inverted light microscope.

**Statistical analysis**

All data are presented as mean ± s.d. for at least three independent measurements. Groups were compared using one-way ANOVA (Tukey’s multiple comparison test), with p<0.05 indicating statistical significance.

**Results and discussion**

**Synthesis of Dextran-MA and γ-CD-NMA**

Dextran functionalized with methacrylate groups (dextran-MA) was obtained by reaction of dextran with glycidyl methacrylate (GMA) in DMSO in the presence of a base (DMAP) as a catalyst 

\[ \gamma \text{-CD-NMA} \]  

After 48 h reaction at room temperature, DMAP was neutralized with hydrochloric acid in order to avoid alkaline hydrolysis of the methacrylic ester, and finally dextran-MA was purified by dialysis. The hydroxyl groups of dextran were ionized at alkaline pH and reacted with methylene carbon of the epoxy group of GMA to form the 3-methacryloyl-1-glyceryl ether of dextran or the respective isomer 2-methacryloyl-1-glyceryl, as shown in scheme 1.
Scheme 1 – Reaction of dextran with GMA in the presence of DMSO and DMAP. The glycidyl group may interact with any of the hydroxyl groups present in the dextran structure, and two region isomers could be created: 2-methacryloyl-1-glyceryl ether and 3-methacryloyl-1-glyceryl ether.

In the reaction mixture, the molar ratio of GMA to glucopyranose units was approximately 1. Based on $^1$H NMR spectra (Figure 1) and having into account that each glucopyranose unit have 3 hydroxyl groups that could react with GMA, the degree of substitution (DS) was determined as $(100X/Y)/3$; X being the average integral of the protons in the double bond present in the added groups (5.75 and 6.2 ppm), and Y the integral of the peak at 4.95-5.1 ppm corresponding to the anomeric proton$^{37}$. The DS obtained was ca. 32%, which indicated that in average 1 hydroxyl group per glucopyranose unit was substituted$^{55}$. These results were in agreement to the high yield of reaction previously reported in the literature, which indicated that more than 90% of the added GMA was incorporated in the polysaccharide$^{37}$.

Figure 1 – $^1$H NMR spectra of dextran as supplied (gray line) and after reaction with GMA (black line) dissolved in deuterated water.
The \( \gamma \)-CD-NMA monomer was prepared according to a previously established protocol that renders one CH\(_2\)=CH- group per \( \gamma \)-CD, as confirmed from \(^1\)H NMR spectrum (Figure 2 B). The DS was estimated from \([100X/(Y/8)]/3\), where X was the average integral of the protons in the vinyl groups (6.2 and 5.8 ppm), and Y the integral of the peak (5.0 ppm) corresponding to 8 protons of the \( \gamma \)-CD (1 proton per glucopyranose unit). The obtained result indicates that ca. 12% of the glucopyranose units were modified, meaning that in average each \( \gamma \)-CD had one NMA group. Thus the MW of \( \gamma \)-CD-NMA can be estimated to be 1378.24 g/mol\(^{15}\) (scheme 2). FT-IR analysis of \( \gamma \)-CD-NMA clearly showed the bands of carboxylic (C=O) and amide II (NH) groups at approximately 1700 cm\(^{-1}\) and 1540 cm\(^{-1}\), respectively, and a small peak due to \( \text{C}=\text{C}\) vinyl groups at 1665 cm\(^{-1}\) (figure 2 A) in agreement with previous reports\(^{15,39}\).

**Hydrogel particles production and characterization**

The spherical particulate hydrogels were obtained under mild conditions on superhydrophobic surfaces\(^{12}\). Fix volumes of dextran-MA solutions containing \( \gamma \)-CD or \( \gamma \)-CD-NMA (Table 1) were dispensed onto the PS superhydrophobic platform and cross-linked under UV light using Irgacure 2959 as photoinitiator. Particle size ranged from 681 to 915 \( \mu \)m (Table 2). Three different networks types were prepared using two dextran-MA concentrations (5% and 10% (w/v)): i) particles formed by a dextran-MA solely network; ii) particles that consist of a dextran-MA network with free \( \gamma \)-CDs; and iii) particles formed by copolymer networks of dextran-MA and \( \gamma \)-CD-NMA. The inter- and intra-chain bonds that may occur in such formulations are depicted in scheme 3.

