

## Free and copolymerized $\gamma$ -cyclodextrins regulate the performance of dexamethasone-loaded dextran microspheres for bone regeneration

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Polymeric particles acting as sources of biological cues to promote tissue regeneration are currently an interesting topic in bone tissue engineering research. In this study, microspheres of dextran-methacrylate (dextran-MA) and  $\gamma$ -cyclodextrins ( $\gamma$ -CD) for the delivery of osteogenic agents were prepared by means of photopolymerization on biomimetic superhydrophobic surfaces. The effects of the incorporation of the  $\gamma$ -CD units as free entities or as structural monomers (acrylamidomethyl- $\gamma$ -cyclodextrin,  $\gamma$ -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  $\gamma$ -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 of inducing 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  $\gamma$ -CDs onto the dextran-MA network may be particularly useful for the development of cytocompatible implantable spherical biomaterials for bone tissue engineering purposes.

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### Introduction

Cyclodextrins (CDs) are appealing biocompatible tools for the design of drug delivery systems based on hydrophilic hydrogels.<sup>1</sup> The capability of CDs to form inclusion complexes with a variety of active substances usually does not diminish, but enhances even when the CDs are trapped in a polymer network.<sup>2,3</sup> Thus, poorly soluble drugs can be homogeneously loaded up to a great extent in hydrophilic networks forming complexes with CDs.<sup>4–6</sup> 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.<sup>1</sup> Different from 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,<sup>7,8</sup> 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.<sup>1,9,10</sup> Recently,  $\gamma$ -CD nanogels obtained *via* direct cross-linking in an emulsified system were shown to be able to load dexamethasone for application as eye drops, being highly biocompatible in *in vivo* studies performed in rabbits.<sup>11</sup>

The aim of this work was to use the superhydrophobic surface technology<sup>12</sup> to produce spherically shaped microgels combining dextran-MA and  $\gamma$ -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  $\gamma$ -CDs and copolymerized  $\gamma$ -CD-NMA (acrylamidomethyl- $\gamma$ -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  $\alpha$ -1,6-linked glucopyranose monomers, widely used as carriers for hydrophilic bioactive agents and as components of scaffolds.<sup>13,14</sup> On the other hand,  $\gamma$ -CDs are highly soluble in water (18.5–39 g per 100 mL at 20–35 °C) allowing the production of hydrogels with high content in freely dispersed  $\gamma$ -CDs.<sup>9</sup> Moreover, monomers of  $\gamma$ -CD have been previously shown to be useful to enhance the uptake of hydrophobic drugs by acrylic

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networks.<sup>15</sup> Despite the potential advantages of combining dextran and CDs, very few binary systems have been reported yet. Fernández *et al.*<sup>16</sup> synthesized a copolymer of dextran and amino derivatives of  $\beta$ -CD to be applied as a thermoprotectant of trypsin. Later, Ramirez *et al.*<sup>17</sup> reported covalently bonded  $\beta$ -CD-dextran as a macromolecular carrier for naproxen, a poorly soluble non-steroidal anti-inflammatory drug. Using click chemistry, Nielsen and co-workers<sup>18</sup> synthesized an open-chain polymer resultant of the combination of alkyne-modified dextran and  $\beta$ -CDs functionalized with azide groups. Using two formulations proposed in such work, Fülöp *et al.*<sup>19</sup> studied their complex forming properties with a model drug and their aggregation ability. Stable nanoassemblies spontaneously formed by mixing dextran- $\beta$ -CD polymers and dextran-adamantyl have been explored for drug delivery due to their low toxicity.<sup>20</sup> However, to the best of our knowledge, dextran-CD networks have not been explored yet for sustained release in tissue engineering applications. In this context, particulate drug delivery systems are especially attractive due to its potential to be implanted *via* minimal invasive maneuvers.<sup>21–24</sup>

Under osteogenic stimuli, some stem cells have the ability to differentiate into osteoblasts, expressing characteristic markers such as bone-specific alkaline phosphatase (ALP).<sup>25</sup> 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.<sup>26,27</sup> 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,  $\beta$ -glycerophosphate ( $\beta$ -GP) and a glucocorticoid such as dexamethasone.<sup>28,29</sup> 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.<sup>30</sup> In fact, the prolonged duration of exposure *in vivo* may cause severe osteoporosis.<sup>31,32</sup>

To prepare the proposed spherical microgels (dextran + CDs) a technique consisting of 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 a complex apparatus and allows obtaining particles with narrow size distribution and 100% encapsulation yield of freely diffusible species.<sup>33</sup> This technology permits also the production of multilayered particles<sup>34</sup> for protein or cell encapsulation under mild conditions.<sup>35</sup> 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-Perfluorodecyltriethoxysilane (PFDTs, 97%), dextran from *Leuconostoc mesenteroids* (MW 100–200 kDa), 4-(N,N-dimethylamido)pyridine (DMAP, 99%), hydrochloric acid (HCl, 37%), acetone, dexamethasone for cell culture, penicillin/streptomycin solution, L-glutamine,  $\beta$ -glycerophosphate disodium salt hydrate ( $\beta$ -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 obtained from Sigma-Aldrich (USA). The BCA kit was purchased from Pierce (USA), the cell proliferation kit (MTT) and the cytotoxicity detection kit (LDH) were supplied by Roche (Switzerland). Paraformaldehyde tablets were obtained 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 10 $\times$  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).  $\gamma$ -Cyclodextrin ( $\gamma$ -CD) Cavamax W8 was from ISP and N-(hydroxymethyl)acrylamide (NMA) from Merck (Germany). Purified water (resistivity >18 M $\Omega$  cm; MilliQ®, Millipore, Spain) was obtained by means of reverse osmosis. All the other chemicals were used as received.

### Polystyrene superhydrophobic surfaces

PS superhydrophobic surfaces were prepared using a phase separation method as described elsewhere.<sup>36</sup> Briefly, a PS solution (70 mg mL<sup>-1</sup>) 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-methacrylate (dextran-MA)

Dextran-MA was synthesized as previously described.<sup>37</sup> Briefly, dextran (25 g) was dissolved in DMSO (225 mL) under a nitrogen atmosphere. Then, DMAP (5 g) was added and, when the dissolution was complete, GMA (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 DMAP. 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). <sup>1</sup>H 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. The IR spectrum of the  $\gamma$ -CD-NMA monomer was recorded over the range 400–4000  $\text{cm}^{-1}$  (Bruker IFS 66V FT-IR, Germany) using the potassium bromide (KBr) pellet technique, and  $^1\text{H}$  NMR spectra of unmodified and modified  $\gamma$ -CD (30  $\text{mg mL}^{-1}$  in deuterated water) were recorded (Mercury 300, 300 MHz, Varian, Australia) and then analyzed using MestReNova software (MestreLab, Spain).

