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ARTICLE TYPE

Surfactant-free polymeric nanoparticles composed of PEG, cholic acid and a sucrose moiety

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Polymer-based nanomedicine is a large and fast growing field that has gained plenty of research attention during recent decades. In the present study, new amphiphilic polymers were designed and synthesized by chemical modification of poly(ethylene glycol) (PEG) conjugated with sucrose and a cholic acid moiety (abbreviated as Suc-PEG-Chol). Two series of polymers with different PEG chain lengths were synthesized and their structures were confirmed by ^{1}H-NMR, ^{13}C-NMR and MALDI-TOF analysis. The fluorescence spectroscopy data of these conjugates showed that they are able to self-assemble in water and the critical association concentration (CAC) value was found to be in the range of 0,06–0,13 g/L. Owing to their amphiphilic characteristics in aqueous solution, polymeric nanoparticles (PNPs) of Suc-PEG-Chol polymers were prepared by a nanoprecipitation method without any surfactants. The particle size distribution was determined by dynamic light scattering (DLS) and the result was 117 nm for Suc-PEG_{2000}-Chol conjugate and 96 nm for the PEG_{4000} analog, both with relatively narrow particle size distribution. All of the obtained PNPs showed negative surface charge and no size dependence on the polymer concentration forming stable nanoparticle suspensions. From the atomic force microscopy (AFM) and scanning electron microscopy (SEM) observations, the PNPs were spherically shaped with a relatively smooth surface. Our results suggest that these PEGylated nanoparticles formulated with cholic acid and sucrose as biocompatible building blocks can be considered a potential candidate for biomedical applications.

1 Introduction

Recent advances and progress in nanoscience have demonstrated that nanomaterials have a great potential for healthcare applications.[1] In the realm of drug delivery, targeted nanomedicines have captured the attention of many scientists who have tried to realize the concept of the "magic bullet", proposed by Paul Ehrlich almost a century ago.[2] Complications arise because drugs have to overcome natural biological hurdles and most infections are localized. Nevertheless, by delivering pharmacologically active agents more effectively and more selectively, nanomedicines aim to improve the pharmacokinetic and pharmacodynamic properties of therapeutic molecules, enhancing efficacy while reducing toxicity.[3] In addition, expensive potentially active substances could be applied efficiently in small amounts. In this light, polymeric nanoparticles (PNPs) play an important role in the "Room at the Bottom" and this family may be considered amongst the most well studied nanomedicines to date.[4-6]

PNPs are frequently defined as solid, colloidal particles having particle size between 10 and 1000 nm. In medical applications well-defined sizes are of great importance, as they play a crucial role in mediating biological effects and *in vivo* fate of the drug delivery system.[7] Therefore to assure the highest potential for *in vivo* applications, PNPs in the size range of 20 to 100 nm are optimal for cellular uptake.[8] These drug carriers are large enough to avoid renal and lymphatic clearance and small enough to avoid phagocytic recognition.

The main advantages of PNPs are their great versatility from a structural point of view. Different physicochemical properties offered by these nanocarriers (size, shape and surface properties) influence their fate in biological systems. Thus, stability, drug release and targeting can be tailored by surface modification, highlighting the importance of well-defined chemistries.[9]

Particle surface chemistry is a key factor in PNPs bioavailability as our body recognizes hydrophobic particles as foreign. To reduce rapid uptake and clearance by cells of the mononuclear phagocytic system (MPS), surface-modified PNPs have been developed to produce ''stealth'' drug delivery nanocarriers.[10] The most common moiety for surface modification is the hydrophilic, non-ionic, biocompatible and FDA-approved polymer polyethylene glycol (PEG).[10,11] PEGylation reduces the likelihood of opsonisation in the bloodstream leading to prolonged blood circulation times. Furthermore, PEG is also the most widely used polymer in delivering anticancer drugs clinically and several PEG-conjugates have already reached late phase clinical trials.[12]

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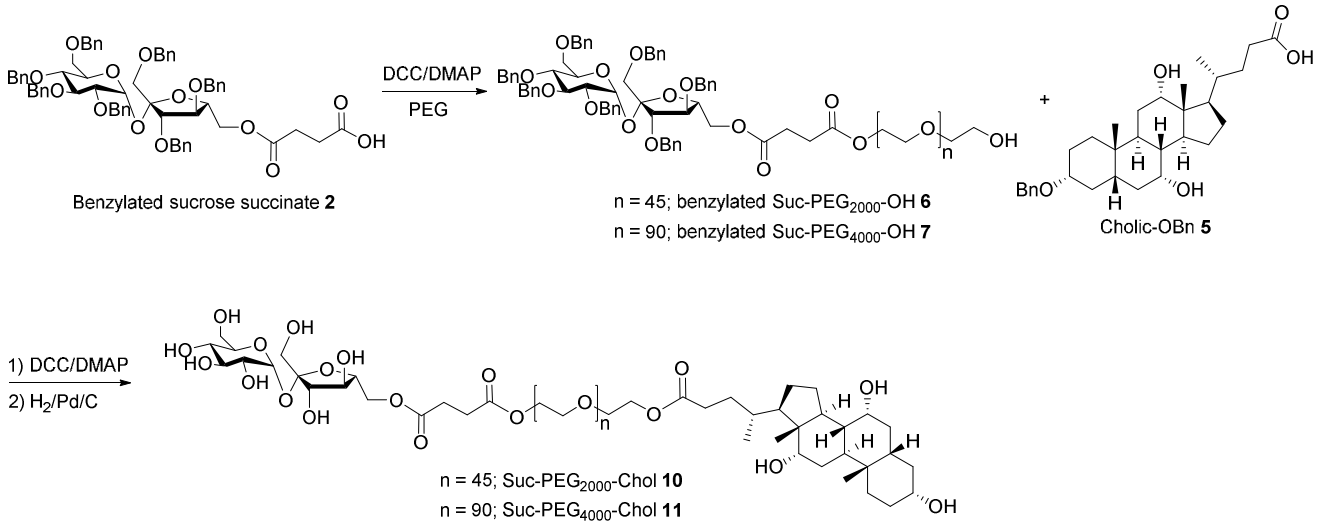


Fig.1 Synthetic scheme for preparing the Suc-PEG-Chol polymer conjugates.

In addition to a PEG coating, most stealth PNPs also have targeting ligands, which are able to facilitate binding through specific over-expressed cell surface receptors.[13] Among them, lectins (carbohydrate-binding proteins) play a complex role in various biological recognition events.[14] Sugar-containing polymers have been developed as vehicles for therapeutics or as therapeutics themselves and some of these compounds displayed impressive gains in binding affinity or *in vivo* efficiency. [15] PNPs displaying saccharide moieties have been developed for liver-specific drug delivery.[16,17] Song et al. showed that disaccharide-modified liposomes enhanced cellular uptake of liposomes into various cancer cell lines via lectins-mediated endocytosis.[18] In order to improve targeting efficiency and reduce side effects, introducing targeting molecules, such as sucrose into PNPs, could enhance the affinity toward cell surface lectins. Moreover, if the rate-determining step for tumour uptake is based on the enhanced permeability and retention (EPR) effect, PNPs decorated with targeting ligands are believed to be internalized easily by cells after accumulation.

An ideal drug delivery vehicle should also have a high drug-loading capacity in order to reduce the dose frequency, improving patient compliance.[19] Drug loading depends on the solubility of the drug molecules in the polymer which is mainly related to intramolecular interactions between these two types of molecules.[19, 20]

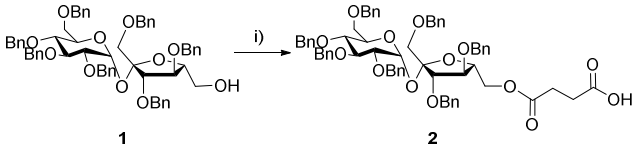
Bile acids are natural compounds with a rigid steroid cyclopentenophenanthrene structure, which has found applications in pharmacology, supramolecular chemistry and nanoscience.[21] Cholic acid is a unique facial amphiphilic molecule and a versatile building block in the design and synthesis of novel polymeric materials. These materials may have improved properties such as rigidity, capacity of self-assemble, biocompatibility, biodegradability and so forth.[22] In addition, the steroid skeleton results in the formation of a cavity, which is a suitable environment for host-guest chemistry.[21]

Herein we report the synthesis of PNPs based on amphiphilic polymeric conjugates composed of a cholic acid, a sucrose moiety and a polymer PEG, to give stealth and important physicochemical properties. In these PNPs carriers, cholic acid will act as a drug incorporation site and the carbohydrate as targeting moiety. We envision that the conjugates constructed with natural building blocks – sugar and cholic acid - should be biocompatible and biodegradable, and due to their structure they will possess high functionality and versatility. Combining molecular entities with distinct properties could provide novel conjugates in which the different molecular segments act cooperatively. The self-assembling behaviour, morphology and particle sizes of PNPs were characterized by fluorescence spectroscopy, scanning electron microscopy (SEM), atomic force microscopy (AFM) and dynamic light scattering (DLS).

