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Research paper

Layer-by-layer surface modification of lipid nanocapsules

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ABSTRACT

Lipid nanocapsules (LNCs) were modified by adsorbing sequentially dextran sulfate (DS) and chitosan (CS) on their surface by the layer-by-layer (LBL) approach. Tangential flow filtration (TFF) was used in intermediate purifications of the LNC dispersion during the LBL process. The surface modification was based on electrostatic interactions between the coating polyelectrolytes (PEs) and the LNCs. Therefore, a cationic surfactant, lipochitosan (LC), was synthesised by coupling stearic anhydride on chitosan, and the surface of LNCs was first modified by this LC by the post-insertion technique. The PEs could be successfully adsorbed on the LNC surface as verified by alternating zeta potential and increase in size. To present a therapeutic application, fondaparinux sodium (FP), a heparin-like synthetic pentasaccharide, was introduced on the LNC surface instead of DS.

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1. Introduction

Since the introduction of layer-by-layer (LBL) surface modification technique [1] to colloidal objects [2], coating of nanosized drug carriers by polyelectrolytes (PEs) has become a strategy of increasing interest during the latest years. The first LBL studies applied synthetic PEs such as poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) [2,3], but later the selection of materials has been expanded toward biocompatible and thus more convenient PEs for pharmaceutical purposes. These macromolecules include chitosan, chitosan sulfate, alginate, dextran sulfate and heparin [4–8]. The goal of an LBL process can be improvement for the stability of the core particles [8,9] or control of the release of an encapsulated substance [6,7,10]. Also, macromolecules such as DNA and RNA, with therapeutic interest, have been used as coating agents [8].

The principle of the LBL coating is to sequentially expose the substrate to be coated to solutions containing either negatively or positively charged PEs. Adsorption of the PEs is based on electrostatic interactions originating from the charged nature of the substrate and the PEs. Thickness and properties of the coating film can be controlled by the number and composition of the layers and the process conditions. After each coating layer, removal of the excess PE in the dispersing medium is crucial to avoid aggregation due to the formation of unwanted PE complexes. In most of the studies, this is done by intermediate ultracentrifugations followed by the replacement of supernatant [2,4,6,7]. To avoid the force of ultracentrifugation leading to particle aggregation in some cases, another used purification approach is ultrafiltration [3,5,11,12]. In this study, tangential flow filtration (TFF) [13] was applied as an intermediate purification method.

Lipid nanocapsules (LNCs) are synthetic lipoproteins, with tuneable size between 20 and 100 nm, and offer a versatile approach to drug delivery [14,15]. LNCs are prepared by a solvent-free, low-energy phase inversion temperature process. Such a method enables a straightforward scale-up of the process with pilot scale equipment, at least up to 50-fold the volume (unpublished data). Their structure can be characterized as a hybrid between polymeric nanocapsules and liposomes (an oily core with a shell consisting of a mixture of lecithin and a PEGylated surfactant) with good dispersion stability (up to 18 months). Because of their structure which includes a semi-rigid shell, LNCs can be further modified by insertion of amphiphilic molecules. The motivation for this kind of post-insertion can be improvement of biodistribution [16–18], targeting profile [19] or creation of a template for further attachment of active targeting or other moieties [20–22].

Chitosan is a natural material possessing great potential to be used in pharmaceutical applications (reviewed *e.g.* in [23]). If hydrophobic chains are introduced to chitosan using a method such as acylation [24,25], the modified chitosan can be used as a hydrophilic cationic surfactant. This study presents synthesis of an amphiphilic chitosan derivative, lipochitosan (LC), and its introduction to the LNC surface by the post-insertion technique. The positive charge provided by LC enabled further modification of the surface by the LBL technique using dextran sulfate (DS) and chitosan oligosaccharide (CS) as model PEs (Fig. 1). Being biocompatible and

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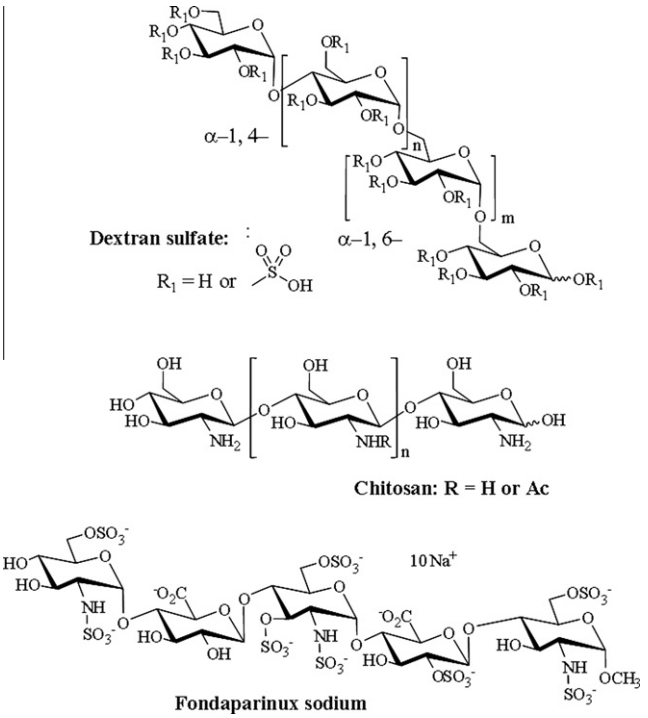

