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PEGylated synthetic surfactant vesicles (Niosomes): novel carriers for oligonucleotides

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Abstract Polyethylene glycol (PEG) modified cationic niosomes were used to improve the stability and cellular delivery of oligonucleotides (OND). PEGylated cationic niosomes, composed of DC-Chol, PEG2000-DSPE and the non-ionic surfactant-Span®, offer some advantages as gene carriers. Complexes of PEGylated cationic niosomes and OND showed a neutral zeta potential with particle size about 300 nm. PEG-modification significantly decreased the binding of serum protein and prevented particle aggregation in serum. The loaded nuclear acid drug exhibited increased resistance to serum nuclease. Compared with cationic niosomes, the PEGylated niosomes showed a higher efficiency of OND cellular uptake in serum. Therefore, in terms of their stable physiochemical properties in storage and physiological environment, as well as low-cost and widely available materials, PEGylated cationic niosomes are promising drug delivery systems for improved OND potency in vivo.

Synthetic surfactant vesicles (niosomes), a self-assembly of non-ionic amphiphiles, are widely studied as an alternative to liposomes because of their similar structures. Compared to their phospholipid liposome counterparts, niosomes offer many advantages for drug delivery. For instance, niosomes have been shown to have great physico-chemical stability; they can be stored up to 84 months without significant morphological changes [1]. Moreover, vast quantities of non-ionic surfactants are commercially available on an industrial scale with high purity. Because of their low cost and superior chemical and storage stabilities [1, 2], niosomes have been used for anti-tumor drug delivery[3–7], diabetic therapy[8–10], diagnostic imaging[11, 12], and vaccine immunizations[13–18] with delivery methods varying from oral[18, 19], transdermal [20–22], vaginal[9] and ophthalmic delivery[23, 24] to specific targeted delivery[25]. Like liposomes, niosomes are also capable of entrapping both hydrophilic and hydrophobic drugs, prolonging circulation of the entrapped drug, and altering the drug's organ distribution.

Although niosomes have been applied in pharmaceutics since the 1980s, only a few reports have focused on their application for gene delivery. Niosomes are biodegradable, biocompatible and nontoxic [26], which allows for them to be safely used in gene therapy. We previously reported the use of cationic niosomes as a potential gene carrier and showed that Span cationic niosomes exhibited positive results for gene delivery [27]. However, positively charged particulates are prone to nonspecific interactions with plasma proteins, which leads to destabilization, dissociation, and rapid clearance of gene/carrier complexes [28]. This largely limits the application of cationic niosomes in biological fluid environments.

Polyethylene glycol is the most widely used hydrophilic polymer for the steric stabilization of nanoparticle drug delivery systems [29]. PEGylated liposomes display increased lifetimes and stability in body fluid environments, showing high accumulation in specific sites [30]. Therefore, we hypothesized that incorporation of PEG will increase the physico-chemical stability of ODN/niosome complexes and make them more attractive for gene delivery. Here we prepared PEGylated cationic niosomes composed of DC-Chol, PEG2000-DSPE and Span®. Modification with PEG2000-DSPE not only significantly prevents niosomes from aggregation in serum but also protects the loaded nuclear acid drug from degradation by

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serum nuclease. Furthermore, complexes of OND and PEGylated cationic niosomes show high cellular uptake efficiency in serum. The development of PEG-modified cationic niosomes may lead to improved efficacy of OND in vivo.

Experimental

Materials

Sorbitan monoesters surfactant, Span®40 (Shanghai Chemical Reagent Co., China), cholesterol and deoxyribonuclease (DNase I, Sigma, USA), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly-(ethyleneglycol)-2000] (PEG2000- DSPE, Lipoid GmbH, Germany), Bovine serum albumin (Huamei Bio-engineering Company, China). DC-Chol, a cationic cholesterol derivative, was synthesized as described by Gao and Huang [31]. Phosphorothioate oligonucleotide (OND), with 15-mer random sequence (5′-CTCAGTTAGGGTTAG-3′), was synthesized by Shanghai Sangon Bio-engineering Technology Company and the 5′ end was labeled with carboxy-fluorescein. All other reagents were of analytical grade supplied by Huadong Medical Co., China.

