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〔**Note**〕

Effect of Hydrophilic Chain Length in Non-Ionic Surfactant on the Physicochemical Characteristics, Gene Expression Efficiency and Cytotoxicity of DNA/Poly-L-Ornithine/Niosome Ternary Complexes for Gene Delivery

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Summary: The aim of this study is to clarify the effect of hydrophilic ethylene oxide (EO) chain length in steareths on the physicochemical characteristics, gene expression efficiency and cytotoxicity of plasmid DNA (pDNA)/poly-L-ornithine (PLO)/niosomes (consisting of non-ionic surfactants [NISs], polyoxyethylene stearyl ethers [steareths]) ternary complexes. Niosomes were prepared with NISs, a series of stearet (steareth-2, steareth-5 and steareth-20) or Tween 80, cholesterol, and octadecylamine of cationic lipid, followed by complexing with pDNA encoding luciferase gene and PLO. The physicochemical characteristics, gene expression efficiency and cytotoxicity of the prepared pDNA/PLO/niosome ternary complexes were evaluated. The particle size and ζ potential of steareth niosomes and pDNA/PLO/steareth niosome ternary complexes decreased with lengthening of the EO chain in steareth. All complexes showed a high pDNA condensation ability, and pDNA/PLO/steareth-2 niosome ternary complexes possessed the highest protective effect in the presence of polyanion, dextran sulfate. The transfection efficiency and cell viability of pDNA/PLO/steareth niosome ternary complexes also decreased with the lengthening of the EO chain. Among the pDNA/PLO/niosome ternary complexes, the pDNA/PLO/steareth-2 niosome ternary complexes showed the highest transfection efficiency and almost no cell damage. In conclusion, it was suggested that the EO chain length in steareths affects the physicochemical characteristics, gene expression efficiency and cytotoxicity of pDNA/PLO/steareth niosome ternary complexes. Furthermore, we pointed out that the pDNA/PLO/steareth-2 niosome ternary complexes might deliver genes efficiently and safely. Further study is required to more elevate the transfection efficiency, stability and safety of pDNA/PLO/steareth-2 niosome ternary complexes, such as using target compounds.

Key words: niosomes; poly-L-ornithine (PLO); polyoxyethylene stearyl ether (steareth); gene delivery; non-ionic surfactant (NIS)

Introduction

There are recent reports on the use of niosomes as well as liposomes as non-viral vectors. Niosomes are closed bilayer vesicles that result from the self-assembly of a non-ionic surfactants (NISs) in aqueous solution[1]. Niosomes have some advantages over liposomes, including biostability, storage stability and cost-effectiveness[2, 3]. Tween and Span are typical NISs used for the preparation of niosomes. Such niosomes have been successfully transfected into the retina and cancer cells[4–7]. On the other hand, it has been reported that the gene vectors composed of cationic surfactants such as ionic surfactants have low dispersion stability and gene transfection efficiency, but they can be improved by adding NISs[8]. Other NISs, polyoxyethylene stearyl ether (steareth or Brij), can also form vesicles[9, 10], and

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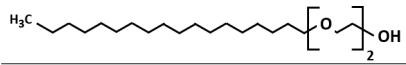
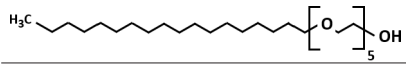
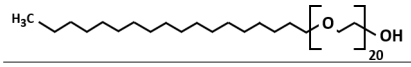
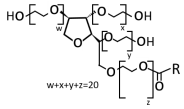
TABLE I. Chemical structures and physicochemical properties of NISs used for the preparation of niosomes in this study.

Chemical structure	Chemical name (Trade name)	Molecular weight (M. W.)	CMC[a] (mM)	HLB[b]
	Polyoxyethylene (2) stearyl ether (Steareth-2)	358	2.5×10^{-4}	4
	Polyoxyethylene (5) stearyl ether (Steareth-5)	490	—	8
	Polyoxyethylene (20) stearyl ether (Steareth-20)	1,096	5.7×10^{-3}	13
	Polyoxyethylene sorbitan monooleate (Tween 80)	1,310	1.0×10^{-2}	15

[a] Critical micelle concentration. The CMC value was obtained from reference [14]. The value was not obtained from reference in steareth-5.
[b] Hydrophilic-lipophilic balance.

their possible application as gene vectors has been suggested, as the niosomes consist of Tween or Span. The characteristics of polyoxyethylene stearyl ether differ according to the length of the hydrophilic ethylene oxide (EO) chain and affect the physicochemical properties of niosomes. The differences may also affect the gene transfection efficiency by niosomes. Therefore, in order to deliver genes effectively, it is important to investigate the effect of EO chain length of polyoxyethylene stearyl ether of niosomes on the physicochemical properties and gene transfection efficiency.

