Synthesis and Characterization of PEG-*graft*-Quaternized Chitosan and Cationic Polymeric Liposomes for Drug Delivery

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ABSTRACT: Poly(ethylene glycol) grafted octadecyl quaternized carboxymethyl chitosan (PEG-*g*-OQC) copolymers were synthesized to both improve the biocompatibility of OQC and form PEGylated cationic polymeric liposomes (CPLs), which composed of the mixture of OQC, cholesterol, and PEG-*g*-OQC. Structure of the copolymers was characterized by using Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), and X-ray diffraction (XRD). The methyl tetrazolium (MTT) assay with L929 cell lines confirmed that PEGylation can decrease the cytotoxicity of OQC. PEGylated CPL nanoparticles (NPs) can be prepared by adding different weight ratio of PEG-*g*-OQC in the mixture. Paclitaxel was successfully incorporated into PEGylated CPLs with high drug encapsulating efficiency (>90%) and drug loading capacity (>15%). Physical stability experiment showed that paclitaxel-loaded PEGylated CPLs was stable with little change of particle size and size distribution in the condition of freeze-dried by adding mannitol or in high temperature and high pressure. Power or reagent of drug-loaded PEGylated CPLs showed a slow steady release profile for paclitaxel. These results show that PEG-*g*-OQC and CPLs have potential application as a drug delivery vehicle. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 1302–1309, 2012

Key words: chitosan; drug delivery; nanoparticles; liposomes

INTRODUCTION

Chitosan, a deacetylation derivative of chitin, is a relatively less toxic and biocompatible cationic polysaccharide. Due to its unique structure and properties, it has been found many applications in drug delivery areas including polymeric excipients for mucosal drug,[1,2] vaccine delivery,[3,4] gene delivery system,[5–8] and antitumor or immune enhancer for hydrophilic macromolecular drugs. However, a lot of applications of chitosan in biomedical fields are limited by its poor solubility in physiological media. To improve its water solubility, many derivatives have been studied, including modification by quaternization of amino groups,[9,10] carboxymethylation,[11] and PEGylation.[12,13]

Trimethyl chitosan (TMC) is a permanently quaternized chitosan derivative, which has been proven to be highly soluble over a wide pH range (pH 1–9) and demonstrated to be a promising drug excipient.[14] Octadecyl quaternized carboxymethyl chitosan (OQC) is a novel quaternized chitosan, synthesized by reaction of carboxymethyl chitosan with glycidyl octadecyl dimethylammonium chloride. It has many desirable properties compared with TMC, such as good solubility in water and organic solvents, perfect crystallization, good thermal stability,[15] and formation with cholesterol for cationic polymeric liposomes.[16] However, the application of OQC has been limited for its relatively high cell cytotoxicity. Therefore, further improvement of the biocompatibility of OQC is desirable. As water soluble, biocompatible, nontoxic, and nonimmunogenic material, PEG can not only enhance biocompatibility but also favorably affect pharmacokinetics and tissue distribution.[17,18] In addition, incorporation of lipid conjugated PEG with liposomes is the most popular way to produce

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long-circulating liposomes nanoparticles.[19] Therefor, PEGylation could possibly provide OQC with further biological function and lower cytotoxicity. Furthermore, it is hypothesized that incorporation of poly(ethylene glycol) grafted octadecyl quaternized carboxymethyl chitosan (PEG-*g*-OQC) with polymeric liposomes can also produce long-circulating polymeric liposomes nanoparticles (NPs).

Herein, PEG-*g*-OQC was synthesized and characterized by Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, and X-ray diffraction (XRD). Additionally, PEGylated cationic polymeric liposomes (CPLs) have been studied. The method for preparing these sterically stabilized polymeric liposomes is similar with PEGylated liposomes.

MATERIALS AND METHODS

Materials

Chitosan (MW $\sim$ 50 kDa, degree of deacetylation >90%) was purchased from Zhejiang Aoxing Biotechnology Co., Ltd. OQC and carboxymethyl chitosan (CMC) are all prepared in our lab (Cancer Institute of Shanghai Jiaotong University). The degree of substitution (DS) of the quaternization group for OQC is 0.73.[15] Poly(eghylene glycol) methyl ether (Mn 2000 Da) and MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were obtained from Sigma-Aldrich (Steinheim, Germany). Maleic anhydride (MA), *N*-hydroxysuccinimide (NHS), and dicyclohexylcarbodiimide (DCC) were obtained from Sinopharm Chemical Reagent Co., Ltd. and used as received. Dulbecco's modified Eagle's medium (DMEM) was obtained from Shanghai Biosun Sci and Tech Co., Ltd. All other chemicals and solvents were of analytical grade.