COS-7 (Transformed African Green Monkey kidney fibroblast) cell line and pGFP-N1 plasmid were kindly provided by Sir Run Run Shaw Hospital, Zhejiang University. Cells were cultured with Dulbecco's Modification of Eagle's Medium (DMEM, Gibco, USA) with 10% fetal bovine serum (Hangzhou Sijiqing Bio-engineering Material Co., China).

Preparation of cationic niosomes and PEGylated cationic niosomes (PEG-NIO)

Niosomes, composed of equal molar Span®40 and DC-Chol, were prepared by a film hydration method. In brief, 20 μmol of lipid mixture was dissolved in chloroform in a pear-shaped flask. It was then attached to a rotary evaporator to dry the organic solvent and kept under vacuum overnight. The dried lipid film was hydrated with 5 ml double distilled water and rotated in water bath at 60 °C for 20 min. The niosomal dispersion was then sonicated for 3 min at 200 W by an ultrasound probe (JY92, Ningbo Scientz Biotechnology Co., China). Lastly, the dispersion was filtered through 0.2μm membrane filter to prepare aseptic samples for cell culture study; non-modified cationic niosomes were obtained and stored at 4 °C. PEGylated cationic niosomes (PEG-NIO) were prepared by adding PEG2000-DSPE to the cationic niosomal dispersion at a concentration of 5 mol% and incubating for 30 min.

Morphology observation using transmission electron microscopy

The morphology of niosomal sample was observed using a JEM-1200EX apparatus (JEOL, Tokyo, Japan). Samples were negatively stained with phosphotungstic acid.

Physical stability of vesicles

Fetal bovine serum (FBS) was added to niosomal samples, with a final serum concentration of 10% (v/v), to mimic cell culture conditions; this concentration is generally used in vitro for cell culture system. Physical stability was determined at 37 °C by measuring turbidity at different time points, spectrophotometrically at 400 nm (TU-1800, Pgeneral, China).

Preparation of DNA (or OND)/PEG-NIO complexes

DNA (or OND)/PEG-NIO complexes were prepared by two methods: *Pre-coating* and *Post-coating* (see Fig. 1). In the *Pre-coating* method DNA was added to a PEG-NIO dispersion and incubated for 30 min. In the *Post-coating* method DNA/Niosome complexes were prepared by adding DNA to cationic niosomal dispersion; PEG2000-DSPE was then added to the DNA/Niosome complex and incubated to obtain the DNA/PEG-NIO complexes.

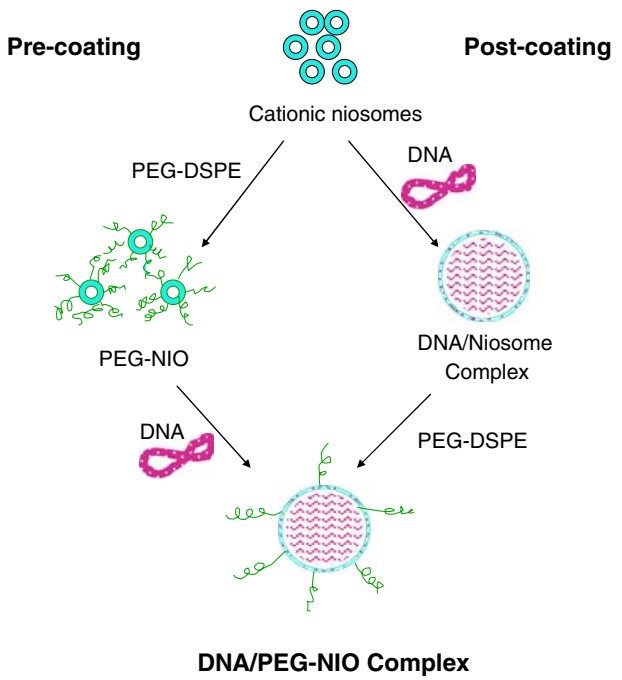

Fig. 1 Two preparative methods employed for DNA/PEG-NIO complexes fabriction: "*Pre-coating*" and "*Post-coating*"

Measurement of particle size and zeta potential

Particle size and zeta potential of niosomes and OND/niosome complexes were determined by laser diffraction spectrometry (Malvern Zetasizer 3000 HS, Malvern, U.K.).

