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Cyclodextrin/PEG based hydrogels for multi-drug delivery

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Abstract

Cyclodextrin–PEG hydrogels were prepared by reaction of hexamethlyene isocyanate-activated β-cyclodextrins with 1.9 kDa NH_2—PEG—NH_2. The reaction was carried out in anhydrous dimethylsulfoxide by using 0.25:1, 0.33:1, 0.5:1, 0.67:1, 1:1, and 2:1 CD/PEG molar ratios. The addition of acetic acid to the reaction mixture was found to slow the cross-linking reaction, yielding homogeneous matrices. The mechanical characterization indicated that the elasticity of the matrices increased as the CD content in the hydrogel increased while the elongation was irrespective of the hydrogel composition. By incubation in water and ethanol, the hydrogels underwent complete swelling in 5–10 min. The water up-take increased logarithmically as the CD/PEG ratio decreased to reach a swelling degree of 800% (swollen hydrogel/dry hydrogel, w/w%). The ethanol uptake increased with a power correlation as the CD/PEG ratio decreased to reach a swelling degree of about 1000% with 0.25:1 CD/PEG hydrogel. Lysozyme, β-estradiol, and quinine were loaded by swell embedding. The lysozyme loading increased as the CD/PEG ratio decreased while the incorporation of β-estradiol and quinine displayed inverse correlation with respect to the CD/PEG ratio. The maximal incorporation (loaded drug/dry hydrogel, w/w%) for lysozyme, β-estradiol and quinine was 2, 0.6, and 2.4%, respectively. Lysozyme was quickly released from the matrices, and the release was faster as the CD/PEG ratio decreased. Also, β-estradiol and quinine release rates were inversely proportional to the CD/PEG ratio, but in these cases, the release profiles were strongly affected by the drug interaction with the hexamethylated β-cyclodextrins in the matrices.

Keywords: Hydrogel; Cyclodextrin; Polyethylene oxide; Drug delivery

1. Introduction

Throughout the years, hydrogels have been gaining increased relevance as drug delivery systems, medical devices, scaffolds for tissue regeneration and substitution, and in several chemical applications as well (Hoffman, 2002; Peppas et al., 2000). In particular, due to their high hydrophilicity, biocompatibility, and suitable loading and release properties, hydrogels have become attractive for the delivery of a variety of low molecular weight drugs and biotech therapeutics such as proteins, peptides and oligonucleotides (Mahato, 2006; Crommelin et al., 2003; Cleland et al., 2001). These systems can, in fact, incorporate drugs under mild conditions by swell embedding, guarantee a non-denaturing environment, and allow for controlled release that may take place by various mechanisms, including diffusion (Case I transport), matrix relaxation (Case II transport), and degradation. For these reasons, hydrogel-based pharmaceutical formulations have been developed for invasive and local applications such as implants and oral, buccal, rectal, ocular, topical, and transdermal delivery (Peppas et al., 2000).

Chemically cross-linked hydrogels have been obtained using a variety of natural and synthetic materials, homo- or co-polymers, and grafted, branched or liner polymers. Therefore, matrices with diverse physicochemical properties, namely stimuli responsiveness, hydrophilic/hydrophobic balance, mechanical properties, biodegradability, or bioadhesivity, can be obtained (Patrickios and Georgiou, 2003; Qiu and Park, 2001; Pillai and Panchagnula, 2001; Hennik and van Nostrum, 2002).

Aimed at obtaining hydrogels with specific physicochemical properties, cyclodextrins have been physically combined or chemically conjugated with various hydrophilic polymers, such

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as polysaccharides, polyglycols and various polyvinyls, and polyacrylates. Structurally organized cyclodextrin-based hydrogels have been prepared by rotaxane crosslinking and controlled hydrolysis, for example. Typical examples are crosslinked α-cyclodextrins with PEG used as a scaffold for fibroblast adhesion and proliferation (Watanabe et al., 2002) and α-cyclodextrin-CL–PEG-CL hydrogels (Zeng-Guo and Sanping, 2003; Ooya et al., 2003). Other hydrogel networks bearing cyclodextrins as pendant groups have been proposed as new biomaterials (Liu and Fan, 2005; Pluemsab et al., 2005). Temperature- and pH-sensitive hydrogels prepared by cyclodextrin crosslinking with acrylates have been demonstrated to be interesting as drug delivery systems as they can load and release drugs under environmental stimuli (Siemoneit et al., 2006; Liu and Fan, 2002; Liu et al., 2004; Zhang et al., 2005).

Though cyclodextrin-based hydrogels have been found useful for a variety of applications, their preparation is often complicate and requires the use of harsh or toxic chemicals, such as several initiators for radical polymerization, cyanogen bromide, hydroxides, and aldehydes, which compromise the network integrity and application safety. Therefore, suitable preparation procedures yielding materials with pharmaceutical properties are paramount for their exploitation as drug delivery systems.

In the present paper, a simple procedure for the preparation of β-cyclodextrin/PEG (CD/PEG) hydrogels for drug delivery is described. In these hydrogels, β-cyclodextrins play multiple roles, acting as crosslinking agents, conveying peculiar structural and physicochemical properties to the matrix, and interacting with hydrophobic drugs, which yield inclusion complexes. By the pharmaceutical point of view, cyclodextrins can control drug release and stabilize biotech drugs such as proteins and peptides, thus improving the therapeutic value of these systems. On the other hand, PEG endows high biocompatibility and hydrophilicity, two main prerequisites of pharmaceutical hydrogels (Peppas et al., 1999).