Hardening of the precursor spherical droplets under UV radiation was affected by the presence of γ-CD units. Droplets without γ-CD showed faster and more successful cross-linking just adding half of the volume of Irgacure 2959 (0.5% w/v) indicated in table 1. It has been previously reported that CDs interact selectively with alkyl side chains. Both dextran-MA and Irgacure 2959 have alkyl groups, namely methyl groups (-CH$_3$), that may form inclusion complexes with γ-CDs in the precursor solution, which in turn may limit the interaction of the photochemically generated free radicals with the monomers and consequently decrease the polymerization efficiency. To overcome this problem, the amount of Irgacure 2959 was increased (Table 1).

The unreacted photoinitiator was quantified in freshly prepared (unwashed) particles. Significant amounts were found in microgels prepared with free γ-CD (Table 2). Oppositely, almost all photoinitiator was consumed during preparation of the formulations composed of dextran-MA solely and dextran-MA + 20%γ-CD-NMA. In the absence of CDs, all Irgacure 2959 molecules and methacrylate groups of dextran-MA are available for reaction. In the dextran-MA and γ-CD-NMA mixtures, the presence of more reactive double bonds caused a greater consumption of photoinitiator. Nevertheless, even under the most unfavorable conditions, more than 90% photoinitiator was consumed in the crosslinking.
Figure 3 – Color of bromine aqueous solutions 5 min after pouring 40 µL of precursor solutions used for particle production or 8 particles of each formulation.

Table 2 – Size and swelling degree of particles prepared with different combinations of dextran-MA and γ-CDs, and the amount of unreacted Irgacure 2959 remnant in each particle formulation after UV photocrosslinking.

<table>
<thead>
<tr>
<th>Code</th>
<th>Particle size (mean ± SD) (µm)</th>
<th>Swelling degree (mean ± SD) (%)</th>
<th>Irgacure 2959 unreacted (% of the initial amount added)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dried particles</td>
<td>Swollen particles</td>
<td></td>
</tr>
<tr>
<td>5% Dextran-MA</td>
<td>681±36</td>
<td>1008±55</td>
<td>223±11</td>
</tr>
<tr>
<td>5% Dextran-MA + 20% γ-CD</td>
<td>683±36</td>
<td>1041±75</td>
<td>248±33</td>
</tr>
<tr>
<td>5% Dextran-MA+ 20% γ-CD-NMA</td>
<td>790±37</td>
<td>1197±78</td>
<td>253±15</td>
</tr>
<tr>
<td>10% Dextran-MA</td>
<td>862±27</td>
<td>1377±81</td>
<td>338±18</td>
</tr>
<tr>
<td>10% Dextran-MA + 20% γ-CD</td>
<td>873±36</td>
<td>1477±70</td>
<td>329±15</td>
</tr>
<tr>
<td>10% Dextran-MA + 20% γ-CD-NMA</td>
<td>915±37</td>
<td>1536±82</td>
<td>242±13</td>
</tr>
</tbody>
</table>

To gain further insight into the polymerization yield, double bonds remaining after cross-linking were quantified by means of bromine test. Bromine reacts very rapidly with alkenes leading to an attenuation of the characteristic yellow-orange color of the bromine solution. As expected, dextran-MA solutions (precursor solutions used to prepare the particles) dramatically altered the color of the bromine solution (Figure 3 A and E); the attenuation being more accentuated for higher polymer concentration solutions. γ-CD-NMA solution also showed a slight lightning of the original color (Figure 3 K), which was not induced by γ-CD solution (Figure 3 J). Such color modifications confirmed the presence of C=C double bonds in dextran-MA and γ-CD-NMA through the methacrylic and acrylic added groups, respectively. By contrast, the particles did not cause modifications in the color of bromine solution (Figure 3 B, C, D, F, G and H) meaning that the UV cross-linking was efficient and almost all C=C bonds disappeared. Therefore, the process implemented to prepare dextran-MA particles enables the obtaining of spherical microparticles with nearly 100% cross-linking yield, with minor amounts of residual photoinitiator. For subsequent experiments, the particles were washed to remove the traces of unreacted photoinitiator. Stereomicroscope images of the particles after being dried are shown in Figure 4. The particles made from 5% dextran-MA solutions were ca. 200 µm smaller than those prepared with 10% polymer (Table 2) due to the lower polymer density.