Determination of serum albumin binding

To measure albumin biding of the cationic niosomes and PEG-NIO, bovine serum albumin (BSA) was added to each and then incubated at 37 °C for an hour. Aliquots of the above suspensions were taken and added to a centrifugal filter device containing an ultra-filtration membrane (MWCO 100,000, Millipore, USA), respectively, and then centrifuged for 5 min at 10,000 × *g*. Unbound protein in the filtrate was determined using BCA Protein Assay Kit (Beyotime® Inst. Biotech., China). The binding efficiency (%) was calculated as follows:

$$Binding\ efficiency(\%) = \left[1 - \left(\frac{BSA_{unbound}}{BSA_{initial}}\right)\right] \times 100$$

Where $BSA_{initial}$ is the total initial protein added to the niosomes and $BSA_{unbound}$ is the unbound protein.

Determination of niosome ability to protect genes from nuclease degradation

DNAse I was added to DNA/PEG-NIO or DNA/niosome complex suspensions and incubated at 37 °C for an hour. The enzyme was then thermally deactivated using a 70 °C water bath. Intact DNA was then released from the complex by addition of polyanionic heparin. Equal volumes of the samples were analyzed by electrophoresis using 1.2% agarose gel.

PEG-NIO mediating OND cellular uptake study

COS-7 cell line containing SV40 T antigen was used for the transfection study. COS-7 cells were seeded in 24-well plates (2×10^5 cells/well) and cultured in DMEM medium with 10% FBS overnight at 37 °C in a 5% CO_2 humidified incubator. Various OND/niosome complexes were prepared as described above. After replacing the DMEM medium with the 50% FBS culture medium, OND/Niosome complexes were added to cells and incubated for 4 h. The culture medium was then removed, cells washed twice with PBS, and then collected by trypsinization and centrifugation (1200 × g, 3 min). The supernatant was discarded and cells washed again twice with PBS. The cells were then resuspended in PBS and analyzed by flow cytometry (Beckman Coulter). Because the 5′ end of OND was labeled with carboxy-fluorescein, the OND uptake by cells can be detected by flow cytometry. The percentage of positive fluorescence cells (α) and mean of fluorescent intensity (MFI) were determined. Cellular uptake efficiencies were evaluated by calculation of the total fluorescent intensity (TFI) according to the formula:

$$TFI = \alpha \times MFI \times 104(\text{amount of detected cells})$$

Statistical analysis

Each experiment was performed in triplicate and the values expressed as mean ± S.D. Statistical analysis were performed using Student's *t*-test.

Result and discussion

Morphology of the niosomes

PEG2000-DSPE is an amphiphilic molecule composed of a hydrophobic phospholipid and hydrophilic PEG chain. The hydrophobic part anchors itself into the bilayer of vesicles while the hydrophilic PEG chains stretch out into aqueous medium and serve as an outer shell. The transmission electron microscopy study showed that PEG-NIO remained intact, with a spherical shape, and displayed little changes in morphology. The size of both cationic niosomes (Fig. 2A) and PEG-NIO (Fig. 2B) vesicles were about 100 nm.

Measurement of particle size and zeta potential

Measured by laser diffraction spectrometry, the average particle size of various formulations ranged from 120–350 nm (Table 1). The average particle size of niosomes and PEG-NIO were 120 and 135 nm, respectively. The small size change indicated that the incorporation of PEG2000-DSPE did not result in a significant morphology change. After loading with nuclear acid, particle size increased to above 300 nm. Furthermore, the size of complexes fabricated by the *Pre-coating* method was slightly larger than that of *Post-coating*. This is due mainly to the different fabrication methods. In the *Pre-coating* method, PEG was first added to prepare PEG-NIO and then OND was added to prepare the OND/PEG-NIO complexes. In this case, the sterically stabilized PEG outer layer, to some degree, hinders the approach of nuclear acid and favors the formation of less compact complexes. Such structure would show a slightly larger size. In addition, zeta potential of cationic niosomes is highly positively charged with an average value of +40 mV. With PEG modification, the zeta potential drastically decreased from +40 to +6 mV for PEG-NIO, near neutral.