Aimed at investigating the influence of the chemical composition on the mechanical, physicochemical, and biopharmaceutical properties of these systems, hydrogels were prepared by using different CD/PEG molar ratios. The structural properties of the hydrogels were characterized by spectroscopic and mechanical characterization and by swelling investigations. In order to gain information about their multifunctional drug delivery potential, biopharmaceutical studies were carried out using drug models with different physicochemical properties: lysozyme, β-estradiol, and quinine.

2. Materials and methods

β-Cyclodextrins (CDs) were a kind gift of Roquette (Lestrem, France). Hexamethylene diisocyanate, β-estradiol, quinine hydrochloride, lysozyme, and anthrone were obtained from Sigma–Aldrich (St. Louis, MO). Diaminopolyethylene glycol 1900 Da [NH_2–PEG–NH_2], picrylsulfonic acid, and all the other reagents of analytical grade were from Fluka Chemie (Buchs, Switzerland). The analytical HPLC reverse phase Jupiter C18 column (250 mm × 4.6 mm) was supplied by Phenomenex (Torrance, CA).

2.1. Cyclodextrin activation

A 50 ml DMSO solution of 190.3 mg/ml β-cyclodextrins (2.85 mmol) was added dropwise to hexamethylene diisocyanate (47.7 ml, 285 mmol). The mixture was heated at 70 °C for 6 h under nitrogen atmosphere and then poured into 500 ml of ice-cooled diethyl ether. The white precipitate was washed three times by ether to eliminate the unreacted hexamethylene diisocyanate and desiccated under vacuum. The product yield was 75 ± 5%. The β-cyclodextrin content in the dry product was determined by the anthrone colorimetric method (Morris, 1948). An aliquot of activated product was dissolved in anhydrous DMSO and reacted with 2 kDa monomethoxyPEG–NH_2 to determine the degree of β-cyclodextrin activation. The reacted amino groups corresponding to the bioconjugated hexamethylene isocyanate moieties were indirectly estimated by the TNBS colorimetric test reported in the literature (Snyder and Sobocinsky, 1975), using a standard solution containing an equimolar concentration of monomethoxyPEG–NH_2 as a reference. The product was finally analysed by ESI-TOF mass spectrometry using an API-TOF Mariner (Applied Biosystems) instrument and ^{1}H NMR using a Bruker Spectrospin 300 MHz.

^{1}H NMR analysis of the reaction product CD-(C_6-NCO)$_5$ in DMSO-d_6 was carried out using a Bruker Spectrospin 300 (300 MHz): δ 7.04 (br s, H of urethane–NHCOO–, 0.86 H); δ 5,71 (br s, 2-OH and 3-OH of cyclodextrin, 2.1 H); δ 4.83 (br d, H1 of cyclodextrin, 1 H); δ 4.43 (br s, 6-OH of unmodified cyclodextrin, 0.38 H); δ 3.62 (br m, H6-H3-H5 of cyclodextrin, 3.6 H); δ 3.33 (br m, H4-H2 of cyclodextrin, 2 H); δ 1.53 (m, –CH_2–C**H_2**–CH_2–NCO, protons of mean methylene of hexamethylene, 2.4 H); δ 1.39 (m, –CH_2–CH_2–C**H_2**–NCO, protons of methylene bound to isocyanate, 2.6 H).

2.2. Hydrogel preparation

The CD/PEG hydrogels were prepared by using a circular 1.5 cm diameter flat bottom vial. Anhydrous DMSO (0.5 ml) containing different amounts of activated β-cyclodextrins (22, 29, 44, 58, 88 and 176 mg) was added under stirring to 0.5 ml of DMSO containing 85 mg of 1.9 kDa NH_2–PEG–NH_2 and 30 μl of acetic acid. The DMSO was extensively washed out by hydrogel incubation in distilled water and finally lyophilized. A preparation set was carried out using 170 mg of NH_2–PEG–NH_2, 175 mg of activated β-cyclodextrins, and acetic acid volumes in the range of 0–60 μl.

Hydrogels for the mechanical characterization were prepared as reported above by using 1 ml of DMSO containing 44, 58, 88, 116, 176, and 352 mg of activated β-cyclodextrins, 1 ml of DMSO containing 170 mg of 1.9 kDa NH_2–PEG–NH_2, and 60 μl of acetic acid.

2.3. FT-IR analysis

Lyophilized hydrogels were milled with KBr and analysed by FT-IR. The spectra were recorded in the range 4000–400 cm^{-1} using a Perkin-Elmer Spectrum BX FTIR Spectrometer. The

analysis was performed with 32 scans/sample and 1 cm^{-1} resolution.

FT-IR spectrum (KBr) showed a broad band at 3432 cm^{-1} (β-cyclodextrins–OH stretchings), a band at 3200 cm^{-1} (N–H stretching of urea and carbamate bonds), a band at 2891 cm^{-1} ($-CH_2-$ alkyl chains stretching), a band at 1654 cm^{-1} (stretching of C=O of carbamate and urea bonds), a band at 1106 cm^{-1} (asymmetric stretching of C–O–C of ethoxyl groups of PEG), and a shoulder at 1020 cm^{-1} (C–O stretching related to cyclodextrins).

