Novel core(polyester)-shell(polysaccharide) nanoparticles:

protein loading and surface modification with lectins

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Bauhinia monandra, Lens culinaris.

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Abstract

This study describes new lectin-decorated or protein-loaded nanoparticles with a hydrophobic poly(ε-caprolactone) (PCL) core and a hydrophilic dextran (Dex) corona.

In this view, a family of block Dex-PCL\textsubscript{n} copolymers was first synthesized, consisting of a Dex backbone to which \( n \) preformed PCL blocks were grafted. The ability of these new copolymers to form nanoparticles was evaluated in comparison with a series of PCL homopolymers of various molecular weights (2,000, 10,000 and 40,000 g/mole). Two different nanoparticle preparation methods have been developed and tested for their efficacy to incorporate proteins. For this, three proteins were used: a model protein, bovine serum albumin (BSA), a lectin from leaves of *Bauhinia monandra* (BmoLL) and *Lens culinaris* (LC) lectin. All these proteins were successfully incorporated in nanoparticles with a mean diameter around 200 nm.

Lectins could also be adsorbed onto the surface of Dex-PCL\textsubscript{n} nanoparticles. Surface-bound BmoLL conserved its hemagglutinating activity, suggesting the possible application of this type of surface-modified nanoparticles for targeted oral administration. Moreover, it was shown that these Dex-PCL\textsubscript{n} nanoparticles had no toxic effect on Caco-2 cells, even at concentrations as high as 660 µg/ml.
1. Introduction

The development of appropriate delivery systems for new macromolecules coming out from the biotech industry is a meaningful challenge for pharmaceutical scientists. Proteins, peptides, oligonucleotides, and genes are very unstable compounds that need to be protected from degradation in the biological environment. Moreover, their efficacy is highly limited by their inability to cross biological barriers and to reach the target site. As such, the future of these molecules as therapeutic agents clearly depends on the design of appropriate carriers for their delivery into the body [1].

Two approaches have been applied to introduce proteins in therapy. In the first case, the protein displays a pharmacological activity and is useful to be encapsulated or incorporated into nanosystems such as liposomes or nanoparticles [2-7]. In the second case, proteins, and more specifically lectins, were bound to the surface of nanocarriers for site-specific drug targeting [8-11]. Indeed, one of the crucial and pervasive troubles in the human therapy is to achieve a satisfactory balance between the toxicity and the therapeutic effect of drugs. Therefore, site-specific delivery could reduce such side effects at non-target sites and increase the efficacy.

In this framework, the development of targeted drug carriers systems could be based on the design of lectin-conjugated nanoparticles. Indeed, it is well known that lectins are involved in cell recognition mechanisms. Lectins are carbohydrate-binding proteins potentially non-immunogenic, originating from plants, microorganisms or animals. They are multivalent molecules, which possess two or more sugar-binding sites for agglutinating plant and animal cells, and for precipitating polysaccharides and other compounds such as glycoproteins, peptidoglycans, or glycolipids. The specificity of lectins is essentially driven by monosaccharides or oligosaccharides that inhibit lectin-
induced agglutination or precipitation reactions [12]. Such specificity, multivalent featured, as well as their non-immunogenic properties render lectins appealing candidates for conceiving site-specific coated nanosystems as carriers for drug delivery [9, 10]. The development of lectin-nanocarrier conjugates was based on the background of understanding at the molecular level the interactions between glycoligands (code word/message) and lectin (receiver/translator), which can trigger a variety of post-binding signaling mechanisms [13]. Moreover, endogenous lectins harbor the potential to be pharmaceuticals in their own right, e.g., in anti-adhesion approaches, immunomodulation or growth controls [2].

Despite the fact that the field of lectin-mediated drug delivery systems is up till now in its early life, some studies in the literature suggested the appeal of such an approach [9-14]. However, technical difficulties are related to the covalent immobilization of lectins to the surface of biodegradable nanoparticles, because of the absence of reactive groups onto their surface. Therefore, the synthesis of novel biodegradable polymers with reactive groups would be a step forward in the design of nanoparticles decorated with ligands.