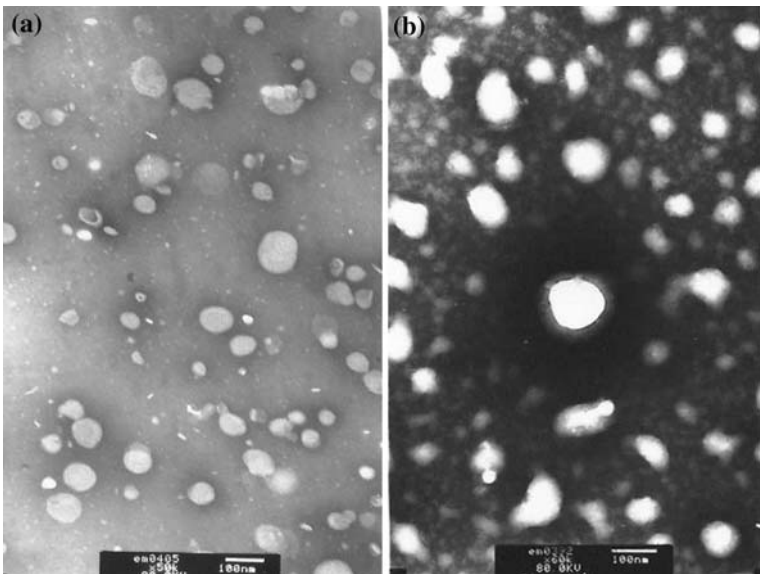

Fig. 2 TEM photograph of cationic niosomes (A) and PEG-NIO (B)

Table 1 Particle size and zeta potential of PEGylated niosomes

	Niosomes	PEG-Nio	OND/Nio	OND/PEG-Nio (Pre-coating)	OND/PEG-Nio (Post-coating)
Size/nm	122.7 ± 7.6	135.1 ± 5.4	305.2 ± 9.0	341.8 ± 10.6	315.4 ± 7.9
Zeta/mV	40 ± 0.5	6 ± 0.2	27 ± 0.6	1 ± 0.2	3 ± 0.2

Physical stability of vesicles

Turbidimetry has been frequently used to study the stability of vesicles [32]. Turbidity of polyelectrolyte complexes is known to increase in the presence of serum albumin [33]. For cationic particles, interaction with negatively charged proteins in vivo always results in aggregation and quick clearance from system circulation. It is well documented that PEGylated liposomes have excellent stability in body fluid environments [30, 34, 35]. Our studies showed that PEG also stabilized cationic niosomes in serum condition (Fig. 3). Turbidity of cationic niosomal suspensions increased to relatively high levels, indicating an increase in particle size induced by aggregation or protein absorption on the particle surface. Meanwhile, the turbidity of PEG-NIO changed little in 6 h and showed only slight increase in 20 h. The cationic niosomes experienced a sharp increase and after 20 h there were precipitates. For cationic niosomes, although a high zeta potential value of +40 mV should generate enough electrostatic repulsion to keep the particles stable in colloidal systems [36], the interaction with serum proteins or opsonins in the body fluid leads to aggregation. In contrast, PEG-NIO was quite stable with the steric effect of the flexible polymer preventing protein-niosomal interactions. Additionally, because neutrally charged surfaces exhibit decreased protein absorption, the neutral particles were more stable in the physiological