### Microgel 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  $\mu\text{L}$  or 100  $\mu\text{L}$  of 0.5% w/v in water) was added to the solutions (Table 1). 5  $\mu\text{L}$  of each solution were dispensed onto the PS superhydrophobic surfaces, and cross-linked under UV light (365 nm; Camag, Switzerland) for 50–60 min. The solid particles were dried in air overnight, washed with water for 48 h and dried again in air at room temperature.

### Microgel characterization

**Quantification of an 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  $\times$  150 mm, 2.6  $\mu\text{m}$ ) column kept at 37 °C, a UV-Vis detector (275 nm, SpectraSystem UV8000 – Thermo Scientific) and acetonitrile : water (90 : 10, 0.5  $\text{mL min}^{-1}$  flow) as the isocratic mobile phase.

**Double bond (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  $\mu\text{L}$ , equivalent volume to prepare 8 particles) and the different formulated cross-linked particles (8 units) were placed in contact with 200  $\mu\text{L}$  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  $\text{mg mL}^{-1}$ ) and 10.7 mL of a solution of NaBr (1.1 g) in 1 M 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 ( $m_i$ ) and the initial weight of the dried particles ( $m_0$ ) as follows:

$$\% \text{Swelling} = \frac{m_i - m_0}{m_0} \times 100 \quad (1)$$

**Particle size.** The sizes of dried and swollen particles were 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 image analysis software (Soft Imaging System®, Münster, Germany).

**Scanning electron microscopy (SEM).** The particles were allowed to swell in water at room temperature. Then, some were collected and allowed to dry in air, and the others were immediately frozen in liquid nitrogen and freeze-dried. 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 a 0.22  $\mu\text{m}$  nylon membrane to remove the non-dissolved drug. The concentration of the dissolved dexamethasone was spectrophotometrically measured at 241 nm (Agilent 8453, Waldborn, Germany).

**Dexamethasone loading.** Ten particles of each formulation were dried in air overnight and then immersed in 10 mL of aqueous solution of dexamethasone (30  $\mu\text{g mL}^{-1}$ ) 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:<sup>38</sup>

$$\text{Loading (aqueous phase)} = (V_s/W_p) \times C_0 \quad (2)$$

where  $V_s$  is the volume of water sorbed by each particle,  $W_p$  is the dried weight of the particles, and  $C_0$  is the concentration of drug in the loading solution.

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

Code	Dextran-MA	$\gamma$ -CD	$\gamma$ -CD-NMA	Irgacure 2959
5% dextran-MA	50 mg	—	—	60 $\mu\text{L}$
5% dextran-MA + 20% $\gamma$ -CD	50 mg	200 mg	—	60 $\mu\text{L}$
5% dextran-MA + 20% $\gamma$ -CD-NMA	50 mg	—	200 mg	60 $\mu\text{L}$
10% dextran-MA	100 mg	—	—	100 $\mu\text{L}$
10% dextran-MA + 20% $\gamma$ -CD	100 mg	200 mg	—	100 $\mu\text{L}$
10% dextran-MA + 20% $\gamma$ -CD-NMA	100 mg	—	200 mg	100 $\mu\text{L}$

**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, 336 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 per well, 1 mL) in DMEM-F12 with 10% FBS and 1% penicillin/streptomycin, and kept in a humidified incubator at 5% CO<sub>2</sub> and 37 °C. After 24 h and 72 h of incubation, aliquots of 100 µL of the culture medium were taken and mixed with 100 µL of the reaction medium contained in the Cytotoxicity Detection kit (LDH). Blank (100 µL of the cell culture medium), negative (an aliquot collected from a well containing just cells) and positive (an aliquot collected from a well to which 20 µL lysis factor was added) controls were also performed. The plates were incubated for 10 min at room temperature protected from light. 50 µL of stop solution was added to each well and the absorbance was measured at 490 nm using a microplate reader (BIORAD Model 680 Microplate Reader). The cytotoxicity was estimated as follows:

$$\% \text{Cytotoxicity} = \frac{\text{abs}_{\text{experiment}} - \text{abs}_{\text{negative control}}}{\text{abs}_{\text{positive control}} - \text{abs}_{\text{negative control}}} \times 100 \quad (3)$$

**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 per 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 with dexamethasone) were placed in 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 (200 mM) 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<sup>-1</sup> of dexamethasone were used to load the formulations under study (Medium 4).

The plates were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and 2 mL of medium were replaced twice a week. The cells were routinely observed using inverted microscopy (×10) in order to discard any contamination. Experiments 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<sup>-1</sup> 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 were incubated again at 37 °C overnight. Aliquots of 200 µL were transferred to 96 well plates and the absorbance was read at 550 nm (BIORAD Model 680 Microplate Reader). The respective calibration curve was constructed using cultures with a 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 the ALP substrate in 96 well plates at 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 a 1 : 1 (v:v) proportion, incubated in darkness for 10 min at room temperature and visualized through an 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.