In the present study, we focused on niosomes consisting of a series of polyoxyethylene stearyl ether (steareth-2, steareth-5 and steareth-20), cholesterol and a cationic lipid (octadecylamine) as new gene delivery niosomes and assessed the effect of the steareth's EO chain length on the physicochemical characteristics, gene expression efficiency and cytotoxicity. Octadecylamine was selected to allow efficient gene transfection[11]. Furthermore, in order to condense the genes more tightly and enhance transfection efficiency, poly-L-ornithine (PLO)[12, 13], which is polycation was used. From the above, we prepared ternary complexes designed from plasmid DNA (pDNA), PLO and niosomes consisting of various steareths (steareth-2, steareth-5 and steareth-20). Tween 80 niosome which is among the most frequently utilized in niosome preparation and shows high gene transfection efficiency was used for comparison purposes[4, 6, 7]. Table I summarized the chemical structure and physicochemical properties of each NIS used for the preparation of niosomes in this study.

Experimental

1. Materials

The plasmid pGL-3 Control Vector encoding the luciferase gene as a reporter gene was purchased from Promega (Madison, WI), amplified in *Escherichia coli* JM109 and purified with a QIAGEN Plasmid Midi kit (Hilden, Germany). Steareth-2, steareth-5 and steareth-20 (the numbers of EO chains are 2, 5 and 20, respectively) were provided by Nihon Emulsion Co., Ltd. (Japan). Tween 80 and cholesterol were purchased from FUJIFILM Wako Pure Chemical Co. (Japan). Octadecylamine was obtained from Tokyo Chemical Industry Co. Ltd. (Japan). Poly-L-ornithine hydrobromide (PLO, 78 kDa) was purchased from Alamanda Polymers Inc. (AI, USA). Human lung adenocarcinoma (A549) cell lines were obtained from Riken cell bank (Japan). SYBR® Gold, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and Opti-MEM® were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents and chemicals were of special grade.

2. Preparation of niosomes

Niosomes were prepared by the thin film hydration method and sonication method by modifying a previously reported process[4, 5]. Each NIS (100 μmol), cholesterol (100 μmol) and octadecylamine (5 μmol) was dissolved in chloroform in a test tube. Chloroform was removed in a vacuum desiccator to form a lipid film. After that, the lipid film was dried under reduced pressure overnight to remove the remaining solvent. The dried lipid film was hydrated with 5 mL of 10 mM HEPES buffer (pH 7.4). The resulting niosome suspension was incubated at 60°C for 20 minutes. The incubated suspension was sonicated on ice for 1 minute in 3 cycles using sonifier® SFX (Branson Ultrasonics. Corp., Danbury, CT, USA). Finally, the suspension was filtrated with a 0.20 μm membrane.

TABLE Ⅱ. Particle size, PDI and ζ potential of each niosome and pDNA/PLO/niosome ternary complex.

Type of NIS	Size (nm)		ζ potential (mV)		PDI	
	Niosome	pDNA/PLO/niosome	Niosome	pDNA/PLO/niosome	Niosome	pDNA/PLO/niosome
Steareth-2	156.3 ± 3.4	163.0 ± 4.6	39.9 ± 1.7	45.0 ± 5.7	0.24 ± 0.03	0.25 ± 0.03
Steareth-5	153.3 ± 8.3	151.2 ± 7.4	16.9 ± 2.8	20.7 ± 5.1	0.28 ± 0.02	0.30 ± 0.02
Steareth-20	132.4 ± 3.0	131.5 ± 1.9	13.3 ± 3.8	19.1 ± 2.9	0.21 ± 0.02	0.22 ± 0.02
Tween 80	77.0 ± 8.7	76.5 ± 4.3	2.3 ± 0.2	4.8 ± 2.2	0.59 ± 0.04	0.54 ± 0.03

Data are shown as the mean ± SD ($n = 3$).