Figure 4 - Stereomicroscope images of the dried particles. Scale bar 500 µm.

For the same content in dextran-MA, copolymerization with γ-CD-NMA increased the particle size, probably due to considerable volume occupied by the γ-CD units in the copolymer mesh. Oppositely, free γ-CDs did not significantly modify the size of dextran-MA solely networks. The low standard deviations obtained in size measurement clearly indicate that the accurate volume of precursor solution dispensed on the superhydrophobic surfaces allows the production of particles with a narrow particle size distribution. After swelling, the increase in particle diameter (300-400 µm and 600-700 µm for 5% and 10% dextran-MA formulations, respectively) confirmed their high ability to uptake...
water. In the 5% dextran-MA particles, γ-CD or γ-CD-NMA did not affect the swelling (225-250% for the three studied formulations) (Table 2). The swelling degree of 10% Dextran-MA particles without and with free γ-CD was around 338±18% and 329±15%, respectively. However, the dextran-MA+γ-CD-NMA copolymer particles exhibited lower swelling degree, approximately 242±13%, which is in agreement with the denser interconnected network that can be created due to the additional covalent linkages that γ-CD-NMA molecules can establish among themselves and with dextran-MA (Scheme 3).

The digital (Figure 4) and SEM (Figure 5 and 6) micrographs confirmed the spherical shape of the formulated gels. Regarding the structure of the particles, the SEM micrographs of the freeze-dried particles (Figure 5) revealed a porous surface due to the dry process used; conversely, the air-dried particles (Figure 6) showed a smooth surface. It is also noticeable that all particles exhibited a heterogeneous internal structure, as visualized in freeze-dried and in air-dried particles. The particles seemed to have a coating layer that probably resulted from the non-homogeneous cross-linking due to a more intense UV photocrosslinking efficiency on the surface than in the inner of the spherically shaped gels.

Dexamethasone loading and release

The loading of a drug in a hydrogel depends on: (i) the easiness of the drug molecules to diffuse into the hydrogel mesh (which is determined by the cross-linking degree and the affinity of water for the network), (ii) the drug concentration in the loading solution and (iii) the affinity of the drug for the network.42,9,38 The maximum solubility of dexamethasone was experimentally determined to be 64.2±1.95 µg/mL. The loading of the particles was carried out by soaking in a 30 µg/mL drug solution to avoid precipitation risk. As explained before, all dextran microgel particles presented high affinity to water (Table 2). The swelling values observed suggest that the mesh sizes of the swollen hydrogels are enough for the free entrance of dexamethasone molecules. Nevertheless, the capability of dextran-MA solely particles to uptake the drug was quite limited (Figure 7).

![Figure 7](image-url) - Dexamethasone loaded by 10 particles of dextran-MA particles immersed in 30 µg/mL drug solution at room temperature.