Copolymers preparation

First, the monohydroxy-terminated PEG (mPEG) was converted to carboxyl-terminated intermediate by esterification with cyclic aliphatic anhydride according to the literature report.[20,21] Briefly, in a 50-mL round-bottomed flask equipped with a reflux condenser, 2.50 g of predried mPEG 2000 Da was dissolved in 20mL of water-free toluene, 2.23 g MA (1 : 5 molar ratio *versus* mPEG) was added under the protection of nitrogen. The reaction mixture was stirred at 70°C for 48 h under the nitrogen atmosphere. Then, toluene and the excess MA were eliminated by distillation and sublimation at 40°C under vacuum. Subsequently, the intermediate mentioned above together with 0.38 g NHS were dissolved in 20 mL dichloromethane with magnetic stirring. The flask was then cooled in an ice-water bath and 0.14 g DCC was added under nitrogen. The reaction mixture was sealed under nitrogen and stirred for 1 h at 0°C, and further 24 h at room temperature. The precipitated 1,3-dicyclohexylurea was removed by filtration. After evaporation to remove dichloromethane, the filtrate was added to acetone and cooled at 4°C for 5 h. The precipitated product was washed and reprecipitated by using acetone for three times to completely remove excess NHS. Finally, the product was dried under vacuum and stored at room temperature under nitrogen.

For copolymers preparation, OQC was dissolved in purified water at concentration of 20 mg/mL and NHS-mPEG was dissolved in water-free dimethyl sulfoxide (DMSO) at concentration of 40 mg/mL. Then, the OQC solution (20 mL) was added to the NHS-mPEG solution (20 mL) and the mixture was stirred at room temperature for 24 h. After reaction, the mixture solution was purified by using dialysis membrane (cut-off 12–14 kDa) for 4 days, and finally lyophilized to obtain white power.

MTT assay

Evaluation of cytotoxicity was done by the MTT assay. L929 cells were seeded at a density of 1×10^4 cells/well, in 96-well flat-bottomed microassay plates, and incubated for 24 h in a fully humidified atmosphere of 5% CO_2. An increasing amount of PEG-*g*-OQC was added and incubated (from 1 to 100 μg/mL) for 48 h at 37°C, respectively. Then, MTT (1 mg/mL, 100 μL/well) was added to the cells and formazan crystals were allowed to form during 4 h and further dissolved in DMSO. Absorbance was measured at 490 nm with an ELISA plate reader.

PEGylated CPLs preparation

Blank PEGylated CPLs and drug-encapsulated PEGylated CPLs were prepared as previously described[16] and according to the literature of liposomes preparation method.[22] For using Reverse-Phase Evaporation (REV) method, OQC, PEG-*g*-OQC, and cholesterol (weight ratio of OQC and cholesterol = 1 : 0.8) were dissolved in dichloromethane at room temperature to obtain the organic phase. Weight ratios of OQC to PEG-*g*-OQC were varied to achieve the NPs with optimal size and size distribution. Then, the purified water was added into above organic phase with stirring (volume ratios of organic phase and aqueous solution was 1 : 2). The mixture was sonicated for 4 min (power 200 W) using a bath-type sonicator, and after evaporation of the organic solvent, the resulting polymeric lipid NPs suspension was obtained. For paclitaxel-loaded PEGylated CPLs, paclitaxel was dissolved in organic phase with polymer and lipids.

Characterization of PEGylated copolymer and PEGylated CPLs

FTIR spectra were recorded with KBr pellets on a Bio-Rad FTS 6000 spectrometer at room temperature. The samples were thoroughly milled with KBr and pressed into pellets. ^{1}H-NMR spectra were recorded on a UNITY Plus-400 spectrometer at 25°C. The samples were dissolved in D_2O solutions. X-ray diffraction patterns of different sample fractions were measured with a Rigaku D/max 2500v/pc with a Cu-Kα source operating at 40 kV and 50 mA at 20°C. The relative intensity was recorded in the scattering range (2θ) of 3–50°.

