Novel drug nanocarriers combining hydrophilic cyclodextrins and chitosan

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Abstract
The aim of this study was to explore the possibility of obtaining nanoparticles (NPs) containing high amounts of cyclodextrin (CD) derivatives such as carboxymethyl-\(\beta\)-CD and sulphobutyl ether-\(\beta\)-CD. The rationale used was to combine the drug solubilizing and stabilizing properties of cyclodextrins (CDs) with the mucoadhesive properties of chitosan (CS) in a unique nanoparticulate drug delivery system. The size of the resulting NPs was affected by the nature of the CDs, ranging between 275 and 550 nm, whereas the zeta potential of the NPs was always positive and close to +35 mV. The positive zeta values, together with the results from NMR studies, suggest that CS is the major compound on the surface of the NPs, while CD molecules are strongly associated with the NP matrix. The empirical composition of the NPs was quantified by elemental analysis and the results indicated that the amount of CD associated with the NPs was strictly dependent on its electrostatic charge. Finally, in vitro stability studies indicated that the presence of CDs in the NP structure can prevent the aggregation of this nanometric carrier system in simulated intestinal fluid. Overall, this new type of NP represents an attractive drug delivery platform of particular interest for the oral administration of drugs with low bioavailability.

1. Introduction
Among the different modalities of drug administration, the oral route continues to be the most attractive one. Unfortunately, a large number of drugs have a low oral bioavailability due to their poor solubility, degradation in the gastrointestinal (GI) compartment and/or low-intestinal permeability. To circumvent these limitations, several oral drug delivery carriers, particularly polymeric nanoparticles (NPs), are currently under study [1]. In principle, nanocarrier systems are characterized by their ability to protect drugs from degradation and to facilitate the interaction of the drug with the mucosa, therefore improving its subsequent absorption. Among the different types of NPs intended for transmucosal drug delivery, those based on the polysaccharide chitosan (CS), are currently under study [1]. In principle, nanocarrier systems are characterized by their ability to protect drugs from degradation and to facilitate the interaction of the drug with the mucosa, therefore improving its subsequent absorption. Among the different types of NPs intended for transmucosal drug delivery, those based on the polysaccharide chitosan (CS) are particularly promising due to their advantageous properties including biocompatibility, mucoadhesion and ability to transiently open the tight junctions of the intestinal barrier. In fact, CS NPs have already shown the capacity to deliver hydrophilic macromolecules both in cellular models and in vivo [2].

Another possible approach to improve oral bioavailability, especially in the case of low-solubility drugs, is based on the formation of complexes between the drug and a natural or synthetic cyclodextrin (CD). CDs represent a family of cyclic oligosaccharides that have been widely employed in the pharmaceutical field to increase the solubility of poorly water-soluble drugs, and to improve their dissolution rate. Due to their cone-shaped structure, CD can form inclusion complexes with a variety of drugs. This complexation process normally results in a modulation of the physicochemical and pharmaceutical properties of the guest molecule (i.e. the drug), such as increased solubility, improved chemical and physical stability and/or enhanced oral absorption. Besides these well-known capabilities of CD, some studies have also disclosed other positive properties of these excipients such as the ability to inhibit the P-glycoprotein function [3, 4] and to enhance the permeability of epithelia [5]. In addition, from a pharmaceutical point of view, a remarkable advantage of CDs

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is their safety status, as several CDs are accepted for parenteral administration [6, 7].

A more recent tendency focuses on incorporating CDs in nanometric particles, a strategy that aims at designing versatile delivery systems that can encapsulate drugs with different physicochemical properties (i.e. hydrophilic and hydrophobic). The most popular approach for the formation of CD-containing NPs consists in the incorporation of previously formed drug-CD inclusion complexes into polymer NPs. In these systems, the presence of CDs generally results in an increase of drug loading of lipophilic [8, 9] and hydrophilic substances [10]. Another option to the formation of nanocarriers from CDs is to design amphiphilic-CDs that self-assemble in the form of stable NPs. Amphiphilic-β-CDs can be obtained by chemical modifications of native β-CD, and NPs can be readily prepared from them by a nanoprecipitation technique [11–13]. The formation of electrostatic aggregates in the nanometric range from CDs has also been described [14–16], although this strategy is mostly restricted to the complexation of cationic CDs with DNA or RNA.

