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Synthesis and application of poly(ethylene glycol)–cholesterol (Chol–PEG^m) conjugates in physicochemical characterization of nonionic surfactant vesicles

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Abstract

Vesicles possessing poly(ethylene glycol) (PEG) chains on their surface have been described as a blood-persistent drug delivery system with potential applications for intravenous drug administration. In this research with different molecular weights (400–10,000 g/mol) of PEG, a series of Chol–PEG^m conjugates were generated by the DCC (*N*,*N*′-dicyclohexylcarbodiimide, DCC)/(4-dimethylaminopyridine, 4-DMAP) esterification method, and confirmed by FT-IR and ^{1}H NMR spectrum. Then their properties in aqueous solution were studied by electron microscopy images, associative behavioral and systematic tensiometric studies over a wide concentration range. In order to elucidate the application of this Chol–PEG^m in vesicles, conventional nonionic surfactant vesicles (niosomes) composed of span 60 and cholesterol were prepared and the influence of various hydrophilic chains of the Chol–PEG^m conjugates was investigated. Results indicated that all the niosomes prepared, with or without Chol–PEG^m composition were similar in micrograph with diameter between 120 nm and 180 nm. The fixed aqueous layer thickness (FALT) around niosomes increased as Chol–PEG^m chain length increase, particularly in the Chol–PEG^{10,000} modified niosomes with 9.33 ± 0.67 nm. *In vitro* release experiments indicated that release rate of nimodipine from Chol–PEG^m modified niosomes was enhanced. Chol–PEG^m modified niosomes showed greater accumulative release than that of plain niosomes over a period of 24 h. These studies have shed some light on the suitability of Chol–PEG^m containing niosome preparation.

Keywords: Niosome; Poly(ethylene glycol)–cholesterol conjugates; Cholesterol; Fixed aqueous layer thickness (FALT); *In vitro* release

1. Introduction

Designing new vehicles for old drugs is an important field of pharmaceutical research, since in many treatments the severe collateral effects of a drug reduce greatly the use of therapeutically compounds. New formulations, in particular liposomes and nonionic surfactant vesicles (niosomes) have brought about increasing interest [1,2]. However, a liposome is provided with a number of serious limitations such as high cost and instability [3], while vesicles prepared from some ionic surfactants are without fine biological acceptability in practice. The low cost, ease of storage, high chemical stability and also large numbers of available vesicles forming nonionic surfactants make niosomes [4–6] more attractive than liposomes for pharmaceutical and industrial applications.

Rather inconveniently these carriers, when given intravenously, were rapidly cleared by the cells of the mononuclear phagocytes system (MPS). Modifications in the composition of the systems, as stealth vesicle drug carriers, can promote changes in structural features and consequently in the mechanical behavior. It could avoid, or at least reduce the uptake by phagocytes and prolong the time of drug at effective concentration in blood circulation. Since pharmacokinetic behavior is strongly dependent on the physicochemical properties of the conjugates used, and affected in turn, by the characteristics of conjugates, in term of composition, molecular weight, hydrophobic/hydrophilic chain length ratio. Hence, a proper physicochemical characterization of aggregational behavior of these systems is a good instrument

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for designing and preparing conjugates with potential in biopharmaceutical field [7,8]. The moieties of poly(ethylene glycol)s (PEGs) or their derivatives with different molecular weight as membrane active agents commonly used within modern pharmaceutical chemistry, could extend in "mushroom" or "brush" chain conformation from the membrane plane towards the water solution, and form thick hydrophilic layers at the membrane surface [9,10]. Many studies have examined the interaction between the prolonged circulation time and the conditions of the PEG-molecule on the surface in terms of their physicochemical properties by PEG-modification [11–13]. The fixed aqueous layer thickness (FALT) was measured and it was reported that the FALT of PEG-modified liposomes increased in comparison with PEG-unmodified liposomes [14,15]. This was responsible for the uptake of foreign particles out of the blood flow, since the hydrophilic layer creates a steric barrier preventing the adsorption of lipoproteins and opsonins to the liposome surface.

Therefore, many studies have been concentrated on the development of a wide variety of amphiphilic associated conjugates containing PEG segment as modifier to drug carriers, such as dimyristoyl phosphatidylethanolamine–bonded PEG (DMPE–PEG) [16] or distearoyl phosphatidylethanolamine/methoxy poly(ethylene glycol) (DSPE–PEG) [17]. However, despite the abundant literature about these widely used associated conjugates in the biomedical field as surface modifiers of some particulate drug carriers like conventional liposomes, emulsion and nanoparticles, the application in niosomes is rare. In addition, the expense of phosphatidylethanolamine derivatives and the difficulties in obtaining large quantities of this lipid, make therapeutic applications of PEG-containing drug carriers impractical.

Cholesterol is a candidate since it confers cohesion on lipid bilayers by increasing the half-life of liposomes *in vivo* [18] and PEGylated cholesterol molecules can be easily synthesized in large amounts on an industrial scale. The synthesis and properties of PEG-liposomes containing two new functionalized PEG cholesteryl derivatives linked via stable carbamate bonds have been reported [19] and others linked by ether and carbonate bonds have been studied previously [20]. In this study, we present poly(ethylene glycol)–cholesterol (Chol–PEG^m) conjugates formed using a simple and inexpensive method which has not been previously reported. Subsequently, the influence of Chol–PEG^m candidates (m is the different molecular weight of PEG at 400–10,000 g/mol) on the physicochemical characterization of niosomes has been extended to evaluate the particle size, morphologies and zeta potential for FALTs as well as *in vitro* releasing capacity monitored by the model substance nimodipine.