Fig. 1. Chemical structures of dextran sulfate (DS), chitosan (CS) and fondaparinux sodium (FP).

highly charged macromolecules, DS and CS are suitable choices for pharmaceutical (self-assembling) systems: these two PEs have been previously used *e.g.* as PE complexes [26] and in LBL coating of nanoparticles [4] and liposomes [12]. Due to the semi-solid structure of LNCs, their purification by a process involving a high stress toward the capsules (ultracentrifugation) might lead to destabilisation of LNCs. Therefore, TFF was introduced in the process to purify the LNC dispersion after each layer. To our knowledge, TFF has not been used as a tool in the LBL coating before. To present a possible therapeutic application, fondaparinux sodium (FP), a synthetic pentasaccharide (Fig. 1) used in the treatment and prophylaxis of venous thromboembolism, was introduced on the LNC surface instead of DS. Treatment by a drug like FP, normally administered subcutaneously, would benefit from oral administration. As LNCs are known to enhance the oral absorption of drug molecules [27], they could serve in avoiding degradation and enhancing absorption of FP in the gastrointestinal tract.

2. Materials and methods

2.1. Materials

Solutol® HS15 (PEG 660 12-hydroxystearate, $M_W \sim 900$ Da) (BASF, Ludwigshafen, Germany), Labrafac® WL 1349 (caprylic/capric acid triglycerides) (Gattefossé S.A., Saint-Priest, France), Lipoid® S75-3 (Lipoid GmbH, Ludwigshafen, Germany), NaCl (Prolabo VWR International, Fontenay-sous-Bois, France) and MilliQ185 water (Waters, Saint-Quentin-en-Yveline, France) were used in the LNC preparation. Chitosan oligosaccharide lactate (CS) (M_W 4000–6000 Da), dextran sulfate sodium salt (DS) (M_W 6500–10,000 Da) and stearic anhydride were from Sigma Aldrich (Steinheim, Germany). Fondaparinux sodium (FP) (arixtra®) was a product of GlaxoSmithKline (Greenford, UK). Sepharose CL-4B, the colorants Cibacron brilliant red 3B-A and Azure A, pancreatin and KH_2PO_4 were from Sigma Aldrich (Steinheim, Germany) and pepsin from Fluka (Buchs, Switzerland). All other used reagents were of analytical grade.

2.2. Methods

2.2.1. Lipid nanocapsule (LNC) preparation and characterization

LNCs were prepared by the phase inversion temperature method described by Heurtault et al. [28]. Briefly, a mixture of Solutol®, Lipoid®, Labrafac®, NaCl and water was heated to 85 °C at a rate of 5 °C/min followed by cooling at the same rate to 65 °C. This cycle was repeated twice. During the last decrease in temperature, at 78 °C (during the phase inversion zone), the system was diluted with cold (0 °C) water leading to formation of stable LNCs.

Size distributions and zeta (ζ) potentials of LNCs were determined with a Zetasizer ZS (Malvern, Worcestershire, UK). Particle sizing was based on photon correlation spectroscopy (PCS); the results were analyzed by CONTIN algorithm, and the sizes were presented based on the volume distributions together with polydispersity indices (PDI). Electrophoretic mobilities were converted to ζ-potentials using Smoluchowski's equation.

2.2.2. Synthesis and characterization of lipochitosan (LC)

Synthesis of LC is schematically presented in Fig. 2. Five grams of CS was dissolved in 100 ml 20% (v/v) aqueous acetic acid at room temperature and then diluted with 200 ml methanol under stirring; 3.5 g stearic anhydride was added gradually to the solution, and the mixture was stirred overnight under reflux. After the removal of the solvent, the residue was washed with acetone and centrifuged (repeated five times) to remove the fatty acid. The obtained brown LC powder was dried under vacuum.

^{1}H NMR spectrum, to identify structures of LC, was recorded with a Bruker AMX-400 instrument (Billerica, USA). To determine the degree of acylation (D_{acyl}) of LC by ^{1}H NMR (400 MHz), about 15 mg of LC was dissolved in 0.7 ml 0.2% HCl/D_2O and kept at room temperature for 4 h to homogenize the solution.

2.2.3. Post-insertion of lipochitosan (LC)

Different concentrations of LNCs (15–115 mg/ml) were incubated with different concentrations of LC (10–40 mg/ml) with constant shaking at 37 °C or room temperature. Progress of the insertion was controlled by size and ζ-potential measurements at different time points (1–24 h). ANOVA was used to test statistical difference between the observed values. The inserted quantity was assayed by a colorimetric method adapted from Muzzarelli [29]. A calibration curve was established by adding 3 ml of 75 μg/ml Cibacron brilliant red 3B-A solution (in water) on 300 μl LC solutions (in water) with final concentrations from 0 to 15 μg/ml. LC insertion was then determined by separating the non-inserted molecules from the LNCs by centrifugation (4000 rpm, 15 min) using a centrifugal device (Pall Nanosep®, M_WCO 10 kDa) (Pall Corporation, Ann Arbor, USA) and analyzing the filtrate with the help of the dye. Spectrophotometric analysis was performed at 575 nm against water with Uvikon 922 spectrophotometer (Kontron Instruments, Montigny Le Bretonneux, France).