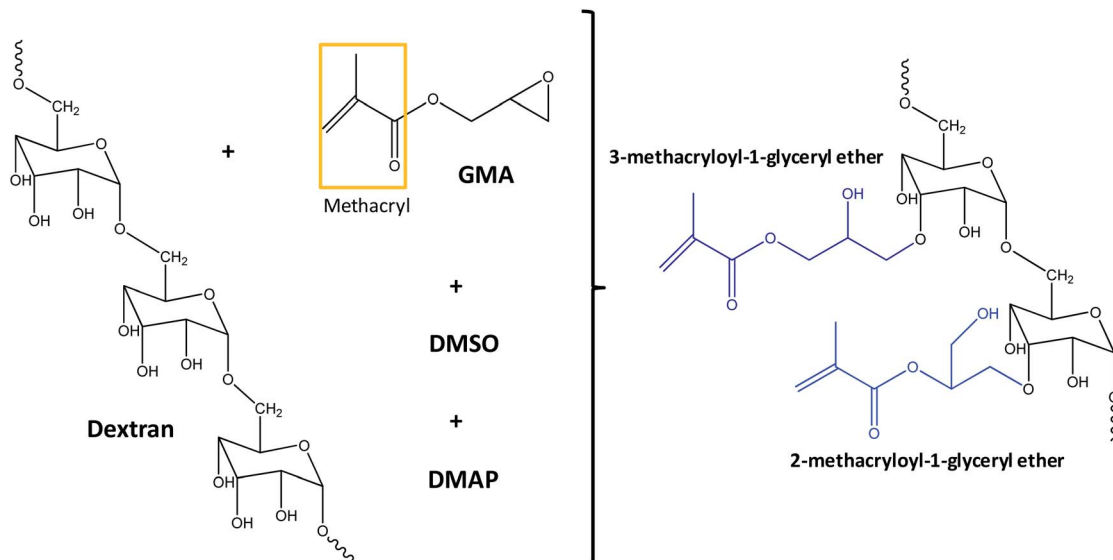
## Results and discussion

### Synthesis of dextran-MA and γ-CD-NMA

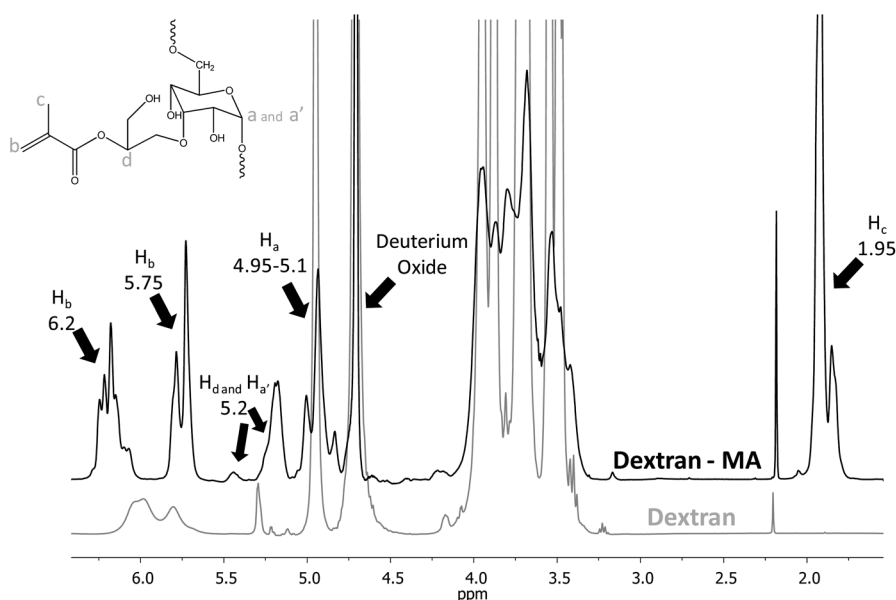
Dextran functionalized with methacrylate groups (dextran-MA) was obtained by reaction of dextran with GMA in DMSO in the presence of a base (DMAP) as a catalyst.<sup>37</sup> After 48 h of reaction at room temperature, DMAP was neutralized with hydrochloric acid in order to avoid alkaline hydrolysis 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-glycerol ether of dextran or the respective isomer 2-methacryloyl-1-glycerol, as shown in Scheme 1.

In the reaction mixture, the molar ratio of GMA to glucopyranose units was approximately 1. Based on <sup>1</sup>H NMR spectra (Fig. 1) and taking into account that each glucopyranose unit has 3 hydroxyl groups that could react with GMA, the degree of substitution (DS) was determined as (100X/Y)/3; X being the





**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.



**Fig. 1**  $^1\text{H}$  NMR spectra of dextran as supplied (gray line) and after reaction with GMA (black line) dissolved in deuterated water.

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.<sup>37</sup> The DS obtained was *ca.* 32%, which indicated that, on average, 1 hydroxyl group per glucopyranose unit was substituted.<sup>55</sup> These results were in agreement with the high yield of reaction previously reported in the literature, which indicated that more than 90% of the added GMA was incorporated into the polysaccharide.<sup>37</sup>

The  $\gamma$ -CD-NMA monomer was prepared according to a previously established protocol that renders one  $\text{CH}_2=\text{CH}$ -group per  $\gamma$ -CD, as confirmed from the  $^1\text{H}$  NMR spectrum (Fig. 2B). 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 on average each  $\gamma$ -CD had one NMA group. Thus the MW of  $\gamma$ -CD-NMA can be estimated to be  $1378.24 \text{ g mol}^{-1}$  (ref. 15) (Scheme 2). FT-IR analysis of  $\gamma$ -CD-NMA clearly showed the bands of carboxylic ( $\text{C}=\text{O}$ ) and amide II ( $\text{NH}$ ) groups at approximately  $1700 \text{ cm}^{-1}$  and  $1540 \text{ cm}^{-1}$ , respectively, and a small peak due to  $\text{C}=\text{C}$  vinyl groups at  $1665 \text{ cm}^{-1}$  (Fig. 2A) in agreement with previous reports.<sup>15,39</sup>

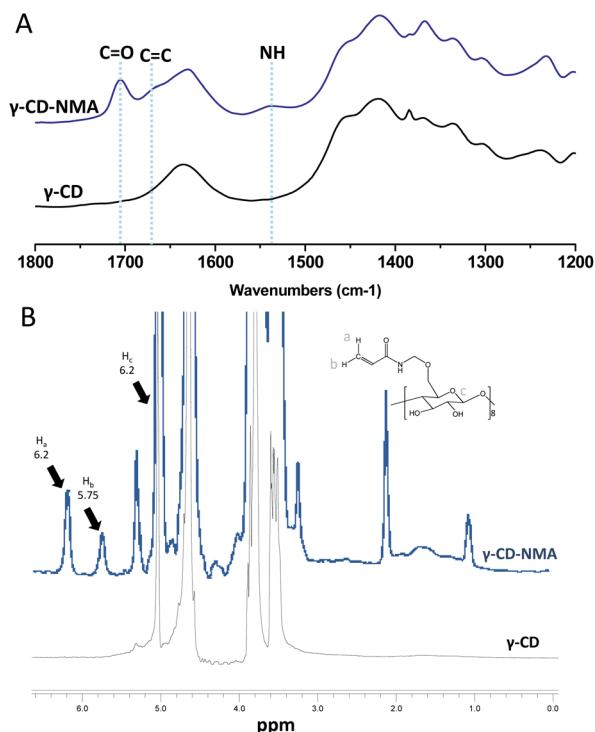


Fig. 2 (A) FT-IR and (B) <sup>1</sup>H NMR spectra of  $\gamma$ -CD (black lines) and  $\gamma$ -CD-NMA (blue lines).