2. Materials and methods

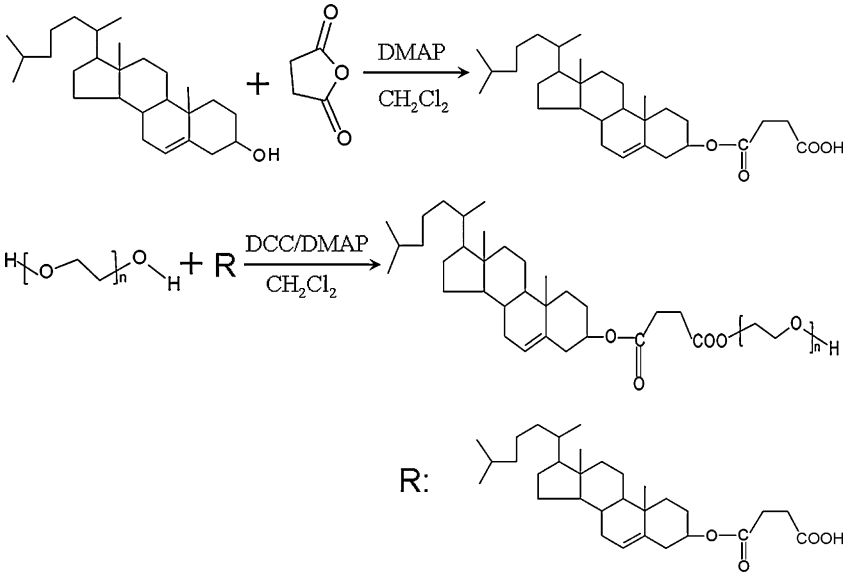
2.1. Materials

N,*N*′-dicyclohexylcarbodiimide was obtained from Sinopharm Chemical Reagent Co., Ltd. The chemical, 4-dimethylaminopyridine, was purchased from the Zhejiang Xianju Pharmaceutical and Chemical Experimental Plant. Cholesterol (China Medicine Shanghai Chemical Reagent Corporation, China) and succinic anhydride (Shanghai Chemical Reagent Co., Ltd., China) were used as received. Poly(ethylene glycol) with M_n 400, 1000, 2000, 4000, 6000 or 10,000 g/mol were precipitated from tetrahydrofuran solution into ether. CH_2Cl_2 was refluxed over P_2O_5 and then distilled. Nimodipine was provided by Shandong Xinhua Pharmaceutical Co., Ltd., China. All other reagents were of AR.

2.2. Methods

2.2.1. General procedures of Chol–PEG^m conjugate synthesis

The synthesis involved two steps of chemical modification on cholesterol by esterification, one with succinic anhydride to



Scheme 1. Chemical reaction scheme for preparing the Chol–PEG^m conjugate.

obtain active carboxylic terminal, and then with PEG to obtain sufficiently hydrophilic terminal according to Scheme 1.

2.2.1.1. Synthesis of succinyl cholesterol. Cholesterol (38.6 g, 0.1 mol) and succinic anhydride (15 g, 0.15 mol) were charged into a round bottom flask in CH_2Cl_2 (200 ml) and 4-DMAP (12.2 g, 0.1 mol) added as catalyst. A slight excess of succinic anhydride was used to ensure that hydroxy terminals were completely reacted. The mixture was stirred at 65 °C for 12 h until ester carboxylic acid was obtained. Once the reaction had cooled down, the unreacted succinic anhydride was easily removed by filtration because unreacted succinic anhydride is insoluble in cold CH_2Cl_2. CH_2Cl_2 in the mixture was removed using rotary evaporator. Finally, the product was washed with copious amounts of water (500 ml) on a filter funnel and dried overnight. TLC analysis (acetone:petroleum ether:acetic acid, 2:6:1) visualized with iodine vapour showed more polar spot.

2.2.1.2. Synthesis of Chol–PEG^m conjugate. The Chol–PEG^m was done in principle as described by Ammazzalorso et al. [21]. PEG (10 mmol), succinyl cholesterol (10 mmol), DCC (10 mmol) and a catalytic amount of DMAP in anhydrous CH_2Cl_2 (200 ml) was stirred at room temperature for 24 h, after which the *N*,*N*′-dicyclohexylurea salts precipitated and were removed by filtration. The filtrate was evaporated under reduced pressure and redissolved in a small volume of CH_2Cl_2 with addition of several drops of tetrahydrofuran. The solution was stored at 4 °C overnight and the white precipitate was collected. The pellet was redissolved in chloroform and then further purified by column chromatography on silica gel H using chloroform/methanol (30:1, v/v) as an eluent. A single spot by TLC analysis (CH_2Cl_2:methanol:ethyl acetate, 7:1:1) was visualized with iodine vapour. Conjugates with different chain lengths were prepared with the same procedure and named as Chol–PEG^{400}, Chol–PEG^{1000}, Chol–PEG^{2000}, Chol–PEG^{4000}, Chol–PEG^{6000}, Chol–PEG^{10,000}, respectively with corresponding PEG.