2.2.4. Tangential flow filtration (TFF)

The filtration was performed with a continuous diafiltration mode (the dispersion volume remained the same during filtration) using a Minimate™ TFF System and a Capsule with Omega ^{TI}10 K Membrane (Pall Corporation, Ann Arbor, USA) run by a peristaltic pump. The flow rate was 40 ml/min, filtration rate 1–2 ml/min and operating pressure 10–20 psi. The LNC dispersion volume was 10 ml, and the volume of water used in purification was 140 ml. The filtration capsule was washed with 0.5 mol/l NaOH and water after each filtration as recommended by the fabricant.

2.2.5. Layer-by-layer (LBL) coating

The LC-inserted LNC dispersion (LNC–LC) was diluted with water to 10 mg/ml before LBL coating. DS and CS were also diluted

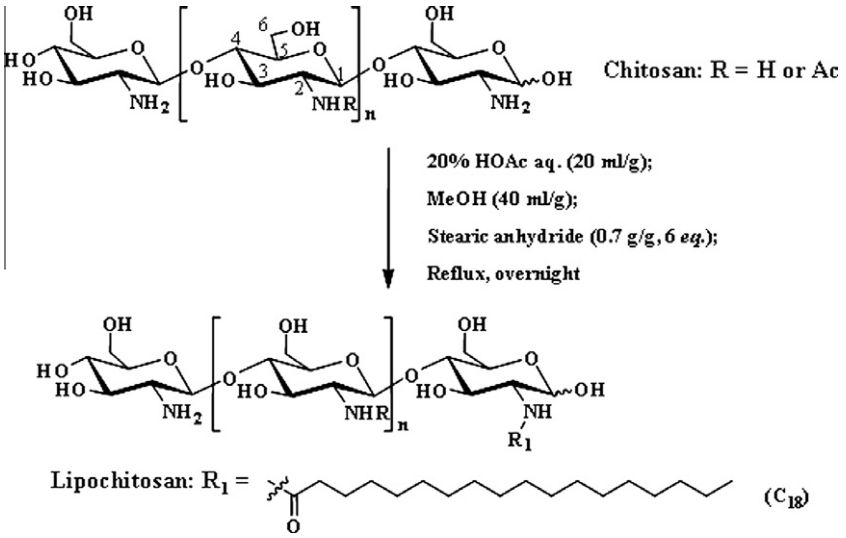

Fig. 2. Acylation of CS with stearic anhydride (R_1 can link on any amine group, not exactly on the position presented in the formula).

in water as 5 mg/ml. To build the next layer after the LC post-insertion, DS solution was added in a stirred dispersion of LNC–LC. After an incubation of 5 min, the dispersion was purified by TFF. Next, CS was added followed by incubation (5 min) and purification. The process was continued up to six layers. Following mass ratios of DS and CS were used (in relation to previous layer): CS:DS 1:0.5 (for a negatively charged layer), DS:CS 1:3 (for a positively charged layer).

After the final applied coating layer, a small volume of the coated LNC dispersion was freeze-dried (Lyovac GT2 (Steris, Germany)/Phoenix P2 C75P (ThermoHaake, Germany)) on a silica plate and analyzed with a JEOL JSM-6301F scanning electron microscope (SEM) (Tokyo, Japan).

2.2.6. Fondaparinux sodium (FP) incorporation

FP was adsorbed by incubating it with LNC–LC in water. Quantity of FP adsorbed on the LNC surface was determined by a spectrophotometric method using the capacity of the molecule to form a complex with Azure A dye [30]. A calibration curve was established by adding 1.5 ml of 0.04 mg/ml Azure A solution (in water) on 500 μl fondaparinux sodium solutions (in water) with concentrations from 0 to 20 μg/ml. Fondaparinux sodium adsorption was then assessed by separating the free drug from the LNCs by centrifugation (4000 rpm, 15 min) using a centrifugal device (Pall Nanosep®, M_WCO 10 kDa) and analyzing the filtrate with the help of the dye. Spectrophotometric analysis was performed at 633 nm against water with Uvikon 922 spectrophotometer (Kontron Instruments, Montigny Le Bretonneux, France).

Stability of these LNCs (LNC–LC–FP and LNC–LC–FP–CS) was studied by incubating them for 4 h in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) under stirring. LNC–LC was also tested as a control. SGF (0.32% pepsin, pH 1.2) and SIF (1% pancreatin, pH 6.8) were prepared according to the US Pharmacopeia [31]. Before PCS analysis, the LNCs were separated from the media by filtration (0.2 μm). Obtained size data was compared to the initial sizes. The stability test was performed in triplicate.