### Hydrogel particle production and characterization

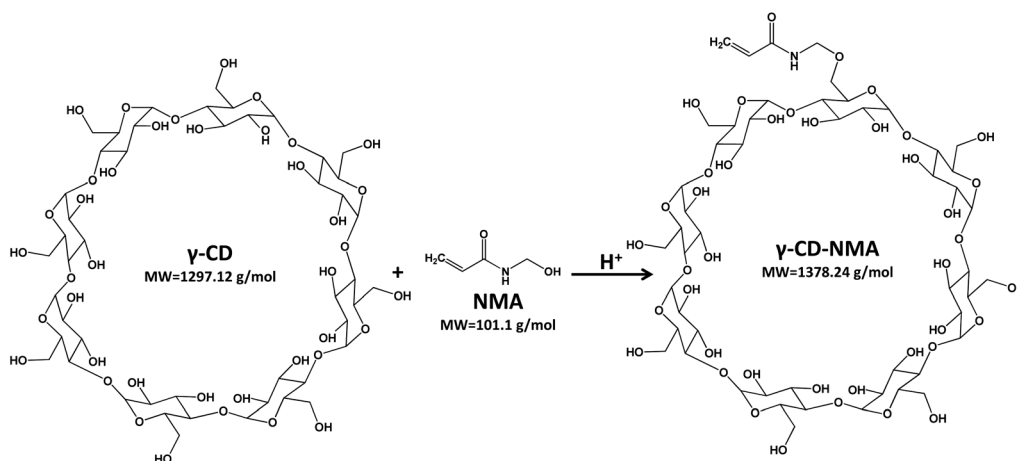
The spherical particulate hydrogels were obtained under mild conditions on superhydrophobic surfaces.<sup>12</sup> Fixed volumes of dextran-MA solutions containing  $\gamma$ -CD or  $\gamma$ -CD-NMA (Table 1) were dispensed onto the PS superhydrophobic platform and cross-linked under UV light using Irgacure 2959 as the photoinitiator. The particle size ranged from 681 to 915  $\mu$ m (Table 2). Three different network 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  $\gamma$ -CD units. Droplets without  $\gamma$ -CD showed faster and more successful cross-linking by 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.<sup>40</sup> Both dextran-MA and Irgacure 2959 have alkyl groups, namely methyl groups ( $-\text{CH}_3$ ), that may form inclusion complexes with  $\gamma$ -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.<sup>41</sup> 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  $\gamma$ -CD (Table 2). In contrast, almost all the photoinitiator was consumed during preparation of the formulations composed of dextran-MA solely and dextran-MA + 20%  $\gamma$ -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  $\gamma$ -CD-NMA mixtures, the presence of more reactive double bonds caused greater consumption of the photoinitiator. Nevertheless, even under the most unfavorable conditions, more than 90% of the photoinitiator was consumed in the crosslinking.

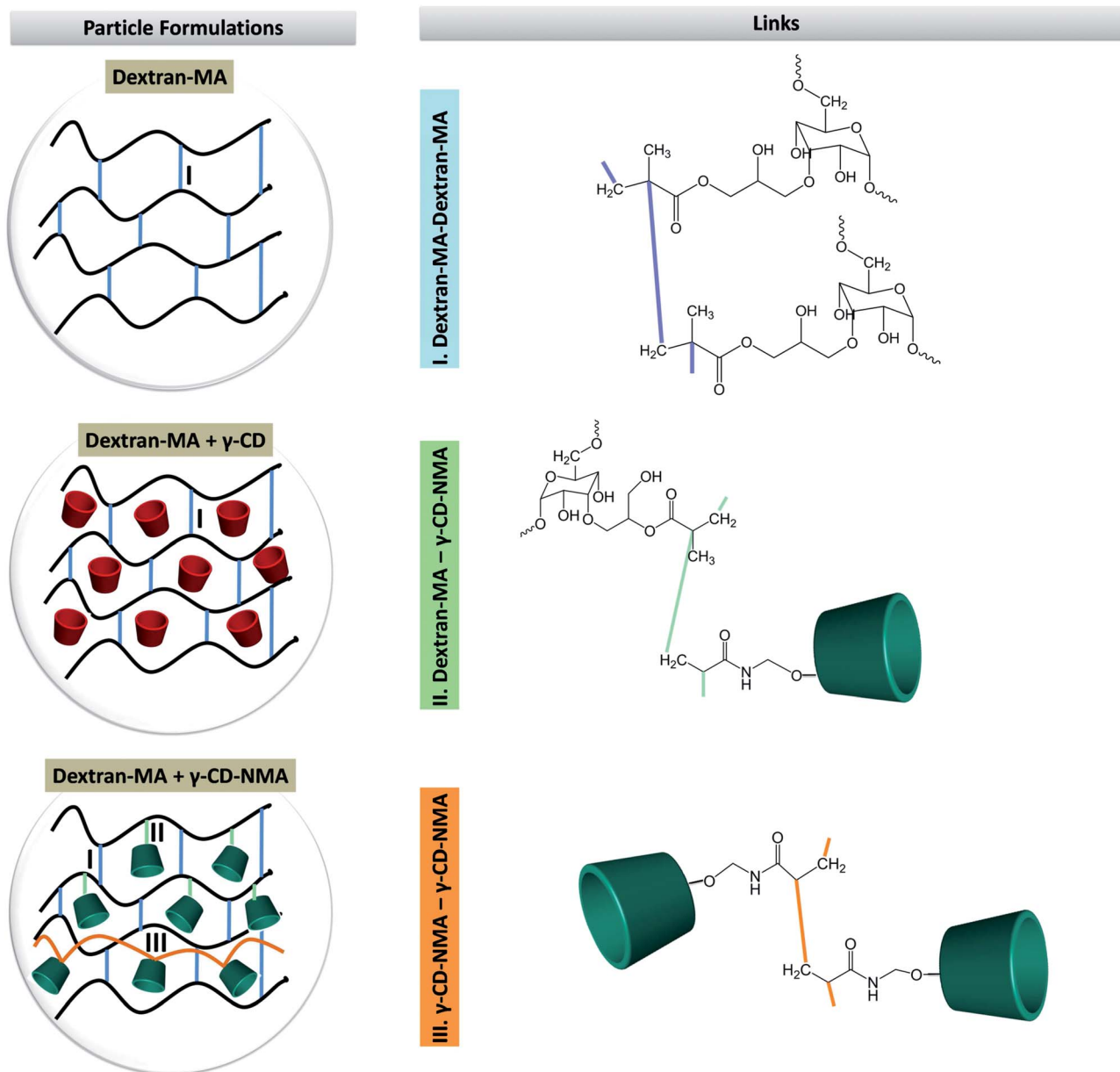
To gain further insight into the polymerization yield, double bonds remaining after cross-linking were quantified by means of a bromine test. Bromine reacts very rapidly with alkenes leading to attenuation of the characteristic yellow-orange color of the bromine solution. As expected, dextran-MA solutions (precursor solutions used to prepare the particles) dramatically altered the color of the bromine solution (Fig. 3A and E); the attenuation being more accentuated for higher polymer concentration solutions. The  $\gamma$ -CD-NMA solution also showed a



Scheme 2 Scheme of the  $\gamma$ -CD monomer ( $\gamma$ -CD-NMA) after reaction with one N-(hydroxymethyl)acrylamide (NMA). The molecular weight of the monomer was estimated assuming that each  $\gamma$ -CD has one NMA group.