2.4. Swelling studies

Lyophilized hydrogels disks (1.5 ± 0.2 cm diameter, 0.15–0.25 cm height) were incubated in distilled water or ethanol at 21 °C. At scheduled times the hydrogels were taken from the incubation solution, rapidly dried on paper to remove the surface solvent, and immediately put into a closed vial and weighted. The swelling degree was expressed as (Ws−Wd)/Wd%, where Wd and Ws are the weight of the dry and swollen hydrogel, respectively.

2.5. Polyethylene glycol release

Lyophilized hydrogels disks (1.5 ± 0.2 cm diameter, 0.15–0.25 cm height) were incubated in 10 ml of distilled water. At scheduled times, 0.5 ml volumes were withdrawn and replaced with fresh water. The polymer content in the samples was determined according to the iodine test reported in the literature (Sim and Snape, 1980).

2.6. Mechanical characterization

A software-controlled dynamometer, TA-XT2*i* Texture Analyzer (Stable Micro Systems, UK), with a 5 kg load cell, a force measurements accuracy of 0.0025%, and a distance resolution of 0.0025 mm (according to the instrument specifications), was used for the mechanical characterization of the gel samples. Penetration tests were performed using a cylindrical steel probe with a surface of 1 mm^2. Hydrogel samples swollen in water were cut into a cylindrical shape (diameter: 2 cm; height: 1 cm). The probe, after reaching the gel surface, was forced to penetrate into swollen hydrogels until rupture was achieved. The pre-test speed was set up at 0.50 mm/s, the test speed at 0.10 mm/s, and the post-test speed at 0.10 mm/s with an acquisition rate of 250 points/s. A trigger force, i.e. the force at which the probe recognizes the gel surface, of 0.005 N was set up. The experiments were carried out in triplicate at room temperature (25 °C). The values reported in the present paper represent the mean values and are within 10% of the mean.

2.7. Drug loading

Dry hydrogel disks (1.5 ± 0.2 cm diameter, 0.15–0.25 cm height) were soaked in 10 ml aqueous solutions containing 10 mg/ml lysozyme or 5 mg/ml quinine hydrochloride, or in suspensions containing 5 mg/ml of β-estradiol. After 24 h incubation, the hydrogels were rapidly washed with distilled water and lyophilised. The residual volume was accurately determined after soaking and the drug concentration of lysozyme was determined by fluorescence (λ_{exc} 295 nm, λ_{em} 350 nm) using a titration standard curve while quinine hydrochloride was estimated by UV (λ_{max} 236 nm, ε_M 21820 M^{-1}). After hydrogel soaking, the β-estradiol suspensions were lyophilised and the drug was dissolved in 1 ml of methanol and analysed by a reverse phase analytical C-18 column isocratically eluted with a 60:40 methanol/water mixture containing 0.05% TFA, and the UV detector set at 280 nm. The area under the peak corresponding to β-estradiol was referred to as the standard titration curve.

The drug loading was calculated as loaded drug weight/dry matrix weight %.

The drug loading efficiency (DLE) was calculated as follows,

$$\mathrm{DLE} = \frac{\mathrm{De}}{\mathrm{Dt}}(\mathrm{w/w}, \%)$$

where De is the experimentally loaded drug in the hydrogel, Dt is the theoretical loaded drug in the hydrogel calculated on the basis of the drug concentration in the loading buffer (10 mg/ml, 3 μg/ml, or 5 mg/ml for lysozyme, β-estradiol, or quinine, respectively) and the volume taken up.

2.8. Drug release studies

Lysozyme- and quinine-loaded dry hydrogels (1.5 ± 0.2 cm diameter, 0.15–0.25 cm height) were incubated in 10 ml of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2, while β-estradiol loaded hydrogels were placed into 10 ml of the same buffer containing 2 mM of β-cyclodextrins. At scheduled times, 1 ml of the release medium was withdrawn for analysis and replaced with 1 ml of fresh buffer. The released lysozyme and quinine content in the collected samples was spectrophotometrically determined, while β-estradiol was determined by RP-HPLC according to the procedure reported above. The data were evaluated according to the semi-empirical equation $M_t/M_\infty = kt^{0.45}$ (Ritger and Peppas, 1987).

3. Results

3.1. Hydrogel synthesis

CD/PEG hydrogels were prepared by β-cyclodextrin activation with hexamethylene diisocyanate followed by reaction with NH_2–PEG–NH_2 in anhydrous DMSO. The β-cyclodextrin conjugation with hexamethylene isocyanate was carried out according to a procedure reported in the literature used to attach CDs to chitosan or to obtain CD–PEG soluble conjugates (Prabaharan and Mano, 2006; Salmaso et al., 2007). The β-cyclodextrin activation was carried out in anhydrous DMSO, in which all the reagents are highly soluble and isocyanate groups do not undergo inactivation. The use of a high diisocyanate/cyclodextrin molar ratio (100:1), corresponding to approximately a 29:1 molar ratio of isocyanate groups to β-cyclodextrin primary hydroxyl groups. This

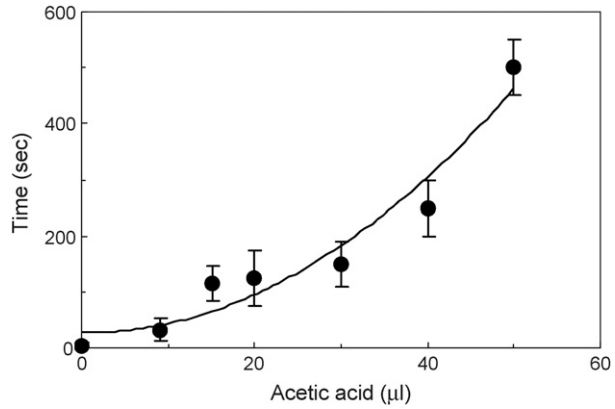

Fig. 1. Hydrogel formation time obtained by using different acetic acid amounts in the reaction mixture.