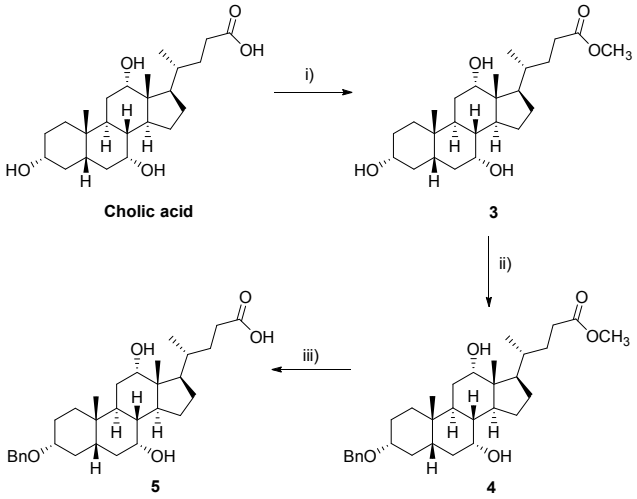
2 Results and discussion

2.1 Synthesis and characterization of polymer conjugates

The Suc-PEG-Chol conjugate was synthesized by covalently binding the appropriate sucrose and cholic acid derivatives to PEG by esterification (Fig. 1). Two series of polymers with different numbers of repeating ethylene oxide units (EO) (n = 45, 90) have been synthesized. In order to obtain solely a sucrose-containing linear polymer, chemoselective derivatization of sucrose was required. The route to selective derivatization of the 6'-position has been developed previously.[23] It allowed us to obtain the fully protected sucrose with only the 6'-hydroxyl unprotected. From this intermediate **1** we prepared the sucrose



Scheme 1 i) Succinic anhydride, DMAP, Et_3N, CH_2Cl_2, rt, 100%.



Scheme 2 C i) MeOH, HCl 33%, reflux, 97%; ii) NaH, BnBr, CH_2Cl_2, 0 °C – r.t., 30 %; iii) LiOH 0.5 N, THF, r.t., 97%.

succinate derivative **2** by reaction with succinic anhydride (scheme 1). An excess of succinic anhydride was used to guarantee that the hydroxyl terminals of **1** were totally reacted, avoiding a more laborious purification. In addition to usual sucrose signals, the ^{1}H-NMR spectrum revealed the succinyl protons as one multiplet centered about 2.5 ppm. PEG chains were then coupled onto the carboxylic group of **2** using DCC/DMAP as coupling agent. Since it has proven difficult to separate the desired functionalized molecules of PEG from the unreacted polymer, the molar number of **2** was 1.2-fold that of the PEG polymer. No dissubstituted product was formed. However, by closely following the reaction by TLC, two major spots with similar R_f values were observed. After purifying with gradient elution flash column chromatography we concluded by ^{1}H-NMR that it was the same desired compound but with different numbers of EO units. Since PEG is a synthetic polymer which shows a certain polydispersity no further attempts at separation were made. The next step to our target consisted of coupling a molecule of cholic acid to the benzylated Suc-PEG-OH moiety synthesized previously. For this, we targeted the 3α-OH for selective protection as its benzyl ether because of the nucleophilic properties of this equatorial hydroxyl group (scheme 2). Furthermore, the reactivity of terminal primary OH groups of the PEG polymer is masked due to both the high flexibility of the backbone chain and the high molecular weight, which turns them less reactive. The synthesis was initiated by preparing the methyl ester of cholic acid, since the ester has a higher solubility in organic solvents than cholic acid itself. [24] Treatment of methyl cholate **3** with benzyl bromide in CH_2Cl_2, in the presence of NaH leads to the compound **4** in a low yield (30%). Keeping the reaction for a longer time in the presence of excess of NaH or changing to a more suitable solvent for SN_2 reactions, as DMF, resulted in the formation of by-products, as supported by previous reports.[25] The methyl ester group was then successfully hydrolysed under mild alkaline conditions, to afford the expected compound **5** in a good yield (97%). To confirm which hydroxyls were modified, the ^{1}H-NMR spectrum of **4** was compared to that of methyl cholate **3**. In methyl cholate **3**, the proton chemical shifts of 3β-H, 7β-H and 12β-H are seen at 3.46, 3.85 and 3.98 ppm, respectively. In compound **4** an upfield shift for the 3β-H (3.22 ppm) was observed, while 7β-H and 12β-H appeared at their original positions, thus confirming the introduction of a benzyl group only at the 3α-position. The complete disappearance of the signal at 3.66 ppm for -OCH_3 established the formation of compound **5**. The coupling of benzylated Suc-PEG-OH with compound **5** was carried out with DCC/DMAP as coupling agent. Subsequent debenzylation by Pd/C catalysed hydrogenolysis in a mixture of EtOH:AcOEt:H_2O (7:7:0.1), gave the desired Suc-PEG-Chol conjugates. Their structures were assigned on the basis of their ^{1}H-NMR, ^{13}C-NMR and mass spectral data. In addition to the usual sucrose and steroidal signals, the broad singlet at 3.40 ppm in the ^{1}H-NMR spectrum may be assigned to the methylene protons of the repeating –OCH_2CH_2O- units in PEG. Signals at 4.2 ppm were assigned to the methylene protons in PEG adjacent to the ester groups. The ^{13}C-NMR spectrum showed three signals at 173.32, 171.96 and 171.87 ppm, which were attributed to carbonyl carbons, -C(=O)O_{Chol}-PEG-, -C(=O)O_{Suc}-PEG- and -C(=O)O_{Suc}-, respectively. The formation of the Suc-PEG-Chol conjugates was further confirmed by its MALDI-TOF mass spectra revealing a molecular weight average of 2748.6 for Suc-PEG_{2000}-Chol and 4875.1 for Suc-PEG_{4000}-Chol. The MALDI-TOF mass spectrum also shows the polydispersed nature of PEG (Fig. S1 and S2). The thermal characteristics of Suc-PEG-Chol polymers were study by DSC (Fig. S3 and S4). The attachment of sucrose and cholic acid moieties decreased the melting temperature of the corresponding PEG. The T_m of PEG_{2000} was reduced from 50 to 39 ºC and PEG_{4000} had a T_m of 55 ºC while the Suc-PEG_{4000}-Chol polymer T_m was reduced to 48 ºC. As expected, the T_m of the polymers increased with increasing molecular weight. The Suc-PEG-Chol polymers can self-associate in an aqueous environment to form micelle like self-aggregates with a hydrophobic cholic acid core surrounded by a hydrophilic sucrose and PEG shell. ^{1}H-NMR spectrum in D_2O was employed to study these conformational states (Fig. S5). The results showed that in D_2O the complete structural resolution was observed. However, the integration between the C-18 methyl protons of cholic acid and the anomeric proton of sucrose did not match with the molecular formula of the target polymer. The number of cholic acid units determined by ^{1}H-NMR was one third of the sucrose moiety, which indicates a hydrophobic core constituted mainly of cholic acid moieties. In order to determine the critical association concentration (CAC), the self-aggregation behaviour of the Suc-PEG-Chol conjugates in aqueous media was investigated by using pyrene as a fluorescence probe, and the results are illustrated in Fig. 2 and 3 (see SI for Suc-PEG_{4000}-Chol data). Figure 2 depicts the fluorescence emission spectra of pyrene obtained at a fixed excitation wavelength of 340 nm against various Suc-PEG_{2000}-Chol concentrations. It shows an increase of fluorescence emission intensity with increasing

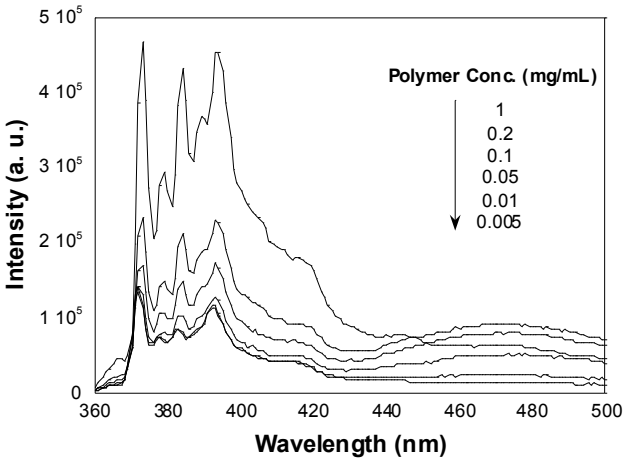


Fig.2 Fluorescence emission spectra of pyrene/Suc-PEG2000-Chol against concentration of Suc-PEG2000-Chol in distilled water.