Previous studies performed in our group have shown the feasibility of forming NPs composed of CS and hydroxypropyl-β-CD [17]. The aim of the present work has been to increase the CD incorporation within the NPs using different types of CD. For this purpose, we selected three different cyclodextrins such as 2-hydroxypropyl-β-CD (HP-β-CD), carboxymethyl-β-CD (CM-β-CD) and sulphobutylether-β-CD (SBE-β-CD) for their incorporation into CS NPs. The resulting NPs were extensively characterized with regard to their size, surface charge, nanostructural composition and stability in a simulated biological medium.

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial sources and used as received. Chitosan (CS, 87% deacetylation degree) was purchased from Pronova Biopolymer (UP CL 113, Norway). Na-carboxymethyl-β-cyclodextrin (CM-β-CD, MW = 1375, substitution degree = 3.00–3.50), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD, MW = 1540, substitution degree = 4.69) and pentasodium tripolyphosphate (TPP) were all purchased from Sigma-Aldrich (Spain). Sulphobutyl ether-β-cyclodextrin sodium salt (SBE-β-CD, MW = 2163, substitution degree = 6.40) was obtained from CyDex, Inc. (USA). Deuterium oxide (D2O) of 99.9% isotopic purity was purchased from Aldrich. All other chemicals were reagent grade or higher.

2.2. Preparation of NPs

CS or CS/CD-based NPs were prepared according to the procedure previously developed by our group [18].

NPs without CDs were spontaneously formed upon addition of 1 ml of TPP aqueous solution (0.15% w/v, polyanionic phase) to 3 ml of the CS solution (0.20% w/v, polycationic phase) under stirring.

CD-containing NPs were prepared differently depending on the type of CD, but volumes of the two phases were always the same as well as for NPs without CDs. (i) CS/HP-β-CD/TPP NPs were prepared by dissolving variable amounts of HP-β-CD either in the polycationic (0.1–1% w/v) or in the polyanionic phases (0.3–3% w/v) described for the preparation of NPs without CDs. NPs were spontaneously formed upon mixing of these polycationic and polyanionic phases under stirring. (ii) CS/SBE-β-CD/TPP NPs were prepared by mixing the CS solution (0.2% w/v) with a polyanionic phase containing SBE-β-CD (0.30–0.45% w/v) or both SBE-β-CD and TPP (0.075% w/v). (iii) CS/CM-β-CD/TPP NPs were prepared by mixing the CS solution (0.2%) with a polyanionic phase containing CM-β-CD (0.15–0.45% w/v) or both CM-β-CD and TPP (0.075–0.15% w/v). The resulting NPs were isolated by ultracentrifugation (16 000 × g, 30 min, 15°C, Beckmann Avanti 30, Beckmann, USA) and resuspended in ultrapure water.

2.3. Physicochemical and morphological characterization of NPs

The mean particle size and the size distribution of the NPs were determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). The determination of the ζ-potential was performed by laser Doppler anemometry (Zetasizer 3000 HS, Malvern Instruments, Malvern, UK) after dilution with KCl 1 mM.

The morphological examination of CS/CD/TPP NPs was performed by transmission electron microscopy (TEM) (CM12 Philips, Eindhoven, Netherlands). All samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar® film for TEM observation.