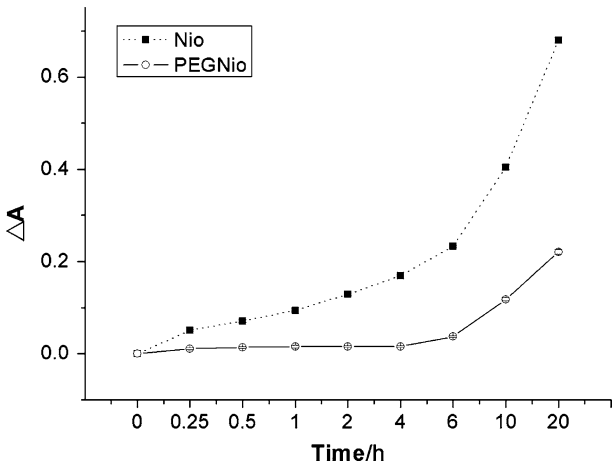

Fig. 3 Turbidity changes of PEGylated niosomes and noisomes

environment compared with cationic particles [37]. These factors contribute to the improved stability of PEG-NIO in serum.

Serum albumin binding study

Positively charged gene carriers readily bind with serum protein, which usually results in rapid clearance from systemic circulation [33]. Albumin is the main component of serum proteins and predominantly responsible for drug

molecule binding [38]. The binding of serum albumin to particles usually results in size increase, aggregation and precipitation [39]. In this study we used bovine serum albumin (BSA) as a serum protein model to evaluate the protein/gene carrier binding efficiency. Our study indicated that the binding efficiency of serum albumin with PEG-NIO was significantly lower than that of cationic niosomes (Fig. 4). A neutral zeta potential and steric outer layer, due to PEG modification, appears to create a physical barrier between protein and gene carrier.

Protection of genes from nuclease degradation

Nuclear acid drugs are readily degraded into small molecules, such as nucleoside and base, by serum nucleases in vivo. Therefore gene carriers are required to stabilize the physicochemical features of gene drugs in physiological environments. Among the niosomal carriers examined in this study, PEG-NIO (in *Post-coating* method) provided the greatest degree of protection for DNA, followed by PEG-NIO (in *Pre-coating* method), and cationic niosomes (Fig. 5). As mentioned above, less compact structures of PEG-NIO/gene complexes result from the *Pre-coating* method comparing with the *Post-coating* method. Therefore, the *Pre-coating* complexes exhibited less protection for DNA than the *Post-coating* complexes. The hydrophilic, sterically stabilized structure of PEG prevents the near-approach of enzymes, and thereby protects DNA from degradation. With their ability to protect genetic materials, PEG-NIO may provide a potential gene carrier for in vivo delivery.

PEG-NIO mediating OND cellular uptake study

A 50% serum concentration, which approximates in vivo physiological serum conditions [40], was used to study the effect of serum on various cationic niosomes mediating OND cellular uptake. Usually, PEGylated particles exhibit, a lower transfer efficiency than cationic particles in non-serum condition because positively charged particles bind more avidly to cells than neutral particles [41]. It should be noted, however, that the efficiencies of OND cellular uptake mediated by cationic niosomes and PEG-NIO (in the *Post-coating* method) were comparable in non-serum condition in this study, without significant difference between these two groups (Fig. 6). This may be explained by the improved physical stability of OND/PEG-NIO complexes, keeping the complex intact longer in cell culture systems and thus increasing OND cellular uptake.

In the complicated body fluid environment, OND cellular uptake efficiencies of all formulations decreased, as was expected. The efficiency of cationic niosomes decreased drastically and showed the lowest level among the three formulations while the PEG-NIO (in the *Post-coating* method) had the highest efficiency of OND cellular uptake (Fig. 6). This indicated that PEGylated niosomes exhibit superior efficiencies in mediating OND cellular delivery in serum compared to cationic niosomes.

In general, there are two methods to prepare gene/PEGylated liposome complexes: *Pre-coating* and *Post-coating*

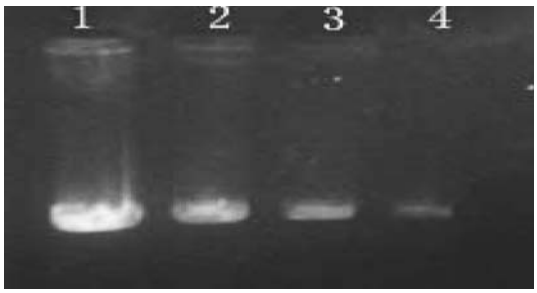

Fig. 5 Agarose gel electrophoresis of DNA degradation by DNase I. (1. DNA/PEG-NIO(Post-coating); 2. DNA/PEG-NIO(Pre-coating); 3. DNA/ NIO; 4. Free DNA)