3. Results and discussion

3.1. Synthesis and characterization of LC

CS (Fig. 1) is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Its modification, such as acylation, can be performed exploiting hydroxyl and/or amino group of D-glucosamine [24,32,33]. Chitosans with higher molecular weight (than used in this study) can also react with stearic anhydride as described but instead of dissolving, they swell in water. Therefore, only low molecular weight chitosans can be treated as "hydrophilic surfactant" candidates. Fig. 3 shows the ^{1}H NMR spectra of the native CS and LC after acylation. In the case of CS, signals from 1.31 to 1.43 ppm, 2.04 ppm and 2.53 ppm were assigned as lactate; signal at 5.4 ppm was H-1 (anomeric proton) of one D-glucosamine among those repeating units of chitosan; signals around 3.13 ppm were attributed to H-2 of D-glucosamine, and other signals such as 4.14 ppm belong to the other protons on sugar backbone. Moreover, the proton assignment of LC was as follows: 0.88 ppm (CH_3), ~1.26 ppm (CH_2 of alkyl chain, overlapped by lactate group peaks), ~2.2 ppm (CH_2 of stearoyl group), 5.4 ppm, 3.90–3.2 ppm, and the rest were attributed to D-glucosamine as with CS. Therefore, comparing with CS, the proton signals of LC confirmed the presence of acyl groups. Calculation of D_{acyl} was based on the proton intensity ratio between the CH_3 of alkyl group (I_{CH3}) and D-glucosamine units. For one CS molecule ($M_W \sim 5000$ Da according to the fabricant), $D_{acyl} = (1/3)\, I_{CH3}:I_{H1} = 4/3 = 1.3$; this means that there were approximately 1.3 stearoyl groups per CS molecule.

3.2. Post-insertion of LC on LNCs

LNCs prepared were homogeneous (PDI ~ 0.03) with size of 50 nm. The post-insertion technique has been originally used for PEGylation or incorporation of targeting molecules on liposome surface [34–37]. The most important parameters governing the insertion profile are time, temperature and concentration of the molecule to be inserted. Therefore, insertion of LC on the surface of the LNCs was first assessed by incubating different amounts of LC together with different concentrations of LNCs at 37 °C (Table 1). Regarding further use, LNC 15 mg/ml was considered too dilute. On the other hand, LNC 115 mg/ml, being the non-diluted original formulation, was considered concentrated as *e.g.* LC could not be dissolved in medium before incubating with LNCs. However, these two LNC concentrations (15 and 115 mg/ml) were included in the tests in order to get a better insight into LNC/LC interplay. An elevated temperature was selected because of more favourable (dynamic) surface properties of LNCs facilitating the insertion [21]. ζ-Potential of the LNCs reached a maximum value of about

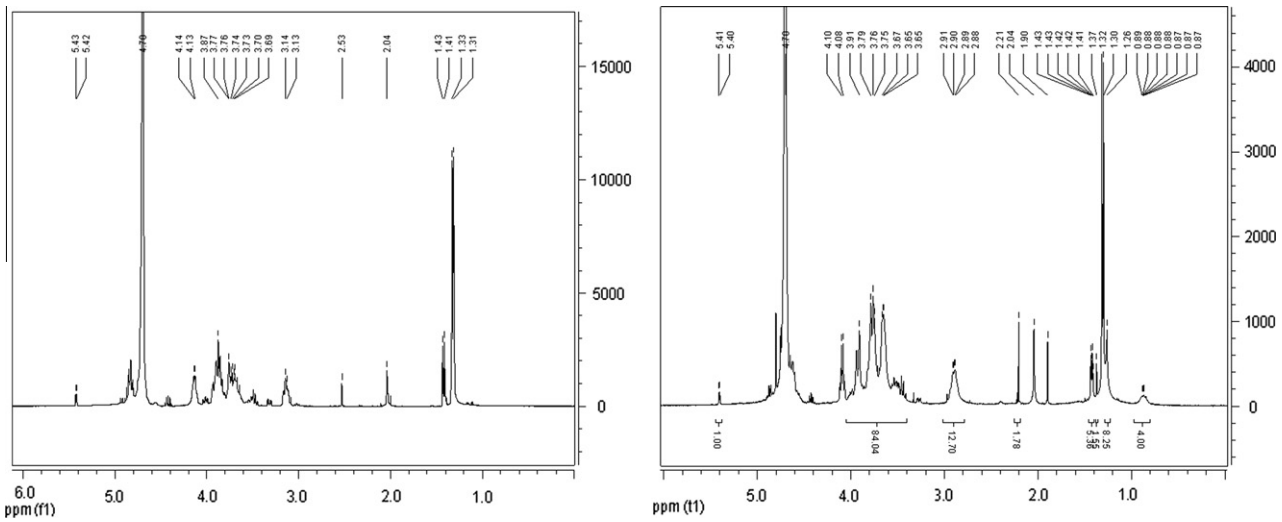

Fig. 3. ^{1}H NMR (400 MHz, D_2O) spectra of CS (left) and LC (right).

Table 1
Size distribution and ζ-potential of LNCs after 24 h incubation with LC at 37 °C. The experiments were performed in triplicate.

LNC 115 mg/ml				LNC 57.5 mg/ml			LNC 15 mg/ml		
LC c(mg/ml)	Size (nm)	PDI	ζ-potential (mV)	Size (nm)	PDI	ζ-potential (mV)	Size (nm)	PDI	ζ-potential (mV)
0	50.1 ± 0.2	0.02	−2.8 ± 0.3	50.1 ± 0.2	0.02	−2.8 ± 0.3	50.1 ± 0.2	0.02	−2.8 ± 0.3
10	52.2 ± 1.8	0.05	19.8 ± 0.9	52.8 ± 0.2	0.05	21.7 ± 4.0	54.4 ± 1.9	0.07	34.2 ± 2.9
20	52.9 ± 2.0	0.08	28.1 ± 0.1	55.0 ± 1.6	0.06	30.6 ± 1.4[a]	56.8 ± 2.2	0.13	31.7 ± 8.4
40	55.4 ± 2.7	0.12	33.3 ± 1.0	56.7 ± 2.0	0.08	32.6 ± 0.6[a]	62.0 ± 4.6	0.19	35.4 ± 2.1

[a] $p > 0.05$ between these values.