2.2.1.3. Structural analysis. IR spectra of succinyl cholesterol, PEG and the reaction products were recorded on a Bruker FT-IR Tensor 27 spectrophotometer (KBr disk). ^{1}H NMR was performed on a 300 MHz Bruker NMR spectrometer using $CDCl_3$ as a solvent.

2.2.2. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

SEM images were obtained at 15.0 kV on a JSM-5310-LV microscope (Jeol, Japan). The sample was sputter coated with an approximately 10 nm thick layer of gold to improve its interface conductivity before testing. TEM observation of Chol–PEG^m dispersed in double distilled water was photographed with an H-7000 transmission electron microscope (Hitachi, Japan) at an accelerating voltage of 75 kV. A drop of the resultant conjugate dispersion was placed onto a carbon-coated copper grid, forming a thin liquid film. The films on the grid were negatively stained by adding immediately a drop of 2% (w/w) phosphotungstic acid, removing the excess solution using a filter paper and followed by a thorough air-drying.

2.2.3. Viscosity measurements

Measurements of the steady-state viscosity were performed on a Brookfield viscometer (Brookfield DV-III) with Rheocak v2.7 data manager. Chol–PEG^m dispersion, at concentrations between 5.0 μg/ml and 50.0 μg/ml in distilled water was loaded in a tubular aluminum sample holder, and the relationship of shear stress–shear rate was treated at a concentration of 50.0 μg/ml. The measurement was carried out at 20 °C with various angular velocities. A typical reaction was performed within a shear rate range of 50–350 s^{-1}. The device registered viscosity values every 20 s.

2.2.4. Surface activity studies

The surface activity of all Chol–PEG^m conjugates and their ability to self-aggregate have been established by performing a systematic tensiometric study in aqueous solution. The critical micelle concentrations of the conjugates in aqueous solution were estimated as the inflexion point of plots of the surface tension versus conjugate concentration. All equipments were interfaced to a computer for both data collection and analysis.

2.2.5. Preparation and characterizations of the Chol–PEG^m incorporating niosomes

Nimodipine-containing vesicles with or without Chol–PEG^m were prepared as follows: Aliquots of chloroform/methanol (6:1, v/v) solutions of nimodipine/span 60/cholesterol and/or Chol–PEG^m (5 μmol:100 μmol:30 μmol:5 μmol) were mixed into a round bottomed flask, which was then evaporated to dryness under reduced pressure by rotary evaporator. The thin lipid film was evacuated in a desiccator and then hydrated with 5.0% (w/v) glucose in a water bath under mechanical agitation at 60 ± 1 °C for 30 min. The vesicle suspension was sonicated for 30 min until optically clear, using a probe type ultrasonic generator (JY 92-II, Scientz Biotechnology. Co., Ltd., Ningbo, China) to obtain niosomes homogeneous in size. The suspension was allowed to cool to room temperature and was then centrifuged to remove any metal particles released from the probe tip. The niosome, without composition of Chol–PEG^m, was expressed as plain niosome. In this regard, PEG-modified niosomes modified with a Chol–PEG^m (400–10,000 g/mol, molecular weight of PEG) were expressed as Chol–PEG^{400}–Niosome (NS), Chol–PEG^{1000}–NS, Chol–PEG^{2000}–NS, Chol–PEG^{4000}–NS, Chol–PEG^{6000}–NS, Chol–PEG^{10,000}–NS.

2.2.6. Particle size and FALT of Chol–PEG^m modified niosomes

The particle sizes, zeta potentials and polydispersity index of the niosomes were measured with a photon correlation spectrometer light scattering apparatus zeta potential/particle sizer 3000HS (Malvern Instruments, UK) and analyzed by the Zetasizer 3000H (MALVERN software). The polydispersity index discloses the quality of the dispersion, from values lower than 0.7 for suitable measurements and good quality of the colloidal suspensions. The fixed aqueous layer thickness of niosomes was

determined by zeta potential measurements of niosomes in NaCl solutions of different ion concentrations. The calculation of FALT (L) was based on the linear correlation between ln ζ (zeta potential) and κ (Debye Huckel-parameter): $\ln\zeta = \ln A - \kappa L$, where A is a constant related to the surface charge density and κ can be expressed in nm^{-1} unit as $\kappa = 3.3\sqrt{C}$, where C is the concentration of NaCl solution [14]. Then ln ζ is plotted against κ and a straight line is obtained. The slope gives L, FALT in nm unit.

2.2.7. In vitro release experiments

Various test niosomes were subjected to release study using the "flow-through" method (USP apparatus 4) on a semi-automated flow-through dissolution–dialysis system (SOTAX CE 7smart, SOTAX Co., Ltd., Switzerland), which is a modified version of the dissolution–dialysis assembly of Shah and Sheth [22]. The sample was placed in a dialysis bag and then transferred to a small volume cell (internal diameter 22.6 mm) through which solvent passed with 4.0 ml/min constant "flow-through" rate at a bath temperature of 37 °C. The eluate was filtered upon leaving the cell and then collected in 4.0 ml automatically using the WinSOTAX Advanced Dissolution Software at preset intervals over a period of 24 h, and then manually compensated by fresh water at each sampling point. A spectrophotometer (SPD-20A, Shimadze Co. Ltd., Kyoto, Japan) at 237 nm was applied to analyze the sample content of nimodipine. The "flow-through" method permits constant optimal sink conditions due to the continuous flow of fresh solvent.