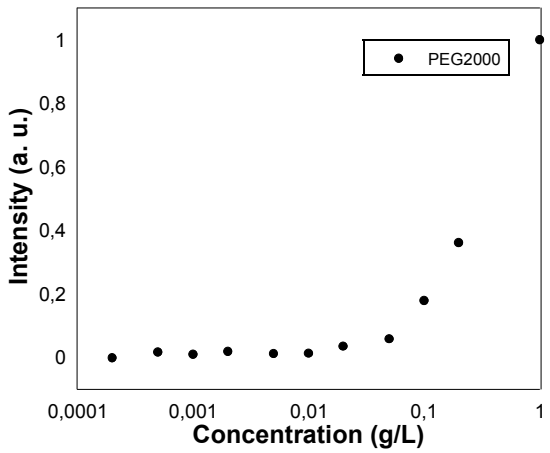


Fig.3 Plots of the overall intensity of the pyrene emissions spectra vs. polymer concentrations.

polymer concentration, which indicated the incorporation of pyrene molecules into the hydrophobic domains of the conjugates. In addition to the monomeric peaks, a broad band appears at longer wavelengths (centered on 470 nm) due to excimer formation. Pyrene excimer reflects a high local molar concentration of pyrene where a ground state and an excited state pyrene ring are ~10 Å from each other.[26] No excimer emission was observed in the absence of polymer. Figure 3 shows the change in overall intensity of the emission spectra as a function of polymer concentration. At a low concentration (c < CAC) a flat region and a sigmoid change in the crossover point were observed and the CAC values were calculated. The CAC value of Suc-PEG_{2000}-Chol conjugate (0.06 g/L) was lower than the Suc-PEG_{4000}-Chol conjugate (0.13 g/L) which is in agreement with the increase in the solubility of the latter due to the presence of a higher PEG chain length.

2.2 Synthesis and characterization of polymeric nanoparticles

PNPs were prepared by the nanoprecipitation method, first developed by Fessi and co-workers.[27] Briefly, a solution of Suc-PEG-Chol polymer dissolved in acetone was injected into deionized water without any surfactant with moderate stirring at room temperature. Nanoparticles were immediately formed and the suspension was maintained under gentle mechanical stirring until complete evaporation of the organic solvent. The physicochemical characteristics of the obtained colloidal suspension of PNPs are summarized in Table 1. The mean diameters of Suc-PEG-Chol nanoparticles was 117 nm for the formulation prepared with PEG_{2000} and 96 nm for the formulation with PEG_{4000} (Fig. S7). The fairly low polydispersity index (PDI) (0.17 to 0.28) demonstrated a narrow particle size distribution. In terms of surface charge, zeta potential becomes less negative with increasing PEG chain length which could be explained by the possible shielding effect of the hydrophilic PEG chains. Another interesting finding was the influence of the polymer concentration on the mean diameter of the nanoparticles. As shown in Figure 4, particle sizes of Suc-PEG_{4000}-Chol PNPs were found to be dependent on the initial concentration in the organic phase and were more pronounced for lower concentrations. On the other hand, particle sizes of Suc-PEG_{2000}-Chol PNPs were scarcely

Table 1 Characterization of the Suc-PEG-Chol nanoparticles by DLS.

Samples	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Suc-PEG_{2000}-Chol	117	0.169	-26
Suc-PEG_{4000}-Chol	96	0.280	-19

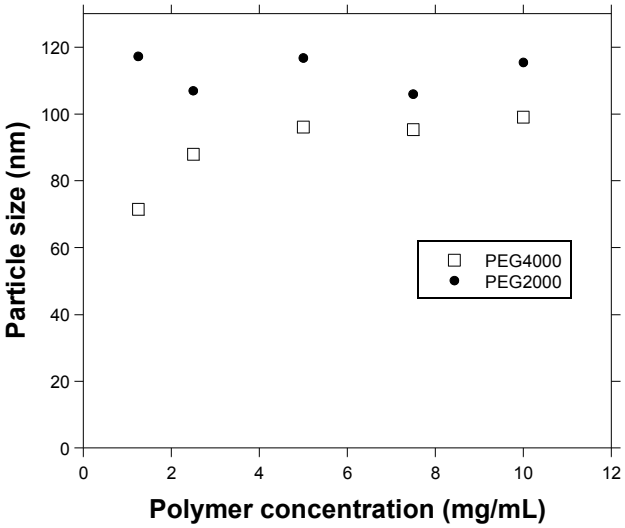


Fig.4 Concentration dependent particle sizes of Suc-PEG-Chol nanoparticles at room temperature.

affected by the concentration of the polymer in the studied range. Therefore, when the concentration was increased, the interaction between the self-assembled nanoparticles was negligible and no aggregation occurred. Furthermore, no change on the mean diameter was observed in suspensions stored at 5 ºC over one month. These findings were also in agreement with the trend of the zeta potential values in the different systems and revealed that the Suc-PEG-Chol polymers formed stable nanoparticle suspensions without aggregation. AFM images of these colloidal suspensions of PNPs revealed that the particles were spherical in shape (Fig. 5). However, the average diameters determined by

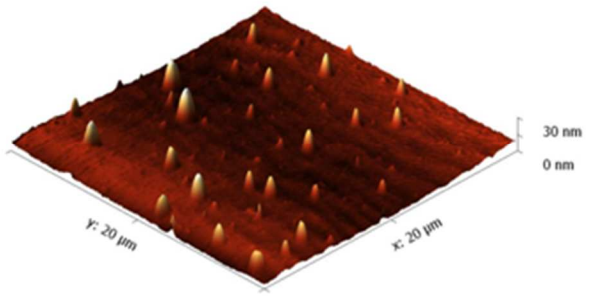


Fig.5 AFM height image of Suc-PEG4000-Chol nanoparticles from a PNPs solution of 0.1mg/mL.

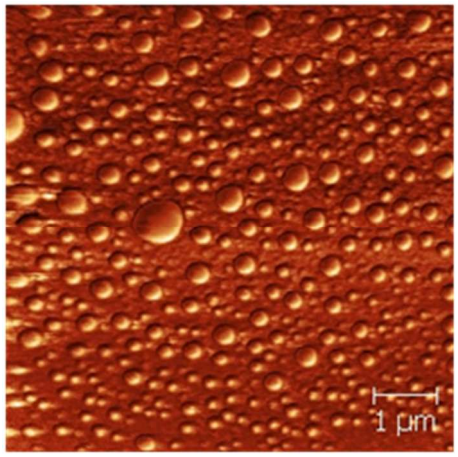


Fig.6 AFM phase image of lyophilized Suc-PEG2000-Chol nanoparticles from a PNPs solution of 0.1mg/mL.

AFM for both conjugates were higher than those measured by DLS. The average nanoparticle diameter was 290 nm for the formulation prepared with PEG_{2000} and 300 nm for the formulation with PEG_{4000} (Fig. S8). Lyophilized PNPs also exhibit the same behaviour. AFM images of lyophilized PNPs prepared with PEG_{2000} revealed an average nanoparticle diameter of 202 nm (Fig. 6). In the case of the lyophilized PNPs prepared with PEG_{4000} it was possible to find on the mica surface a "snapshot" of the aggregation process (Fig. 7). The average nanoparticle diameter was 112 nm which indicates that no aggregation occurred during the lyophilization process even without any cryoprotectant. From these results we noticed some influence from the water evaporation entailed in the preparation of the AFM sample on the size of the PNPs. In fact, the aggregation may be caused by the hydrophilicity of the mica substrate which could have traces of water even after evaporation, causing nanoparticles to form agglomerates.[28] SEM was also used to study the surface morphology of the PNPs with higher resolution as shown in Fig.8. The lyophilized Suc-PEG_{4000}-Chol PNPs size found from the SEM images tallies with that detected by DLS previous to lyophilization. It was not possible to examine PEG_{2000} conjugates by SEM due to their low melting point.

3 Conclusions

The present study demonstrated the synthesis and self-aggregation behaviour of Suc-PEG-Chol polymers in an aqueous milieu. Applying various technical procedures, we found that

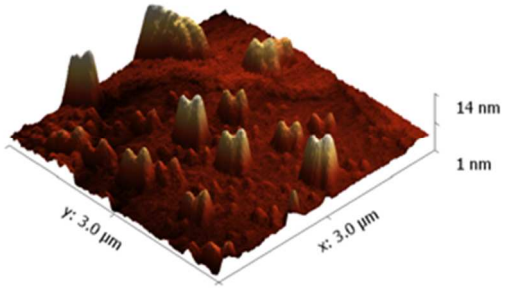


Fig.7 AFM height image of lyophilized Suc-PEG4000-Chol nanoparticles from a PNPs solution of 0.1mg/mL.

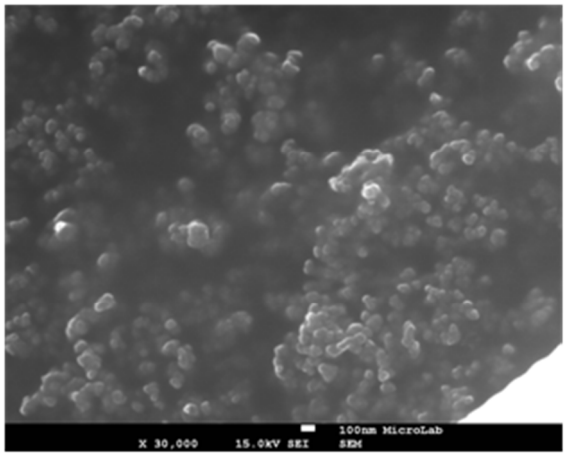


Fig.8 SEM image of Suc-PEG4000-Chol nanoparticles.

PNPs could be formed from Suc-PEG-Chol conjugates by the nanoprecipitation method without any surfactant being necessary. The PNPs showed a spherical morphology and a size distribution of about 90 – 120 nm which is suitable for drug delivery systems. Furthermore, these particles were constituted mainly of a hydrophilic outer shell of PEG and sucrose and a hydrophobic core of cholic acid, as designed. Although further investigation on the *in vivo* effect of Suc-PEG-Chol PNPs is required, the findings of our study opened the possibility of introducing this type of PNPs into various biomedical fields. The presence of reactive functional groups in sucrose and cholic acid offers great opportunity for chemical modification which affords the possibility for incorporating additional therapeutic and diagnostic moieties. Drug delivery systems based upon these PNPs are under study.