allowed for extensive β-cyclodextrin functionalization and avoided the formation of cross-linked products. The colorimetric assays used to evaluate the conjugation degree demonstrated that a mean of five hexamethylene isocyanate moieties were attached to each β-cyclodextrin molecule. The ^{1}H NMR signals were assigned according to the data reported in the literature for β-cyclodextrins (Schneider et al., 1998). Both ^{1}H NMR and mass spectrometric analysis (spectra not reported) confirmed the activation degree. Mass spectrometry analysis gave a 375 *m*/*z* signal which was assigned to $[CD–hexamethylene–NH–COOH + 5H^{+}]^{5+}$ (CD–hexamethylene–NH–COOH calculated molecular mass 1875 Da).

The addition of activated β-cyclodextrins to a DMSO solution of NH_2–PEG–NH_2 yielded instantaneous hydrogel formation. The gelation time was determined by measuring the stirring end-time of a mini magnetic stirrer located into the reaction mixture.

The addition of acetic acid into the hydrogel forming mixture was found to slow the gelation process. Fig. 1 shows that the gelation time obtained by using a 1:1 CD/PEG molar ratio increases as the acetic acid in the reaction mixture volume increases.

In order to obtain hydrogels with different reticulation degrees, different CD/PEG molar ratios were used: 0.25:1, 0.33:1, 0.5:1, 0.67:1, 1:1, and 2:1. The crosslinking reaction was carried out by using 30 μl of acetic acid in order to reach complete gelation in 3 min.

Table 1
Mechanical properties (fracture stress, fracture strain and elastic modulus) of hydrogels obtained using 2:1, 1:1, 0.67:1, 0.5:1, 0.33:1, and 0.25:1 CD/PEG molar ratios

CD/PEG	Fracture stress (kPa)	Fracture strain (%)	Elastic modulus (kPa)
2:1	406	53	195.2
1:1	185	32	39.6
0.67:1	102	60	23.6
0.5:1	32	45	12.7
0.33:1	29	41	6.6
0.25:1	15	46	4.8

The FT-IR analysis of the hydrogels showed the typical signals corresponding to the formation of cyclodextrins, PEG, and hexamethylene chains linked through a carbamate and urea bond. The absence of the signal at 2200 cm^{-1} (corresponding to the –NCO stretching) indicated that these groups were reacted or hydrolysed.

3.2. Mechanical properties

The penetration tests described in Fig. 2 and the results summarized in Table 1 showed that the mechanical properties of the hydrogels are strongly affected by the CD/PEG composition. The elastic modulus and the fracture stress decreased as the CD content in the hydrogel decreased, indicating that the hydrogel elasticity and rigidity increase as the CD content increases. The fracture strain could not be correlated to the gel composition; a critical deformation yield for the CD/PEG systems was in the range of 30–60%.

3.3. Polyethylene glycol release and hydrogel swelling

Fig. 3 reports the PEG release profiles obtained by hydrogel incubation in water. The amount of released PEG slowly increased as the CD/PEG molar ratio decreased from 2:1 up

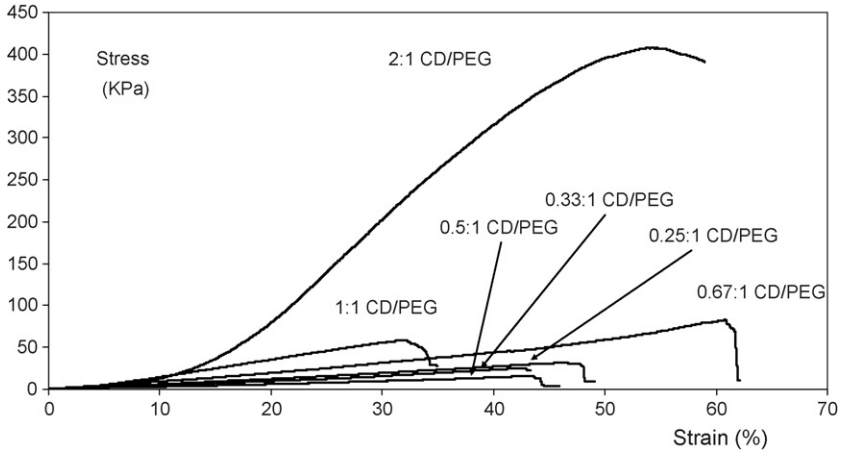

Fig. 2. Stress strain curves for the CD/PEG hydrogels at the different ratios prepared.