The average particle size and size distribution were determined by Dynamic Light Scattering Nanoparticle Size Analyzer LB-550 (Horiba) and quasielastic laser light scattering with a Malvern Zetasizer (Malvern Instruments Limited, United Kingdom). Each measurement was repeated three times, and an average value was obtained and used. The morphologies of PEGylated CPLs were observed using a transmission electron microscopy (TEM) (JEOL-100CXII, Japan). TEM observation of PEGylated CPLs was carried out at an operating voltage of 200 kV in bright-field mode and by electron diffraction. To prepare TEM samples, dilute suspensions (0.1 mg/mL) of NPs in solution were dropped onto carbon-coated copper grid, and air-dried at room temperature.

Drug loading efficiency and drug release *in vitro*

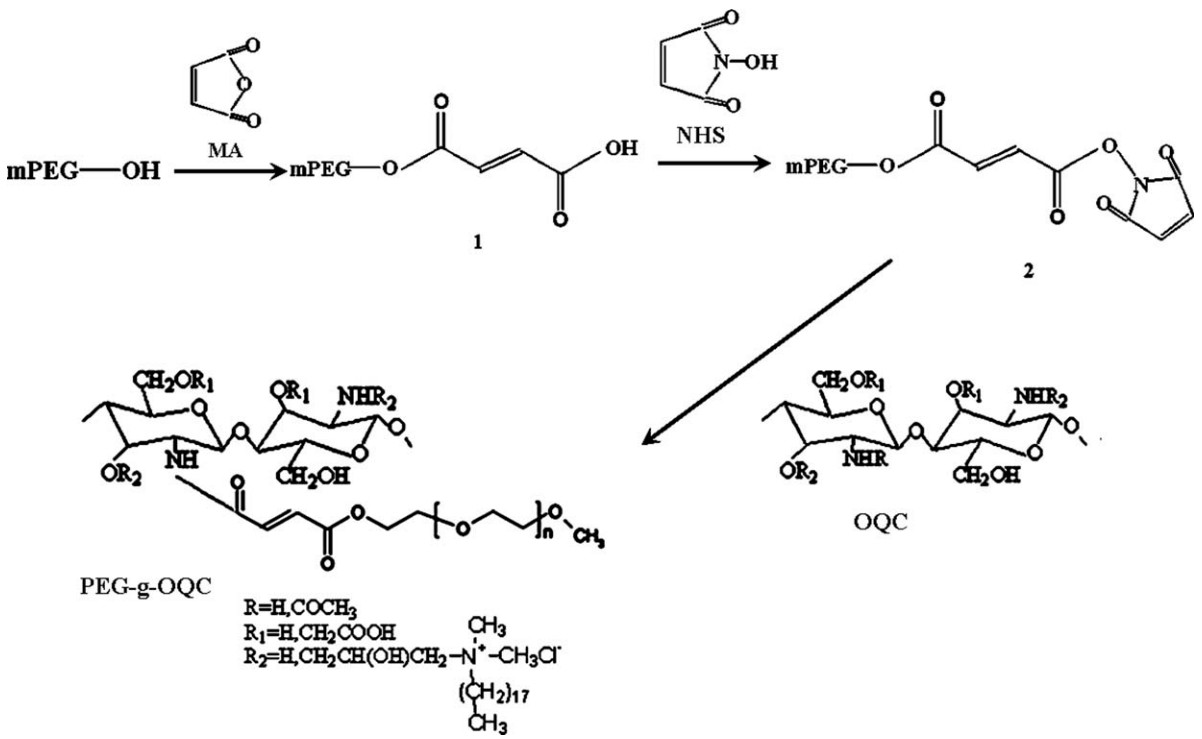
The measurement of paclitaxel content in PEGylated CPLs was performed by a UV assay. After preparing paclitaxel-loaded PEGylated CPLs, the mixture solution was centrifuged to remove un-encapsulated paclitaxel (4000 rpm for 10 min). Drug content was determined by calculating the weight of paclitaxel encapsulated in PEGylated CPLs.

The *in vitro* release of paclitaxel was characterized as described in reports.[23,24] Briefly, 0.7 mL of paclitaxel-loaded PEGylated CPLs dispersion, which contained 4.7 mg/mL PEGylated CPLs and 0.32 mg/mL paclitaxel, were added into dialysis membrane (cut-off 12–14 kDa) and incubated in 8 mL of PBS (pH 7.4) at 37°C under shaking at 80 rpm. The amount of paclitaxel released was determined by measuring paclitaxel concentration in PBS solution out of dialysis membrane. After 1, 3, 24, 48, 102, and 168 h, the incubation was replaced with 8 mL of fresh PBS buffer. The amount of paclitaxel released at certain set times was determined by UV/vis spectrophotometer at 230 nm (with PBS as reference).[25] Each experiment was repeated three times.