3. Preparation of pDNA/PLO complexes and pDNA/PLO/niosome ternary complexes

In order to prepare pDNA/PLO complexes with a fixed mass ratio of 1:4, 100 μg/mL pDNA solution (0.6 mL) was added to 100 μg/mL PLO solution (2.4 mL) while stirring. The mixture was incubated for 30 minutes at room temperature to form complexes. Then, in order to prepare pDNA/PLO/niosome ternary complexes with a mass ratio of 1 : 4 : 30, which showed high DNA condensation ability and transfection efficiency in a preliminary study, pDNA/PLO complex suspension (0.5 mL) was added to each niosome suspension (2.2 mL) while stirring. The mixture was further incubated for 30 minutes at room temperature to form ternary complexes.

4. Measurement of particle size, PDI and ζ potential

The mean particle size, polydispersity index (PDI) and ζ potential of niosome and pDNA/PLO/niosome ternary complexes were measured using a Zetasizer 3000HSA (Malvern, UK).

5. Agarose gel retardation assay

Each pDNA/PLO/niosome ternary complex (containing 0.1 μg pDNA), pDNA/PLO complex suspensions and pDNA solution was loaded on 1% agarose gel, and electrophoresed in Tris-acetate-EDTA buffer for 40 minutes at 100 V. After electrophoresis, the gel was stained with SYBR® Gold for 30 minutes. The stained gel was imaged using Gel Doc EZ (Bio-Rad Laboratories Ltd., Maryland, USA). The protective effect of prepared complexes against the polyanion, which was dextran sulfate (DS), was also assessed. An equal volume of 1% DS solution was added to pDNA/PLO/niosome ternary complex and pDNA/PLO complex suspensions. These mixtures were incubated for 30 minutes at room temperature, and electrophoresis was then performed as described above.

6. Evaluation of gene transfection efficiency

Before the transfection experiment, A549 cells were seeded in a 24-well plate at a density of 1.0×10^5 cells/well in 0.5 mL of DMEM supplemented with 10% FBS, and then incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced with 0.5 mL of Opti-MEM®, and the suspension of each complex was applied (500 ng of pDNA in complex per well) to each well. After 3 hours of incubation at 37°C, the test medium was replaced with fresh DMEM and incubated for an additional 45 hours. After removing the medium, the cells were lysed with cell lysis solution. The luminescence intensity generated by luciferase was measured using a Luciferase assay system (Promega) according to the manufacturer's protocol. The total protein content was quantified with a Pierce® BCA Protein Assay kit (Thermo Fisher Scientific). The Luciferase gene expression efficiency was expressed as relative luminescent units (RLU) per mg of protein.

7. Evaluation of cell viability by MTT assay

The suspension for each complex was applied to A549 cells according to the procedure described in the previous section. After 48 hours of incubation, the medium was replaced with DMEM (0.5 mL) containing MTT (0.5 mg/mL) and incubated for 4 hours at 37°C. After removing the medium, dimethyl sulfoxide was added to each well to dissolve the resulting formazan. The absorbance of the solution at a wavelength of 450 nm was measured using a Multiskan Ascent Plate Reader (MTX Lab System, FL, USA). The cell viability was expressed as a percentage of the absorbance of the treated cells to the untreated cells.

8. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The significance of differences between groups was analyzed by a one-way analysis of variance (ANOVA) with Tukey's post hoc test and Student's t-test. P values of <0.05 were considered to indicate statistical significance.

Results and Discussion

1. Particle size, PDI and ζ potential of niosomes and pDNA/PLO/niosome ternary complexes

As shown in Table Ⅱ, the average particle size and ζ potential of steareth niosomes were decreased with lengthening of the EO chain in steareth; those of steareth-2 niosome were the highest. Although the particle size and ζ potential of Tween 80 niosome were smaller