30 mV after a certain LC concentration (40 mg/ml for 115 mg/ml LNC; 20 mg/ml for 57.5 mg/ml LNC; 10 mg/ml for 15 mg/ml LNC). With increasing LC concentrations, adsorption continued as observed by increases in sizes and PDIs. This adsorption increased the surface density of LC but as the initially PEGylated surface of LNC provides long blood circulation times [38] and good (steric) dispersion stability [14], attainment of a steady ζ-potential level could be a decent indicator of an adequate LNC–LC formulation for further use. Therefore, to have a LNC formulation concentrated enough, with a ζ-potential >30 mV and a narrow size distribution (PDI < 0.1), LNC 57.5 mg/ml incubated with 20 mg/ml LC was selected for further studies (no significant difference was found between the ζ-potential values obtained with 20 and 40 mg/ml LC). Next, the effects of incubation time and temperature were studied. After 1 h of incubation at 37 °C, the ζ-potential was already above 30 mV together with a slight size increase from 50 nm to 53 nm. At room temperature, the size increase was similar but the ζ-potential distribution suggested another smaller peak at −3 mV in addition to that of about 30 mV. The ζ-potential distribution was the same even after 24 h incubation at room temperature. It is most probable that the −3 mV peak resulted from the original LNCs in which LC was not adsorbed. Thus, an elevated temperature seemed to be a prerequisite for a successful post-insertion. Traditionally, in the case of liposomes, a temperature rise enables a transfer between phospholipids of the surface and the lipophilic moiety of an amphiphilic molecule [36,37]. In the case of LNCs, it has been assumed that rather than by the phospholipids of the surface (Lipoid®), the incorporation of amphiphilic molecules is controlled by the conformation of the PEG chains (Solutol®) which is more favourable at an elevated temperature [21]: the "brush" conformation at an elevated temperature allows better penetration of the amphiphilic molecules between the PEG chains. On the other hand, regardless the temperature, while PEGylating liposomes, at a certain point, the PEG density at the surface creates a steric barrier that resists further post-insertion [36].

LC insertion efficiency was determined by exploiting the capacity of the amino groups of chitosan to adsorb anionic dyes [39]. Indeed, a linear calibration curve could be established for the LC concentration range 0–15 μg/ml with a sulfonate containing dye, Cibacron brilliant red 3B-A. After incubation of 57.5 mg/ml LNC with 20 mg/ml LC, centrifugal filtration enabled the separation of the non-inserted LC. Analyzing the filtrate, the LNCs were calculated to contain 70 ± 9% ($n = 3$) of the LC used in the post-insertion. This corresponds to about 11.6% (mol/mol) of the excipients forming the LNC shell (LC, Solutol® and Lipoid®). Observed incorporation value was in line with a previous study presenting a 7% (mol/mol) incorporation of DSPE-PEG_{2000}-maleimide on the LNC surface [21]. In the case of liposomes, the post-inserted system is dynamic undergoing a change of the molecules in and out between the liposome membrane and the micelles of the dispersion medium [36,37]. Instead, in the LNC surface, the insertion is more stable as the incorporated molecules remain among the semi-rigid shell [21]. The LC-inserted LNCs (LNC–LC) were stable at least up to 5 months (4 °C) with no modification in size or ζ-potential (data not shown).

To treat LNC–LC for further use, the excess LC was removed by TFF. TFF (or cross-flow filtration) has been found as a very practical method to purify nanoparticulate dispersions from excess material [40–47]. It is more gentle and continuous process-based and more easily scaled up than *e.g.* the traditional (dead-end) ultrafiltration [11] or ultracentrifugation [2]. In TFF, the dispersion is pumped tangentially along the surface of the membrane. Filtrate is formed when an applied pressure forces part of the dispersion medium through the membrane. The retained particles do not block the

membrane because they are swept away with the tangential flow. In this study, a filtration capsule with relatively low molecular weight cut-off (10 kDa) was selected because of an observed phenomenon that a fraction of semi-rigid LNCs could deform and pass a membrane with higher cut-off (data not shown). The TFF equipment was run with the filtrate line completely open, and the operating pressure was raised to obtain a filtration rate of 1–2 ml/min. A suitable rate was attained when the pressure was 10–20 psi. These kinds of pressures have been shown to result in efficient filtration with no excessive membrane fouling [40]. To assess the efficacy of the filtration, LC was detected (as described earlier) from the filtrate. No LC was detected in the end of the TFF cycle, 140 ml (seven diafiltration volumes) (Fig. 4). The same filtration volume was then used for all the further filtrations. Size, PDI and ζ-potential remained unchanged after the TFF.