RESULTS AND DISCUSSION

Copolymer preparation and characterization

MA was chosen for esterification reaction of mPEG in preference to succinic anhydride because it has



Scheme 1 Synthesis route to PEG-*g*-OQC.

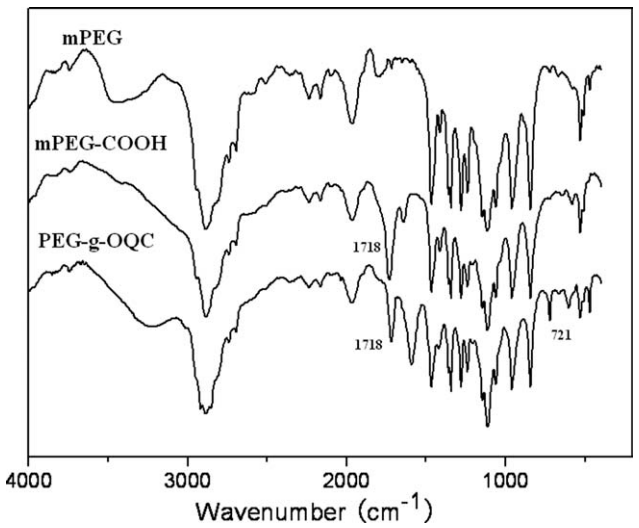


Figure 1 FTIR spectra of mPEG, mPEG-COOH, and PEG-*g*-OQC.

been demonstrated to be more reactive to PEG hydroxyl group, and can be eliminated straightforward via sublimation after reaction.[20,21] After activation, PEGs were coupled to the amino groups of OQC, forming a graft copolymer, i.e., PEG-*g*-OQC. The degree value of PEG chains for use in our experiments is 13.6 mol % sugar unit^{-1}. The synthetic route is shown in Scheme 1.

The FTIR spectra of mPEG, mPEG-COOH, and PEG-*g*-OQC are shown in Figure 1. In the spectra of mPEG-COOH and PEG-*g*-OQC, the appearance of absorption peak at 1718 cm^{-1} applied the existence of COO^-, which attributed to the vibration of C=O. Characteristic peaks of long carbon chain at 2852–2922 cm^{-1} for quaternary ammonium salt and 721 cm^{-1} for chitosan can be found in spectra of PEG-*g*-OQC, which also exist in spectra of OQC.[15]

^{1}H-NMR spectra of graft copolymer and corresponding intermediate mPEG-COOH are shown in Figure 2. ^{1}H-NMR resonance signals are assigned accordingly. The curve of mPEG-COOH (2 kDa) showed resonance signals at ~ 4.2 (—CH_2—O C (=O)—), ~ 6.2, and 6.4 ppm (doublet, —C(=O) CH=CH C(=O)—), which are not present in the spectrum of HO-terminated mPEG containing no maleic moiety in other report.[20] For grafted copolymer PEG-*g*-OQC, signals at ~ 6.45 (C=C), ~ 3.2 (—CH_3), and ~ 3.3–4.2 (—CH_2) can be also observed, which indicated the existence of mPEG-COOH. The slight shift of signal at ~ 6.45 (C=C) compared with