The main aim of this work was to engineer a system with enhanced drug-network interactions by means of the incorporation of free γ-CD or the copolymerization with γ-CD-NMA. The polarity and size of dexamethasone make it suitable to form inclusion complexes with γ-CDs.11,9 Thus, a greater uptake by the formulations that contained CDs (free or attached to dextran backbone) was hypothesized. In fact, the particles containing γ-CD-NMA were the most effective: 5% dextran-MA + 20% γ-CD-NMA particles captured ca. 15.24 µg/mg and 10% dextran-MA + 20% γ-CD-NMA ca. 8.69 µg/mg (Table 3). Microgels with free γ-CD showed an intermediate behavior, probably because free γ-CDs were partially removed during the washing step but also due to the absence of the cooperative contributions that can take place when the CDs are forming part of the polymer network. The amount of dexamethasone loaded by simple equilibrium between the aqueous phase of the network and the loading solution, can be estimated to be close to 0.067 µg/mg for 5% dextran-MA microgels and 0.101 µg/mg for 10% dextran-MA microgels (Table 3).
Experimental loading data were slightly greater than these amounts for dextran-MA microgels prepared in the absence of γ-CDs (Table 3), which means that unspecific hydrophobic interactions of dexamethasone with dextran weakly contribute to the loading. To gain an insight into the role of the cyclodextrin units in the loading process, the partition coefficient, $K_{N/W}$, between the microgel particles and the drug loading solution was estimated from the following expression $^{38}$:

$$ Loading (total) = \frac{(V_p + K_{N/W}V_p)\times C_0}{W_p} $$

where $V_p$ is the volume of dried polymer and the other symbols maintain the same meaning as in eq. 2. The values of $K_{N/W}$ for 5% dextran-MA + 20% γ-CD-NMA particles and 10% dextran-MA + 20% γ-CD-NMA particles were as high as 505 and 287, respectively, while the respective formulations prepared with free γ-CD had $K_{N/W}$ values of 82 and 46, respectively (Table 3). These findings clearly prove the advantage of the immobilization of γ-CDs by means of the copolymerization, and also that the greater the relative content in γ-CD (either free or copolymerized), the higher the increase in affinity of the network for dexamethasone. Compared to a previously reported macrogel of cross-linked γ-CDs units that exhibited $K_{N/W}$ values in the 60-180 range$^3$, the microgels prepared by copolymerization of dextran-MA with γ-CD-NMA are clearly advantageous. This finding may be related to the higher degree of swelling that dextran chains communicate to the networks, which should facilitate the access of dexamethasone to the γ-CDs.

Tuning the time of immersion in the loading solution and the amount of particles, it is possible to regulate the amount of dexamethasone loaded in order to attain concentrations able to induce osteogenic differentiation (40-400 ng/mL) with a low mass of particles.

Regarding dexamethasone release (Figure 8), if the drug only interacts with dextran-MA, the release rate should be practically independent of the γ-CD proportion in the hydrogel, except if the mesh size becomes altered$^{43}$. Conversely, if drug-γ-CD complexation occurs, the release rate would be controlled not only by the diffusion of the free drug (hosted in the aqueous phase) but also by the stability constant of the complexes. In such case, the greater the content in γ-CDs, the slower the release$^{43}$. Dexamethasone release from air-dried microgel particles is depicted in figure 8. Copolymerization of dextran-MA with γ-CD-NMA was advantageous in terms of amount of dexamethasone loaded and ability to sustain the release for a long period of time. Disregarding dextran-MA proportion, the particles exhibited a rapid release in the first 24 h (45-47% release) followed by a more sustained release for at least 10 days. Moreover, the release of dexamethasone from copolymerized dextran-MA and γ-CD-NMA particles became much slower when a certain drug concentration in the medium was reached, even under sink conditions, as observed previously by Rosa dos Santos et al.$^{44}$ for soft contact lenses with β-CD pendant groups. This finding indicates that equilibrium between drug release and drug reabsorption by the microgel particles is attained. To trigger the release of the drug, at certain time points (24, 120, 264, 336 and 432 h), 1 mL of fresh medium was added to the initial volume of release medium (2 mL) in order to disturb the equilibrium. 5% dextran-MA + 20% γ-CD-NMA and 10% dextran-MA + 20% γ-CD-NMA particles released 71% and 77% dexamethasone in 11 days. Differently, particles containing free γ-CDs exhibited a faster delivery (ca. 60%) in the first 24 h, reaching 90% released after 11 days. The amounts of dexamethasone released from dextran-MA solely were too low for precise quantification (in agreement with the previous low loading).

In sum, γ-CD covalently integrated in the hydrogel network provided an unique way to control the loading and the release of dexamethasone, which may be useful for the design of osteogenic systems. Comparing the three studied formulations, the copolymerization of γ-CD-NMA with dextran allows increasing the amounts of dexamethasone released and ability to sustain the release for a long period of time.