4 Experimental

4.1 Reagents and materials

All reactions were performed under a dry argon atmosphere. Cholic acid (Chol), sucrose (Suc), *N,N′*-Dicyclohexylcarbodiimide (DCC), succinic anhydride (SC), 4-Dimethylaminopyridine (DMAP), pyrene and 10% palladium on activated carbon (Pd/C) were purchased from Aldrich and used as received. PEG_{2000} and PEG_{4000} were supplied from Aldrich and dried under vacuum over P_2O_5 to remove the residual water. All solvents were distilled prior to use from an appropriate drying agent. Column chromatography was performed on silica gel from Macherey-Nagel (Kieselgel 60 M). All reaction were monitored

by thin layer chromatography (TLC), which was conducted on aluminium-backed silica gel Merck 60 F254 plates, and compounds were visualized by UV light (254 nm) and/or staining with a solution of concentrated H_2SO_4/MeOH 2:8 or in a solution of phosphomolybdic acid (5 g) in EtOH (95 mL) and subsequent heating. Hydrogenation reactions were performed in a Parr Shaker apparatus. Melting points were measured on an Electrothermal capillary melting point apparatus or by DSC. Optical rotations were measured on an Optical Activity AA-1000 polarimeter (0.5 dm cell) at 589 nm and values are given in units of 10^{-1}deg.cm^3.g^{-1} at 20 ºC. Elemental analyses were performed on Thermo Finnigan-CE Flash EA 1112 CHNS series analyser. Mass spectra were measured by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) on a Bruker Autoflex with α-cyano-4-hydroxycinnamic acid (4-HCCA) matrix. NMR spectra were recorded on a Bruker AMX-400 MHz spectrometer operating at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei. $CDCl_3$ (99.50 % isotopic purity) and DMSO-*d6* (99.80 % isotopic purity) were purchased from Aldrich.

4.2 Synthesis and characterization of polymer conjugates

The synthetic routes for polymers are shown in Fig. 1.

1',2,3,3',4,4',6-Hepta-*O*-benzyl-6´-*O*-succinyl-sucrose (2)

To a solution of 1',2,3,3',4,4',6-Hepta-O-benzyl-sucrose **1** [22] (3.35 g, 3.44 mmol, 1 eq) in dry CH_2Cl_2 (60 mL) were added succinic anhydride (0.70 g, 6.88 mmol, 2 eq), 4-*N,N*-dimethylaminopyridine (0.43 g, 3.44 mmol, 1 eq) and triethylamine (0.30 mL). The mixture was kept for 4 h at room temperature, until no more of the starting material was detected by TLC (Hex:AcOEt 3:1). The reaction mixture was diluted with CH_2Cl_2 (100 mL), washed successively with HCl 0.1 M (6 x 10 mL), brine (3 x 10 mL), and H_2O (2 x 10 mL) and dried with anhydrous Na_2SO_4. The solvent was evaporated to give **2** (3.67 g, 3.4 mmol, 100 %) as a colorless oil. R_f 0.39 (Hex:AcOEt 3:2). $[\alpha]_D^{20}$ +26.4 (*c* 1.0 in $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.41-7.20 (m, 33H, Ph), 7.18-7.10 (m, 2H, Ph), 5.69 (d, *J* = 4.0 Hz, 1H, H-1), 4.96 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.85 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.80 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.70 – 4.30 (m, 14H, H-5', H-6', C**H**$_2$-Ph), 4.21 – 4.13 (m, 1H, H-4'), 4.11 – 4.04 (m, 2H, H-5, H-3'), 3.97 (t, *J* = 8.0 Hz, 1H, H-3), 3.76 (d, *J* = 10.9 Hz, 1H, H-1'$_a$), 3.66 (t, *J* = 9.6 Hz, 1H, H-4), 3.60 – 3.48 (m, 3H, H-2, H-6$_a$, H-1'$_b$), 3.46 – 3.38 (m, 1H, H-6$_b$), 2.59 – 2.45 (m, 4H, C**H**$_2$C**H**$_2$COOH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.89 (**C**OOH), 171.95 (**C**(=O)O), 138.92 (C(Ph)), 138.27 (C(Ph)), 138.00 (C(Ph)), 137.94 (C(Ph)), 137.74 (C(Ph)), 137.70 (C(Ph)), 128.41(**C**-H(Ph)), 128.35 (**C**-H(Ph)), 128.30 (**C**-H(Ph)), 128.13 (**C**-H(Ph)), 128.09 (**C**-H(Ph)), 127.97 (**C**-H(Ph)), 127.83 (**C**-H(Ph)), 127.72 (**C**-H(Ph)), 127.66 (**C**-H(Ph)), 127.56 (**C**-H(Ph)), 127.51 (**C**-H(Ph)), 104.51 (C2'), 89.97 (C1), 83.99 (C5'), 81.82 (C3), 81.47 (C4'), 79.56 (C2), 77.99 (C3'), 77.55 (C4), 75.60 (**C**H$_2$-Ph), 74.99 (**C**H$_2$-Ph), 73.49 (**C**H$_2$-Ph), 73.36 (**C**H$_2$-Ph), 73.17 (**C**H$_2$-Ph), 72.85 (**C**H$_2$-Ph), 72.25 (**C**H$_2$-Ph), 71.00 (C1'), 70.34 (C5), 68.25 (C6), 64.88 (C6'), 29.26 (**C**H$_2$COOH), 28.82 (**C**H$_2$CH$_2$COOH). Found: C, 72.85; H, 6.46. $C_{65}H_{68}O_{14}$ requires C, 72.74; H, 6.39%.

Methyl 3α,7α,12α-trihydroxy-5β-cholan-24-ate (3)

A solution of commercial available cholic acid (14 g, 33.5 mmol) in methanol (65 mL) was acidified with hydrochloric acid 33% (0.5 mL) and the mixture was refluxed for 20 min. The solution was allowed to cool to r.t. and the solvent was concentrated to 40 mL in a rotary evaporator and then cooled to 5 ºC. The resulting crystals were separated from the mother liquor and rinsed with cold MeOH to give **3** (13.74 g, 32.5 mmol, 97 %) as white needles. R_f 0.33 ($CHCl_3$:MeOH 9:1). mp 153 – 154 ºC (lit. 154 - 155 ºC) [23]. $[\alpha]_D^{20}$ + 26.0 (*c* 1.0, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.02-3.93 (m, 1H, H-12β), 3.90-3.80 (m, 1H, H-7β), 3.67 (s, 3H, OCH_3), 3.52-3.39 (m, 1H, H-3β), 2.45-2.31 (m, 1H, H-23α), 2.30-2.13 (m, 3H, H-4α, H-9α, H-23β), 2.00-1.85 (m, 3H, H-6β, H-14α, H-16α), 1.84-1.73 (m, 4H, H-1α, H-4β, H-17α, H-22α), 1.72-1.63 (m, 2H, H-2β, H-15β), 1.62-1.47 (m, 4H, H-6α, H-8β, H-11), 1.47-1.33 (m, 4H, H-2α, H-5β, H-20, H-22β), 1.33-1.23 (m, 1H, H-16β), 1.18-1.05 (m, 1H, H-15α), 1.03-0.93 (m, 1H, H-1β), 0.99 (d, *J* = 5.6 Hz, 3H, 21-CH_3), 0.89 (s, 3H, 19-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.84 (**C**(=O)O), 73.09 (C12), 71.96 (C3), 68.47 (C7), 51.51 (C25), 47.01 (C17), 46.43 (C13), 41.66 (C14), 41.47 (C5), 39.49 (C8), 39.46 (C4), 35.31 (C1), 35.29 (C20), 34.76 (C10), 34.63 (C6), 31.11 (C23), 30.92 (C22), 30.33 (C2), 28.16 (C11), 27.51 (C16), 26.38 (C9), 23.22 (C15), 22.46 (C19), 17.31 (C21), 12.48 (C18).