In this respect, very recently, a family of new Dex-PCL\textsubscript{a} copolymers was synthesized with the aim of forming core (polyester)/corona (polysaccharide) nanoparticles [15]. These copolymers combine the advantages of the hydrophobic PCL polymers for drug encapsulation, and those of Dex for nanoparticles surface modification and ligand covalent binding. The aim of this work was to explore the feasibility of protein entrapment into these new materials, as well as their surface modification with lectins. Furthermore, with the scope of possible applications for oral protein delivery, the cytotoxicity of the Dex-PCL\textsubscript{a} with regard to Caco-2 cells in culture was investigated.
2. Materials and methods

2.1 Materials and copolymer synthesis and characterization

Block Dex-PCL\textsubscript{n} copolymers bearing an average of 3, 5.5 or 7 PCL side chains (named respectively Dex-PCL\textsubscript{3}, Dex-PCL\textsubscript{5.5}, and Dex-PCL\textsubscript{7}) were synthesized as previously described [15]. Briefly, preformed monocarboxylic PCL chains with low molecular weight (2100 g/mole) (named PCL2K) were first obtained by uncatalyzed ring-opening polymerization of ε-caprolactone. They were then reacted under anhydrous conditions with Dex (5000 g/mole, Fluka) using carbonyldiimidazole (Sigma-Aldrich) as coupling agent. Dex, PCL and Dex-PCL\textsubscript{n} copolymers were further characterized by gel permeation chromatography (GPC) using a triple detection system (Viscotek, Houston, Texas, US). Polystyrene standards (Polymer Laboratories, Shropshire, UK) were used to determine molecular weights from universal calibration, using two GMH-HR H columns mounted in series and heated at 60°C. The mobile phase was N, N dimethylacetamide (DMAC) containing 0.4% LiBr at a flow rate of 0.5 mL/min. The injected volumes were 100 µl. Sample concentrations ranged from 5 to 10mg/mL. The number n of polyester chains grafted per Dex macromolecules was calculated by subtracting the determined MW of Dex (5000 g/mole) from the MW of Dex-PCL\textsubscript{n} copolymers and dividing by the MW of polyester grafts (2100 g/mole).

PCL homopolymers (MW 10,000 Da and 40,000 Da) named PCL 10K and PCL 40K were furnished by Aldrich (USA). Bovine serum albumin (BSA, fraction V), sodium cholate (SC), and LC were purchased from Sigma (USA). BmoLL was purified and characterized by Coelho and Silva [16]. All the other chemical reagents, of analytical grade, were obtained from Merck (Darmstadt, Germany).
2.2 Preparation of protein loaded-nanoparticles

Nanoparticles made of Dex-PCL_{n} copolymers, loaded or not with proteins (BSA, BmoLL or LC), were manufactured following two different preparation methods.

Firstly, 4 mg of insoluble Dex-PCL_{n} copolymers were suspended in 1 ml dichloromethane. After addition of 200 µL of water and under vigorous stirring during 1 min, a W_{1}/O emulsion was formed. This broad emulsion was sonicated (CV 145 sonicator, Vibra Cell, France) at 40 W during 20 sec in an ice bath, and 4 ml of a sodium cholate solution (0.1%, w/v) were added under stirring by vortex for 3 min. The resulting W_{1}/O/W_{2} emulsion was sonicated as described above for 30 sec. The solvent was removed by evaporation under reduced pressure and the nanoparticles were recovered by centrifugation (Beckman L7-55 centrifuge, USA) at 144,000 g for 30 min and washed twice with water. Nanoparticles were diluted with 2 ml of 5% glucose, lyophilized and stored at 4°C. The efficiency of protein entrapment in Dex-PCL_{5.5} nanoparticles was evaluated for BSA, BmoLL and LC lectins. For this, the proteins were dissolved in the internal aqueous phase at different concentrations (1.05, 12.25 and 20.8 mg/ml). The protein theoretical loading L was calculated as following:

\[ L (\%) = 100 \frac{m_p}{m_p + m_{cp}} \]

Where \( m_p \) and \( m_{cp} \) are respectively the weights of protein and of Dex-PCL_{n} copolymer used for the nanoparticle preparation.