Table 3 – Dexamethasone loaded by the microgels and network/water partition coefficient ($K_{N/W}$). Mean values and, in parenthesis, standard deviation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Total amount of drug loaded (µg/mg)</th>
<th>Drug loaded in aqueous phase (µg/mg)</th>
<th>$K_{N/W}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Dextran-MA</td>
<td>0.28 (0.37)</td>
<td>0.067</td>
<td>7.3 (12.2)</td>
</tr>
<tr>
<td>5% Dextran-MA + 20% γ-CD</td>
<td>2.54 (0.50)</td>
<td>0.070</td>
<td>82.4 (16.8)</td>
</tr>
<tr>
<td>5% Dextran-MA + 20% γ-CD-NMA</td>
<td>15.24 (0.50)</td>
<td>0.075</td>
<td>505.5 (16.7)</td>
</tr>
<tr>
<td>10% Dextran-MA</td>
<td>1.08 (0.47)</td>
<td>0.101</td>
<td>32.8 (15.7)</td>
</tr>
<tr>
<td>10% Dextran-MA + 20% γ-CD</td>
<td>1.47 (0.42)</td>
<td>0.098</td>
<td>45.8 (14.0)</td>
</tr>
<tr>
<td>10% Dextran-MA + 20% γ-CD-NMA</td>
<td>8.69 (0.31)</td>
<td>0.073</td>
<td>287.2 (10.5)</td>
</tr>
</tbody>
</table>
Cytotoxicity of the hydrogel particles

A preliminary screening of the cytocompatibility of dexamethasone-loaded and non-loaded particles formulations was carried out against a human cell line of osteoblasts. The formulations were placed in direct contact with cells previously adhered to the bottom of the wells. The LDH released was evaluated and correlated with the percentage of live/dead cells (Figure 9). At 24 h, dexamethasone-loaded dextran-MA particles exhibited lower cell compatibility (one-way ANOVA, Tukey’s multiple comparison test, p<0.05) when compared with the same non-loaded formulations. However, such differences were not observed for the other formulations at this time point. At 72 h cell viability was close to 100% for all formulations, which indicates that the materials and the drug released do not cause relevant cytotoxic effects. These findings are in agreement with the well-known biocompatibility of the starting materials (dextran and γ-CD), and also confirm that there are not leakage of unreacted monomers that could trigger unfavorable cell reactions.

Effect of hydrogel particles on Human-Adipose-derived Stem Cells (hASCs)

In vitro proliferation. The hASCs were seeded on the well bottom and allowed to proliferate for 12 days under direct contact with particles loaded or not with dexamethasone (Figure 10). The proliferation was in average higher in the presence of dexamethasone, being the differences more accentuated at day 7 (p<0.05). Previous studies demonstrated that dexamethasone either alone or in combination with β-GP + ascorbic acid stimulates the human bone marrow mesenchymal stem cells proliferation46. Such evidences can be correlated with the ability of glucocorticoids, at low concentrations, to stimulate the proliferation and/or differentiation of osteoprogenitor cells36,31. Moreover, ascorbic acid solely also demonstrated capability to stimulate the proliferation of mesenchymal stem cells and its derived cell types, namely osteoblasts, adipocytes, chondrocytes, etc.47, while β-GP solely exerted the opposite effect46. The similar cell proliferation observed in the negative controls 1 and 2 revealed that the combination of β-GP and ascorbic acid does not alter cell proliferation (no significant statistic differences), probably due to their antagonistic effect. On the other hand, the greater cell number recorded in positive control 2 compared to that obtained in negative control 2 (p<0.05) at days 7 and 12 indicates that the presence of dexamethasone have a stimulating effect on hASCs proliferation.
**In vitro osteogenic differentiation.** The osteoinductive potential of the particles was evaluated recording ALP activity. ALP is an enzyme present in osteoblasts membrane (early osteogenic marker) involved in ions phosphate hydrolysis stimulating the mineralization through the formation of hydroxyapatite crystals.