3.3. Layer-by-layer coating

Suitable starting conditions for an LBL coating process, based on the electrostatic interactions, were evaluated by checking ζ-potential of LNC–LC as a function of NaCl concentration and pH (Fig. 5). ζ-Potential of LNC–LC decreased strongly even at a low concentration of NaCl (0.02 mol/l). With increasing concentrations, the ζ-potential values approached zero but the LNC–LC remained stable with no modification in size or PDI. This kind of behaviour probably results from the surface of LNC–LC which consists of PEG chains (steric stabilization) and LC molecules imbedded with relatively low surface density (fast decrease of ζ-potential). For comparison, electrostatically stabilized nanoparticles, such as poly(lactic acid) nanoparticles, possess high charge density which enables the conservation of the ζ-potential when the salt concentration increases but at certain concentration aggregation occurs [11]. Interestingly, ζ-potential remained constant throughout the studied pH range although chitosans are known to lose their charge at basic pH values (pKa around 6.5). To avoid the ζ-potential-decreasing effect of increased ionic strength, pH of the test solutions was modified simply by NaOH/HCl (resulting in low ionic strengths). It is possible that the added NaOH amounts were not high enough to screen the amino groups of LC. Based on these properties of LNC–LC, the LBL coating was decided to be performed in water.

The surface of LNC–LC was modified by sequential adsorption of PEs on the surface. Biocompatible PEs, DS and CS were used in the process. The LBL coating was performed on dilute LNC dispersions (10 mg/ml) to avoid excessive excipient consumption.

DS contains on average 2.3 negatively charged units per monomer, whereas CS has at the most 1 positively charged amino unit per monomer (Fig. 1) [26]. Therefore, more CS is needed to overcome

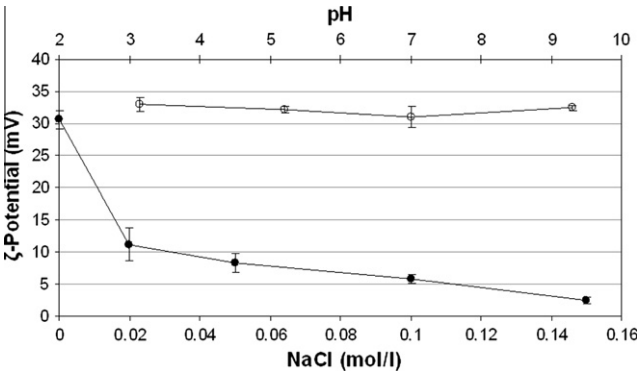

Fig. 5. ζ-potential of the LNC–LC as a function of NaCl concentration (●) and pH (○).

the charge of DS at each step. Different amounts of CS (for positively charged layers) and DS (for negatively charged layers), to give different mass ratios of CS:DS (or DS:CS), were tested and, indeed, when building up a positively charged layer, at least triple the amount (mass) of CS was needed to reverse the charge of the dispersion to a ζ-potential of around 20 mV. On the contrary, it was observed that starting from LNC–LC, half of the amount of DS compared to LC was needed to achieve negative ζ-potentials. Thus, all the positive coating layers were built up with CS solutions giving a CS:DS mass ratio 3:1 and all the negatively charged layers with DS solutions giving a CS:DS mass ratio 1:0.5 (in both cases comparing to the CS or DS mass used for the previous layer). Evolution of ζ-potential in such an LBL process is presented in Fig. 6. Correspondingly, size and PDI of the same process together with a SEM image after the final layer are presented in Fig. 7. No remarkable size increase was observed during the two first layers (LC and DS). From the third layer on (CS), the particle size began to increase resulting to about 200 nm size after the sixth layer. The size evolution behaviour can be explained, again, by the surface of the post-inserted LNC–LC which consists of the chains of PEG and the relatively low surface density of inserted LC chains. DS molecules adsorbed on the chitosan part of the LC chains and as more charged PEs, they reversed the surface charge even at lower amount compared to amount of the surface-located LC. A drastic size increase was not observed because the size was, at this point, still determined partly by the majority of the PEG chains on the LNC surface. The steric stabilization provided by PEG also enabled gradual addition of CS on LNC–LC–DS, passing by the neutral ζ-potential, without causing aggregation (data not shown). However, as already mentioned, to reverse the negative charge provided by DS, triple the amount of CS was needed, and the CS amount started to accumulate

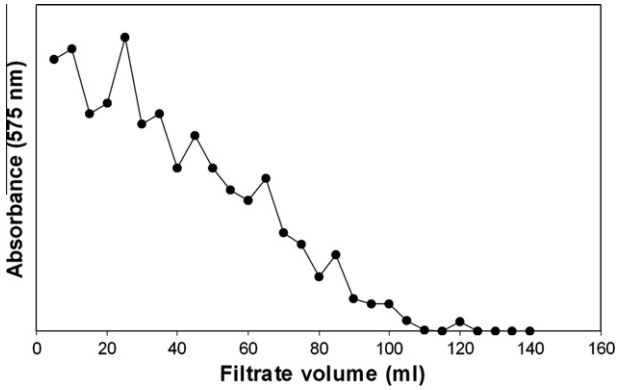

Fig. 4. Detection of LC from the TFF filtrate. Each 5 ml aliquot of the filtrate was analyzed separately.