As the copolymers used in this method were insoluble in both water and dichloromethane phases, it was named “interfacial migration-double emulsion” (IM-DE).

Secondly, an “interfacial migration-simple emulsion” (IM-SE) technique was used for comparison with the IM-DE method. Briefly, 1.0 ml of a polymer solution (4 mg/ml) in dichloromethane was added to 200 µL of water and 4.0 ml of a sodium cholate (SC)
solution (0.1%, w/v) under vigorous stirring by vortex (3 min). The broad o/w emulsion was sonicated (CV 145 sonicator, Vibra Cell, France) at 40 W during 20 sec in an ice bath. The solvent was eliminated by evaporation under reduced pressure. The nanoparticles were recovered by centrifugation and treated in the same conditions as previously described in the IM-DE method.

2.3 nanoparticle characterization

After their preparation, the morphology of the nanoparticles was analyzed, and their size distribution, density and zeta potential were measured.

The size distribution of nanoparticles was determined in water at 20°C by photon correlation spectroscopy (PCS) using a Nanosizer N4-MD® (Coulter, France). The Zeta potential (ζ) of nanoparticles dispersed in 10 mM NaCl solutions was measured using a Zetasizer 4® (Malvern, UK). The density of the nanoparticles was estimated according the sucrose gradient method [17]. For this, nanoparticle samples (0.5 ml) were placed at the top of a sucrose gradient and then submitted to centrifugation (L-55 centrifuge, Beckaman, USA) at 122,000 g (20°C, 15 min). The nanoparticles could be detected with the naked eye in the sucrose gradient. They settle at a certain depth in the tubes, accordingly to their density. Aliquots were withdrawn and their density was determined in a densimeter (DMA 45, Instrulab France). Morphological examination of nanoparticles was performed by scanning electronic microscopy (SEM, JOEL, Japan) at 20 KV. For this, lyophilized samples of nanoparticles were resuspended in water and placed on a glass surface, which were fixed on metallic supports with carbon-glue. After drying, the samples were directly coated with colloidal gold using a gold sputter module.
in a high-vacuum evaporator (JFC-1100, JOEL, Japan) before observation with the scanning electron microscope.

2.4 Determination of protein entrapment efficiency

The amount of non-entrapped protein in the dispersion medium was determined by the Lowry-Peterson protein assay [18]. For this, the nanoparticle suspensions were centrifugated immediately after their preparation at 87,000 g for 15 min (L7-55 centrifuge, Beckman, USA). The supernatants were centrifuged again at 144,000 g for 30 min in order to make the smallest nanoparticles sediment. The resulting supernatants were recovered for protein dosage. The protein entrapment efficiency E was determined as following:

\[ E (\%) = 100 \left( \frac{m_s}{m_p} - 1 \right) \]

Where \( m_s \) is the total amount of protein detected in the supernatant, after the second centrifugation. Each sample was assayed in triplicate.