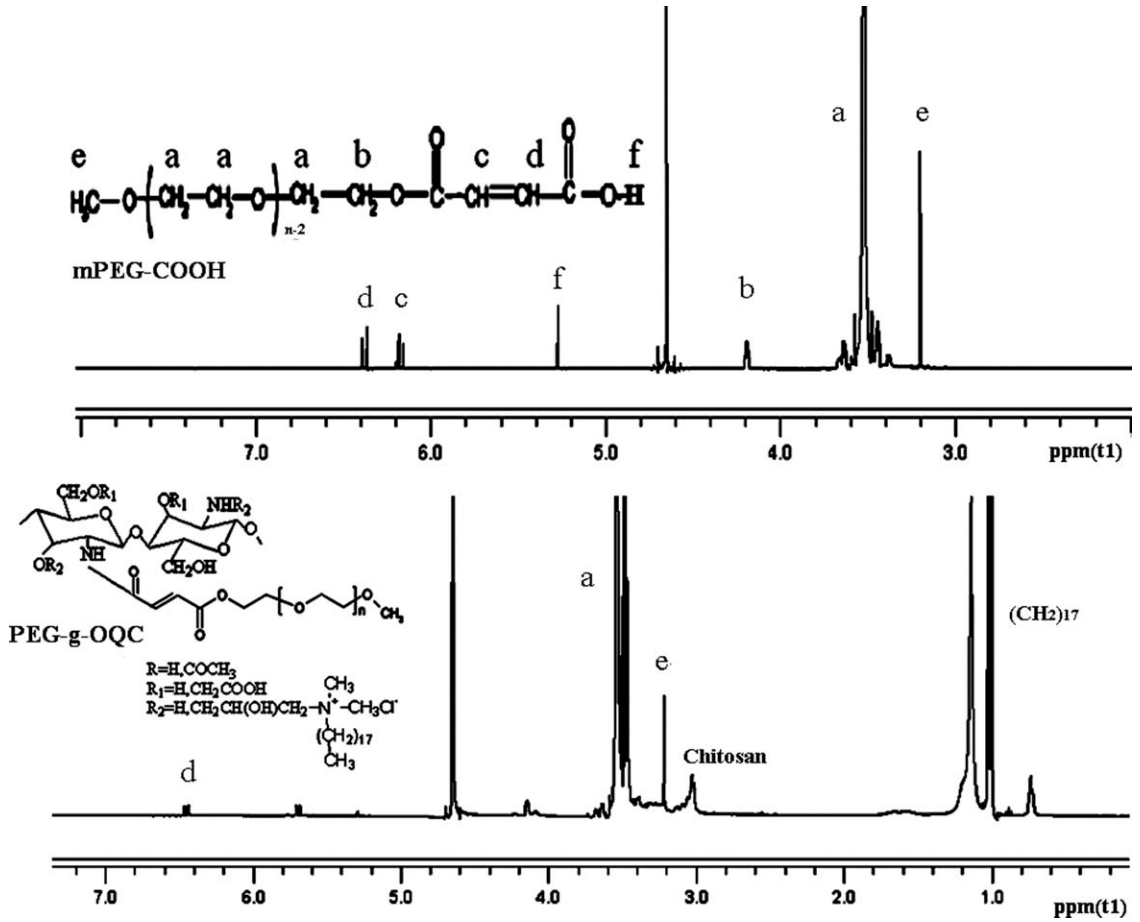


Figure 2 ^{1}H-NMR spectrum of mPEG-COOH and PEG-*g*-OQC.

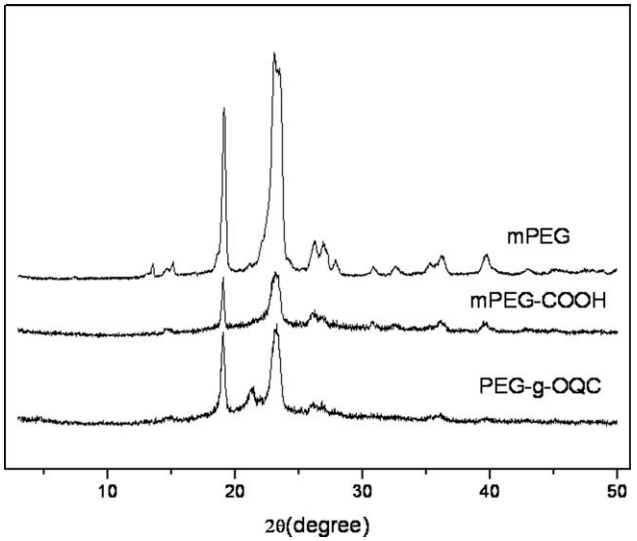


Figure 3 X-ray diffraction patterns of mPEG, mPEG-COOH, and PEG-*g*-OQC.

spectra of mPEG-COOH also implied that the environment of the double bond has changed, and likely the proton shielding effect of local chemical environment to the two methane groups become closer. Additionally, the most intensive signals at $\sim$ 1.0–1.3 and $\sim$ 3.0–3.8 were attributed to long carbon chain of the quaternary ammonium salt and methyl groups of chitosan.[15] These results are consistent with the expected chemical structure of the copolymers.