3. Results and discussion

3.1. Synthesis and characterization of Chol–PEG^m

The main aim of this study was to obtain functionalized PEGylated cholesterol derivatives conferring steric stabilization to niosomes, which are easier to obtain on an industrial scale than PEG–PE [23,24]. The direct reaction of the hydroxy group at three-position of cholesterol with the various long chain poly(ethylene glycol) was difficult for providing steric resistance. Therefore, succinyl cholesterol was produced in the first step to obtain free carboxylic terminal for the following reaction. The excess of succinic anhydride was used to ensure that equilibrium was obtained. In the second step, Chol–PEG^m conjugates were prepared using the corresponding PEG as reagent and 4-dimethylaminopyridine as catalyst. Dicyclohexylcarbodiimide was added as condensation agent removing the water formed during esterification by its reaction to dicyclohexylurea, which could be precipitated from the reaction mixture.

To detect if esterification occurred, the FT-IR spectra was measured and the spectra of succinyl cholesterol, PEG and Chol–PEG^m were displayed. It was observed that the carbonyl stretching vibration of ester linkage for succinyl cholesterol (Fig. 1A) and Chol–PEG^{2000} (Fig. 1C) was located at 1730 cm^{-1} and 1735 cm^{-1}, which was absent in the original PEG (Fig. 1B). These results indicated the formation of ester by the reaction between the hydroxyl group in PEG and the carboxylic group of succinyl cholesterols. At 1109 cm^{-1} and 1112 cm^{-1} in the

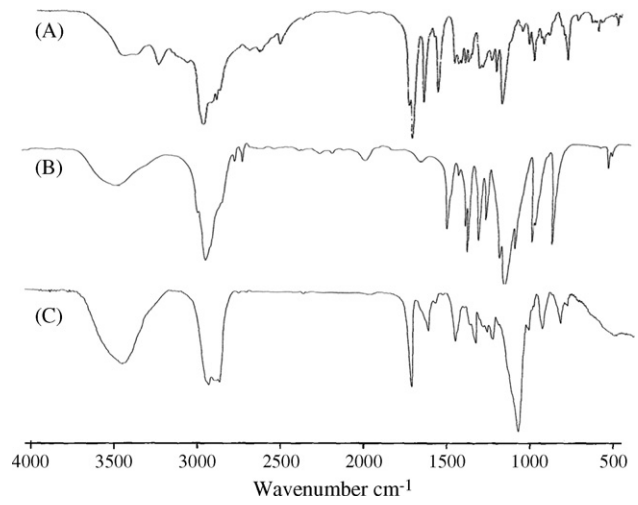


Fig. 1. FT-IR transmittance spectra for (A) succinyl cholesterol, (B) PEG^{2000} and (C) Chol–PEG^{2000}.

fingerprint region corresponding to –C–O–C– stretching vibration in PEG^{2000} and Chol–PEG^{2000}, respectively. The band at 2946 cm^{-1} (Fig. 1A), 2889 cm^{-1} (Fig. 1B) and 2934 cm^{-1} (Fig. 1C) were the characteristic vibration of C–H stretches. –OH stretching bands of succinyl cholesterol, PEG^{2000} and Chol–PEG^{2000} consisted of a broad band centered at 3411 cm^{-1} (Fig. 1A), 3424 cm^{-1} (Fig. 1B) and 3425 cm^{-1} (Fig. 1C), respectively. All these results confirmed that cholesterol had been reacted into place on the PEG backbone.

The ^{1}H NMR spectrum of the Chol–PEG^m was also resolved in Fig. 2. The six- and three-position protons in cholesterol were found at 5.35 ppm and 4.58 ppm, respectively. Signals at 4.24 ppm assigned to the methylene protons in PEG adjacent to the succinyl group appeared upon the reaction. The signals at 3.71–3.54 ppm were attributed to the repeating units in PEG. A multiplet was present at 2.64 ppm, corresponding to the methylene proton of succinyl group (–CH_2–C=O). In addition, the signals appearing at 2.32 ppm contributed to the four-position of the cholesterol, and all of the other methylene protons in cholesterol were at 2.29–0.67 ppm. These results further indicated that

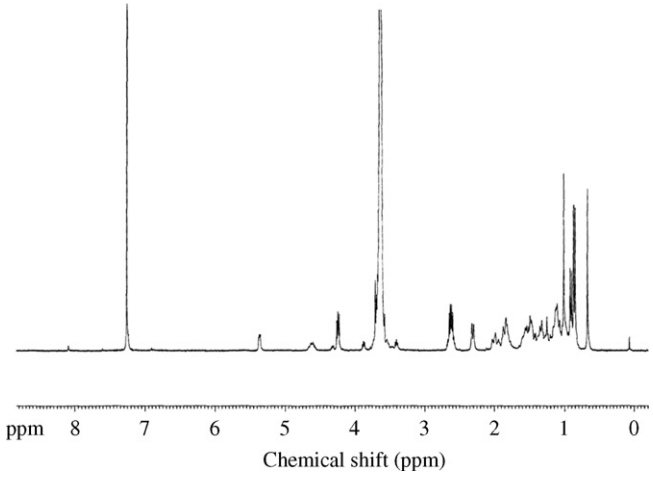


Fig. 2. 300 M ^{1}H NMR spectrum of Chol–PEG^{2000} in $CDCl_3$.