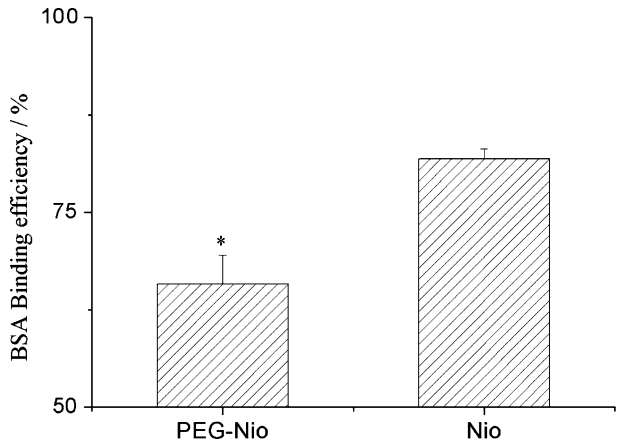

Fig. 4 Serum albumin/niosomes binding efficiency

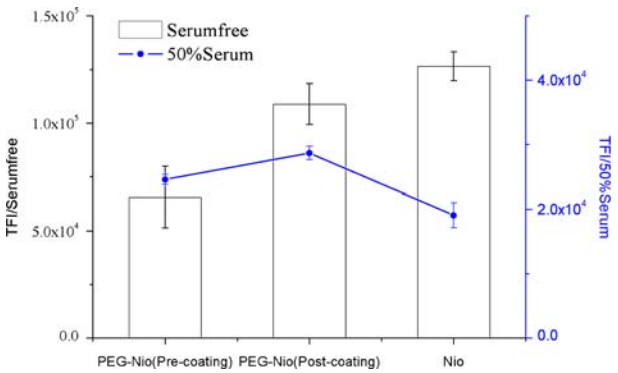

Fig. 6 Cellular uptake efficiency of OND mediated by various niosomes in serum-free and 50% serum conditions

[42]. In this study, PEGylated niosomes were prepared using these methods. Results showed that PEGylated niosomes (in the *Post-coating* method) had improved capability of gene delivery. As mentioned above, this improvement in efficiency could be due to structural differences of these two complexes. A similar result was also reported for PEGylated liposomal gene carriers [42].

Additionally, although animal studies were not conducted in our study, similar results might be expected in vivo for the PEGylated niosomes, compared with that of cationic niosomes, due to the protection of gene drugs by PEG and a longer half-life of PEGylated particles [43]. This would increase drug accumulation in sites of action by the enhanced permeability and retention (EPR) effect.

PEGylated niosomes have shown a promise in mediating OND cellular delivery in vivo. In our preliminary study of DNA transfection, however, PEGylated niosomes appeared compromised in DNA transfection of pGFP-N1, which is a green-fluorescent-protein-coding plasmid. Endosome escape is a key step for DNA to take its action. Although Span® may play some role in destabilizing biomembranes and gene transfection, there was far than enough for facilitating endosome escape. DOPE, the most widely used helper lipid in gene delivery for endosome escape, facilitates phase transition from lamellar phase to inverted hexagonal phase [44]. It was reported that lipopolyplexes could not transfect cells without the addition of DOPE to the formulation [45]. DOPE is usually incorporated into cationic liposomes at considerably high concentrations (about 30~50 mol%) to achieve its function. However, it could not be incorporated into niosomes in such high concentrations because it forms liposomes with cholesterol, instead of niosomes. We incorporated DOPE in niosomes at a low concentration of 10 mol%, but it did not improve DNA transfection significantly (Fig. 7A and B). At such a low concentration it could not facilitate the bilayer phase transition.