2.4. Elemental analysis of the NPs

Elemental analysis of the starting materials (i.e. CS and the CDs) and the NPs was performed on freeze-dried samples (Telstar Cryodos freeze-drier, Telstar Industrial SL, Spain). Samples of 1–10 mg were analyzed by an Elemental Analyzer (Fisons model EA 1108, Thermo Finnigan, Italy). For all samples, the elemental composition on C, H and N was determined. For the SBE-β-CD (pure component) and CS/SBE-β-CD NPs, the corresponding composition in S was also determined. The precision of the analysis was within ±0.3% and the reproducibility was within ±0.2%. The CS content of the NP sample was analyzed by the N content of the sample in comparison with pure CS. For CD content determination, the C content of the samples arising from CS was calculated and subtracted from the observed value. This excess of C was inputted to calculate the amount of CD present in the samples. In the case of the CS/SBE-β-CD/TPP NPs, the S content was also used for CD quantification. The composition values obtained were very similar regardless of the element used (S or C) for calculating the composition in CD of the samples and, thus, the data here reported represent an average of the two results. The remaining fraction of the NP composition was assigned to counterions (i.e. TPP, Na, Cl etc.) For CS and CD, the incorporation efficiency, a parameter
that represents the percentage of each starting material that was actually incorporated into the NPs, was calculated as follows. Incorporation efficiency $(\%) = 100^\circ$ is the empirical amount of compound in the NPs/amount of compounds in the starting materials, where the empirical amount of the compounds in the NPs was calculated as the final mass of the NPs obtained and the percentage of the material of interest according to the NP composition as determined by elemental analysis. The amounts of compounds in the starting materials was the mass weighted for this particular material for NP preparation.

2.5. NMR studies

All the NMR spectra were acquired at 25 °C on an Inova-750 Varian spectrometer operating at 750 MHz. The signals of pure CS and CM-β-CD were assigned by a combination of 1D proton and 2D-TOCSY experiments (data not shown). NPs $4/1/1$ (CS/CM-β-CD/TPP, w/w/w) were isolated, resuspended in $D_2O$ at a concentration of 10 mg ml$^{-1}$ and analyzed by liquid-state proton NMR and diffusion ordered spectroscopy (DOSY). DOSY experiments were acquired to aid in the interpretation of the structure of the carriers; this technique allows the calculation of the diffusion coefficients of compounds displaying a signal in the proton spectra. Further, to detect which components of the NPs were exposed to the external water phase, a water-LOGSY experiment (water-ligand observed via gradient spectroscopy) [19, 20] was performed on isolated samples resuspended in a 90/10 (v/v) $H_2O/D_2O$ solvent mixture. The water-LOGSY was acquired with a 4 s relaxation delay between scans. The consistency of the water-LOGSY NOEs detected with the solvent was checked by determining the build up of these NOEs upon variation of the mixing time from 50 ms to 2 s across several experiments. Each experiment was repeated twice.

2.6. Stability study in intestinal medium

Selected NP formulations were freshly prepared, isolated and resuspended at 0.1% (w/v) in a simulated intestinal medium (USP-XXVI, pH 6.8 without pancreaticin). The tested formulations were: CS/TPP 4/1 (w/w), CS/CM-β-CD/TPP 4/1/1 (w/w/w) and CS/HP-β-CD/TPP 4/2/1 (w/w/w, HP-β-CD in the TPP phase). The NPs were incubated at 37 °C under agitation (100 rpm), and samples were collected at time points of 0, 30, 60 and 120 min. The size distribution of the NPs was measured by PCS. Each experiment was performed in triplicate.

2.7. Statistics

Data from different experimental groups were compared by a one-way ANOVA (GraphPad Prism v.4.00 GraphPad Software, Inc. San Diego, CA). Bonferoni tests were used for post-hoc contrast. A significance level of $\alpha \leq 0.01$ was considered throughout this study.

3. Results and discussion

We have previously shown the interest of NPs composed of CS and HP-β-CD for the delivery of hydrophobic drugs [17]. The results from this previous work suggested that these carriers effectively combine some beneficial characteristics of CS NPs and CDs in a unique carrier. More precisely, CS/CD/TPP NPs should combine the excellent capacity of CS nanocarriers to load hydrophilic drugs [18] with a higher efficiency when encapsulating hydrophobic molecules [17]. In the present work, we have optimized these carriers, and designed other CS NP carriers containing CDs with very different physicochemical properties. We hypothesized that the physicochemical characteristics of the CDs were critical in terms of controlling their interaction with CS and, thus, we selected a group of CDs with markedly different properties: HP-β-CD, SBE-β-CD and CM-β-CD (figure 1). HP-β-CD is a neutral molecule, whereas SBE-β-CD and CM-β-CD are polyatomic. SBE-β-CD and CM-β-CD differ in the number of charges per molecule, and in the range of pH at which they are charged. SBE-β-CD presents an average of 6.4 sulphobutyl anionic groups per molecule with a pKa $< 1.8$. CM-β-CD has approximately 3.5 carboxylic groups (pKa = 3.0 ± 0.2) per cyclodextrin ring.