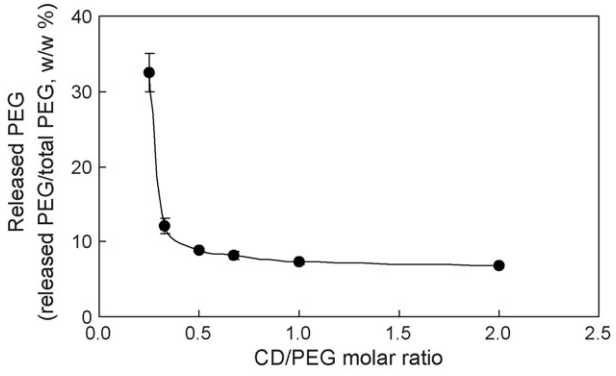

Fig. 3. Polyethylene glycol released after 24 h incubations of the hydrogels in distilled water. The amount of released polyethylene glycol has been calculated as (w/w)% of the polymer initially present in the hydrogels. The polyethylene glycol release and the standard deviations (±S.D.) were calculated on the basis of five experiments.

to 0.33:1, while an abrupt PEG release was obtained with the 0.25:1 CD/PEG hydrogel.

By incubation in water and ethanol, the lyophilised matrices swelled rapidly, regardless of their composition. After 5–10 min incubation in water or ethanol, all hydrogels reached equilibrium. Fig. 4 shows that the hydrogel swelling degree at equilibrium depends on both solvent and matrix composition. Either water or ethanol up-take increased as the CD/PEG molar ratio decreased. In water, the swelling degree was logarithmically correlated to the CD/PEG ratio ($y = -365.85\ln(x) + 347.95$, $R^2 = 0.97$) while the ethanol swelling degree showed a power correlation with the CD/PEG ratio ($y = 248.03x^{-0.8821}$, $R^2 = 0.97$).

3.4. Drug loading and release

The drug loading and release studies were carried out using circular hydrogels with 1.5 ± 0.2 cm diameter and 0.15–0.25 cm height. After swelling, the hydrogel size increased to 2.0–2.3 cm diameter and 0.5–0.7 cm height. The slight size differences of the swollen hydrogels were in the range of the experimental error (standard deviation).

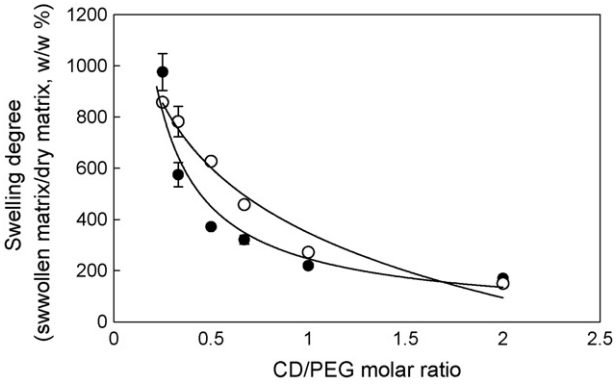

Fig. 4. Swelling degree obtained by incubation of hydrogels with different CD/PEG compositions in water (◯) and ethanol (●). The swelling degree was calculated as % of increased weight as compared to the dry matrix weight. The mean swelling degree and the standard deviations (±S.D.) were determined on the basis of five experiments.

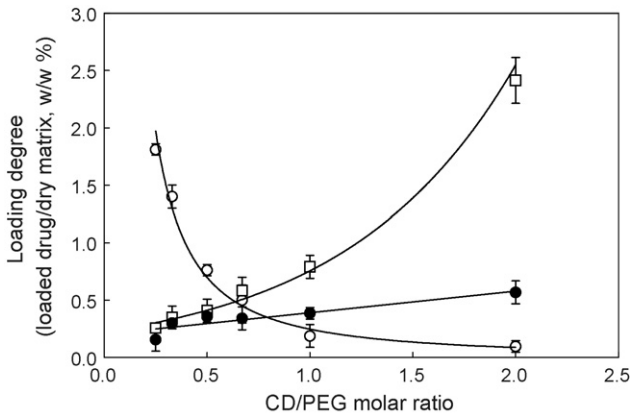

Fig. 5. Lysozyme (◯), β-estradiol (●), and quinine (□) loading in the CD/PEG matrices after 24 h soaking in medium containing the three drug models. The amount of loaded drug was indirectly calculated on the basis of the unloaded molecules and expressed as (w/w)% of the dry matrix weight. The mean loading values and standard deviations (±S.D.) were calculated on the basis of the results obtained with five experiments.

Fig. 5 reports the lysozyme, β-estradiol, and quinine loading in the various hydrogels.

The lysozyme loading increased as the CD/PEG ratio decreased according to a power correlation ($y = 0.247x^{-1.5}$, $R^2 = 0.98$). The calculated protein concentration in the buffer that was taken up was lower than in the external solution, indicating that the network hindered the protein penetration into the hydrogel. However, the lysozyme loading efficiency (DLE) increased from 6% to 20% as the CD component in the hydrogel decreased.

The best fit obtained with hydrophobic β-estradiol loading was linearly correlated with the increase of CD content in the matrix ($y = 0.1888x + 0.2018$, $R^2 = 0.93$). The β-estradiol loading efficiency (DLE) rose from 8700% to 125000% as the CD/PEG molar ratio increased. In the hydrogels, the β-estradiol/β-cyclodextrin molar ratio was in the range of 6–15%.

Quinine loading increased exponentially as the CD content in the matrix increased ($y = 0.224e^{1.2x}$, $R^2 = 0.98$). In the hydrogels, the quinine/β-cyclodextrin molar ratio was in the range of 11–21% and the loading efficiency (DLE) rose from 6% to 290% as the CD component increased.