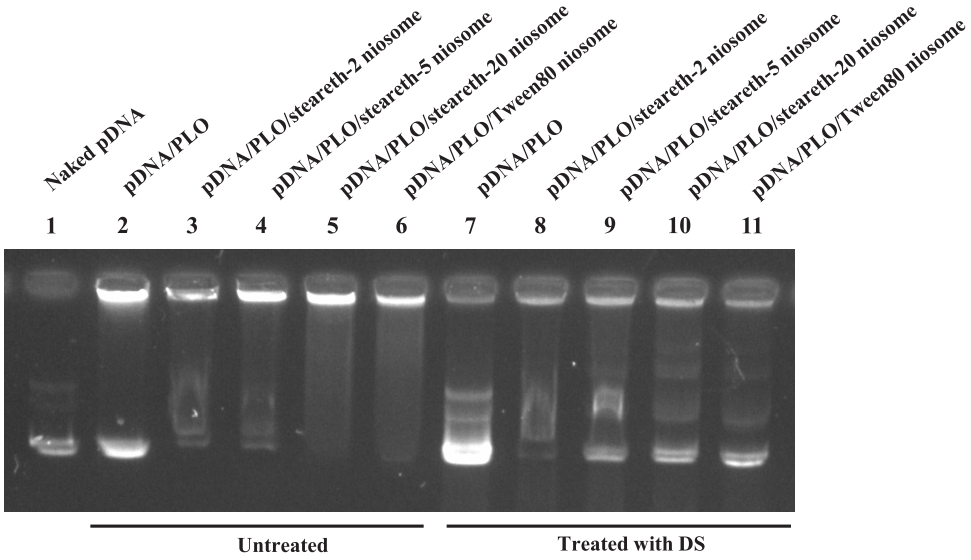


Fig. 1. Gel retardation and protective effect of pDNA/PLO complexes and pDNA/PLO/niosome ternary complexes against anionic polysaccharides (DS). Lane 1, Naked pDNA; lanes 2 and 7, pDNA/PLO complex; lanes 3 and 8, pDNA/PLO/steareth-2 niosome ternary complex; lanes 4 and 9, pDNA/PLO/steareth-5 niosome ternary complex; lanes 5 and 10, pDNA/PLO/steareth-20 niosome ternary complex; lanes 6 and 11, pDNA/PLO/Tween 80 niosome ternary complex. The weight ratios of pDNA/PLO complex and each of the pDNA/PLO/niosomes ternary complexes were 1 : 4 and 1 : 4 : 30, respectively. Lanes 2–6, Samples were not treated with DS (Untreated). Lanes 7–11, Samples were treated with DS.

in comparison to steareth niosomes, the PDI value was higher, indicating that a wide particle size distribution and aggregation of niosomes were obtained. The average particle size and ζ potential of pDNA/PLO/niosome ternary complexes were also decreased with lengthening of the EO chain in steareth.

2. The pDNA condensation ability and protective effect of pDNA/PLO complexes and pDNA/PLO/niosome ternary complexes

In the absence of DS treatment, partial pDNA migrated in the gel in pDNA/PLO complexes (Fig. 1, lane 2). On the other hand, pDNAs were completely retarded in all pDNA/PLO/niosome ternary complexes, indicating that their complexes had high pDNA condensation ability (Fig. 1, lanes 3–6). When pDNA/PLO complexes were treated with DS, pDNA was almost completely dissociated from the complexes (Fig. 1, lane 7). However, the complete dissociation of pDNA was not observed in the case of pDNA/PLO/niosome ternary complexes (Fig. 1, lanes 8–11). As a result, these results revealed that combination with some cationic compounds can give pDNA/PLO/niosome ternary complexes a high pDNA condensation ability and protective effect against anionic polysaccharides. In addition, pDNA/PLO/steareth-2 niosome ternary complexes showed the highest protective effect (Fig. 1, lane 8).

3. Transfection efficiency and cytotoxicity by pDNA/PLO/niosome ternary complexes

The transfection efficiency of pDNA/PLO/steareth niosome ternary complexes was decreased with lengthening of the EO chain in steareth; that of pDNA/PLO/steareth-2 niosome ternary complexes were the highest and were even more higher than pDNA/PLO/Tween 80 niosome ternary complexes (Fig. 2A). Gene vectors with a particle size of approximately 100–200 nm and positive charge promote cellular uptake[15]. On the other hand, gene vectors containing NISs and polyethylene glycol inhibit interaction with the cells depending on the EO chain length rather than the particle size[16]. Therefore, a longer EO chain inhibits the interaction between pDNA/PLO/niosome ternary complexes and the cell membrane through steric hindrance, resulting in decreased transfection efficiency. The cell viability in pDNA/PLO/steareth-2 niosome ternary complexes was approximately 90%, while that in pDNA/PLO/steareth-20 niosome ternary complexes decreased to approximately 4% (Fig. 2B). The result in this study suggests that cell membrane damage is not due to the larger positive charge of the complex, but is closely related to surfactants property, which is hypothesized that NISs with higher hydrophobic content and lower HLB show low membrane solubilization[17].