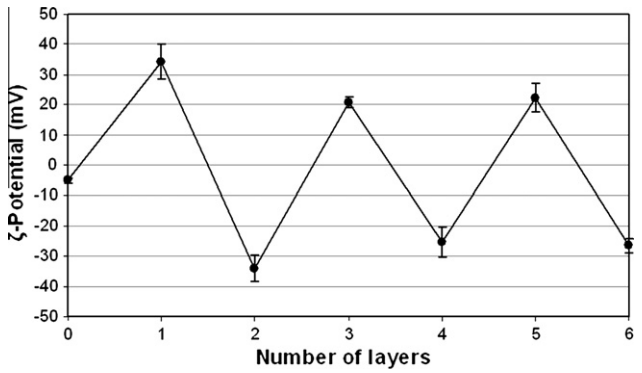

Fig. 6. Evolution of ζ-potential as a result of LC post-insertion and during the LBL process. Layer '0': LNC; '1': LNC–LC; '2': LNC–LC–DS; '3': LNC–LC–DS–CS, etc.

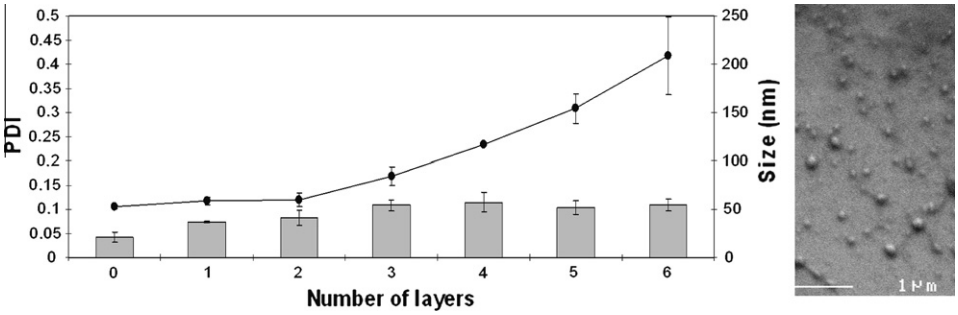

Fig. 7. Left: size (circles) and PDI (bars) after the LC post-insertion and during the LBL process; right: a SEM image of the LNCs with six coating layers.

PE bulk on the surface. Starting from this third layer, the process was governed by the dynamics of interaction between DS and CS [26,48] with increased role of electrostatic stabilization. Another fact explaining the remarkable overall size increase (from 50 to 200 nm) might relate to the small size of the used PEs (5–10 kDa) that do not necessarily form uniform layers on the surface when the PE amounts increase during the formation of each layer. In a "traditional" LBL process, bulky PEs, such as PAH and PSS (*e.g.* 70 kDa), are adsorbed providing somewhat constant size increase from the first layer [2]. Despite the size increase, PDIs remained around 0.1 throughout the LBL process indicating narrow size distributions. Thus, the purification by TFF was successful: the excess material was removed and the aggregation/LNC break-up was avoided.

In an LBL process, a risk exists that the PEs desorb from the surface and form PE complexes instead of cumulating on the surface of the core particles [49]. To study this eventual phenomenon, different proportions of CS and DS solutions were mixed together and size and ζ-potential of the formed complexes were determined. However, the parameters measured were not comparable to those obtained from the LBL process, *e.g.* the size reaching up to 500 nm with certain DS and CS amounts (data not shown). Additionally, an LBL coated dispersion was applied to a Sepharose CL-4B column in order to separate possible PE complexes and LNCs. PCS analysis of separated fractions (1 ml each) showed that the LBL coated LNCs passed the column intact (21–26 ml eluted) and after that, no other particle populations were detected. Also, taking into account that the size distributions during the LBL process were very narrow (low PDIs) and did not contain peaks for different particle populations (separate LNCs and PE complexes), it can be assumed that the LBL process was successful resulting in a colloidal system where LNCs were incorporated inside shells consisting of DS and CS.

3.4. Incorporation of FP on the LNC–LC

Heparin and its derivatives as well as similar synthetic macromolecules, used as anticoagulants, are one of the most used active substances in hospital practice [50]. However, their administration by parenteral injections limits patient compliance and present restrictions and risks in terms of pharmacokinetics and pharmacodynamics, not to mention laborious production of these drugs (sterility, etc.). Therefore, various strategies to formulate non-invasive heparin and heparin-like drugs have been reported [51]. The most convenient route of administration would be the oral one but it sets some obstacles for these macromolecules: enzymatic degradation, instability for the acidic pH of the stomach, and limited absorption. As heparin and macromolecules with similar structure possess high (negative) charge density, they can be used as PEs in LBL processes [52]. If CS is used as a positively charged PE, it can possibly protect the heparin-like molecule toward the enzymatic degradation and acidic pH as well as enhance absorption due to its mucoadhesive properties [53].

Different amounts of a synthetic pentasaccharide, FP, were adsorbed on the surface of LNC–LC. The chemical structure of FP is analogous to that of DS (and heparin). Table 2 summarizes the characteristics of these LNCs. The amounts of FP were compared to the amount of LC in the LNC surface taking into account that 70% of LC used was inserted on the surface. As in the case of DS, a minor amount of FP in relation to LC was needed to reverse the charge of the LNCs. Despite the structural similarity of FP and DS (charge density), the attained ζ-potential values were about −16 mV, thus less negative than with DS. Probably the smaller FP molecules could not fully cover the LC chains and thus screen all their charges. Interestingly, near the point of neutral surface (LC:FP 1:0.18), a large LNC size could be observed. It is probably that when there was no electrostatic repulsion between the LC chains, their conformation was extended out from the surface resulting in increase in the particle size. It should be mentioned that even at neutral surface charge, the LNC dispersion was stable (low PDI, no observed aggregation) emphasizing the steric stabilization provided by the PEG chains co-located on the surface. Otherwise, the size increase was constant when more FP was adsorbed.