3.1. CS/CD/TPP NPs formation process

3.1.1. Conditions for NP formation. First of all, we explored the conditions that would allow us to form CS/CD NPs. For this scope, we adopted a technique developed in our laboratory based on the ionic gelation of CS with TPP [18]; recently this technique was shown to allow the incorporation of HP-β-CD [17]. As we hypothesized, the experimental conditions required for the formation of the different types of CS/CD NPs were clearly affected by the nature of the CD incorporated. In agreement with our previous results [17], we observed that during NP preparation, HP-β-CD could be included either in the CS or in the TPP solution. Within the range of concentrations tested in this particular experiment, i.e. 0.1–1% and 0.3–3% w/v respectively for HP-β-CD added in the CS or in the TPP phase, CS NPs containing HP-β-CD could be prepared under the same conditions used for the control formulation (i.e. NPs without CDs). From our previous work, it was already known that such NPs can be formed even at HP-β-CD concentrations as high as 1 and 3 mg ml$^{-1}$ respectively in the CS or in the TPP phase) [17]. Altogether, these results suggest that the presence of HP-β-CD has no critical impact in the NP formation process and, therefore, no particular limit of CD concentration needs to be respected for the preparation of these NPs.

The situation observed with negatively charged CDs was quite different as SBE-β-CD and CM-β-CD can readily interact with CS, leading to the formation of NPs. In other words, NPs can be formed by the direct incorporation of these anionic CDs to the CS solution either with or without the crosslinking agent TPP. This is reasonably explained by the formation of ionic intermolecular linkages between the negative groups present in both TPP and CDs, and the positively charged amino groups of CS [18, 21]. Similarly
to the case in which CS is crosslinked with TPP, excessive amounts of polyanionic CDs led to the formation of aggregates instead of a NP suspension (tables 2a and 3a) [18].

The important role that the electrical charge of CDs has in NP formation is illustrated by the different formulation conditions required to prepare CS NPs integrating CDs with distinct charge densities. SBE-CD has 0.02958 moles of negative charges per gram (calculated from manufacturers’ specifications), and forms NPs in the concentration range 0.45–0.75% (w/v) in the absence of TPP. On the other hand, CM-CD bears 0.02181 negative charges per gram (calculated from manufacturers’ specifications) and, therefore, higher concentrations are necessary to form NPs in the absence of TPP: 0.75–1.20% (w/v).

In the case of NPs containing both TPP and polyanionic CDs, a cooperative effect was observed. On one hand, lower amounts of CD and TPP were required for NP formation than in formulations where only one polyanion is added. Moreover, aggregates were also formed with smaller quantities of CD and TPP than in systems prepared only with one of these polyanions. For example, CS/TPP NPs without CDs are formed in the following range of CS/TPP (w/w) ratios: 4/0.67–4/1.3 [18]. With CS/CD/TPP carriers, NPs can be formed with 4/0.5 CS/TPP (w/w) ratios and most compositions aggregate at CS/TPP (w/w) ratios of 4/1. As an exception, these NPs can be formed with low amounts of the weak polyanion CM-CD (CS/CM-CD/TPP 4/1/1).

3.1.2. Incorporation efficiency of the components in the NPs formation process. The fractions of the initial materials that end up forming part of the NPs are indicative of the particle formation process, and the mechanism of components interaction. We define the incorporation efficiency of a material as the percentage of the starting material that is incorporated into the NPs. These values were calculated by determining the yield of the NP formation process for each formulation and the composition of the NPs (more details about NP composition in section 3.3).

The incorporation efficiency of CS and CD for NPs prepared from different theoretical CS/CD/TPP mass ratios can be seen in figures 2(a) and (d). As expected, under the experimental conditions of this study the incorporation efficiency of HP-CD within the NPs is quite low (≤3%) (figures 2(a) and (b)) and it is not affected by the incorporation approach (either in the CS or the TPP phase). Interestingly, the presence of HP-CD enhanced the incorporation efficiency of CS. Indeed, the incorporation efficiency of CS in the absence of CD was close to 40%, whereas this value increased significantly in the presence of CD; moreover, this increase was similar irrespectively of whether the CD was added to the CS or to the TPP phase. This result suggests that a certain interaction occurs between HP-CD and CS, which affects the integration of both compounds in the form of NPs. Given the non-ionic nature of this CD, we hypothesized that hydrogen bonding or hydrophobic forces could be presumably involved in the interaction occurring between the two compounds [22].