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

Code	Particle size (mean $\pm$ SD) ( $\mu$ m)		Swelling degree (mean $\pm$ SD)(%)	Irgacure 2959 unreacted (% of the initial amount added)
	Dried particles	Swollen particles		
5% dextran-MA	681 $\pm$ 36	1008 $\pm$ 55	223 $\pm$ 11	0.48
5% dextran-MA + 20% $\gamma$ -CD	683 $\pm$ 36	1041 $\pm$ 75	248 $\pm$ 33	8.12
5% dextran-MA + 20% $\gamma$ -CD-NMA	790 $\pm$ 37	1197 $\pm$ 78	253 $\pm$ 15	1.06
10% dextran-MA	862 $\pm$ 27	1377 $\pm$ 81	338 $\pm$ 18	0.99
10% dextran-MA + 20% $\gamma$ -CD	873 $\pm$ 36	1477 $\pm$ 70	329 $\pm$ 15	2.40
10% dextran-MA + 20% $\gamma$ -CD-NMA	915 $\pm$ 37	1536 $\pm$ 82	242 $\pm$ 13	0.87



**Scheme 3** Photocrosslinking of the dextran-MA solution drops may result in three distinct covalent bonds: (A) dextran-MA-dextran-MA; (B) dextran-MA- $\gamma$ -CD-NMA; (C)  $\gamma$ -CD-NMA- $\gamma$ -CD-NMA.

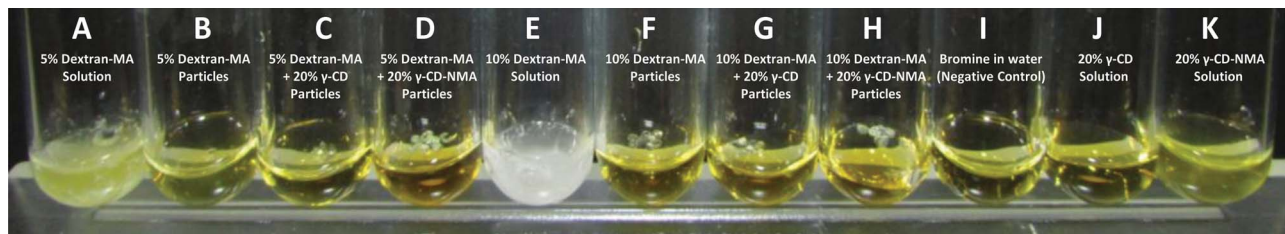


Fig. 3 Color of bromine aqueous solutions 5 min after pouring 40  $\mu\text{L}$  of precursor solutions used for particle production or 8 particles of each formulation.

slight lightening of the original color (Fig. 3K), which was not induced by  $\gamma$ -CD solution (Fig. 3J). Such color modifications confirmed the presence of C=C double bonds in dextran-MA and  $\gamma$ -CD-NMA through the methacrylic and acrylic added groups, respectively. By contrast, the particles did not cause modifications in the color of bromine solution (Fig. 3B–D and F–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 the residual photoinitiator. For subsequent experiments, the particles were washed to remove the traces of the unreacted photoinitiator.

Stereomicroscope images of the particles after being dried are shown in Fig. 4. The particles made from 5% dextran-MA solutions were *ca.* 200  $\mu\text{m}$  smaller than those prepared with the 10% polymer (Table 2) due to the lower polymer density.

For the same content in dextran-MA, copolymerization with  $\gamma$ -CD-NMA increased the particle size, probably due to the considerable volume occupied by the  $\gamma$ -CD units in the copolymer mesh. In contrast, free  $\gamma$ -CDs did not significantly modify the size of dextran-MA solely networks. The low standard deviations obtained in size measurements 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 the particle diameter (300–400  $\mu\text{m}$  and 600–700  $\mu\text{m}$  for 5% and 10% dextran-MA formulations, respectively) confirmed their high ability for water uptake. In the 5% dextran-MA particles,  $\gamma$ -CD or  $\gamma$ -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  $\gamma$ -CD was around  $338 \pm 18\%$  and  $329 \pm 15\%$ , respectively. However, the dextran-MA +  $\gamma$ -CD-NMA copolymer particles exhibited a lower swelling degree, approximately  $242 \pm 13\%$ , which is in agreement with the denser interconnected network that can be created due to the additional covalent linkages that  $\gamma$ -CD-NMA molecules can establish among themselves and with dextran-MA (Scheme 3).

The digital (Fig. 4) and SEM (Fig. 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 (Fig. 5) revealed a porous surface due to the dry process used; conversely, the air-dried particles (Fig. 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 part 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

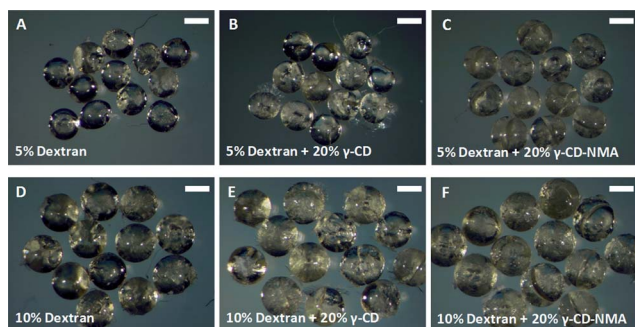


Fig. 4 Stereomicroscope images of the dried particles. The scale bar is 500  $\mu\text{m}$ .