Before the charge reversal point, all the introduced FP was adsorbed on the LNC surface (in Table 2, quantity of FP adsorbed (%) refers to the total amount of FP used in each formulation). Increasing the drug amount, the adsorption rate decreased but the accumulation of FP on the LNC surface still continued. This kind of adsorption of surfactants and other molecules on solid surfaces is reviewed *e.g.* in [54]. Therapeutic doses of FP are in the range of 2.5–10 mg, depending on the indication [55]. Even with the feed mass ratio LC:FP 1:0.36, 51 µg of FP could be found on 1 mg of LNC. Thus, in that case, 49–194 mg of LNCs would be needed in one dose. A possible formulation could be imaginable as the LNCs can be prepared with concentrations even higher than 100 mg/ml.

To evaluate the addition of another layer, different amounts of CS were added to dispersions of LNC–LC–FP (LC:FP ratio 1:0.36) purified by TFF. The FP:CS ratio 1:1 resulted in LNCs with size and ζ-potential 94.9 ± 5.2 nm and 19.9 ± 1.1 mV, respectively. When the CS amount was doubled, the size increased to 104.0 ± 6.1 nm and ζ-potential to 34.1 ± 1.0 mV. The PDIs remained below 0.07 indicating that the homogeneity of the LNCs could be retained. Thus, a formulation with narrow size distribution of LNCs containing FP and an outer layer of CS could be prepared.

In a previous study, LNCs were found to be stable in the gastrointestinal tract [56]. To evaluate stability of the LNC–LC–FP and the LNC–LC–FP–CS in the gastrointestinal environment, these LNCs formulations were incubated in SGF and SIF. After incubation and subsequent elimination of the media components by filtration, the LNC sizes remained almost unchanged indicating adequate stability and thus probably protection of the FP molecules before absorption. Ratios of initial size vs. size after incubation were following: LNC–LC–FP in SGF 1.04 ± 0.03; in SIF 0.92 ± 0.06; LNC–LC–FP–CS in SGF 1.22 ± 0.03; in SIF 1.20 ± 0.05. As also the LNC–LC sizes remained

Table 2
FP adsorption on the LNC surface. The experiments were performed in triplicate.

Mass ratio LC:FP	Size (nm)	PDI	ζ-potential (mV)	Quantity of FP adsorbed	Mass FP (μg) adsorbed/mg LNC[a]
1:0.09	70.6 ± 3.1	0.07	13.4 ± 1.8	100%	22
1:0.18	109.5 ± 9.2	0.04	−3.8 ± 0.5	89 ± 5%	39
1:0.36	89.3 ± 2.1	0.04	−16.4 ± 1.7	59 ± 3%	51
1:0.71	95.2 ± 3.5	0.04	−15.0 ± 1.8	49 ± 5%	85
1:1.42	99.6 ± 4.0	0.04	−14.9 ± 1.1	47 ± 4%	166
1:2.85	108.1 ± 5.2	0.04	−14.8 ± 1.0	36 ± 3%	254

[a] calculated from the average% quantity of FP adsorbed.

nearly unchanged (1.10 ± 0.06 in SGF; 1.01 ± 0.08 in SIF) Thus, these results were in line with the previous study [56]. Forthcoming studies will evaluate the pharmacodynamic properties of this kind of formulations in biological environment.

Summarizing the studies with FP, a hydrophilic therapeutic molecule could be incorporated in an LNC formulation with the help of electrostatic interactions. The therapeutic molecule was further covered (protected) by another layer (CS). As lipophilic drug molecules can be encapsulated inside the oily core of LNCs [27,57], this LBL approach could enable the formulation of drug carriers containing both lipophilic and hydrophilic therapeutic molecules.

4. Conclusions

A cationic lipopolysaccharide, lipochitosan, was synthesized by coupling stearic anhydride on low molecular weight chitosan. Lipochitosan was attached on the surface of lipid nanocapsules by the post-insertion technique. This kind of amphiphilic molecule could be of interest when formulating drug delivery systems because of its non-toxic components and positive charge. In the post-inserted lipid nanocapsules, the surface consisted of PEG chains and lipochitosan molecules (11.6% mol/mol surface coverage). Taking advantage of the formed positive surface charge, polyelectrolytes such as dextran sulfate and chitosan were sequentially adsorbed on the lipid nanocapsules by the layer-by-layer approach. Also, introduction of a model therapeutic polyelectrolyte, fondaparinux sodium, in the layer-by-layer process was assessed. Tangential flow filtration was applied to purify the nanocapsule dispersion after each coating layer. Success of the coating process was observed by the alternating ζ-potential and increase in size. The results showed that the tangential flow filtration was a convenient method to be combined with the layer-by-layer technique and the nanocapsule integrity could be retained even in the course of successive purification steps. In general, combination of lipid nanocapsules and tangential flow filtration is an interesting approach in the formulation and production of colloidal drug delivery systems as no toxic solvents or harsh conditions are needed, and all the process steps can be easily scaled up.

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