XRD is one kind useful method to demonstrate the structure of graft copolymers in solid state. The XRD patterns of mPEG, mPEG-COOH, and PEG-*g*-OQC copolymers were detected as illustrated in Figure 3. It was known that homopolymers of PEG is highly crystalline with a well-defined crystal structure, and chitosan is a semicrystalline polymer.[26] OQC also had good crystalline properties and showed narrow peak at $2\theta = 5^\circ$ and 20°, which had been reported in our former study.[27] In XRD curve of PEG-*g*-OQC copolymers, there existed three sharp signals at $2\theta = 19^\circ$, 21.4°, and 23.2° besides the weak and broad peak in the $2\theta = 15^\circ$–25° region. The disappearance of signal at $2\theta = 5^\circ$ belonging to quaternary ammonium salt sequences of OQC indicated that the introduction of PEG branch suppressed the crystallization of OQC. Meanwhile, the new sharp signals coincided with the diffraction peaks of OQC crystals and PEG crystals, respectively, which demonstrated crystalline phases of OQC and PEG in the graft copolymers. Thus the PEG-*g*-OQC copolymer was formed as micophase-separated crystalline materials (i.e., crystalline OQC within graft copolymers).

Cytotoxicity

MTT assay was performed to determine the cytotoxicity of PEG-*g*-OQC. Figure 4 shows the cytotoxicity of PEG-*g*-OQC in different concentrations below 100 μg/mL. It can be seen that all L929 cells treated with copolymer of PEG-*g*-OQC with different concentrations present good viability after 48-h incubation. In general, this critical concentration of 100 μg/mL was highly above the concentration for use *in vitro* experiments.[28] Furthermore, the safety of the copolymers was corroborated by observing the morphological change of the cells with microscopy. In comparison with MTT assay of our former study for OQC, PEGylation can decrease the cytotoxicity and increase the biocompatibility of OQC to a great extent.[15] This demonstrated that PEG-*g*-OQC had a low cellular toxicity and were suitable for application of drug delivery.

Characterization of PEGylated CPLs

OQC and cholesterol had been used to prepare new polymeric liposomes, which had similar structure with conventional liposomes formed from phosphatidylcholine and cholesterol (PC/Chol).[16] It has been known that attachment of the flexible and water-soluble PEG polymer to the liposome surface increases the blood circulation time significantly, and that PEG functions as a steric barrier can prevent proteins of the immune system from adsorbing onto the liposome surface.[19,29,30] Using PEG-*g*-OQC, OQC, and cholesterol, PEGylated CPLs can be prepared by Thin-Layer Evaporation or Reverse-Phase Evaporation method.[16]

PEGylated CPLs were generally spherical in shape with narrow particle size distribution. The spherical shape had become more irregular with a little increasing in diameter after conjugation with PEG [Fig. 5(a,b)]. The hydrodynamic diameter of

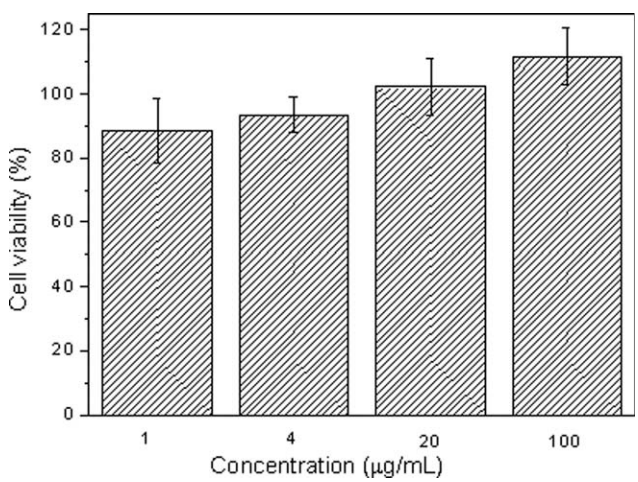


Figure 4 Cytotoxicity of PEG (2k)-*g*-OQC copolymers measured by MTT assay after 48-h incubation with L929 cells. Each point represents the mean ± SD of seven experiments.