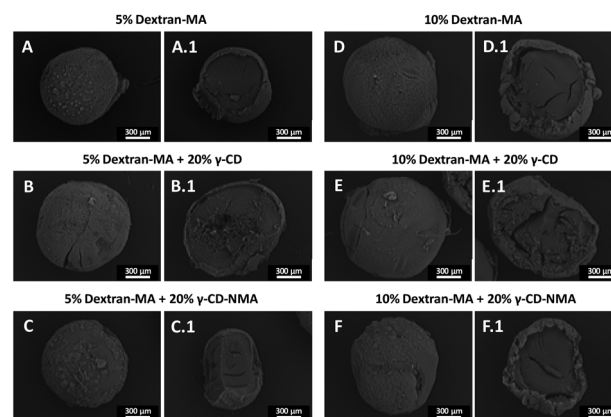


Fig. 5 SEM micrographs of swollen freeze-dried particles and their respective cross-sections.



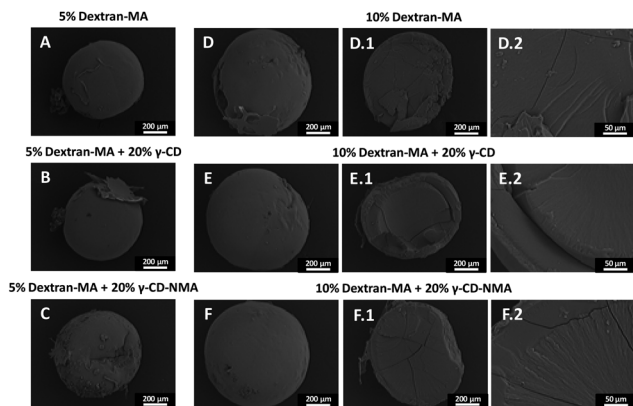


Fig. 6 SEM micrographs of the air-dried particles, cross-sections and respective magnifications.

solution and (iii) the affinity of the drug for the network.<sup>9,38,42</sup> The maximum solubility of dexamethasone was experimentally determined to be  $64.2 \pm 1.95 \mu\text{g mL}^{-1}$ . The loading of the particles was carried out by soaking in a  $30 \mu\text{g mL}^{-1}$  drug solution to avoid the 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 (Fig. 7).

The main aim of this work was to engineer a system with enhanced drug-network interactions by means of the incorporation of free  $\gamma$ -CD or the copolymerization with  $\gamma$ -CD-NMA. The polarity and size of dexamethasone make it suitable to form inclusion complexes with  $\gamma$ -CDs.<sup>11,9</sup> Thus, a greater uptake by the formulations that contained CDs (free or attached to the dextran backbone) was hypothesized. In fact, the particles containing  $\gamma$ -CD-NMA were the most effective: 5% dextran-MA + 20%  $\gamma$ -CD-NMA particles captured *ca.*  $15.24 \mu\text{g mg}^{-1}$  and 10% dextran-MA + 20%  $\gamma$ -CD-NMA *ca.*  $8.69 \mu\text{g mg}^{-1}$  (Table 3). Microgels with free  $\gamma$ -CD showed an intermediate behavior, probably because free  $\gamma$ -CDs were partially removed during the

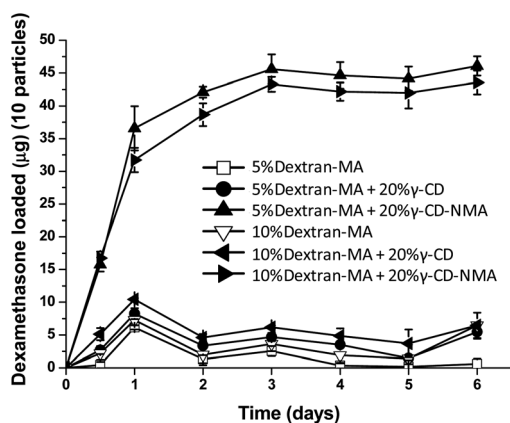


Fig. 7 Dexamethasone loaded by 10 particles of dextran-MA particles immersed in  $30 \mu\text{g mL}^{-1}$  drug solution at room temperature.

washing step but also due to the absence of the cooperative contributions that can take place when the CDs form part of the polymer network. The amount of dexamethasone loaded by simple equilibrium between the aqueous phase of the network and the loading solution, which leads the drug concentration within the particles to be approximately equal to that of the loading solution, and can be estimated to be close to  $0.067 \mu\text{g mg}^{-1}$  for 5% dextran-MA microgels and  $0.101 \mu\text{g mg}^{-1}$  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  $\gamma$ -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:<sup>38</sup>

$$\text{Loading}(\text{total}) = \frac{(V_s + K_{N/W}V_p) \times C_0}{W_p} \quad (4)$$

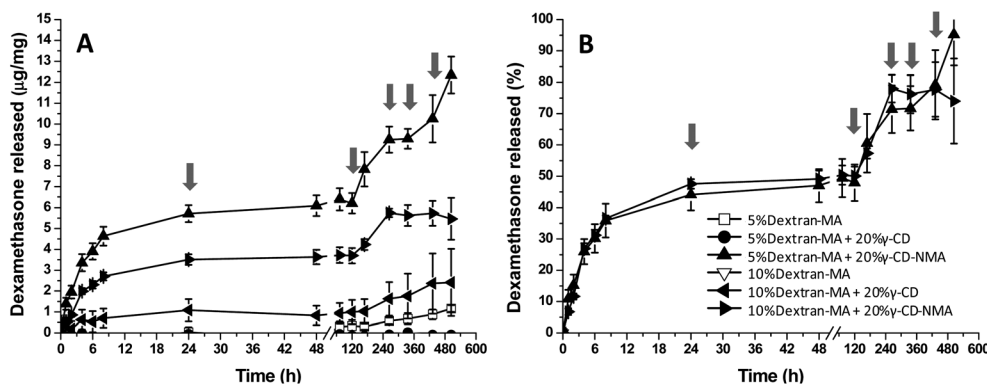
where  $V_p$  is the volume of the dried polymer and the other symbols maintain the same meaning as in eqn (2). The values of  $K_{N/W}$  for 5% dextran-MA + 20%  $\gamma$ -CD-NMA particles and 10% dextran-MA + 20%  $\gamma$ -CD-NMA particles were as high as 505 and 287, respectively, while the respective formulations prepared with free  $\gamma$ -CD had  $K_{N/W}$  values of 82 and 46, respectively (Table 3). These findings clearly prove the advantage of the immobilization of  $\gamma$ -CDs by means of copolymerization, and also that the greater the relative content in  $\gamma$ -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  $\gamma$ -CD units that exhibited  $K_{N/W}$  values in the 60–180 range,<sup>9</sup> the microgels prepared by copolymerization of dextran-MA with  $\gamma$ -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  $\gamma$ -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 which are able to induce osteogenic differentiation ( $40\text{--}400 \text{ ng mL}^{-1}$  (ref. 30)) with a low mass of particles.