For both series of NPs prepared with anionic CDs and without TPP, the incorporation efficiency of the CDs was very variable and proportional to the amount of CD in the starting materials (figures 2(c)–d). For CS/SBE-CD NPs, the incorporation efficiency of the CD changed with formulation conditions between 28% for the 4/3/0 (CS/SBE-CD/TPP, w/w/w) and 97% for the 4/5/0. The high incorporation efficiency of this CD is indicative of its strong affinity for CS. For CS/CM-CD NPs, the CD incorporation efficiency values increased from 12% for the 4/5/0 (CS/CM-CD/TPP, w/w/w) composition to 48% for the 4/8/0 one. These more moderate values of incorporation are consistent with lower affinity of this CD for CS than that observed for SBE-CD. An additional observation was that irrespective of the type of CD, its incorporation and the production yield of the resulting NPs are directly related to the amount of CD added to the preparation medium. Additionally, this increment of the CD incorporation was accompanied by an increase of CS.
Table 1a. Physicochemical properties of CS/HP-β-CD/TPP NPs. HP-β-CD added to the CS phase (mean ± S.D., n = 6). *—Significant differences from control values (CS/CD/TNP 4/0/1 NP formulation, α < 0.01). (N.D.: not determined.)

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Concentration HP-β-CD (%w/v)</th>
<th>Size (nm)</th>
<th>Polydispersity</th>
<th>ζ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/CD/TNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/0/1</td>
<td>0</td>
<td>361 ± 20</td>
<td>0.36-0.44</td>
<td>+35.1 ± 0.3</td>
</tr>
<tr>
<td>4/2/1</td>
<td>0.10</td>
<td>309 ± 54</td>
<td>0.34-0.46</td>
<td>+35.8 ± 0.4</td>
</tr>
<tr>
<td>4/4/1</td>
<td>0.20</td>
<td>385 ± 12</td>
<td>0.35-0.45</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/8/1</td>
<td>0.40</td>
<td>456 ± 27*</td>
<td>0.29-0.46</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/12/1</td>
<td>0.60</td>
<td>433 ± 45*</td>
<td>0.37-0.63</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/16/1</td>
<td>0.80</td>
<td>323 ± 18</td>
<td>0.25-0.35</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/20/1</td>
<td>1.00</td>
<td>334 ± 34</td>
<td>0.29-0.31</td>
<td>+35.5 ± 0.1</td>
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</table>

Table 1b. Physicochemical properties of CS/HP-β-CD/TPP NPs. HP-β-CD added to the TPP phase (mean ± S.D., n = 6). *—Significant differences from control values (CS/CD/TNP 4/0/1 NP formulation, α < 0.01). (N.D.: not determined.)

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Concentration HP-β-CD (%w/v)</th>
<th>Size (nm)</th>
<th>Polydispersity</th>
<th>ζ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/CD/TNP</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/0/1</td>
<td>0</td>
<td>361 ± 20</td>
<td>0.36-0.44</td>
<td>+35.1 ± 0.3</td>
</tr>
<tr>
<td>4/2/1</td>
<td>0.30</td>
<td>405 ± 39</td>
<td>0.37-0.43</td>
<td>+34.4 ± 0.1</td>
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<tr>
<td>4/4/1</td>
<td>0.60</td>
<td>446 ± 37*</td>
<td>0.45-0.55</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/8/1</td>
<td>1.20</td>
<td>408 ± 22</td>
<td>0.40-0.60</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/12/1</td>
<td>1.80</td>
<td>376 ± 31</td>
<td>0.35-0.45</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/16/1</td>
<td>2.40</td>
<td>385 ± 13</td>
<td>0.31-0.49</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/20/1</td>
<td>3.00</td>
<td>373 ± 10</td>
<td>0.36-0.44</td>
<td>+37.9 ± 0.1*</td>
</tr>
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</table>

Table 2a. Physicochemical properties of CS/SBE-β-CD NPs (mean ± S.D., n = 6). *—Significant differences from control values (CS/CD/TNP 4/0/1 NP formulation, α < 0.01). (N.D.: not determined.)