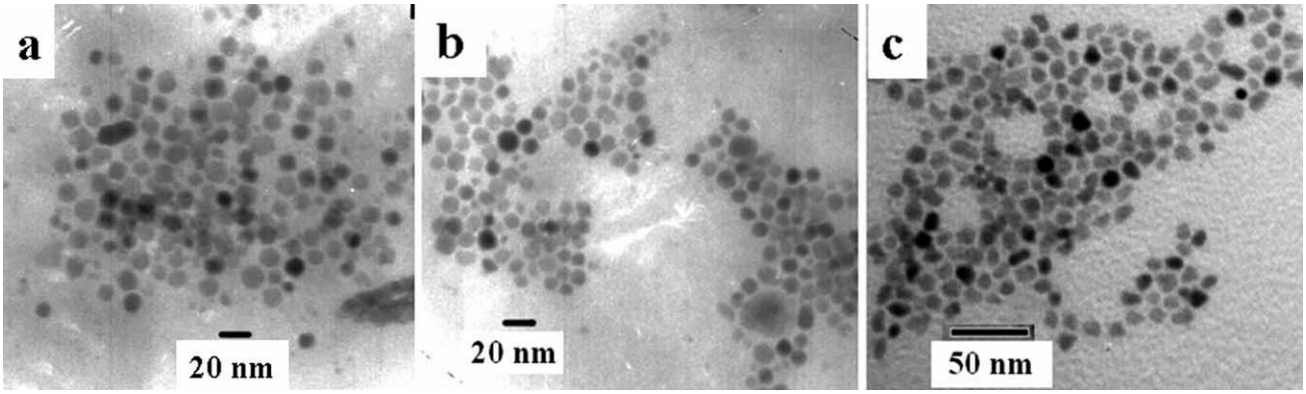


Figure 5 Transmission electron micrographs of PEGylated polymeric liposomes. (a) Black CPLs, (b) black PEGylated polymeric liposomes, and (c) paclitaxel-loaded PEGylated CPLs

PEGylated CPLs with weight ratio 1 : 4 of PEG-*g*-OQC to OQC is 93.1 ± 5.4 nm (polydispersity index of 0.380), which is greater than the TEM diameter because of the hydration of polymeric liposomes bilayer in aqueous solution. Zeta potential of the same PEGylated CPLs was only 6.3 ± 1.6 mV, which is lower than the value of PEG-*g*-OQC (19.3 ± 1.6 mV), OQC (38.1 ± 2.8 mV), and CPLs (27.8 ± 2.1 mV). From Table I it can be seen that zeta potentials of CPLs have been decreased by adding PEG-*g*-OQC. The reason is that high molecular weight PEG (2000 Da) covalently attached to the polymeric lipid headgroup undergoes steric block to the polymeric liposomes surface.[30]

The influence of the weight ratios of PEG-*g*-OQC and OQC on the formation of CPLs was also discussed. From analysis of particle size, the size of the vesicles made from PEG-*g*-OQC and cholesterol was larger than that from mixed samples of PEG-*g*-OQC, OQC, and cholesterol (Table I). With decrease of PEG-*g*-OQC content in the mixture (for the weight ratio of 1 : 0, 1 : 3, and 1 : 4), the mean particle size of CPLs decreased first, but there are no clear difference in particle size and size distribution when the weight ratios were 1 : 5 and 1 : 6. It can be concluded that PEGylated CPLs can be formed with suitable and different particle size with narrow particle size distribution by adding PEGylated copolymer. These weight ratios of PEG-*g*-OQC to OQC are often selected in preparation of PEG liposomes.[31,32]

Lipophilic drug, paclitaxel,[33] was selected to evaluate the drug loading capacity of PEGylated CPLs. The calibration curve for UV/vis absorbance for paclitaxel gives an equation of the form:

$$C_i = 0.3138 + 449323A_U,\ R = 0.999 \qquad (1)$$

where C_i is the drug concentration (μg/mL), A_U is the UV/vis absorbance of the drug.

Paclitaxel can be encapsulated effectively in PEGylated polymeric liposomes NPs, which the drug encapsulation efficiency and loading capacity were above 90 and 15%, respectively. The size of paclitaxel-loaded PEGylated CPLs in PBS solution was close to 102.0 nm with a fairly narrow-size distribution of polydispersity index 0.145 (Table I). The TEM picture also reveals the spherical shape and homogeneous size distribution of the drug-loaded NPs.

TABLE I
The Hydrodynamic Diameter of CPL with Different PEG-*g*-OQC and OQC Weight Ratio

Samples with different weight ratio	Mean diameter (nm)	Polydispersity (μ/Γ2)	Zeta potential (mV)
1 : 0	220.2 ± 5.5	0.113	19.3 ± 1.6
1 : 3	141.2 ± 8.7	0.309	5.4 ± 1.4
1 : 4	93.1 ± 5.4	0.380	6.3 ± 1.6
1 : 5	128.1 ± 0.2	0.344	7.7 ± 0.6
1 : 6	106.7 ± 1.1	0.357	13.6 ± 1.0

TABLE II
The Hydrodynamic Diameter of Paclitaxel-Loaded PEGylated CPLs at Different Condition

Sample	Mean diameter (nm)	Polydispersity (μ/Γ2)
Sample untreated	83.1 ± 0.482	0.232
Sterilized (under 3 kgf/cm^2, 100°C)	104.0 ± 0.571	0.326
Refrigeration (−20°C)	1434.6 ± 0.6	0.360
High temperature (in 70°C for 15 min)	84.7 ± 0.483	0.233
High temperature (in 100°C for 15 min)	103.9 ± 0.569	0.324
Freeze-dried (no mannitol)	252.2 ± 0.622	0.387
Freeze-dried (adding mannitol)	167.9 ± 0.486	0.236

The weight ratio of PEG-*g*-OQC/OQC is 1 : 4.