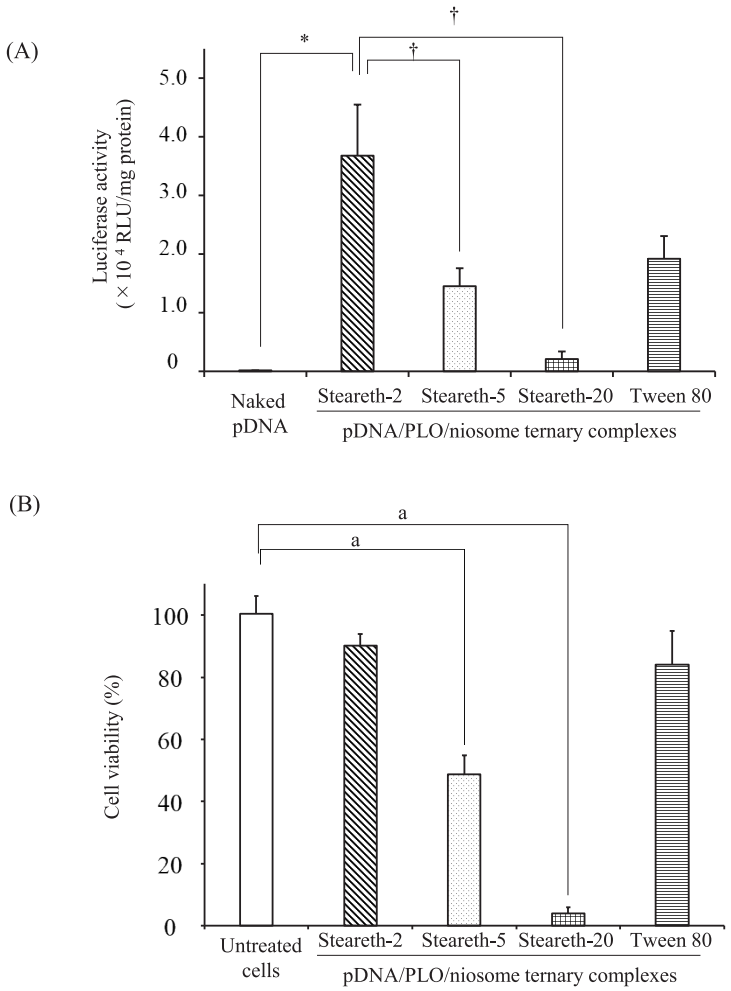


Fig. 2. Transfection efficiency and cell viability of each pDNA/PLO/niosome ternary complex at a weight ratio of 1∶4∶30 in A549 cells. (A) Transfection efficiency. Data are shown as the mean ± SD ($n = 3$). The significance of differences was analyzed by a one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.01$ in comparison to naked pDNA; $^{\dagger}p < 0.05$ in comparison to pDNA/PLO/steareth-2 ternary complexes. (B) Cell viability. Data are shown as the mean ± SD ($n = 3$). The significance of differences was analyzed with Student's t-test. $^{a}p < 0.01$ in comparison to untreated cells.

Conclusion

We attempted to develop gene vectors combining various steareth niosomes and polycation. The EO chain length in steareths affects the physicochemical characteristics, gene expression efficiency and cytotoxicity of pDNA/PLO/steareth niosome ternary complexes. Among pDNA/PLO/steareth niosome ternary complexes, the complex using steareth-2 niosome showed better protective effect, efficacy and safety. Moreover the gene expression efficiency of pDNA/PLO/steareth-2 niosome ternary complexes was higher than pDNA/PLO/Tween 80 niosome ternary complexes. Further study is required to more elevate the transfection efficiency, stability and safety of pDNA/PLO/steareth-2 niosome ternary complexes, such as using target compounds (ligands, specific antibodies, etc.). In addition, it is supposed that pDNA/PLO/steareth-2 niosome ternary complexes are probably transfected into the cells via endocytosis after the complexes bind to the cell membrane, but the mechanism is still unclear in this study. The study of transfection mechanism is also needed.