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Concentration SBE-β-CD (%w/v)</th>
<th>Size (nm)</th>
<th>Polydispersity</th>
<th>ζ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/CD/TNP</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>4/0/1</td>
<td>0</td>
<td>361 ± 20</td>
<td>0.36-0.44</td>
<td>+35.1 ± 0.3</td>
</tr>
<tr>
<td>4/1/0</td>
<td>0.15</td>
<td>359 ± 14*</td>
<td>0.37-0.43</td>
<td>+30.8 ± 0.0*</td>
</tr>
<tr>
<td>4/3/0</td>
<td>0.45</td>
<td>549 ± 14*</td>
<td>0.37-0.43</td>
<td>+30.8 ± 0.0*</td>
</tr>
<tr>
<td>4/4/0</td>
<td>0.60</td>
<td>344 ± 11</td>
<td>0.25-0.36</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/5/0</td>
<td>0.75</td>
<td>275 ± 52*</td>
<td>0.12-0.28</td>
<td>+21.2 ± 0.3*</td>
</tr>
<tr>
<td>4/6/0</td>
<td>0.90</td>
<td>280 ± 52*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A clear solution was obtained.

b Precipitation of aggregates was observed.

incorporation. Indeed, for NPs prepared with SBE-β-CD, the incorporation efficiency of CS ranged from a modest 16% (for the CS/SBE-β-CD/TNP 4/3/0 formulation) to a very high value of 86% (for the 4/5/0 formulation). For NPs prepared with CM-β-CD, CS incorporation efficiency ranged between 9% (for 4/5/0 formulation) and 63% (for the 4/8/0 formulation).

For NPs formed with SBE-β-CD and TPP, the incorporation efficiency of the CD and the CS was higher than that observed for the same formulation without TPP. These results indicate that TPP acts cooperatively with polyanionic CDs in the NP formation process.

In summary, the incorporation of the neutral cyclodextrin HP-β-CD, which is supposed to weakly interact with CS, is low and does not affect significantly the NP formation process. On the other hand, the incorporation of anionic cyclodextrins, which can readily crosslink CS, is very high and markedly influences the yield of the NP formation process.

3.2. Characterization of NPs: size, zeta potential and morphology

Tables 1a–3b show the physicochemical properties of the different NPs, including also the ones prepared without any CD (used as control). It is well known that the size of CS NPs largely depends on the concentration of CS, the concentration of TPP and the ratio CS/TPP [17, 18, 23]. Overall, within the range of conditions explored in this work, the size of the resulting NPs varied between 275 and 549 nm and their polydispersity index between 0.3 and 0.7. In all the series prepared, the mean diameter of the NPs varied accordingly to the concentration of CDs added during the preparation process.

As shown in tables 1a and 1b, the incorporation of low amounts of HP-β-CD to the NP formation medium, either to the CS phase or to the TPP phase, led to an initial increase (α < 0.01) in the size of the NPs (from 361 nm to approximately 450 nm). This initial increase was surprisingly followed by a reduction in particle size when the concentrations of HP-β-
CD reached a certain limit. The addition of small amounts of CD facilitated the incorporation of CS (see section 3.1.2), thus resulting in the formation of larger particles. However, the increase of the HP-β-CD/CS ratio above 4/4 leads to more compact nanostructures and, hence, to a reduction in the particle size.

A different behavior was observed for the anionic CDs. In the case of CS/SBE-β-CD/TPP NPs the size decreased from 549 to 275 nm as the CS/CD/TPP ratio varied from 4/3/0 to 4/5/0 (table 2a). Overall, the variation in particle size was not remarkable for the formulations made of CS/CM-β-CD/TPP, whose size changed from 420 nm (4/5/0 CS/CM-β-CD/TPP) to 363 nm (4/8/0 CS/CM-β-CD/TPP). For both series of formulations there was a clear correlation between the incorporation efficiency of the anionic CDs (figures 2c and d) and their particle size (tables 2a–3b). Consequently, their reduction in size could be understood by the increasing ionic interactions between CS and these CDs and their possible effect in compacting the polymer matrix. An alternative explanation might be that increasing concentrations of these anionic CDs might lead to the formation of a higher number of nuclei of precipitation upon their contact with CS. This higher number of precipitation nuclei could result in a higher number of NPs of smaller particle size.