Methyl 3α-*O*-benzyl, 7α, 12α-dihydroxy-5β-cholan-24-oate (4)

Methyl 3α, 7α, 12α-trihydroxy-5β-cholan-24-oate **3** (2 g, 4.74 mmol, 1 eq) was dissolved in dry CH_2Cl_2 (15 mL) and then cooled to 0 ºC in an ice-water bath. To this solution, NaH (0.375 g, 9.48 mmol, 2 eq) was added under argon flush and the mixture was stirred for 30 min. BnBr (1.13 mL, 9.48 mmol, 2 eq) was added dropwise and the reaction was left at room temperature and stirred for 12 h. The reaction was quenched by adding crushed ice and extracted with CH_2Cl_2 (2 x 50 mL). The combined extracts were washed with brine (2 x 20 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The resulting crude product was purified by column chromatography using AcOEt/Hexane (1:1 and then 2:1) as eluent to give pure **4** (0.72 g, 1.4 mmol, 30%) as a colourless oil that foamed under vacuum. R_f 0.68 (AcOEt/Hex 2:1). mp 49.2 ºC. $[\alpha]_D^{20}$ +34.0 (*c* 1.0, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.39 - 7.29 (m, 4H, Ph-H), 7.28 - 7.22 (m, 1H, Ph-H$_{para}$), 4.60-4.50 (m, 2H, C**H**$_2$-Ph), 4.01-3.94 (m, 1H, H-12β), 3.88-3.79 (m, 1H, H-7β), 3.66 (s, 3H, OC**H**$_3$), 3.28-3.17 (m, 1H, H-3β), 2.43-2.32 (m, 1H, H-23α), 2.30-2.13 (m, 3H, H-4α, H-9α, H-23β), 2.01-1.73 (m, 8H, H-1α, H-2β, H-4β, H-6β, H-14α, H-16α, H-17α, H-22α), 1.71-1.47 (m + H_2O, 5H, H-6α, H-8β, H-11, H-15β), 1.46-1.22 (m, 5H, H-2α, H-5β, H-20, H-16β, H-22β), 1.21-1.07 (m, 1H, H-15α), 1.02-0.90 (m, 1H, H-1β), 0.98 (d, *J* = 5.8 Hz, 3H, 21-C**H**$_3$), 0.89 (s, 3H, 19-C**H**$_3$), 0.69 (s, 3H, 18-C**H**$_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.72 (**C**(=O)O), 139.20 (**C**(Ph)), 128.31 (**C**-H$_{meta}$(Ph)), 127.57 (**C**-H$_{ortho}$(Ph)), 127.31 (**C**-H$_{para}$(Ph)), 78.65 (C3), 72.86 (C12), 69.71 (**C**H$_2$-Ph), 68.27 (C7), 51.51 (C25), 47.10 (C17), 46.52 (C13), 42.02 (C14), 41.44 (C5), 39.65 (C8), 36.26 (C4), 35.22 (C1), 35.12 (C20), 35.02 (C10), 34.61 (C6), 31.04 (C23), 30.88 (C22), 28.42 (C11), 27.39 (C16), 27.27 (C2), 26.75 (C9), 23.19 (C15), 22.64 (C19), 17.32 (C21), 12.59 (C18). Found: C, 74.81;

H, 9.58. $C_{32}H_{48}O_5$ requires C, 74.96; H, 9.44%.

3α-*O*-benzyl, 7α, 12α-dihydroxy-5β-cholic acid (5)

Methyl 3α-*O*-benzyl, 7α, 12α-dihydroxy-5β-cholan-24-oate **4** (0.75 g, 1.46 mmol) was dissolved in THF (25 mL). A solution of 0.5 N aqueous lithium hydroxide (12 mL) was added and the solution was stirred at room temperature for 6 hours. The solution was then acidified with HCl 10% and it was extracted with CH_2Cl_2 (2 x 50 mL). The organic extract was washed with brine (3 x 10 mL) followed by H_2O (2 x 10 mL), dried with anhydrous Na_2SO_4, and concentrated in vacuum to give **5** (0.726 g, 1.46 mmol, 97 %) as a colourless oil that foamed under vacuum. R_f 0.40 (AcOEt/Hex 2:1). mp 64.2 ºC. $[\alpha]_D^{20}$ +37.7 (*c* 1.0, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.38 - 7.32 (m, 4H, Ph-H), 7.31 - 7.26 (m, 1H, Ph-H_{para}), 4.58 (s, 2H, C**H**$_2$-Ph), 4.01-3.94 (m, 1H, H-12β), 3.82-3.72 (m + THF, 1H, H-7β), 3.33-3.21 (m, 1H, H-3β), 2.50-2.38 (m, 1H, H-23α), 2.36-2.18 (m, 3H, H-4α, H-9α, H-23β), 1.98-1.76 (m, 8H, H-2β, H-4β, H-6β, H-14α, H-16α, H-17α, H-22α), 1.73-1.34 (m, 9H, H-2α, H-5β, H-6α, H-8β, H-11, H-15β, H-20, H-22β), 1.30-1.22 (m, 1H, H-16β), 1.19-1.06 (m, 1H, H-15α), 1.05-0.91 (m, 1H, H-1β), 1.01 (d, *J* = 6.1 Hz, 3H, 21-C**H**$_3$), 0.88 (s, 3H, 19-C**H**$_3$), 0.69 (s, 3H, 18-C**H**$_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.35 (**C**(=O)OH), 138.70 (C(Ph)), 128.34 (C-H_{meta}(Ph)), 127.71 (C-H_{ortho}(Ph)), 127.49 (C-H_{para}(Ph)), 78.98 (C3), 73.09 (C12), 69.79 (**C**H_2-Ph), 68.35 (C7), 47.04 (C17), 46.50 (C13), 42.00 (C14), 41.41 (C5), 39.39 (C8), 35.95 (C4), 35.52 (C20), 35.20 (C1), 34.94 (C10), 34.50 (C6), 30.91 (C23), 30.77 (C22), 28.08 (C11), 27.63 (C16), 26.80 (C2), 26.47 (C9), 23.22 (C15), 22.37 (C19), 17.14 (C21), 12.48 (C18). Found: C, 74.69; H, 9.29. $C_{31}H_{46}O_5$ requires C, 74.66; H, 9.30%.

General procedure 1 for DCC-mediated coupling reactions

To an ice-cold solution of CH_2Cl_2 containing the carboxylic acid component (1.2 eq or 2.0 eq) were added 4-*N,N*-dimethylaminopyridine (1.2 eq or 2.0 eq) and DCC (2.4 eq or 4.0 eq). The mixture was stirred for 20 min. and then the appropriate PEG compound (1.0 eq) was added. After the addition, the reaction mixture was allowed to warm up to room temperature and was stirred for 48 h. The solution was cooled overnight in a refrigerator (4 ºC). After removal of the *N,N*-dicyclohexylurea precipitates by filtration through a plug of celite, the filtrate was concentrated on a rotary evaporator. The crude product material was purified by column chromatography using a step gradient of MeOH (1-10 %) in $CHCl_3$. Compounds were visualized with phosphomolybdic acid by TLC analysis ($CHCl_3$: MeOH 9:1).

Benzylated Suc-PEG$_{2000}$-OH (6)

According to the general method **1**, 1',2,3,3',4,4',6-Hepta-*O*-benzyl-6´-O-succinyl-sucrose **5** (0.64 g, 0.6 mmol) dissolved in dry CH_2Cl_2 (20 mL), 4-*N,N*-dimethylaminopyridine (73 mg, 0.6 mmol), DCC (0.25 g, 1.2 mmol) and PEG_{2000} (1 g, 0.5 mmol) were reacted for 48 h. Flash chromatography with $CHCl_3$:MeOH (1-10 %) afforded product **6** (1.12 g, 0.37 mmol, 74 %) as a white waxy solid. R_f 0.46 and 0.58 ($CHCl_3$:MeOH 9:1). ^{1}H-RMN (400 MHz, $CDCl_3$): δ 7.33-7.18 (m, 33H, Ph-H), 7.14-7.09 (m, 2H, Ph-H), 5.63 (d, *J* = 3.5 Hz, 1H, H-1), 4.91 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.80 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.76 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.66 – 4.25 (m, 14H, H-5', H-6', C**H**$_2$-Ph), 4.23 – 4.16 (m, 2H, -C(=O)OC**H**$_2$CH$_2$O-), 4.13 – 4.04 (m, 3H, H-5, H-3', H-4'), 3.95 (t, *J* = 9.3 Hz, 1H, H-3), 3.64 (m and Brs, H-4, PEG backbone), 3.55 – 3.40 (m, 5H, H-2, H-6, H-1'), 2.60 – 2.52 (m, 4H, -C**H**$_2$C**H**$_2$COO-PEG). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.17 (**C**(=O)O-PEG), 171.99 (**C**(=O)O), 138.83 (C(Ph)), 138.46 (C(Ph)), 138.24 (C(Ph)), 138.04 (C(Ph)), 138.01 (C(Ph)), 137.89 (C(Ph)), 137.75 (C(Ph)), 128.33(**C**-H(Ph)), 128.29 (**C**-H(Ph)), 128.27 (**C**-H(Ph)), 128.25 (**C**-H(Ph)), 128.23 (**C**-H(Ph)), 127.91 (**C**-H(Ph)), 127.89 (**C**-H(Ph)), 127.88 (**C**-H(Ph)), 127.85 (**C**-H(Ph)), 127.77 (**C**-H(Ph)), 127.69 (**C**-H(Ph)), 127.63 (**C**-H(Ph)), 127.56 (**C**-H(Ph)), 127.50 (**C**-H(Ph)), 127.46 (**C**-H(Ph)), 104.59 (C2'), 90.08 (C1), 83.68 (C5'), 81.99 (C4'), 81.86 (C3), 79.66 (C2), 78.23 (C3'), 77.59 (C4), 75.51 (**C**H$_2$-Ph), 74.82 (**C**H$_2$-Ph), 73.32 (**C**H$_2$-Ph), 72.82 (**C**H$_2$-Ph), 72.58 (**C**H$_2$-Ph), 72.55 (**C**H$_2$-Ph), 72.47 (**C**H$_2$-Ph), 70.88 (C1'), 70.55 (PEG backbone), 70.23 (C5), 69.00 (PEG backbone), 68.39 (C6), 65.53 (C6'), 63.73(-C(=O)O**C**H$_2$CH$_2$O-), 61.66 (PEG backbone), 28.77 (-**C**H$_2$**C**H$_2$-).