Regarding dexamethasone release (Fig. 8), if the drug only interacts with dextran-MA, the release rate should be practically independent of the  $\gamma$ -CD proportion in the hydrogel, except if the mesh size becomes altered.<sup>43</sup> Conversely, if drug- $\gamma$ -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 a case, the greater the content in  $\gamma$ -CDs, the slower is the release.<sup>43</sup> Dexamethasone release from air-dried microgel particles is depicted in Fig. 8. Copolymerization of dextran-MA with  $\gamma$ -CD-NMA was advantageous in terms of the amount of dexamethasone loaded and ability to sustain the release for a long period of time. Disregarding the dextran-MA proportion, the particles exhibited a rapid release in the first 24 h (45–47%

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

Formulation	Total amount of drug loaded ( $\mu\text{g mg}^{-1}$ )	Drug loaded in the aqueous phase ( $\mu\text{g mg}^{-1}$ )	$K_{N/W}$
5% dextran-MA	0.28 (0.37)	0.067	7.3 (12.2)
5% dextran-MA + 20% $\gamma$ -CD	2.54 (0.50)	0.070	82.4 (16.8)
5% dextran-MA + 20% $\gamma$ -CD-NMA	15.24 (0.50)	0.075	505.5 (16.7)
10% dextran-MA	1.08 (0.47)	0.101	32.8 (15.7)
10% dextran-MA + 20% $\gamma$ -CD	1.47 (0.42)	0.098	45.8 (14.0)
10% dextran-MA + 20% $\gamma$ -CD-NMA	8.69 (0.31)	0.073	287.2 (10.5)

**Fig. 8** Dexamethasone release profiles in water referred to the particle mass (A) and to the total amount of dexamethasone loaded (B). Arrows indicate the moment at which an additional volume (1 mL) of release medium was added.

release) followed by a more sustained release for at least 10 days. Moreover, the release of dexamethasone from copolymerized dextran-MA and  $\gamma$ -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.*<sup>44</sup> for soft contact lenses with  $\beta$ -CD pendant groups. This finding indicates that the 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%  $\gamma$ -CD-NMA and 10% dextran-MA + 20%  $\gamma$ -CD-NMA particles released 71% and 77% dexamethasone in 11 days. Differently, particles containing free  $\gamma$ -CDs exhibited a faster delivery (*ca.* 60%) in the first 24 h, reaching 90% release after 11 days. The amount of dexamethasone released from dextran-MA solely was too low for precise quantification (in agreement with the previous low loading).

In sum,  $\gamma$ -CD covalently integrated in the hydrogel network provided a 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  $\gamma$ -CD-NMA with dextran allows the increase in the amount and the period of time during which dexamethasone is released. The drug:CD decomplexation that rapidly occurs when free complexes enter into contact with physiologic fluids<sup>45</sup> is slowed down due to the anchorage of  $\gamma$ -CD-NMA onto the dextran-MA chains (Scheme 3B. Dextran-MA-

$\gamma$ -CD-NMA links) and due to the entanglement of dextran-MA and  $\gamma$ -CD-NMA networks (Scheme 3A. Dextran-MA-dextran-MA + B.  $\gamma$ -CD-NMA- $\gamma$ -CD-NMA).

### Cytotoxicity of the hydrogel particles

A preliminary screening of the cytocompatibility of dexamethasone-loaded and non-loaded particle 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 (Fig. 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  $\gamma$ -CD), and also confirm that there is no 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

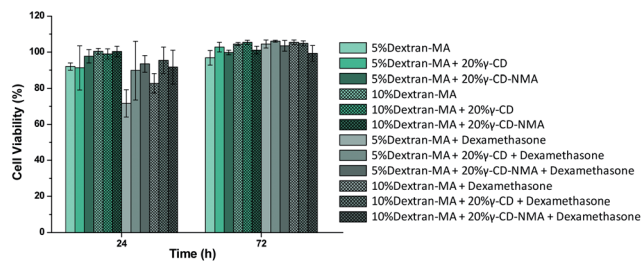


Fig. 9 Viability of SaOs-2 cells after being in direct contact with dexamethasone loaded and non-loaded particles for 24 and 72 h ( $n = 4$ , error bars represent standard deviations).

contact with particles loaded or not with dexamethasone (Fig. 10). The proliferation was on average higher in the presence of dexamethasone, the differences being more accentuated at day 7 ( $p < 0.05$ ). Previous studies demonstrated that dexamethasone either alone or in combination with  $\beta$ -GP + ascorbic acid stimulates the human bone marrow mesenchymal stem cell proliferation.<sup>46</sup> Such evidence can be correlated with the ability of glucocorticoids, at low concentrations, to stimulate the proliferation and/or differentiation of osteoprogenitor cells.<sup>46,31</sup> Moreover, ascorbic acid solely also demonstrated capability of stimulating the proliferation of mesenchymal stem cells and their derived cell types, namely osteoblasts, adipocytes, chondrocytes, *etc.*,<sup>47</sup> while  $\beta$ -GP solely exerted the opposite effect.<sup>46</sup> The similar cell proliferation observed in the negative controls 1 and 2 revealed that the combination of  $\beta$ -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 has a stimulating effect on hASC proliferation.

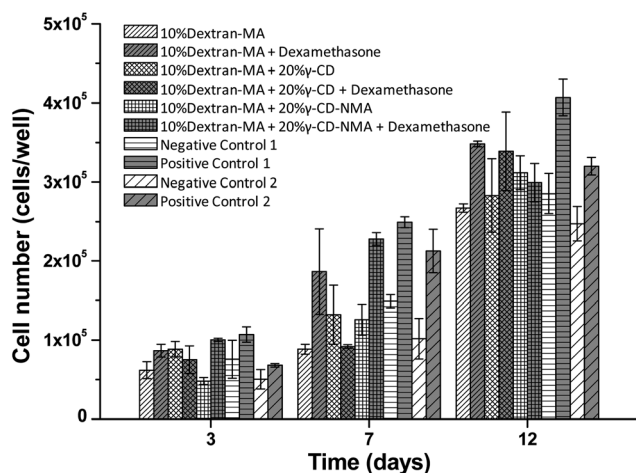


Fig. 10 Proliferation of hASCs cultured in direct contact with dexamethasone loaded and non-loaded particles and in the respective controls up to day 12. Cell number per well was calculated using a calibration curve of known cell numbers ( $n = 3$ , bars represent standard deviations).