Fig. 6 shows the release profiles of the three drug models from the various matrices. In all cases the lysozyme release was rapid and complete in 10 h. The release rate from the hydrogels prepared with 0.25:1, 0.33:1, 0.5:1, 0.67:1 and 1:1 CD/PEG molar ratios increased as the CD component in the matrix decreased. A linear fit was obtained by plotting the released drug/loaded drug fraction (M_t/M_∞) towards the $t^{0.45}$, and the linearity was maintained up to 0.7–0.85 M_t/M_∞ with 0.94–0.98 R^2. As an exception, the matrix consisting of a 2:1 CD/PEG molar ratio showed a burst release in 30 min, corresponding to 75% of the loaded drug.

The β-estradiol release studies were performed by using β-cyclodextrin-containing release buffer in order to increase the drug solubility and guarantee sink conditions. The β-estradiol release rate increased as the CD content in the matrices decreased. The drug release was found to fit a bi-modal

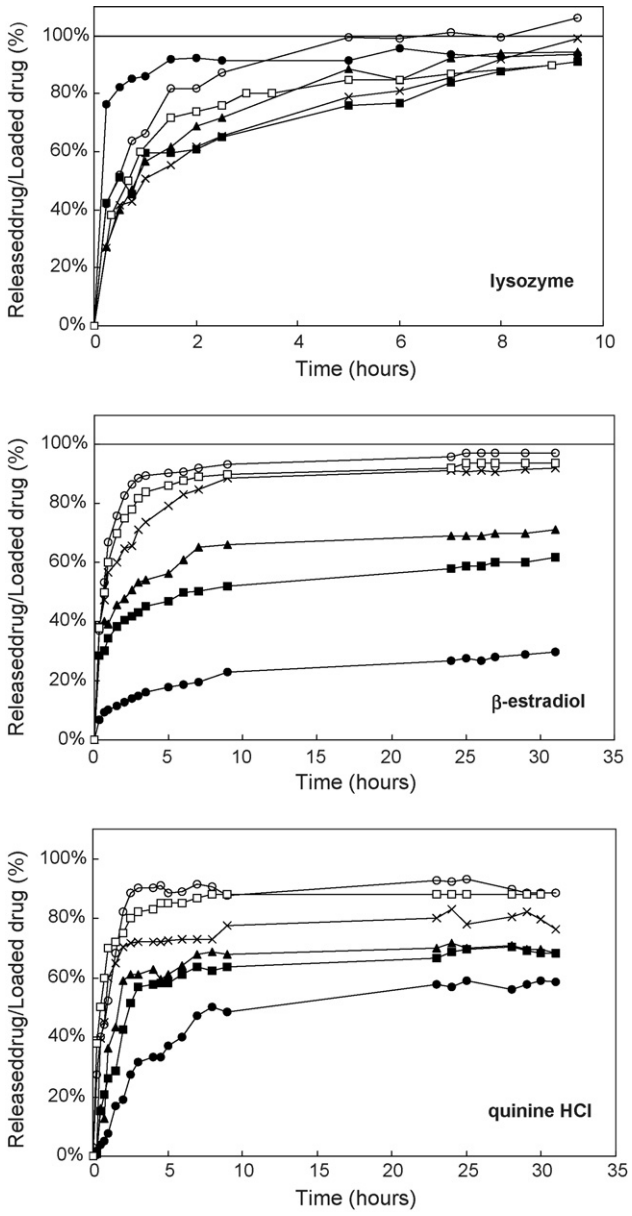

Fig. 6. Lysozyme, β-estradiol and quinine release profiles from hydrogels prepared using different CD/PEG molar ratios: 2:1 (●), 1:1 (■), 0.67:1 (▲), 0.5:1 (×), 0.33:1 (□) and 0.25:1 (◯). The figure reports the mean values calculated at each point with five matrices per hydrogel composition. Standard deviation (±S.D.) was in the range of ±2 to 6% of the reported values.

behaviour. During the first phase (0–4 h), linear $(M_t/M_\infty)/t^{0.45}$ plots with 0.94–0.98 R^2 were obtained. After 4 h, the β-estradiol release from the hydrogels with low CD content (0.25:1, 0.33:1 and 0.5:1 CD/PEG molar ratios) was negligible. On the contrary, the hydrogels containing higher CD amounts (0.67:1, 1:1 and 2:1 CD/PEG molar ratios) displayed a slow but constant drug release, and linear $(M_t/M_\infty)/t^{0.45}$ plots with 0.93–0.99 R^2 were obtained.

Similar to β-estradiol, quinine was released according to a bi-modal behaviour, and the release rate increased as the CD content in the hydrogel decreased. The data obtained during the first phase (0–3 h) were found to fit linear $(M_t/M_\infty)/t^{0.45}$ plots. After 3 h, the drug release from the matrices obtained with 0.25:1, 0.33:1, 0.5:1, 0.67:1 and 1:1 CD/PEG molar ratios was negligible, while the drug was slowly released from the matrix obtained with 2:1 CD/PEG molar ratio. In the latter case the drug was constantly released up to 30 h, when about 55% of the loaded drug was released.

4. Discussion

The preparation of chemical CD/PEG hydrogels was carried out according to a simple procedure which, compared to other methods reported in the literature, avoids the use of radical initiators or other potentially toxic reagents. The unreacted isocyanate groups in the hydrogels are rapidly inactivated by hydrolysis during the matrix washing step, making this material chemically inert (Ivanova et al., 2006). Finally, the β-cyclodextrin decoration with alkyl chains allowed for the expansion of the hydrophobic cyclomaltoheptaose cavity, which can enhance the affinity for hydrophobic drugs, as already observed with soluble hexamethylene–PEG modified CD derivatives (Salmaso et al., 2007).