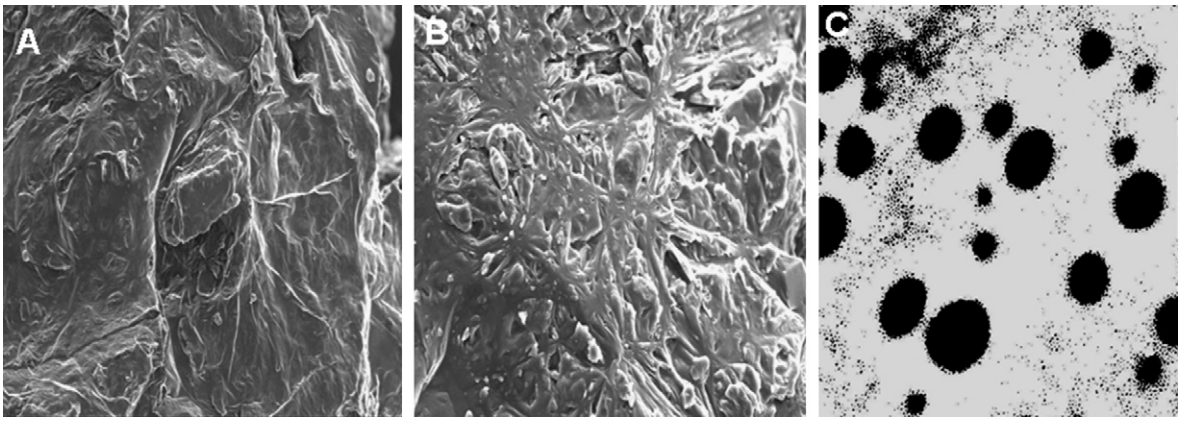


Fig. 3. SEM micrographs of (A) PEG^{2000} and (B) Chol–PEG^{2000} at 500× magnification. (C) TEM image of Chol–PEG^{2000} in double distilled water at 40,000× magnification.

the esterification had definitely occurred. The functionalization ratios were determined from the ^{1}H NMR signal integration (4.24 ppm) of the methine group with 0.98, 0.94, 0.10, 0.95, 0.97 and 1.02, respectively, for according PEG with molecular weights of 400, 1000, 2000, 4000, 6000 and 10,000 g/mol.

3.2. SEM and TEM determination of Chol–PEG^{m}

The morphology of Chol–PEG^{m} conjugates was amorphous from the scanning electron micrographs. This demonstrated there were no crystal structures formed. Fig. 3A and B showed the SEM images of PEG^{2000} and Chol–PEG^{2000}. The TEM images (Fig. 3C) of Chol–PEG^{m} conjugates (with PEG number average molecular weights of 400–10,000 g/mol) in double distilled water were similar to each other. After dissolving in double distilled water, Chol–PEG^{m} formed spherical micelles of uniform size in the water.

3.3. Rheological studies

The shear stress–shear rate relationship of a 50.0 μg/ml Chol–PEG^{m} dispersion in distilled water was shown in Fig. 4, where it was clear that the rate of increase of shear stress was decreased with the increase in shear rate showing a typical Newtonian viscosity behavior. The Herschel–Bulkley model [25] took account of both the yield stress and the shear thinning behavior of Chol–PEG^{m} conjugates and fitted the experimental data very well (correlation coefficient exceeded 0.99 for all conjugates prepared). Therefore, this model was appropriate to describe the rheological properties for the phenomenon of shear thinning observed. Shear thinning results from the tendency of the applied force to disturb the long chains from their favored conformation, causing elongation in the direction of shear [26]. Although, PEG is a nonionic hydrophilic chain, the presence of the cholesteryl group may result in hydrophobic associations. In addition, all the Chol–PEG^{m} conjugates synthesized dispersion in distilled water were of very low viscosity, and the rheological characteristics of the investigated samples were markedly influenced by the concentration of the compound. The highest viscosity was observed for Chol–PEG^{10,000} at the highest concentration with viscosity of 1.98 ± 0.11 cps, 2.13 ± 0.09 cps, 2.34 ± 0.06 cps, 3.09 ± 0.12 cps, 5.46 ± 0.10 cps and 8.98 ± 0.22 cps for Chol–PEG^{400}, Chol–PEG^{1000}, Chol–PEG^{2000}, Chol–PEG^{4000}, Chol–PEG^{6000} and Chol–PEG^{10,000}, respectively. Fig. 5 shows

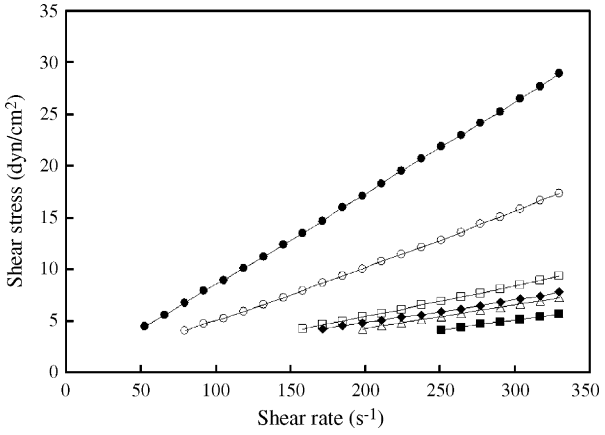


Fig. 4. Shear stress versus shear rate for 50.0 μg/ml (■) Chol–PEG^{400}, (△) Chol–PEG^{1000}, (♦) Chol–PEG^{2000}, (□) Chol–PEG^{4000}, (◯) Chol–PEG^{6000} and (●) Chol–PEG^{10,000} suspension in distilled water at 20 °C.