As can be observed in Figure 11, the ALP activity increased over time. At day 3 the levels were similar for all formulations. At day 7 the dexamethasone-loaded formulations showed an early ALP activity peak with statistically significant differences (p<0.05) when compared with the respective non-loaded formulations or the negative controls. For dexamethasone-loaded 10% dextran-MA and 10% dextran-MA + 20% γ-CD the results were similar to those obtained with the positive control (1 and 2). By contrast, the presence of γ-CD-NMA in the particles induced a higher ALP activity (statistically different to positive control 1; p<0.05) probably due to the increased capacity of the particles to load dexamethasone and release it in a sustained way during the period required to induce the cell differentiation. During the experiment, part of the cell culture medium was replaced with fresh one in order to guarantee the feeding of the cells and the clearance of waste products. Such medium renovation may be advantageous because it should trigger the release of more drug from the particles in order to maintain a constant level of dexamethasone in the medium, as observed in the *in vitro* release studies.

In all studied time points, the non-loaded particles presented an ALP activity similar to that of negative controls (undifferentiated cells), which means that the materials *per se* did not have osteoinductive properties. Moreover, the incorporation of β-GP + ascorbic acid to the culture medium without dexamethasone did not cause any effect on ALP activity (negative control 2). Therefore, dexamethasone plays a key-role in osteogenesis induction being maximum between days 3 and 7. Previous studies demonstrated that hASCs stimulated by medium supplement with β-GP, ascorbic acid and 10 or 100 nM of dexamethasone also showed an ALP peak after 7 days in culture\(^27\). The quantitative results about differentiation of the hASCs to osteoblasts were also visualized by means of ALP staining after 12 days of cell culture (Figure 12). Cells in contact with dexamethasone-loaded particles exhibited a more intense red color. Again, the drug-loaded 10% dextran-MA + 20% γ-CD-NMA particles caused the highest red staining (similar to that cells in positive control 2, which was supplemented with the maximum amount of dexamethasone that particles can release). This is explained by the increased capacity of such particles to load dexamethasone. The non-loaded formulations and the negative controls did not induce significant increase in ALP activity.

![Figure 11 – ALP activity in hASCs induced by dexamethasone loaded and non-loaded particles and the respective positive and negative controls after 3, 7 and 12 days in culture. The results are expressed in mean ± SD with n=3 for each bar.](image-url)

Distinct polymeric systems encapsulating dexamethasone have been successfully reported for osteogenic differentiation, namely PLGA scaffolds\(^48,49\), microparticles\(^23\) and nanoparticles\(^50\), carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles\(^25,51,52\), polycaprolactone\(^53\) and poly(L-lactide-co-caprolactone)/collagen fibers\(^54\); however none studied the combination of polymers with cyclodextrins and the respective role in loading and release of such corticoid drug and the effect on osteogenic cell differentiation. The particles developed in this work demonstrate high potential to be applied in *in situ* bone regeneration avoiding the use of unstable proteins (e.g. growth factors) or even genetic manipulation of cells, and using a simple and cost effective method.
Conclusions
Dextran-MA photocrosslinked spherical particles can be obtained using the superhydrophobic surfaces methodology and their performance to load hydrophobic drugs is improved via copolymerization with γ-CDs. Copolymers networks of γ-CDs (γ-CD-NMA) and 10% dextran-MA are ca. 7 times more effective in loading dexamethasone than the formulations with free γ-CDs and dextran-MA solely, meaning that the anchorage of CDs is advantageous. The in vitro release of dexamethasone from dextran-MA + 20% γ-CD-NMA particles can be sustained for at least 10 days due to the hindered drug-CD decomplexation inside the network. These particles show an excellent cytocompatibility and are able to induce the osteogenic differentiation of hASCs after 3-7 days in culture. In sum, the improved characteristics of the developed system could be useful for application in bone regeneration.

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Notes and references
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Figure 12 - ALP staining of hASCs in direct contact with non-loaded and loaded particles, and the respective controls at 12 days. Scale bar 100 µm.