In order to prevent instantaneous hydrogel formation, which yielded macroscopically heterogeneous matrices, acetic acid was added to the NH_2–PEG–NH_2 solution before the addition of the activated cyclodextrins. The partial protonation of the PEG amino groups reduced the reactivity of the hexamethylene isocyanate groups, slowing down the gel formation. The gelation time increased exponentially as the acetic acid content in the reaction mixture increased. No gel formation was observed with a 20:1 acetic acid/NH_2–PEG–NH_2 molar ratio, probably because under these conditions the isocyanate inactivation prevents the reaction with NH_2–PEG–NH_2.

The influence of the chemical composition on the mechanical, physicochemical and biopharmaceutical properties of the matrices was evaluated by preparing hydrogels using different CD/PEG molar ratios. PEG release studies showed that in the case of hydrogels obtained with 2:1, 1:1, 0.67:1, and 0.5:1 CD/PEG molar ratios, about 90–95% of PEG was chemically incorporated into the matrix. Since in these preparations the number of PEG amino groups in the reaction mixture is lower than the isocyanate groups, these results indicate that under the reaction conditions a significant fraction of isocyanate moieties were not derivatized with PEG. When 0.33:1 and 0.25:1 CD/PEG molar ratios were used, only the PEG corresponding to the amino groups stoichiometrically exceeding the isocyanate functions was released from the matrix, indicating that when low CD/PEG molar ratios are used, extensive isocyanate conjugation was achieved. However, no quantitative information about the amount of PEG forming bifunctional crosslinks and the fraction forming pendant chains by single end-side chain conjugation could be obtained. Indeed, the IR data could provide only qualitative information about the β-cyclodextrin–hexamethylene–PEG conjugation.

Mechanical studies were undertaken to gain information about the effect of the CD/PEG molar ratio on the structural and biopharmaceutical properties of the hydrogels. Mechanical characterization performed by penetration test is, in fact, useful to characterize the elastic properties of hydrogels (Jones et

al., 1996; Tamburic et al., 1996). In the case of the CD/PEG hydrogels, the elastic modulus decreased as the CD content decreased, indicating that the hexamethylated-cyclodextrins act as crosslinkers. Therefore, the elasticity of the hydrogels increased as the CD content increased. Furthermore, the fracture stress data show that a looser and more flexible matrix is obtained as the CD/PEG molar ratio used in the hydrogel fabrication decreased, though the fracture strain data indicate that the deformability of the hydrogels was irrespective of the matrix composition.

Stress/strain properties of the hydrogels are mainly dictated by the presence of physical interactions and chemical bridging. The increased stress resistance obtained by increasing the CD content in the hydrogels may be explained with the formation of physical or chemical reticulation sites. In the CD/PEG hydrogels, both physical interactions and chemical bridging are expected to increase as the CD content increases. Indeed, cyclodextrins have been reported to reduce the flexibility of hydrogel networks by promoting microenvironmental aggregations (Liu and Fan, 2005). On the other hand, as the CD content in the reaction mixture increases, the single side-end PEG binding becomes dominant over the bridging reaction.

The swelling profiles of hydrogels in water and ethanol are in good agreement with the mechanical data. In fact, the solvent uptake was found to decrease as the CD content in the matrix increased, confirming that a looser and weaker network is obtained when a low CD/PEG molar ratio is used. On the contrary, matrices with a high CD/PEG molar ratio possess higher rigidity and lower swellability. This behaviour is in agreement with the crosslinking role of hexamethylated-cyclodextrins. However, the different swelling degrees at equilibrium cannot be solely attributed to the physical/chemical reticulation. Indeed, the hydrogel swelling is determined by the opposing hydrophilic/hydrophobic balance of the hydrogels conveyed by the hydrophobic hexamethylene-decorated CDs and the hydrophilic polymer. Therefore, the higher swelling degree in water of hydrogels with lower CD content is attributable to the higher hydrophilic/hydrophobic balance, and the solvent uptake tends to plateau as the CD/PEG ratio increases. The influence of the hydrophilic/hydrophobic nature of the hydrogel components is also more evident in the case of ethanol, which is less polar than water. The low ethanol uptake obtained with high CD/PEG molar ratio hydrogels can be explained by the low solubility of the cyclodextrins in this solvent. In such a case, PEG plays a key role in counterbalancing the poor solubility of the cyclodextrins, and a dramatic swelling is obtained when a critical CD/PEG ratio is reached.

The biopharmaceutical properties of the CD/PEG hydrogels were evaluated by using matrices with similar sizes. Indeed, the different weights of hydrogels with different compositions were balanced by the different swelling degrees. These operative conditions were chosen to avoid the simultaneous modification of several parameters, namely, CD and PEG content or size and weight of the dry matrixes, which could make the result interpretation difficult. The studies were performed by using three different drug models: lysozyme, quinine, and β-estradiol. These molecules were selected because of their different molecular size, structural complexity, and hydrophilic/hydrophobic character. Lysozyme is a highly hydrophilic macromolecule (mol wt. 14.3 kDa), quinine is a small hydrophilic molecule (mol wt. 324 Da) forming weak inclusion complexes with β-cyclodextrins (stability constant K_s 221), while β-estradiol is a hydrophobic compound (mol wt. 272 Da) forming stable inclusion complexes with the cyclopolysaccharides (inclusion constant K_c 77947 M^{-1}) (Liu et al., 2003; Caliceti et al., 2003). As expected, the drug loading and the release profiles were strictly related to both matrix composition and drug physicochemical properties.