Benzylated Suc-PEG$_{4000}$-OH (7)

According to the general method **1**, 1',2,3,3',4,4',6-Hepta-*O*-benzyl-6´-O-succinyl-sucrose **5** (0.64 g, 0.6 mmol) dissolved in dry CH_2Cl_2 (20 mL), 4-*N,N*-dimethylaminopyridine (73 mg, 0.6 mmol), DCC (0.25 g, 1.2 mmol) and PEG_{4000} (2 g, 0.5 mmol) were reacted for 48 h. Flash chromatography with $CHCl_3$:MeOH (1-10 %) afforded product **7** (1.77 g, 0.35 mmol, 70 %) as a white waxy solid. R_f 0.42 ($CHCl_3$:MeOH 9:1). ^{1}H-RMN (400 MHz, $CDCl_3$): δ 7.39-7.20 (m, 33H, Ph-H), 7.17-7.11 (m, 2H, Ph-H), 5.66 (d, *J* = 3.4 Hz, 1H, H-1), 4.93 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.82 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.79 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.72 – 4.28 (m, 14H, H-5', H-6', C**H**$_2$-Ph), 4.26 – 4.17 (m, 2H, -C(=O)OC**H**$_2$CH$_2$O-), 4.15 – 4.06 (m, 3H, H-5, H-3', H-4'), 3.97 (t, *J* = 9.4 Hz, 1H, H-3), 3.66 (m and Brs, PEG backbone, H-2, H-4, H-6, H-1'), 2.66 – 2.52 (m, 4H, -C**H**$_2$C**H**$_2$COO-PEG). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.15 (**C**(=O)O-PEG), 171.97 (**C**(=O)O), 138.89 (C(Ph)), 138.30 (C(Ph)), 138.10 (C(Ph)), 138.07 (C(Ph)), 137.95 (C(Ph)), 137.81 (C(Ph)), 128.37(**C**-H(Ph)), 128.34 (**C**-H(Ph)), 128.31 (**C**-H(Ph)), 128.30 (**C**-H(Ph)), 128.28 (**C**-H(Ph)), 127.95 (**C**-H(Ph)), 127.93 (**C**-H(Ph)), 127.92 (**C**-H(Ph)), 127.89 (**C**-H(Ph)), 127.81 (**C**-H(Ph)), 127.73 (**C**-H(Ph)), 127.67 (**C**-H(Ph)), 127.61 (**C**-H(Ph)), 127.540 (**C**-H(Ph)), 127.50 (**C**-H(Ph)), 104.65 (C2'), 90.09 (C1), 83.74 (C5'), 82.01 (C4'), 81.87 (C3), 79.69 (C2), 78.23 (C3'), 77.60 (C4), 75.50 (**C**H$_2$-Ph), 74.83 (**C**H$_2$-Ph), 73.33 (**C**H$_2$-Ph), 72.82 (**C**H$_2$-Ph), 72.55 (**C**H$_2$-Ph), 72.48 (**C**H$_2$-Ph), 72.47 (**C**H$_2$-Ph), 70.53 (PEG backbone), 70.28 (C5), 69.00 (PEG backbone), 68.39 (C6), 65.53 (C6'), 63.73 (-C(=O)O**C**H$_2$CH$_2$O-), 61.69 (PEG backbone), 28.77 (-**C**H$_2$**C**H$_2$-).

Benzylated Suc-PEG$_{2000}$-Chol (8)

According to the general method **1**, 3α-*O*-benzyl, 7α, 12α-dihydroxy-5β-cholic acid **5** (0.34 g, 0.68 mmol) dissolved in dry CH_2Cl_2 (20 mL), 4-*N,N*-dimethylaminopyridine (82 mg, 0.68 mmol), DCC (0.29 g, 1.36 mmol) and benzylated Suc-PEG_{2000}-

OH **6** (1.03 g, 0.34 mmol) were reacted for 48 h. Flash chromatography with $CHCl_3$:MeOH (1-10 %) afforded product **8** (0.77 g, 0.22 mmol, 65 %) as a white waxy solid. A single purple spot was visualized by staining with a solution of concentrated H_2SO_4/MeOH 2:8 by TLC analysis. R_f 0.56 ($CHCl_3$:MeOH 9:1). ^{1}H-RMN (400 MHz, $CDCl_3$): δ 7.35-7.18 (m, 38H, Ph-H), 7.15-7.08 (m, 2H, Ph-H), 5.63 (d, *J* = 3.5 Hz, 1H, H-1), 4.91 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.80 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.76 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.66 – 4.26 (m, 14H, H-5', H-6', C**H**$_2$-Ph), 4.26 – 4.24 (m, 4H, -C(=O)OC**H**$_2$CH$_2$O-), 4.13 – 4.03 (m, 3H, H-5, H-3', H-4'), 3.98 – 3.91 (m, 2H, H-3, H-12β$_{chol}$), 3.85 – 3.78 (m, 1H, H-7β$_{chol}$), 3.74 (Brs, PEG backbone), 3.55 – 3.41 (m, 6H, H-2, H-4, H-6, H-1'), 3.26 – 3.17 (m, 1H, H-3β$_{chol}$), 2.61 – 2.51 (m, 4H, -C**H**$_2$C**H**$_2$-), 2.45 – 2.34 (m, 1H, H-23α$_{chol}$), 2.33 – 2.16 (m, 3H, H-4α$_{chol}$, H-9α$_{chol}$, H-23β$_{chol}$), 2.01 – 1.46 (m, 13H, H-1α$_{chol}$, H-2β$_{chol}$, H-4β$_{chol}$, H-6$_{chol}$, H-8β$_{chol}$, H-11$_{chol}$, H-14α$_{chol}$, H-15β$_{chol}$, H-16α$_{chol}$, H-17α$_{chol}$, H-22α$_{chol}$), 1.45 – 1.24 (m, 5H, H-2α$_{chol}$, H-5β$_{chol}$, H-16β$_{chol}$, H-20$_{chol}$, H-22β$_{chol}$), 1.20-1.06 (m, 1H, H-15α), 1.01-0.92 (m, 1H, H-1β$_{chol}$), 0.97 (d, *J* = 6.2 Hz, 3H, 21-C**H**$_{3chol}$), 0.88 (s, 3H, 19-C**H**$_{3chol}$), 0.68 (s, 3H, 18-C**H**$_{3chol}$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.16 (C24$_{chol}$), 172.17 (**C**(=O)O-PEG), 171.99 (**C**(=O)O), 139.21 (C(Ph)), 138.86 (C(Ph)), 138.49 (C(Ph)), 138.26 (C(Ph)), 138.07 (C(Ph)), 138.04 (C(Ph)), 137.92 (C(Ph)), 137.78 (C(Ph)), 128.34 (**C**-H(Ph)), 128.30 (**C**-H(Ph)), 128.27 (**C**-H(Ph)), 128.26 (**C**-H(Ph)), 128.24 (**C**-H(Ph)), 127.92 (**C**-H(Ph)), 127.90 (**C**-H(Ph)), 127.88 (**C**-H(Ph)), 127.86 (**C**-H(Ph)), 127.78 (**C**-H(Ph)), 127.70 (**C**-H(Ph)), 127.64 (**C**-H(Ph)), 127.57 (**C**-H(Ph)), 127.49 (**C**-H(Ph)), 127.25 (**C**-H(Ph)), 104.61 (C2'), 90.09 (C1), 83.70 (C5'), 82.02 (C4'), 81.88 (C3), 79.69 (C2), 78.61 (C3$_{chol}$), 78.25 (C3'), 77.62 (C4), 75.51 (**C**H$_2$-Ph), 74.82 (**C**H$_2$-Ph), 73.34 (**C**H$_2$-Ph), 72.83 (**C**H$_2$-Ph), 72.75 (C12$_{chol}$), 72.56 (**C**H$_2$-Ph), 72.48 (**C**H$_2$-Ph), 70.94 (C1'), 70.54 (PEG backbone), 69.64 (**C**H$_2$-Ph$_{chol}$), 69.17 (PEG backbone), 69.02 (PEG backbone), 68.42 (C6), 68.18 (C7$_{chol}$), 65.54 (C6'), 63.75 (-C(=O)O**C**H$_2$CH$_2$O-), 63.40 ((-C$_{chol}$(=O)O**C**H$_2$CH$_2$O-), 47.05 (C17$_{chol}$), 46.49 (C13$_{chol}$), 41.99 (C14$_{chol}$), 41.42 (C5$_{chol}$), 39.65 (C8$_{chol}$), 36.21 (C4$_{chol}$), 35.19 (C1$_{chol}$), 35.08 (C20$_{chol}$), 34.99 (C10$_{chol}$), 34.56 (C6$_{chol}$), 31.10 (C23$_{chol}$), 30.76 (C22$_{chol}$), 28.79 (-**C**H$_2$**C**H$_2$-), 28.43 (C11$_{chol}$), 27.38 (C16$_{chol}$), 27.23 (C2$_{chol}$), 26.73 (C9$_{chol}$), 23.12 (C15$_{chol}$), 22.62 (C19$_{chol}$), 17.30 (C21$_{chol}$), 12.57 (C18$_{chol}$).