2.5 In vitro BSA release kinetics from Dex-PCL\(_a\) nanoparticles

Samples of 10 mL of BSA-loaded nanoparticles were suspended in 100 mL of phosphate buffer saline (PBS, pH 7.4) containing 0.1% (w/v) sodium azide and incubated at 37°C under moderate magnetic stirring. At different time intervals, 1.5 mL samples were withdrawn and centrifuged at 144,000 g for 30 min. The amount of released BSA in the supernatant was determined as described in section 2.4.
2.6 Adsorption of BmoLL onto Dex-PCL\textsubscript{n} nanoparticles

The adsorption of BmoLL and LC lectins onto the surface of Dex-PCL\textsubscript{5.5} nanoparticles has been determined as follows: an amount of 4 mg of lyophilized unloaded Dex-PCL\textsubscript{5.5} nanoparticles was diluted in 1 ml of 10 mM phosphate buffered saline (pH 7.4) containing 30 µg of lectin, and incubated overnight under magnetic stirring at room temperature. Then, the lectin over coated nanoparticles were centrifuged (Beckman L7-55 centrifuge, USA) at 144,000 g for 30 min and washed twice with water in order to remove free lectin. The amount of adsorbed lectin was calculated from the values of the dosed lectin in the supernatant.

Finally, the haemagglutinating activity and the specificity for D (+)-galactose of BmoLL on the surface of Dex-PCL\textsubscript{5.5} nanoparticles have been determined. In short, the pellet of nanoparticles with surface-adsorbed BmoLL was resuspended in 500 µl of 10 mM PBS and serially diluted for determination of haemagglutinating (HA) activity [19]. The specificity of BmoLL was assessed by determining the HA activity in the presence of 3 mM D (+)-galactose. Briefly, samples of BmoLL lectin (50µL) were two fold serially diluted in 0.15M NaCl, in microtiter plates. Glutaraldehyde-treated rabbit erythrocytes in suspension (2.5% v/v in 0.15M NaCl, 50µL) were added. The titer was read after 45 min. The HA titer is defined as the inverse of the highest sample dilution that exhibited the last visible red cell agglutination, and is expressed as HA units.

2.7 Evaluation of the cytotoxicity of PCL and Dex-PCL\textsubscript{n} nanoparticles on Caco-2 cells

The cytotoxicity of Dex-PCL\textsubscript{n} nanoparticles and their constituents, expressed as cell viability, was evaluated on cells of human colon carcinoma (Caco-2) by using the
colorimetric technique with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Row material and nanoparticles were placed in contact with Caco-2 cells at different concentrations. The cells were grown as monolayers in Dubelcco’s modified Eagle’s MEM medium (DMEM) containing 25 mM glucose (Eurobio, France), supplemented with 10% heat-inactivated fetal calf serum (Boehringer, Germany) and 1% nonessential amino acids. For viability experiments, cells were grown in flasks until 80-100% confluence during 15 days. Then, cells were seeded at a density of $7 \times 10^4$ per well and cultured in 96-well plates (Corning Glass Works, USA). Dex-PCL$_n$ nanoparticles were firstly diluted in PBS at concentrations ranging from 50 to 750 µg/ml and then incubated with the cells for 72 h. The viability of Caco-2 cells was also measured after treatment with BmoLL in phosphate buffered saline (pH 7.4) at concentrations ranging from 30 to 500 µg/ml. After 72 h incubation with the different samples (nanoparticles or lectins), a solution of 0.05% MTT /well was added and plates were incubated for 4 hours. The supernatant was removed, DMSO (50 µl/well) was added and read at 590 nm. The cells were cultured and all experiments were carried out at 37°C in a 5% CO$_2$ atmosphere. Different concentrations of nanoparticles were tested in quadruplicate and repeated three times in separate experiments. Results are expressed as the mean ± SD of viable cells.

3. Results and Discussion

The linkage of preformed PCL chains onto Dex backbone allowed obtaining new amphiphilic materials with controlled structures. Thus, Fig. 1 shows the GPC chromatograms of the starting material (Dex and PCL) and of two Dex-PCL$_n$ copolymers, bearing respectively 3 and 5.5 PCL pending chains. It could be concluded that Dex-PCL$_n$
copolymers were pure without residual Dex and PCL and that they presented a higher MW than these starting materials. This clearly indicated that the coupling reaction took place. Moreover, MW calculation by GPC allowed determining the number of grafted PCL side chains.