References

1) F. Uchegbu, S. P. Vyas, Non-ionic surfactant based vesicles (niosomes) in drug delivery, *Int. J. Pharm.*, **172**, 33–70 (1998).

2) G. Abdelbary, N. El-gendy, Niosome-encapsulated gentamicin for ophthalmic controlled delivery, *AAPS PharmSciTech*, **9**, 740–747 (2008).
3) R. Rajera, K. Nagpal, S. K. Singh, D. N. Mishra, Niosomes: A controlled and novel drug delivery system, *Biol. Pharm. Bull*., **34**, 945–953 (2011).
4) Y. Huang, Y. Rao, J. Chen, V. C. Yang, W. Liang, Polysorbate cationic synthetic vesicle for gene delivery, *J. Biomed. Mater Res*., **96**, 513–519 (2011).
5) O. Paecharoenchai, N. Niyomtham, L. Leksantikul, T. Ngawhirunpat, T. Rojanarata, B. Yingyongnarongkul, P. Opanasopit, Nonionic surfactant vesicles composed of novel spermine-derivative cationic lipids as an effective gene carrier *in vitro*, *AAPS PharmSciTech*, **15**, 722–730 (2014).
6) G. Puras, M. Mashal, J. Zárate, M. Agirre, E. Ojeda, S. Grijalvo, R. Eritja, A. Diaz-Tahoces, G. Martínez Navarrete, M. Avilés-Trigueros, E. Fernández, J. L. Pedraz, A novel cationic niosome formulation for gene delivery to the retina, *J. Controlled Release*, **174**, 27–36 (2014).
7) G. Puras, G. Martínez-Navarrete, M. Mashal, J. Zárate, M. Agirre, E. Ojeda, R. Eritja, A. Diaz-Tahoces, M. Avilés-Trigueros, E. Fernández, J. L. Pedraz, Protamine/DNA/niosome ternary nonviral vectors for gene delivery to the retina: The role of protamine, *Mol. Pharm*., **12**, 3658–3671 (2015).
8) J. You, M. Kamihira, S. Iijima, Surfactant-mediated gene transfer for animal cells, *Cytotechnology*, **25**, 45–52 (1997).
9) Z. S. Bayindir, N. Yuksel, Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery, *J. Pharm. Sci*., **99**, 2049–2060 (2010).
10) R. C. Pasquali, M. P. Taurozzi, N. Sacco, C. Bregni, Birefringent emulsions stabilized with steareth-2 and steareth-21, *Lat. Am. J. Pharm*., **27**, 839–844 (2008).
11) D. Wang, N. Jing, Q. Lin, Stearylamine liposome as a new efficient reagent for DNA transfection of eukaryotic cells, *Biochem. Biophys. Res. Commun*., **226**, 450–455 (1996).
12) E. Ramsay, J. Hadgraft, J. Birchall, M. Gumbleton, Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in-vitro, *Int. J. Pharm*., **210**, 97–107 (2000).
13) M. Tokunaga, M. Nagao, M. Nagata, N Hazemoto., T. Yotsuyanagi, DNA transfection mediated by synthetic polycationic peptides, *J. Pharm. Sci. Technol*., *Jpn*., **63**, 71–78 (2003).
14) S. K. Hait, S. P. Moulik, Determination of critical micelle concentration (CMC) of nonionic surfactants by donor-acceptor interaction with iodine and correlation of CMC with hydrophile-lipophile balance and other parameters of the surfactants, *J. Surfactants Deterg*., **4**, 303–309 (2001).
15) P. Li, S. Chen, Y. Jiang, J. Jiang, Z. Zhang, X. Sun, Dendritic cell targeted liposomes-protamine-DNA complexes mediated by synthetic mannosylated cholestrol as a potential carrier for DNA vaccine, *Nanotechnology*, **24**, 295101 (2013).
16) T. W. Kim, Y. J. Kim, H. Chung, I. C. Kwon, H. C. Sung, S. Y. Jeong, The role of non-ionic surfactants on cationic lipid mediated gene transfer, *J. Controlled Release*, **82**, 455–465 (2002).
17) M. P. Vinardell, M. R. Infante, The relationship between the chain length of non-ionic surfactants and their hemolytic action on human erythrocytes, *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol. Endocrinol*., **124**, 117–120 (1999).