**In vitro osteogenic differentiation.** The osteoinductive potential of the particles was evaluated by recording ALP activity. ALP is an enzyme present in the osteoblast membrane (early osteogenic marker) involved in ion phosphate hydrolysis stimulating the mineralization through the formation of hydroxyapatite crystals.

As can be observed in Fig. 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%  $\gamma$ -CD the results were similar to those obtained with the positive control (1 and 2). By contrast, the presence of  $\gamma$ -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 a 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  $\beta$ -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 at the maximum between days 3 and 7. Previous studies demonstrated that hASCs stimulated by medium supplement with  $\beta$ -GP, ascorbic acid and 10 or 100 nM

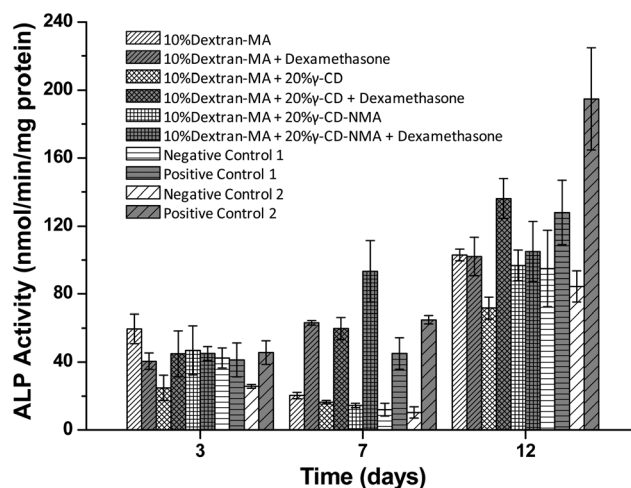


Fig. 11 ALP activity in hASCs induced by dexamethasone loaded and non-loaded particles and the respective positive and negative controls after 3, 7 and 12 days in culture. The results are expressed in mean  $\pm$  SD with  $n = 3$  for each bar.



of dexamethasone also showed an ALP peak after 7 days in culture.<sup>27</sup> The quantitative results about differentiation of the hASCs to osteoblasts were also visualized by means of ALP staining after 12 days of cell culture (Fig. 12). Cells in contact with dexamethasone-loaded particles exhibited a more intense red color. Again, the drug-loaded 10% dextran-MA + 20%  $\gamma$ -CD-NMA particles caused the highest red staining (similar to those cells in positive control 2, which were 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.

Distinct polymeric systems encapsulating dexamethasone have been successfully reported for osteogenic differentiation, namely PLGA scaffolds,<sup>48,49</sup> microparticles<sup>23</sup> and nanoparticles,<sup>50</sup> carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles,<sup>25,51,52</sup> polycaprolactone<sup>53</sup> and poly(*L*-lactide-*co*-caprolactone)/collagen fibers;<sup>54</sup> however none studied the combination of polymers with cyclodextrins and their respective roles in loading and release of such a 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 surface methodology and their performance to load hydrophobic drugs is improved *via* copolymerization with  $\gamma$ -CDs. Copolymer networks of  $\gamma$ -CDs ( $\gamma$ -CD-NMA) and 10% dextran-MA are *ca.* 7 times more effective in loading dexamethasone than the formulations with free  $\gamma$ -CDs and dextran-MA solely, meaning that the anchorage of CDs is advantageous. The *in vitro* release of dexamethasone from dextran-MA + 20%  $\gamma$ -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. To summarize, the improved characteristics of the developed system could be useful for applications in bone regeneration.

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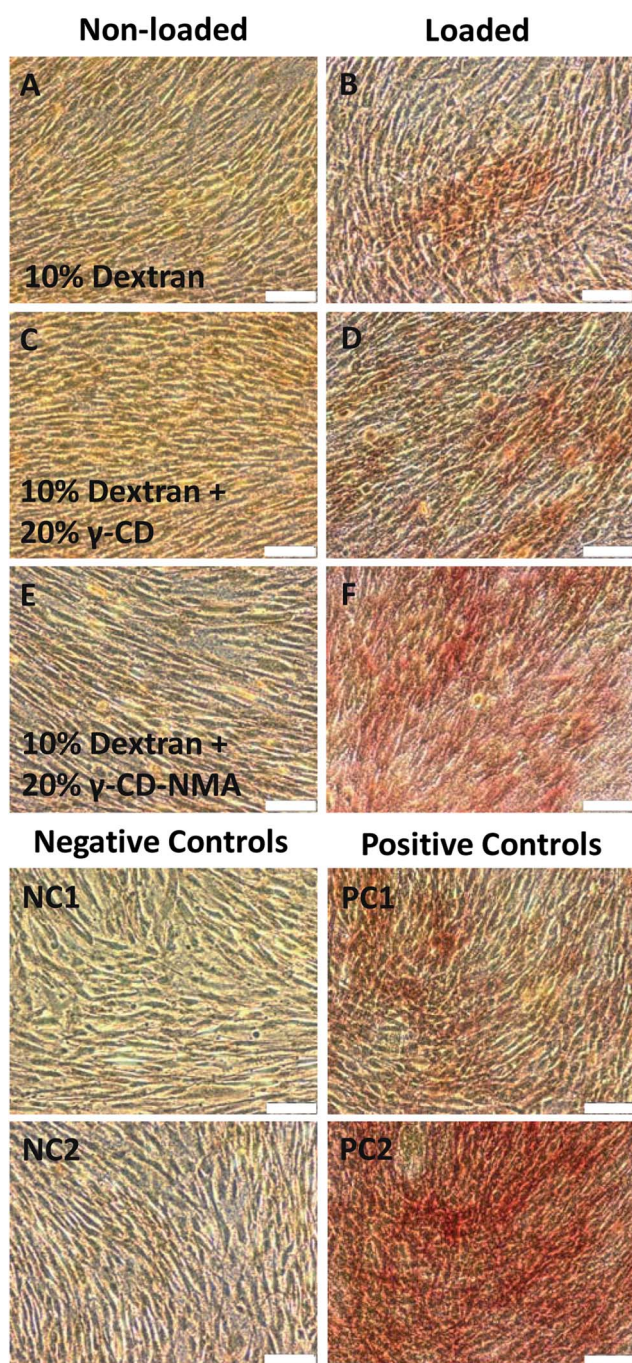


Fig. 12 ALP staining of hASCs in direct contact with non-loaded and loaded particles, and their respective controls at 12 days. The scale bar is 100  $\mu$ m.



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