Nanoparticles made of preformed (co)polymers are generally prepared following methods such as emulsification-solvent evaporation, nanoprecipitation or salting-out, in which the (co)polymers are first dissolved in an organic solvent. The series of Dex-PCL\textsubscript{n} copolymers synthesized here were found to be insoluble in the organic solvents commonly used for the preparation of nanoparticles, such as acetone, THF, ethyl acetate, methylene chloride, or chloroform. This is the reason why we have developed here an original "interfacial migration - solvent evaporation" method to form core/corona protein-loaded nanoparticles using the newly synthesized family of insoluble Dex-PCL\textsubscript{n} copolymers (Fig. 2). Two cases were considered: IM-DE (Fig. 2A) and IM-SE (Fig. 2B). In the first case, a W/O/W emulsion was formed and in the second one, an O/W simple emulsion was obtained. Although insoluble in water and in methylene chloride, the copolymers were able to self-organize at the interface between these two immiscible phases. The water-soluble Dex backbone would have the tendency to orientate towards the aqueous phase, whereas the grafted hydrophobic PCL side chains would deploy in the organic phase. Stable emulsions were thus formed after vigorous stirring of the two immiscible phases in the presence of copolymers. After solvent evaporation, SEM studies showed that spherical nanoparticles below 200 nm were formed and no polymer aggregates were observed in the nanosuspensions (Fig. 3). Advantageously, due to the amphiphilic properties of the Dex-PCL\textsubscript{n} copolymers, nanoparticles could be formed even in the absence of additional surfactants such as SC. This is considered as an advantage, as no additional washing steps are needed. On the contrary, the homopolymer PCL failed to produce such nanoparticles
by this method, as only large aggregates and films, detectable with the naked eye, were obtained.

The influence of the type of copolymer on the mean diameter and the zeta potential of unloaded nanoparticles produced by the IM-DE method was determined (Fig. 4). All polymers lead to the formation of nanoparticles with a mean diameter around 200 nm (Fig. 4a). The polydispersity index was inferior to 0.17, indicating that the nanoparticles had a narrow size distribution. Zeta potential was strongly related to the chemical composition of the polymer which formed the nanoparticles (Fig. 4b). Depending on their MW, PCL homopolymers led to the formation of nanoparticles with Zeta potential values comprised between -38 and -28 mV. The Dex-PCL₇ copolymers with the highest PCL content (n=7) produced nanoparticles with the lowest zeta potential. On the contrary, Dex-PCL₃ and Dex-PCL₅.₅ nanoparticles had significantly increased zeta potential values (-20 and -18 mV, respectively). Possibly, these results could be explained on the basis of the formation of a neutral Dex coating layer at the nanoparticles’ surface, as schematized in Fig. 2. The higher the Dex content in the copolymers (i.e. the lower the number n of grafted PCL side chains), the better the protective effect of Dex at the surface.

The same conclusions could be drawn from the study of the nanoparticles made by the IM-SE method. Nanoparticles with a narrow size distribution and with a mean diameter less than 200 nm were obtained, whatever the copolymer (Table 1). Moreover, no significant difference could be detected on the zeta potential values of Dex-PCLₙ nanoparticles obtained by IM-SE or IM-DE methods (data not shown). The density of unloaded Dex-PCLₙ nanoparticles was around 1.1 g/cm³, whatever the preparation method or the nature of the copolymer used.
The stability of unloaded Dex-PCL<sub>n</sub> nanoparticles with regard to morphological changes and size distribution evolutions was studied by SEM (Fig. 3) and QELS. When stored at 4°C, all nanoparticles were stable during at least 15 days, as neither size evolution nor morphological changes were detected. However, this was not the case for storage at 37°C, as at this temperature, optical microscopy observations have detected that Dex-PCL<sub>3</sub> nanoparticles had lost their integrity, whereas Dex-PCL<sub>7</sub> nanoparticles formed aggregates (data not shown). Only Dex-PCL<sub>5.5</sub> nanoparticles were stable over 15 days of storage at 37°C. In conclusion, an optimal copolymer composition is required to achieve a good stability of the nanosuspension. Remarkably, the diameter of Dex-PCL<sub>5.5</sub> nanoparticles stored at room temperature during one year period was constant (less than 5% variation). No aggregates were observed, whereas under the same conditions, PCL nanoparticles partially aggregated.