The reduced sizes of the NP formulations containing CDs are very interesting in view of their potential application as transmucosal carriers. Indeed, it is well known that particles in the small nanometric range are easily transported more efficiently through biological barriers [2] and, therefore, the reduction achieved in particle size (25% difference between the smallest formulation and the control) could result in more efficacious drug carriers for oral drug delivery.

With respect to the zeta potential of the three series of NPs, it was interesting to observe that all the series exhibited positive charge values close to +35 mV (tables 1a–3b). The fact that even the NPs modified with anionic CDs showed positive zeta
Incorporation efficiency of CS and CD into the final NPs (mean ± S.D., n = 3). (a) CS/CD/TPP NPs: (a) CS/HP-β-CD/TPP, HP-β-CD dissolved in the CS phase; (b) CS/HP-β-CD/TPP, HP-β-CD dissolved in the TPP phase; (c) CS/SBE-β-CD/TPP; (d) CS/CM-β-CD/TPP. *—Significant differences from control values (CS/CD/TPP 4/0/1 NP formulation, α < 0.001).

Figure 2. Incorporation efficiency of CS and CD into the final NPs (mean ± S.D., n = 3). CS ( ), CD ( ), (a) CS/HP-β-CD/TPP, HP-β-CD dissolved in the CS phase; (b) CS/HP-β-CD/TPP, HP-β-CD dissolved in the TPP phase; (c) CS/SBE-β-CD/TPP; (d) CS/CM-β-CD/TPP. *—Significant differences from control values (CS/CD/TPP 4/0/1 NP formulation, α < 0.001).

The morphological appearance of CS/CD/TPP NPs is shown in the TEM micrographs presented in figures 3(a) and (c). In general, particles sizes measured by TEM are smaller than those determined by photon correlation spectroscopy, which is attributed to the loss of water during the drying step prior to electron microscopy. It could also be observed that NPs containing HP-β-CD had an irregular morphology (figure 3(a)), whereas those containing SBE-β-CD or CM-β-CD exhibited a round shape (figures 3(b) and (c)).

3.3. Chemical composition of NPs

Different approaches have been proposed in the literature for the quantitative analysis of CD content in particulate carriers. Most of them are colorimetric reactions of the CD with an appropriate reagent (e.g. fading of phenolphthalein reaction or phenol-sulphuric acid reaction) [10, 24]. These methods are convenient, but limited by the necessity of separating CD from the particle matrix. In order to avoid this separation process, in this work, the chemical composition of the NPs was determined by elemental analysis. Using this technique, the composition of the NPs could be determined easily by comparing the C–N mass ratios (or the C–N–S mass ratios) of CS and CD with those of the NPs (figures 4(a) and (d)). A similar approach has recently been reported by us for the determination of CD in NPs [25].

CS/TPP (4/1) NPs were analyzed and taken as a reference for the CD-containing formulations. Their composition was 74.5% CS and 25.5% counterions, values that are close relation to the theoretical ratios at which the materials were mixed (4/1). The percentage of HP-β-CD in NPs prepared with this CD was low but increased with higher theoretical mass ratios of the CD in the starting materials (figures 4(a) and (b)). For NPs where the HP-β-CD was included in the CS phase, the maximum content of CD was around 13%; when the HP-β-CD was included in the TPP phase, it was 19%. Taking into account that NPs are expected to load therapeutic molecules in
the form of complexes with this CD [17], these results support the interest of this system for drug delivery applications.

As expected, the anionic CDs could be incorporated into the NPs in high amounts: up to 58.4% and 69% (w/w) of the final composition of SBE-β-CD and CM-β-CD-containing NPs corresponding to the respective CDs incorporated (figures 4(c) and (d)).