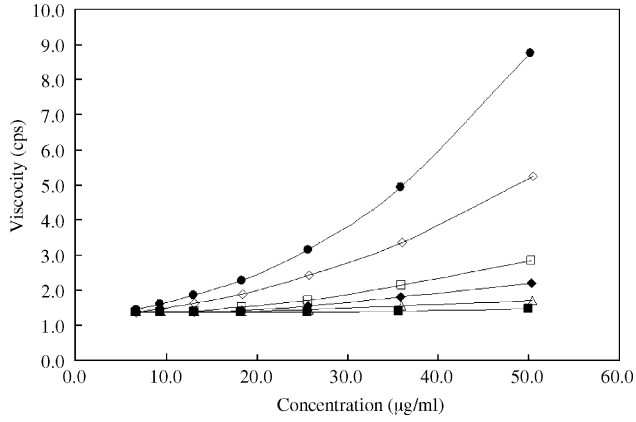


Fig. 5. Change in viscosity versus concentration of the (■) Chol–PEG^{400}, (△) Chol–PEG^{1000}, (♦) Chol–PEG^{2000}, (□) Chol–PEG^{4000}, (◊) Chol–PEG^{6000} and (●) Chol–PEG^{10,000} suspended in distilled water at 20 °C.

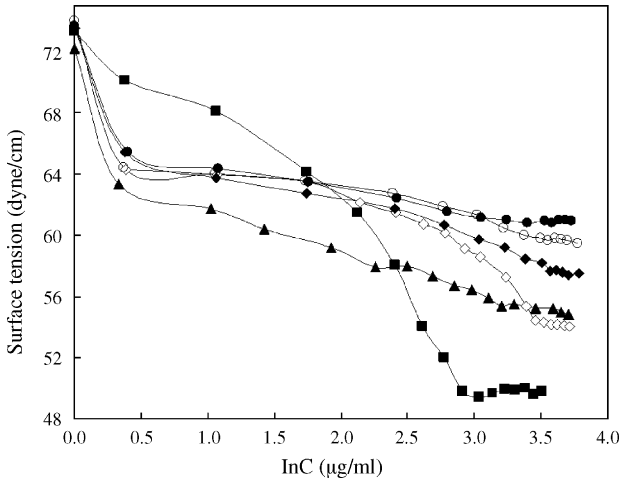


Fig. 6. Plots of surface tension as a function of the Chol–PEG^m conjugate concentration for (■) Chol–PEG^{400}, (▲) Chol–PEG^{1000}, (◇) Chol–PEG^{2000}, (♦) Chol–PEG^{4000}, (◯) Chol–PEG^{6000} and (●) Chol–PEG^{10,000} at 20 °C.

the variation in the viscosity according to the conjugate concentration for Chol–PEG^m. This study revealed a marked associative behavior: the viscosity of Chol–PEG^m was systematically higher than that of the non-modified PEG^m, and the evolution with the concentration was not linear. This particular rheological property was explained by the incompatibility between various groups, for example, hydrophilic and hydrophobic groups, within the same macromolecule. Depending on the importance of the repulsion and attraction forces (steric, electrostatic, etc.) these compounds may have a more or less marked antagonistic character [27,28]. Suitable combinations of a grafted layer and a membrane barrier structure with steric effect are essential to particulate drug carriers.

3.4. Surface tension data

Fig. 6 shows the surface tension of different Chol–PEG^m conjugate dispersion over the concentration of 0–45.0 μg/ml. The Chol–PEG^m with different PEG chain has exerted a surface activity with large variation to the critical micelle concentration (c.m.c.), and the results were shown in Table 1. The c.m.c. values of Chol–PEG^m were estimated from the break point of surface tension as a function of conjugate concentration. From Table 1, in terms of the different chain lengths, the highest critical surface tension goes to Chol–PEG^{400}, and the higher critical surface tensions in turn goes to Chol–PEG^{1000}, Chol–PEG^{2000}, Chol–PEG^{4000}, Chol–PEG^{6000} and the lowest Chol–PEG^{10,000}. Addition of the PEG^{10,000} chain in the Chol–PEG^{10,000} skeleton decreased the surface tension value of about 10 dyne/cm (mN/m), with respect to pure water, while the presence of the PEG^{400} chain in the conjugate backbone lead to a decrease of about 20 dyne/cm. These trends were in line with the macromolecular nature of the conjugates. In fact, the surface activity of the Chol–PEG^m can be attributed to its weak amphiphilic nature while the cholesteryl tail confers to the conjugate a very hydrophobic character. The chain in Chol–PEG^{10,000} was longest which may be in particular had higher flexibility, giving a difficulty to align on the surface, and so surface tension was the highest. As it can be seen from the surface tension plateau values, the ability to decrease the surface tension was brought about by the equilibrium of its surface-active analytes comprising both hydrophobic and hydrophilic structural regions. Thus, in aqueous solution, these species diffused towards the air/liquid interface and were preferentially adsorbed at the surface, thereby lowering the surface tension of the solution [29,30].