Based on these considerations, Dex-PCL<sub>5.5</sub> copolymer was chosen for the preparation of protein-loaded nanoparticles. In the particular case of BmoLL, we previously determined that the nanoparticle preparation steps (vortex, ultrasounds, exposure to the organic solvent) did not affect its haemagglutinating activity (results not shown).

The entrapment of three proteins, BSA, BmoLL and LC, into Dex-PCL<sub>5.5</sub> nanoparticles prepared by the IM-DE and IM-SE methods was successfully achieved (Tables 1 and 2). In the case of BSA, encapsulation efficiencies reached 30-32 wt% with Dex-PCL<sub>5.5</sub> and PCL 2K with a carboxylic end-group (Table 1). The commercially available PCL 10K entrapped only 21-23 wt% BSA. Fig. 5 shows a typical release profile of BSA from Dex-PCL<sub>5.5</sub> nanoparticles. In the first day, 28% of the total amount of loaded BSA was released, independently of the nanoparticles’ preparation method (IM-DE.
or IM-SE). After three days, the quantity of BSA released from the nanoparticles prepared by the IM-DE method reached a plateau at about 65% of the total amount entrapped, whereas the nanoparticles made following the IM-SE method released almost 90% of the total amount of BSA associated to the nanoparticles. These differences are possibly related to the location of the protein in the nanoparticles. The amount of BSA in the nanoparticles’ top layers might be higher when these were made by the IM-SE method, compared to the IM-DE method. In this latter case, the protein was previously dissolved in the inner aqueous phase. Similar incomplete human serum albumin release was also reported in the case of core (poly(lactic acid))- corona (poly(ethylene glycol)) nanoparticles [20].

Interestingly, the Zeta potential of the BSA-loaded nanoparticles reached -9 to -1 mV (Table 1). These values are significantly increased compared to the unloaded nanoparticles (Fig. 4b), suggesting the possible presence of the protein at the nanoparticles’ surface.

Following the same IM-DE method, BmoLL and LC lectins were entrapped with high yields (53 and 80 wt%, respectively) (Table 2). These results suggest the strong affinity of this lectin for the Dex-PCL₅.₅ nanoparticles. Moreover, even the IM-SE method allowed to associate the lectins to this type of nanoparticles, but with a lower efficiency (59 and 23 wt% for respectively, BmoLL and LC) (Table 2). IM-DE method appears more efficient than the IM-SE method for lectin incorporation. Possibly, the presence of an aqueous inner phase containing the lectin is beneficial for its incorporation.

LC and BmoLL have sugar-binding specificities for glucose and galactose, respectively. This could be one explanation for the efficient association of LC to the Dex-coated nanoparticles. This is the reason why lectin-decorated nanoparticles have been
considered further, by direct interaction of the lectin with the polysaccharide-coated nanoparticles, where the lectin has a strong affinity for the sugars in the coating layer. Lectin-coated nanoparticles could thus be prepared just by mixing nanoparticles suspensions with lectin solutions. Practically, unloaded Dex-PCL₅.₅ nanoparticle suspensions prepared following the IM-DE method were incubated with a lectin solution at a concentration of 30 µg/ml. Under these conditions, respectively 25% (± 0.4) and 40.4% (± 1.5) of BmoLL and LC were associated with the nanoparticles. Additionally, it was found that the lectin on the nanoparticles’ surface maintained its heamagglutinating activity (HA). Indeed, BmoLL adsorbed onto the nanoparticles promoted HA at a concentration of 1.4 µg/ml. Moreover, HA was totally inhibited when the nanoparticles were placed in contact with free galactose, which has specificity for BmoLL. These findings corroborate well the adsorption of active BmoLL at the surface of Dex-PCL₅.₅ nanoparticles.