Benzylated Suc-PEG$_{4000}$-Chol (9)

According to the general method **1**, 3α-*O*-benzyl, 7α, 12α-dihydroxy-5β-cholic acid **5** (0.34 g, 0.68 mmol) dissolved in dry CH_2Cl_2 (15 mL), 4-*N,N*-dimethylaminopyridine (82 mg, 0.68 mmol), DCC (0.29 g, 1.36 mmol) and benzylated Suc-PEG$_{4000}$-OH **7** (1.7 g, 0.34 mmol) were reacted for 48 h. Flash chromatography with $CHCl_3$:MeOH (1-10 %) afforded product **9** (1.53 g, 0.28 mmol, 82 %) as a white waxy solid. A single purple spot was visualized by staining with a solution of concentrated H_2SO_4/MeOH 2:8 by TLC analysis. R_f 0.40 ($CHCl_3$:MeOH 9:1). ^{1}H-RMN (400 MHz, $CDCl_3$): δ 7.37-7.17 (m, 38H, Ph-H), 7.15-7.09 (m, 2H, Ph-H), 5.64 (d, *J* = 3.5 Hz, 1H, H-1), 4.92 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.81 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.80 (d, *J* = 10.9 Hz, 1H, C**H**$_2$-Ph), 4.69 – 4.26 (m, 14H, H-5', H-6', C**H**$_2$-Ph), 4.25 – 4.16 (m, 4H, -C(=O)OC**H**$_2$CH$_2$O-), 4.14 – 4.04 (m, 3H, H-5, H-3', H-4'), 3.99 – 3.91 (m, 2H, H-3, H-12β$_{chol}$), 3.86 – 3.79 (m, 1H, H-7β$_{chol}$), 3.78 – 3.39 (m and Brs, PEG backbone, H-2, H-4, H-6, H-1'), 3.27 – 3.17 (m, 1H, H-3β$_{chol}$), 2.63 – 2.52 (m, 4H, -C**H**$_2$C**H**$_2$-), 2.45 – 2.35 (m, 1H, H-23α$_{chol}$), 2.34 – 2.11 (m + H_2O, 3H, H-4α$_{chol}$, H-9α$_{chol}$, H-23β$_{chol}$), 2.01 – 1.73 (m, 8H, H-1α$_{chol}$, H-2β$_{chol}$, H-4β$_{chol}$, H-6β$_{chol}$, H-14α$_{chol}$, H-16α$_{chol}$, H-17α$_{chol}$, H-22α$_{chol}$), 1.71 – 1.24 (m, 10H, H-2α$_{chol}$, H-5β$_{chol}$, H-6α$_{chol}$, H-8β$_{chol}$, H-11$_{chol}$, H-15β$_{chol}$, H-16β$_{chol}$, H-20$_{chol}$, H-22β$_{chol}$), 1.20-1.06 (m, 1H, H-15α$_{chol}$), 0.99-0.91 (m, 1H, H-1β$_{chol}$), 0.98 (d, *J* = 6.1 Hz, 3H, 21-C**H**$_{3chol}$), 0.89 (s, 3H, 19-C**H**$_{3chol}$), 0.69 (s, 3H, 18-C**H**$_{3chol}$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.12 (C24$_{chol}$), 172.12 (**C**(=O)O-PEG), 171.94 (**C**(=O)O), 139.14 (C(Ph)), 138.79 (C(Ph)), 138.41 (C(Ph)), 138.20 (C(Ph)), 138.00 (C(Ph)), 137.97 (C(Ph)), 137.85 (C(Ph)), 137.71 (C(Ph)), 128.28 (**C**-H(Ph)), 128.25 (**C**-H(Ph)), 128.21 (**C**-H(Ph)), 128.19 (**C**-H(Ph)), 127.87 (**C**-H(Ph)), 127.85 (**C**-H(Ph)), 127.84 (**C**-H(Ph)), 127.81 (**C**-H(Ph)), 127.73 (**C**-H(Ph)), 127.65 (**C**-H(Ph)), 127.59 (**C**-H(Ph)), 127.52 (**C**-H(Ph)), 127.44 (**C**-H(Ph)), 127.41 (**C**-H(Ph)), 127.20 (**C**-H(Ph)), 104.55 (C2'), 90.04 (C1), 83.63 (C5'), 81.95 (C4'), 81.82 (C3), 79.62 (C2), 78.57 (C3$_{chol}$), 78.18 (C3'), 77.55 (C4), 75.46 (**C**H$_2$-Ph), 74.77 (**C**H$_2$-Ph), 73.28 (**C**H$_2$-Ph), 72.77 (**C**H$_2$-Ph), 72.69 (C12$_{chol}$), 72.50 (**C**H$_2$-Ph), 72.42 (**C**H$_2$-Ph), 70.88 (C1'), 70.48 (PEG backbone), 69.58 (**C**H$_2$-Ph$_{chol}$), 69.11 (PEG backbone), 68.96 (PEG backbone), 68.33 (C6), 68.11 (C7$_{chol}$), 65.48 (C6'), 63.69 (-C(=O)O**C**H$_2$CH$_2$O-), 63.34 ((-C$_{chol}$(=O)O**C**H$_2$CH$_2$O-), 46.97 (C17$_{chol}$), 46.42 (C13$_{chol}$), 41.91 (C14$_{chol}$), 41.36 (C5$_{chol}$), 39.57 (C8$_{chol}$), 36.14 (C4$_{chol}$), 35.13 (C1$_{chol}$), 35.03 (C20$_{chol}$), 34.93 (C10$_{chol}$), 34.51 (C6$_{chol}$), 31.03 (C23$_{chol}$), 30.69 (C22$_{chol}$), 28.73 (-**C**H$_2$**C**H$_2$-), 28.36 (C11$_{chol}$), 27.33 (C16$_{chol}$), 27.16 (C2$_{chol}$), 26.65 (C9$_{chol}$), 23.07 (C15$_{chol}$), 22.57 (C19$_{chol}$), 17.24 (C21$_{chol}$), 12.51 (C18$_{chol}$).

General procedure 2 for hydrogenation

A solution of the benzylated compound in EtOH:AcOEt:H_2O (7:7:0.1) was saturated with argon for 10 min. After that, Pd-charcoal activated hydrogenation catalyst (Pd 10% wt) was added. The reaction mixture was shaked under 40 psi of H_2 for 24 h. The suspension was filtered through a plug of celite and the residue washed with MeOH. The mixture was then concentrated under reduced pressure.

Suc-PEG$_{2000}$-Chol (10)

According to the general method **2**, benzylated Suc-PEG$_{2000}$-Chol **8** (0.76 g, 0.22 mmol) dissolved in EtOH:AcOEt:H_2O (7:7:0.1) (30 mL) and Pd/C 10% (0.20 g) were reacted for 24 h. Pure **10** (0.62 g, 0.22 mmol, 100 %) was obtained as a white waxy solid. mp 38.60 ºC. ^{1}H-RMN (400 MHz, DMSO-d_6): δ 5.6 (d, *J* = 8.0 Hz, 1H, -O**H**), 5.40 – 5.33 (m, 1H, -O**H**), 5.1 (d, *J* = 3.6 Hz, 1H, H-1), 5.08 – 5.01 (m, 1H, -O**H**), 4.91 – 4.61 (m, 3H, -O**H**), 4.39 – 4.27 (m, 2H, H-6'$_a$, -O**H**), 4.15 – 4.06 (m, 6H, H-6'$_b$, -C(=O)OC**H**$_2$CH$_2$O), 3.99 (d, *J* = 3.2 Hz, 1H, -O**H**), 3.92 – 3.85 (m, 1H, H-3'), 3.83 – 3.73 (m, 2H, H-4', H-12β$_{chol}$), 3.72 – 3.64 (m, 2H, H-5, H-5'), 3.63 – 3.24 (m and Brs, H-3, H-6, H-1', H-7β$_{chol}$, PEG backbone), 3.21 – 3.12 (m, 2H, H-2, H-3β$_{chol}$), 3.08 (t, *J* = 9.3 Hz, 1H, H-4), 2.57 – 2.54 (m, 4H, -C**H**$_2$C**H**$_2$-), 2.36 – 2.26 (m, 1H, H-23α$_{chol}$), 2.26 – 2.07 (m, 3H, H-4α$_{chol}$, H-9α$_{chol}$,