Table 1
Values of c.m.c. for the Chol–PEG^m in distilled solution

Chol–PEG^m conjugates	c.m.c. value (μM)
Chol–PEG^{400}	12.70 ± 2.03
Chol–PEG^{1000}	4.67 ± 0.18
Chol–PEG^{2000}	3.43 ± 0.11
Chol–PEG^{4000}	3.17 ± 0.17
Chol–PEG^{6000}	2.44 ± 0.08
Chol–PEG^{10,000}	1.55 ± 0.07

c.m.c. values of Chol–PEG^m were estimated from the break point of surface tension as a function of conjugate concentration at 20 °C.

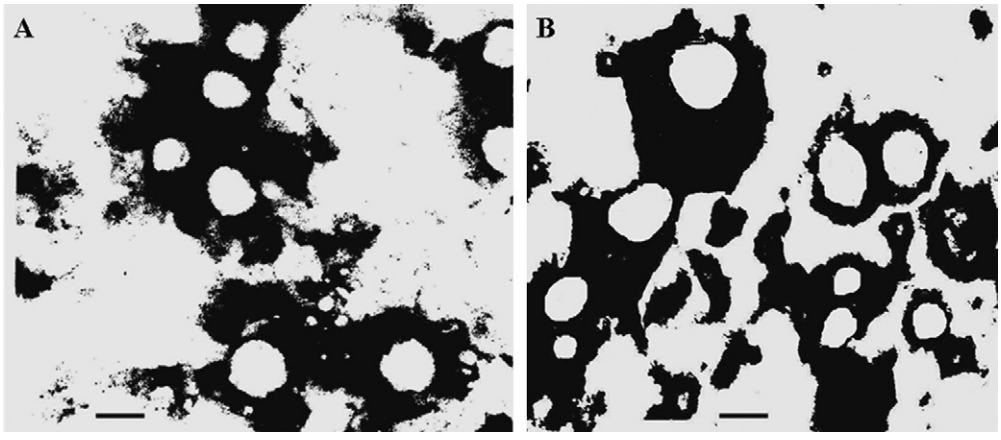


Fig. 7. The negative staining TEM photograph of the niosome system. (A) Nimodipine/span 60/cholesterol (5:100:30 molar ratio) vesicles and (B) nimodipine/span 60/cholesterol/Chol–PEG^{2000} (5:100:30:5 molar ratio) vesicles. Scale bar: 100 nm.

Table 2
Physicochemical parameters of Chol–PEG^m modified niosomes and plain (not Chol–PEG^m modified) niosomes

	Particle size (nm)	Zeta potentials (mV)	P.I.[a]	FALT[b] (nm)
Plain niosome	120.4 ± 4.3	−52.1 ± 3.4	0.33 ± 0.071	0.68 ± 0.04
Chol–PEG^{400}–NS	121.6 ± 5.5	−47.7 ± 5.7	0.36 ± 0.072	1.23 ± 0.48
Chol–PEG^{1000}–NS	145.2 ± 2.8	−38.2 ± 1.7	0.38 ± 0.049	1.30 ± 0.15
Chol–PEG^{2000}–NS	164.4 ± 3.2	−32.2 ± 3.0	0.40 ± 0.042	2.80 ± 0.15
Chol–PEG^{4000}–NS	162.9 ± 5.1	−26.8 ± 2.1	0.36 ± 0.046	2.91 ± 0.47
Chol–PEG^{6000}–NS	176.9 ± 2.6	−23.8 ± 1.2	0.34 ± 0.037	5.31 ± 0.13
Chol–PEG^{10,000}–NS	166.1 ± 5.3	−5.9 ± 0.8	0.36 ± 0.056	9.33 ± 0.67

Each data were expressed as the mean ± S.D. ($n \geq 3$).
[a] P.I.: polydispersity index.
[b] FALT: fixed aqueous layer thickness.

3.5. Physicochemical characteristics of plain niosomes and Chol–PEG^m modified niosomes

All of the niosomes prepared were apparently white, soft and homogenous dispersion and did not show any changes in appearance, homogeneity when modified with Chol–PEG^m. The size and morphology of the niosomes can be determined by dynamic light scattering and observed by negative staining-transmission electron microscopy (Fig. 7). The TEM images of Chol–PEG^m modified niosomes (with PEG number average molecular weights of 400–10,000 g/mol) were spherical and similar to each other. The average diameter of the niosomes prepared was between 120 nm and 180 nm. Compared with plain niosomes, the average diameter of niosomes modified with Chol–PEG^m was greater (Table 2), which suggested that the PEG chains were located on the surface of the niosomes. While the increase was different in the case of different Chol–PEG^m modified niosomes, which might be due to the different surface density of the layer of PEG chains. A higher density of stealth agent on the niosome surface may favor the interaction between niosomes, increasing the average diameter value. Indeed a small increase of the polydispersity index (Table 2) was seen to confirm this hypothesis [31,32].

Due to the amphiphilic nature of the Chol–PEG^m introduced in the external aqueous phase, it was expected that the corresponding niosomes would be coated by them. This expectation was certainly in agreement with the zeta potential values observed for particles prepared with different chain length of Chol–PEG^m conjugates in Table 2. Indeed, this reduction in the surface charge of niosomes, which was decreased with the increase of the chain length of Chol–PEG^m conjugates indicating the presence of PEG chains on the surface, may have been due to the PEG-aqueous layer. The slipping plane is moved to a point further out from the surface where the charge density is much smaller than that on the surface, and therefore lower zeta potentials were recorded [33].