Because of its high hydrophilicity and molecular weight, lysozyme was expected to localize in the hydrated fraction of the matrices without significant interaction with the hexamethylated β-cyclodextrins. The inverse correlation between the lysozyme loading and CD/PEG ratio was in agreement with the swellability results discussed above. The low loading efficiency indicates that the hydrogels are formed by tight networks that prevent protein diffusion into the matrix. The loading efficiency increased as the CD/PEG ratio decreased, confirming that looser matrices are generated by using low CD/PEG molar ratios. However, it should remind that lysozyme is a cationic molecule and replsion charges with free amino groups of the hydrogel may interfere with the drug loading.

On the other hand, β-estradiol loading was found to increase proportionally with the CD content in the hydrogel, because of its ability to form inclusion complexes with the cyclopolysaccharides. Interestingly, despite the fact that only a small fraction of cyclodextrin hosts the β-estradiol (6–15% of the available cyclopolysaccharides), the loading efficiency was exceptionally high and increased significantly (up to 125,000%) as the cyclodextrin content increased, demonstrating that the presence of hydrophobic clusters in the matrix enhances the inclusion of hydrophobic drugs.

Unexpectedly, the quinine loading efficiency rose from 6% to 290% as the CD content in the matrix increased, though this hydrophilic molecule, which forms weak inclusion complexes with β-cyclodextrins, was expected to localize mainly in the hydrated fraction of the matrix. Actually, the decoration of β-cyclodextrins with hexamethylene moieties could increase the quinine association, as already reported with other drugs, thus enhancing its loading (Prabaharan and Mano, 2006).

The lysozyme, β-estradiol and quinine releases were differently affected by the hydrogel composition. Lysozyme, for example, was rapidly released from all the matrices by diffusion from the swollen hydrogels (Case I transport). Indeed, linear $(M_t/M_\infty)/t^{0.45}$ plots were obtained and the matrix relaxation time (about 5–10 min) was much shorter than the diffusion process (a few hours). Furthermore, the decreased release rate obtained by increasing the CD content was in good agreement with the swelling and loading results, which demonstrated that a tighter network slowing the diffusion/penetration processes is obtained as the CD content in the hydrogel increases. The anomalous rapid release from the matrix constituted by the highest CD/PEG molar ratio can be ascribed to the high network tightness, which prevents the protein penetration into the core of the matrix during the loading step. Therefore, in this case the

drug is essentially confined on the hydrogel surface from where it is rapidly released.

Drugs forming complexes with β-cyclodextrins showed different release behaviours as compared to lysozyme. The release profiles obtained with β-estradiol and quinine indicate that the hydrogel composition has a dramatic effect on the drug release. In the case of β-estradiol, for example, high CD concentrations in the hydrogels significantly slow the drug release rate, probably because of the high drug affinity for the β-cyclodextrins. On the other hand, the effect of the cyclodextrin complexation seems to be minimized when the PEG content in the hydrogel becomes predominant. Similar to β-estradiol, the quinine release was strongly affected by the cyclodextrin content, although in this case the slow release due to the formation of complexes with the modified β-cyclodextrins was counterbalanced by the high diffusivity through the swelled matrix caused by the hydrophilic character of this small molecule. Therefore, a higher released drug fraction as compared to β-estradiol occurred during the first phase. However, both β-estradiol and quinine release rates decreased significantly during the second phase, yielding incomplete release. The bi-modal diffusion behaviour obtained with β-estradiol and quinine may be due to microstructural changes of the matrix, which occur after 3–4 h incubation or, more likely, to the presence of β-estradiol/β-cyclodextrin as well as quinine/β-cyclodextrin complexes with different stability constants. Probably, the drug can strongly interact with assembled cyclodextrin clusters, formed in the hydrogels with high CD content, which prevents the complete release of the drug.

5. Conclusions

The results reported in the present study show that random chemical hydrogels with peculiar and adaptable physicochemical, mechanical, and biopharmaceutical properties can be easily obtained following a few simple chemical steps.

Hydrogels constituted by PEG and cyclodextrins, two materials already used in several pharmaceutical formulations, possess the main prerequisites for pharmaceutical applications. Furthermore, they have multifunctional drug delivery properties, as they can be properly designed to deliver either hydrophilic or hydrophobic molecules, which may even be simultaneously loaded into the matrices. The simultaneous presence of CD and PEG may provide for the stabilization of biotech drugs and their maintenance under physiological conditions thus preventing their denaturation. Interestingly, these systems allow for the molecular dispersion of hydrophobic drugs into hydrophilic biocompatible networks, and provide for proper drug release that may be modulated by modification of the hydrogel composition. Of note, the amphiphilic nature of polyethylene glycol can be exploited to yield high loading of hydrophobic drugs. Low water soluble molecules could be, in fact, loaded into the hydrogels by using ethanol or another organic solvent solution. Furthermore, the drug release can be properly controlled by modulating the CD/PEG composition, and a sustained release can be achieved when high CD/PEG molar ratios are used.

Therefore, the potential versatility of these materials makes them promising in drug delivery, where innovative alternatives to traditional formulations are strongly demanded in order to obtain specific therapeutic functions.

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