H-23β_{chol}), 2.02 – 1.92 (m, 1H, H-14α_{chol}), 1.83 – 1.55 (m, 6H, H-1$\alpha_{chol,}$ H-6$\beta_{chol,}$ H-15β_{chol} , H-16α_{chol}, H-17α_{chol}, H-22α_{chol}), 1.53 – 0.95 (m, 12H, H-2α_{chol}, H-4$\beta_{chol,}$ H-5β_{chol}, H-6α_{chol}, H-8β_{chol}, H-11$_{chol}$, H-15α_{chol}, H-16β_{chol}, H-20$_{chol}$, H-22β_{chol}), 0.93 – 0.74 (m, 1H, H-1β_{chol}), 0.91 (d, *J* = 6.3 Hz, 3H, 21-C**H**$_{3chol}$), 0.79 (s, 3H, 19-C**H**$_{3chol}$), 0.57 (s, 3H, 18-C**H**$_{3chol}$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.32 (C24$_{chol}$), 171.96 (-**C**(=O)O-PEG), 171.87 (-**C**(=O)O), 104.25 (C2'), 91.74 (C1), 79.17 (C5'), 76.39 (C3'), 74.73 (C4'), 72.84 (C3), 72.78 (C5), 71.62 (C2), 70.97 (C12$_{chol}$), 70.42 (C3$_{chol}$), 70.03 (C4), 69.77 (PEG backbone), 68.33 (-C(=O)O**C**H$_2$CH$_2$O-),), 68.21 (-C(=O)O**C**H$_2$CH$_2$O-), 66.22 (C7$_{chol}$), 65.93 (C6'), 63.47 (PEG backbone), 63.04 (PEG backbone), 61.64 (C-1'), 60.72 (C-6), 46.08 (C17$_{chol}$), 45.76 (C13$_{chol}$), 41.51(C5$_{chol}$), 41.36 (C14$_{chol}$), 39.75 (C4$_{chol}$)*, 39.47 (C8$_{chol}$)*, 35.30 (C1$_{chol}$), 35.00 (C20$_{chol}$), 34.86 (C6$_{chol}$), 34.37 (C10$_{chol}$), 30.70 (C22$_{chol}$), 30.65 (C23$_{chol}$), 30.40 (C2$_{chol}$), 28.51 (C11$_{chol}$), 28.45(-**C**H$_2$**C**H$_2$-), 27.25 (C16$_{chol}$), 26.19 (C9$_{chol}$), 22.80 (C15$_{chol}$), 22.61 (C19$_{chol}$), 16.86 (C21$_{chol}$), 12.30 (C18$_{chol}$).*DEPT

Suc-PEG$_{4000}$-Chol (11)

According to the general method **2**, benzylated Suc-PEG$_{4000}$-Chol **9** (0.90 g, 0.16 mmol) dissolved in EtOH:AcOEt:H$_2$O (7:7:0.1) (30 mL) and Pd/C 10% (0.25 g) were reacted for 24 h. Pure **11** (0.67 g, 0.14 mmol, 88 %) was obtained as a white waxy solid. mp 47.58 ºC. ^{1}H-RMN (400 MHz, DMSO-d_6): δ 5.60 (d, *J* = 8.0 Hz, 1H, -O**H**), 5.45 – 5.35 (m, 1H, -O**H**), 5.13 (d, *J* = 3.6 Hz, 1H, H-1), 5.10 – 5.01 (m, 1H, -O**H**), 4.91 – 4.63 (m, 3H, -O**H**), 4.39 – 4.28 (m, 2H, H-6'$_a$, -O**H**), 4.20 – 4.06 (m, 6H, H-6'$_b$, -C(=O)OC**H$_2$**CH$_2$O), 3.95 – 3.86 (m, 1H, H-3'), 3.85 – 3.76 (m, 2H, H-4', H-12β_{chol}), 3.75 – 3.24 (m and Brs, H-3, H-5, H-6, H-1', H-5'H-7β_{chol}, PEG backbone), 3.22 – 3.14 (m, 2H, H-2, H-3β_{chol}), 3.13 – 3.03 (m, 1H, H-4), 2.60 – 2.54 (m, 4H, -C**H$_2$**C**H$_2$**-), 2.38– 2.27 (m, 1H, H-23α_{chol}), 2.25 – 2.09 (m, 3H, H-4α_{chol}, H-9α_{chol}, H-23β_{chol}), 2.05 – 1.93 (m, 1H, H-14α_{chol}), 1.85 – 1.57 (m, 6H, H-1$\alpha_{chol,}$ H-6$\beta_{chol,}$ H-15β_{chol} , H-16α_{chol}, H-17α_{chol}, H-22α_{chol}), 1.52 – 0.97 (m, 12H, H-2α_{chol}, H-4$\beta_{chol,}$ H-5β_{chol}, H-6α_{chol}, H-8β_{chol}, H-11$_{chol}$, H-15α_{chol}, H-16β_{chol}, H-20$_{chol}$, H-22β_{chol}), 0.91 – 0.76 (m, 1H, H-1β_{chol}), 0.92 (d, *J* = 6.3 Hz, 3H, 21-C**H**$_{3chol}$), 0.76 (s, 3H, 19-C**H**$_{3chol}$), 0.59 (s, 3H, 18-C**H**$_{3chol}$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.36 (C24$_{chol}$), 172.00 (**C**(=O)O-PEG), 171.90 (**C**(=O)O), 104.24 (C2'), 91.76 (C1), 79.19 (C5'), 76.42 (C3'), 74.76 (C4'), 72.87 (C3), 72.79 (C5), 71.64 (C2), 71.02 (C12$_{chol}$), 70.42 (C3$_{chol}$), 70.05 (C4), 69.80 (PEG backbone), 68.35 (-C(=O)O**C**H$_2$CH$_2$O-),), 68.23 (-C(=O)O**C**H$_2$CH$_2$O-), 66.26 (C7$_{chol}$), 65.95 (C6'), 63.50 (PEG backbone), 63.07 (PEG backbone), 61.68 (C-1'), 60.75 (C-6), 46.11 (C17$_{chol}$), 45.79 (C13$_{chol}$), 41.38(C5$_{chol}$), 41.06 (C14$_{chol}$), 35.30 (C1$_{chol}$), 35.00 (C20$_{chol}$), 34.86 (C6$_{chol}$), 34.40 (C10$_{chol}$), 30.72 (C22$_{chol}$), 30.65 (C23$_{chol}$), 30.40 (C2$_{chol}$), 28.56 (C11$_{chol}$), 28.48(-**C**H$_2$**C**H$_2$-), 27.27 (C16$_{chol}$), 26.19 (C9$_{chol}$), 22.79 (C15$_{chol}$), 22.63 (C19$_{chol}$), 16.89 (C21$_{chol}$), 12.32 (C18$_{chol}$).

Crystalline properties characterization by differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were carried out on a Setaram DSC 131 scanning calorimeter equipped with a thermal analysis data system. Samples of 10 mg were placed in aluminum pans and sealed. The probes were heated from -130 ºC to 100 ºC at a rate of 10 ºC/min under nitrogen atmosphere.

Fluorescence spectroscopy

Pyrene emission fluorescence spectra were obtained by using a spectrofluorophotometer (SPEX Fluorolog Spectrofluorimeter). All emission spectra were collected with 2 nm slit bandwidth for excitation and 1 nm for emission, with correction files. The excitation wavelength was 340 nm. The samples were prepared as follows: a known amount of pyrene dissolved in acetone was added to a series of 4 mL vials and the acetone was then evaporated overnight in a vacuum desiccator. The pyrene concentration was then adjusted to give a final concentration of 6.0 x 10^{-7} M in 3 mL of aqueous polymer solutions concentrations from 1 mg/mL to 1 x 10^{-4} mg/mL. The resulting solutions were left overnight at room temperature to equilibrate the pyrene and the polymeric nanoparticles.

4.3 Preparation of Suc-PEG-Chol PNPs

Polymeric nanoparticles (PNPs) were prepared by the nanoprecipitation method. Briefly, accurately weighed 20 mg of the polymer conjugate was dissolved in acetone (2 mL). The resulting polymer solution was added drop-wise into 4 mL magnetically stirring (360 rpm) aqueous solution and agitated at room temperature until complete evaporation of the organic solvent. The obtained nanoparticle suspensions were freeze-dried at 0.5 kPa. The dry powder was stored at 4ºC. Millipore water was used to prepare nanoparticle solutions.

4.4 Characterization of Suc-PEG-Chol PNPs

Particles size and zeta potential

The size distribution and zeta potential of the produced nanoparticles were measured by dynamic light scattering (DLS) using a laser light scattering instrument (SZ-100 nanopartica, Horiba) at 90º scattering angle. Refractive index was 1.330 and temperature was kept at 25 ºC during measuring process. All the tests were run 3 times and took mean values.

Surface morphology – scanning electron microscopy (SEM) and atomic force microscopy (AFM)

The morphology of prepared nanoparticles was observed under an atomic force microscopy (AFM) and a scanning electron microscope (SEM). AFM images were taken by the vibrating mode in air on a TT-AFM instrument from AFM Workshop. A drop of an aqueous nanoparticle solution (0.1 mg/mL) was deposited onto freshly cleaved mica lamella and these dried overnight at 4 ºC or freeze-dried.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Supporting information provides experimental data for molecules **2-11**: ^{1}H-NMR. MALDI-TOF analysis and DSC of conjugates **10** and **11**. Determination of CAC for Suc-PEG4000-Chol conjugate. Particle size distributions and AFM images of Suc-PEG-Chol nanoparticles. See DOI: 10.1039/b000000x/

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Surfactant-free polymeric nanoparticles composed of PEG, cholic acid and sucrose moiety

New amphiphilic polymers synthesized from a sucrose-containing conjugate exhibited interesting self-assembly properties in water. Owing to their amphiphilic characteristics polymeric nanoparticles were prepared by nanoprecipitation method without any surfactants. These nanoparticles formulated with biocompatible building blocks can be considered a potential candidate for drug delivery applications.