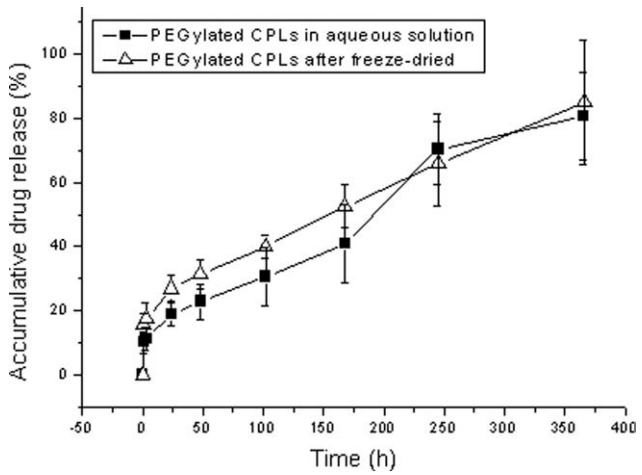


Figure 6 *In vitro* release of paclitaxel in PBS (pH 7.4) at 37 ± 0.5°C.

Physical stability of CPL

The stability of liposomes NPs is a crucial factor for their exploitation in drug delivery systems. Particle size and size distribution of CPLs was analyzed by particle size analyzer in different storage conditions. Drug-loaded PEGylated CPLs were used to compare the influence of different temperature and storage condition (freeze-dry and refrigeration) on the final physicochemical properties of the NPs (Table II). It can be seen that the particle size and size distribution of paclitaxel-loaded PEGylated CPLs has changed little after treating in high temperature at 70°C and 100°C for 15 min, and after sterilizing by high temperature and high pressure. But after treated by refrigeration at −20°C, the particle size has increased a lot. These results also can be seen from turbidity observation. On the other hand, the turbidity and particle size both increase when the NPs suspension was freeze-dried and dispersed in solution again. But for freeze-dried by adding protective agent (mannitol), paclitaxel-loaded PEGylated CPLs had remained stable and the solution including NPs was transparent. The stability study results reaffirmed the greater stability of polymeric liposomes[16] and it makes clear that the drug-loaded polymeric liposome can be preserved under high temperature or by freeze-drying.

In vitro drug release

The *in vitro* drug release profile of paclitaxel-loaded CPLs in purified water and redispersed by purified water after freeze-dried are shown in Figure 6. The release of paclitaxel from CPLs is expressed as the cumulative weight fraction of paclitaxel released from polymeric liposomes NPs into PBS buffer with the time extended. At initial burst release time of the first day, 19.0% and 26.8% of drug had been released from paclitaxel-loaded CPLs stored in solution and by freeze-dried with adding mannitol, respectively. After 2 weeks, 80–86% of total drug had been released from these CPLs. There is no distinct difference in drug release profile for paclitaxel-loaded CPLs NPs stored by two different forms. It can be concluded that both samples showed a long drug release action for lipophilic drug paclitaxel and their release behaviors in aqueous solution are similar.

CONCLUSIONS

PEG-*g*-OQC copolymers were successfully synthesized and characterized by FTIR, ^{1}H-NMR spectroscopy, and XRD measurements. PEGylation can decrease the cytotoxicity of OQC to a great extent by using MTT assay with L929 cell lines. OQC, PEG-*g*-OQC, and cholesterol can form stable PEGylated CPLs NPs by method for preparing PEGylated liposomes. Paclitaxel can be encapsulated in PEGylated CPLs with high drug encapsulating efficiency (>90%) and drug loading capacity (>15%). The particle size of these drug-loaded NPs in aqueous solution is about 102.0 nm with a narrow-size distribution of polydispersity index 0.145. Paclitaxel-loaded PEGylated CPLs can be stored in the condition of low temperature under 50°C or through freeze-dried by adding mannitol, which the drug-loaded NPs has little drug leakage and change of particle size. Power and reagent of drug-loaded PEGylated CPLs have a slow steady release profile for paclitaxel. The present PEGylated CPLs NPs are a promising carrier candidate for antitumor drugs.

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