Furthermore, when a small amount of cetyltrimethyl ammonium bromide (CTAB), a cationic surfactant which was reported to facilitate endosome escape [46], was incorporated into PEGylated niosomal formulations at a concentration of 10 mol%, a higher transfection efficiency was achieved (Fig. 7C). However, CTAB exhibits the inherent cytotoxicity characteristic displayed by cationic surfactants. Observation by light microscope confirmed CTAB cytotoxicity by the presence of both shrunken and dead cells. Therefore, the balance of CTAB efficiency and safety is still a challenge to medical application. Thus, the development of a suitable helper for endosome escape is needed to achieve efficient DNA transfection with PEGylated niosomes. Although PEGylated cationic niosomes show compromised efficiency for DNA transfection, they exhibit a promising potency for OND delivery in vivo.

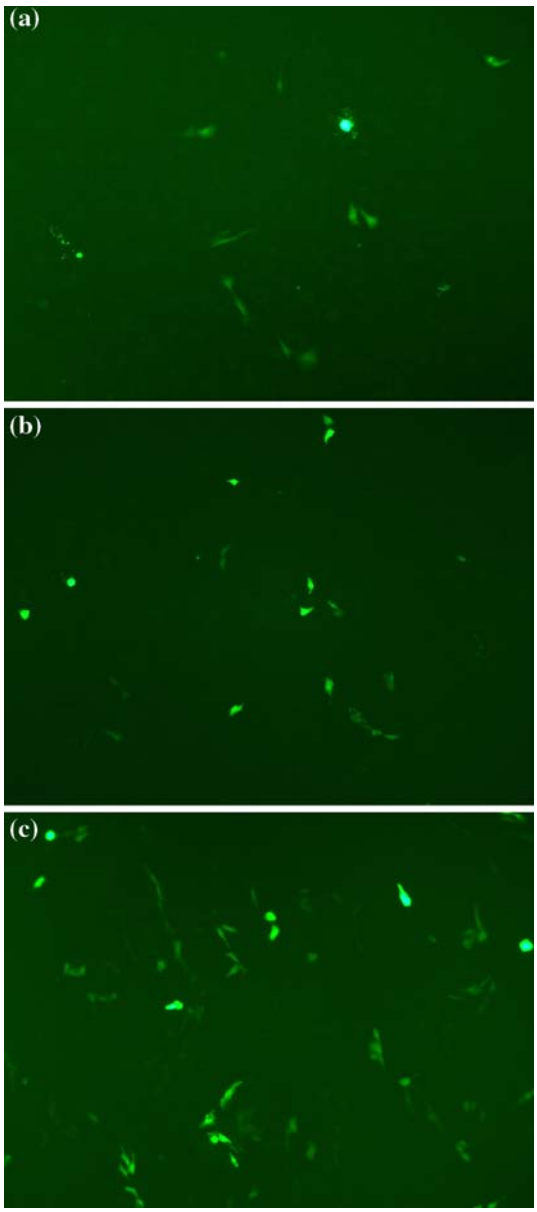

Fig. 7 Green fluorescent protein expression in COS7 cell line with PEGylated niosomes (A. without incorporation of DOPE and CTAB; B. with incorporation of DOPE; C. with incorporation of CTAB) mediating pEGFP gene transfection

Concluding remarks

Gene therapeutic drugs face the significant barriers of degradation in physiological environments [47] and poor cellular uptake [28]. As a promising alternative for liposomes, cationic niosomes showed a high efficiency in OND cellular delivery in our previous study [27]. However, as discussed above, cationic niosomes appear to be at a disadvantage when exposed in body fluid environments. Based on our previous study, we improved the potency of cationic niosomal gene carriers using PEG-modification,

which not only increased the particle stability in serum but also enhanced the nuclease resistance of the loaded gene drug. Compared with cationic niosomes, complexation of PEGylated cationic niosomes with OND enhanced the cellular uptake of OND in serum. The presence of PEG coated on the cationic niosomes not only contributes to the neutral zeta potential but also prevents serum proteins and nucleases from approaching the cargo. Moreover, this hydrophilic and flexible polymer provides steric repulsion among particles [48]. In conclusion, PEGylated niosomes are novel potential carriers for systemic in vivo delivery of OND having excellent and unique properties for drug delivery.

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