### 3.4. Structural composition of the NPs

Once the composition of the NPs was known, we aimed at characterizing the architectural disposition of CS and CD in these systems. For this purpose, we used liquid-NMR techniques as previously reported for other nanocarriers [19, 26]. The formulation CS/CM-β-CD/TPP4/1/1 was selected as a model for the characterization of these systems.

To analyze the composition in the outer shell of the model NPs, we performed a 1D-water-LOGSY [20, 27, 28], an experiment that can show nuclear Overhauser effect contacts between the external water phase and other molecules in close contact with it. The water-LOGSY spectrum of the selected NPs is shown in figure 5(a). Well-isolated signals from CS at 1.9 ppm (methyl group of the acetylated monomers) and 2.9 ppm (proton axial to CS amine group) could be easily identified in this spectrum. We also observed signals from the sugar ring that could correspond to CS or CM-β-CD (3.3–3.8 ppm). However, we could not identify any characteristic signal in the range of 3.9–4.2 ppm, which would be associated to CM-β-CD. The absence of CM-β-CD in the spectrum suggests that this compound is not in contact with the external water phase. On the other hand, the presence of some CS in the spectrum indicates that a fraction of chains of this polymer do interact with the external water phase and, furthermore, that they are endowed of sufficient rotational mobility to give a signal in liquid-state NMR.

In order to confirm that the signals observed in the proton NMR spectrum stand for protons attached to the NPs, we performed an NMR diffusion experiment. The results in figure 5(b) show that CS signals were not filtered out in the diffusion experiment. This indicates a particularly slow diffusion for CS, a result that confirms that CS is attached to the NPs and does not diffuse as molecules in solution.

In summary, the results arising from these experiments are consistent with a model of the NPs in which some chains of the CS polymer are located on the external side and exposed to the aqueous medium. On the other hand, CM-β-CD seems to be well entrapped into the NP polymeric network. These findings were in good agreement with the positive zeta potential values of all the studied NPs. A schematic representation of the structure deduced for these carriers is presented in figure 6.
3.5. Stability study in intestinal medium

A critical aspect in the design of NPs for transmucosal drug delivery is the assessment of their stability in biological fluids. For example, in the case of PLA and PLGA NPs, it is well known that they suffer an aggregation process in gastric fluids that limits their ability to interact with the intestinal mucosa [29]. This aggregation process could be significantly prevented by the use of polyethylene glycol or by the association of poloxamers to the NP structure [29, 30]. To the best of our knowledge, CD derivatives have not been employed for NP stabilization. Nevertheless, some works have shown other properties that suggest a possible role of CDs in particle stabilization: namely β-cyclodextrins derivatives were able to reduce NP opsonization when used as surface-modifying agents [31].

The NPs tested for this experiment were the control 4/1 (CS/TPP) formulation and two formulations containing cyclodextrins: 4/2/1CS/HP-β-CD/TPP NPs (HP-β-CD added in the TPP phase) and 4/1/1CS/CM-β-CD/TPP NPs.

Control CS NPs (without CDs) showed quick particle aggregation upon incubation in the simulated intestinal medium (figure 7). This process has been connected to the reduction of the NPs’ surface charge due to the deprotonation of CS amines in alkaline medium, and also to the dissociation of the CS molecules in a rich-ion environment. Interestingly, the CD-containing NPs became stable when incubated in the intestinal medium for up to 120 min (figure 7). Our previously discussed physicochemical characterization data suggest that NP-containing CDs present a more compact inner structure (smaller size, higher incorporation of the materials to the NP structure). Taking into account these observations, our current hypothesis is that this change in the inner structure of the nanocarriers could be the reason behind the improved stability of these carriers in simulated biological fluids.

4. Conclusions

In this work, we present a new drug nanocarrier consisting of a core rich in CDs and a coating rich in CS. This nanocarrier is prepared by a very mild ionic crosslinking technique, suitable for the incorporation of delicate compounds. Due to their core nature, these nanostructures have the capacity of carrying both, hydrophilic and hydrophobic compounds. The nature of the entrapped CD has a marked effect on the physicochemical properties of the resulting NPs and also in the affinity of these molecules for CS. These CD-containing NPs have shown an improved stability in intestinal fluid, thus being promising carriers for the oral administration of drugs.

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