Further evidence of the effect that the incorporation of PEG has on the surface of niosomes was the observed FALT value. Several results suggested that FALT was one of the important factors that defined the pharmacokinetics of the PEG-modified vesicles. Namely, an increase of the FALT led to improvements of the escape ability from RES and circulation in the blood [34]. As shown in Table 2, the FALT around the niosomes increased with the increase in PEG-molecular weight, but the FALT value of Chol–PEG^{1000} was similar to Chol–PEG^{2000}. The FALT of the Chol–PEG^{10,000} modified niosomes increased remarkably, and was 9.33 ± 0.67 nm. Modeling of PEG-lipid has shown that at least two regimes can be identified, "mushrooms" (isolated grafts) and "brushes" (extended chain conformations determined by the interaction between neighboring chains [35]. In Chol–PEG^m modified niosomes, the phenomenon could suggest that, because, PEG lipid with a large molecular weight constructed more complete "mushroom" structures and the increased density of the PEG on the niosome membrane was induced to change from the mushroom structure to the brush structure as previous reports [14].

3.6. In vitro drug release study

Dissolution profiles of the investigated Chol–PEG^m modified niosome and plain niosome were determined using the USP 4 "flow-through" method. Mean values ($n = 3$) were used to construct the dissolution curves, but standard deviation bars were omitted to avoid overlapping. Fig. 8 gave the releasing behaviors *in vitro* of niosomes modified by Chol–PEG^m and plain niosomes. The release data indicated predominately dif-

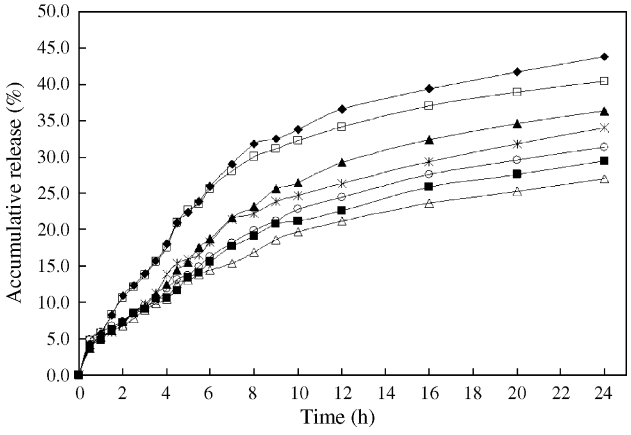


Fig. 8. *In vitro* cumulative release profiles of nimodipine from plain niosome and Chol–PEG^m modified niosome at 37 °C using dialysis–dissolution method, for (△) plain niosome, (■) Chol–PEG^{400}–NS, (◯) Chol–PEG^{1000}–NS, (*) Chol–PEG^{2000}–NS, (▲) Chol–PEG^{4000}–NS, (□) Chol–PEG^{6000}–NS and (♦) Chol–PEG^{10,000}–NS.

fusional drug release mechanism with sustained and prolonged drug release. The cumulative release of nimodipine in plain niosomes was $27.02 \pm 1.33\%$ for 24 h, and the rate of drug releasing was enhanced with the increase of chain length of Chol–PEG^m in niosomes. Chol–PEG^{10,000}–NS showed the greatest accumulative release of $43.86 \pm 3.95\%$ over a period of 24 h. The difference could be explained by the fact that the PEG chains on the niosome surface acting as a hydrophilic shield surrounding niosomes, as a consequence, the surface tension of the particles was reduced [36]. The Chol–PEG^m chain has the cholesteryl group at the terminal. It was considerable that Chol–PEG^m can be easily incorporated into lipid bilayer. The incorporation of Chol–PEG^m into lipid bilayer could increase the surface hydrophilicity of niosomes, and inhibit niosomes aggregating. A plausible explanation in agreement with the decreasing tendency of zeta potential and increasing the FALT was the enhanced extension of a "hairy layer" of water-soluble PEG moiety on the surface of niosomes (Table 2). Due to the incorporation of hydrophilic PEG, the surface layer and even the inside as well were somewhat laden with water, and probably the swollen state of the particle enhanced faster release and achieved a higher over-all drug release [37].

4. Conclusion

Cholesterol confers cohesion on lipid bilayers and PEGylated cholesterol seemed quite appropriate. The novel and simple method presented here is suitable for producing Chol–PEG^m conjugates. Steady-state rheological measurements revealed that all the investigated samples exhibited Herschel–Bulkeley flow behavior, and the Chol–PEG^m produced were amphiphilic molecules with surface activity. The physicochemical characterisation of niosomes prepared by span 60/cholesterol, with or without Chol–PEG^m modification included an investigation of their properties, such as zeta potential, size and morphology. The images of TEM indicated that the niosomes were spherical in shape and homogeneous in size. FALTs around niosomes were estimated by a method based on the zeta potential, and these increased with the chain length of Chol–PEG^m. The drug release rate was enhanced in Chol–PEG^m modified niosomes for the hydrophilic shield around the drug carrier. Taken together, these results suggested that Chol–PEG^m could be used to modify niosomes as a new drug carrier. However, to confirm the behavior of drug release, the speed of clearance, and the final biodistribution of Chol–PEG^m modified niosomes *in vivo*. These issues of delivery and assimilation will be pursued in our future work.

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