These encouraging results suggest the possibility to use such type of lectin-coated Dex-PCLₐ nanoparticles for oral delivery systems. Therefore, the cytotoxicity of unloaded Dex-PCL₅.₅ nanoparticles was studied on human colon carcinoma cells (Caco-2). Long incubation times (three days) were chosen in order to detect any possible toxicity. Fig. 6 shows the Caco-2 cell viability as a function of the concentration of the empty nanoparticles prepared by the IM-SE or the IM-DE method. Cellular viability was maintained at levels higher than 80% in the case of IM-De method, even at high concentrations (300 µg/ml). Cellular viability of the nanoparticles prepared by the IM-SE method was close to 70%, whatever their concentration. We also checked that BmoLL had no toxic effect on Caco-2 cells at the concentrations from 15 to 500 µg/ml. Fig. 6 shows
that the cellular viability remained close to 90%, allowing to conclude that BmoLL could be considered non cytotoxic.

4. Conclusion

New Dex-PCL\textsubscript{n} copolymers were found to be able to form nanoparticles with a mean diameter around 200 nm and a narrow size distribution, when prepared following an “interfacial migration/solvent evaporation” method. Zeta potential measurements strongly suggested that the Dex-PCL\textsubscript{n} nanoparticles were core-corona type with dextran chains placed preferentially outer the surface of a caprolactone core. A model protein (BSA), as well as two lectins (LC and BmoLL), were successfully loaded in Dex-PCL\textsubscript{n} nanoparticles, which open interesting perspectives for these new nanoparticles as protein carriers with controlled release and modulated biodistribution.

Furthermore, lectins were successfully adsorbed onto the surface of Dex-PCL\textsubscript{n} nanoparticles, maintaining their specificity and their haemagglutinating activity. The interaction was promoted by the specific affinity of the lectins for the nanoparticles' polysaccharidic coating.

Given the lack of cytotoxicity of these new nanoparticles for Caco-2 cells, they could be useful as site-specific drug delivery systems for oral administration.

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Table 1.

Influence of the type of polymer on the mean diameter, BSA encapsulation efficiency and Zeta potential of Dex-PCL\textsubscript{5.5}, PCL\textsubscript{2K} and PCL\textsubscript{10K} nanoparticles loaded with BSA. The final polymer concentration was 1mg/ml. The nanoparticles were prepared following the IM-DE method. Each experiment was repeated thrice.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>BSA theoretical loading (wt %)</th>
<th>Mean Diameter ± SD (nm)</th>
<th>Polydispersity Index</th>
<th>Entrapment Efficiency (wt %)</th>
<th>Zeta Potential (mV)</th>
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</thead>
<tbody>
<tr>
<td>Dex-PCL\textsubscript{5.5}</td>
<td>5</td>
<td>188±58</td>
<td>0.17</td>
<td>32</td>
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<tr>
<td></td>
<td>38</td>
<td>172±41</td>
<td>0.09</td>
<td>33</td>
<td>-2.4</td>
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<tr>
<td></td>
<td>51</td>
<td>211±64</td>
<td>0.15</td>
<td>31</td>
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<tr>
<td>PCL 2K</td>
<td>38</td>
<td>189±44</td>
<td>0.09</td>
<td>31</td>
<td>-8.9</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>189±48</td>
<td>0.12</td>
<td>31</td>
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</tr>
<tr>
<td>PCL 10K</td>
<td>38</td>
<td>177±53</td>
<td>0.16</td>
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<td>-4.9</td>
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<tr>
<td></td>
<td>51</td>
<td>185±65</td>
<td>0.05</td>
<td>23</td>
<td>-3.5</td>
</tr>
</tbody>
</table>
Table 2.

Influence of the preparation method on the particle mean diameter and lectin entrapment efficiency in Dex-PCL$_{5.5}$ nanoparticles. The polymer concentration was 1 mg/ml, and the lectin-polymer ratio was 5 wt%. The initial concentration of BmoLL and *Lens culinaris* lectin was 50 µg/ml. Each experiment was repeated thrice.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Preparation Method</th>
<th>Mean Diameter ± SD (nm)</th>
<th>Entrapment efficiency (wt %)</th>
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</thead>
<tbody>
<tr>
<td><em>Lens Culinaris</em></td>
<td>IM-SE</td>
<td>114 ± 40</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>IM-DE</td>
<td>128 ± 51</td>
<td>80</td>
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<tr>
<td><em>Bauhinia Monandra</em></td>
<td>IM-SE</td>
<td>97 ± 34</td>
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<tr>
<td></td>
<td>IM-DE</td>
<td>103 ± 39</td>
<td>53</td>
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**Figure captions**

**Figure 1.** Gel permeation chromatograms of Dex-PCL$_3$ and Dex-PCL$_{5.5}$ copolymers and of the starting materials (Dex and PCL2K) which led to their formation.

**Figure 2.** Hypothetical mechanisms of protein entrapment into core-corona nanoparticles prepared using insoluble amphiphilic Dex-PCL$_n$ copolymers: IM-DE (A) and IM-SE (B) preparation methods.

A: sonication of insoluble Dex-PCL$_n$ copolymers in the presence of an aqueous protein solution and methylene chloride, leading to the formation of a W/O emulsion. Part of the copolymer remains insoluble.

B: sonication of the W/O emulsion in the presence of an aqueous phase, leading to W/O/W emulsion formation.

C and E: solvent evaporation, leading to nanoparticle formation.

D: sonication of insoluble Dex-PCL$_n$ copolymers in the presence of an aqueous protein solution, leading to the formation of a O/W emulsion.

**Figure 3.** Evaluation of the morphology of unloaded Dex-PCL$_{5.5}$ nanoparticles by scanning electron microscopy.

**Figure 4.** Mean diameter (a) and surface charge (b) of unloaded nanoparticles prepared by IM-DE method, for new Dex-PCL$_n$ nanoparticles compared with PCL nanoparticles: (1) Dex-PCL$_7$, (2) Dex-PCL$_3$, (3) Dex-PCL$_{5.5}$, (4) PCL 2K, (5) PCL 10K, (6) PCL 40K. Error bars represent SD. Each experiment was repeated thrice.
**Figure 5.** In vitro release profiles of BSA from Dex-PCL$_{5.5}$ nanoparticles prepared by IM-SE (●) and IM-DE (○) methods. The concentration of BSA was 642 µg/ml of nanoparticle suspension. Each experiment was repeated thrice.

**Figure 6.** Evaluation of the cytotoxicity on the human colon carcinoma cells (Caco-2) of unloaded Dex-PCL$_{5.5}$ nanoparticles produced by IM-SE (●) and IM-DE (○) methods and BmoLL (●). Cellular viability expressed in % was calculated as $(N_t/N_c) \times 100$, where $N_t$ and $N_c$ are the number of surviving cells in the treated group and in the untreated group, respectively. Lyophilized nanoparticles were resuspended in 200 µl of phosphate buffered saline (pH 7.4). Serial dilutions from 10.31 to 660 µg of particle/ml were added in each well. Cell viability was determined by MTT reduction after 72 h of incubation with the cells. Different concentrations of nanoparticles were tested in quadruplicate and repeated three times in separate experiments. Results are expressed as the mean ± SD of viable cells.
Fig. 4
Fig. 5
Fig. 6