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Cationic niosomes as gene carriers: preparation and cellular uptake *in vitro*

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Cationic niosomes of sorbitan monoesters were prepared using film hydration method and tested for their effect on delivery of antisense oligonucleotides (OND) in a COS-7 cell line. These formulations showed positive results on cellular uptake of antisense oligonucleotides. Especially, cationic niosomes of Span® 40 and 60 have a more significant effect.

Niosomes, the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed bilayer structures, are superior in cost, chemical stability and storage compared with liposomes (Uchegbu and Vyas 1998). Niosomes have been applied in pharmaceutics since the 1980s, and niosomes of sorbitan monoesters have been studied as drug delivery system for 5-fluorouracil (Namdeo and Jain 1999), doxorubicin (Uchegbu et al. 1996), colchicine (Hao et al. 2002) and insulin (Varshosaz et al. 2003).

In this study cationic niosomes of sorbitan monoesters (Span® 20, 40, 60, 80) containing the cationic lipid (3β[*N*-(*N′*,*N′*-dimethylaminoethan)-carbamoyl]cholesterol, DC-Chol) were prepared using the film hydration method with sonication which formed complexes with OND as gene carrier. DC-Chol, a cholesterol derivative with low toxicity, was the first cationic lipid used for clinical trial (Caplen et al. 1995). We used it as a substitute for cholesterol that was the most common membrane additive in niosomal systems. Characterization of niosomes was performed by measurements of particle sizes and zeta potential. The average diameters of niosomes of spans® ranged from 100 to 160 nm. With incorporation of the cationic lipid of DC-Chol, the zeta potentials of niosomes of spans® were all above +30 mV which was admitted as electrostatic stabilization level (Heurtault et al. 2003). The results are listed in the Table.

The cytotoxicity of cationic niosomes was determined using a modification of the MTT (dimethyl-thiazol-diphenyl-tetrazolium-bromide) method (Mosmann 1983). Cationic niosomes showed low cellular toxicity; the percentage of the cells viability was above 80% in all the test concentrations. The percentage of the cell viability was above 90% when the lipid concentration was less than 40 μM.

Binding efficiency of the self-assemble complexes of OND/niosomes was examined using agarose gel electrophoresis. When the charge ratio (+/−) was 4 or higher, cationic niosomes could efficiently form complexes with OND. From the gel electrophoresis assay, no OND strap was visible in the lanes of the tested complexes.

The cellular uptake in an *in vitro* study was carried out in a COS-7 (African green monkey) cell line. The results are shown in the Figure. Niosomes of Spans® were effective in mediating cellular uptake of OND. Compared with cationic liposomes (CL) composed of equimolar amounts of soybean phospholipids and DC-Chol, niosomes of Span® 40 and 60 showed a significant enhancement in this study ($P < 0.05$) while those of Span® 20 and 80 also had some positive effects but without significant difference.

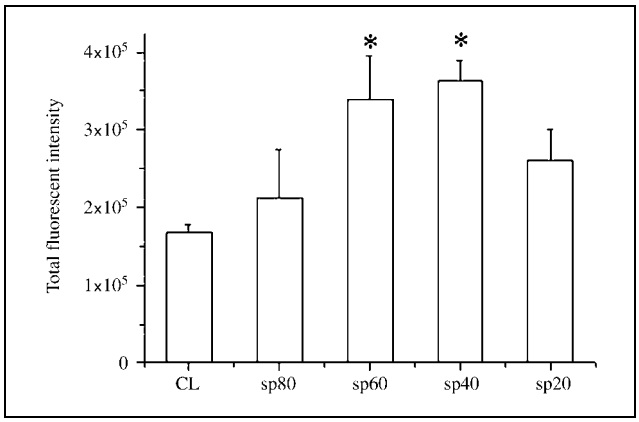

Fig.: The cellular uptake of OND mediated by cationic niosomes of Spans®. Each experiment was performed in triplicate and the values were expressed as mean ± S.D. Statistical analyses were performed using Student's t-test. (* $P < 0.05$)

Niosomes have been widely applied in anticancer, anti-infective, anti-inflammatory and diagnostic imaging drug delivery (Uchegbu and Vyas 1998), but attempts to use them as gene carriers are new. To our knowledge, this cationic niosomes of Spans® were firstly used for gene delivery. In this niosomal gene delivery system, DC-Chol, a cationic cholesterol derivative, is a contributing component for this gene carrier. Firstly, the incorporation of cholesterol serves to facilitate the formation of niosomes and to stabilize the bilayer (Uchegbu and Vyas 1998). Secondly, it offers positive charge to bind nucleic acid drugs and the accessibility to cellular membrane that is the prerequisite to mediating cellular endocytosis (Gao and Huang 1991). And thirdly, vesicle surface positive charged by the cationic cholesterol molecule leads to electrostatic stabilization in colloidal systems (Heurtault et al. 2003). Additionally a

Table: Particles size and zeta potential of niosomes of Spans®

	Sp80-nio	Sp60-nio	Sp40-nio	Sp20-nio
Size/nm	162.9 ± 15.2	104.5 ± 11.6	135.8 ± 17.3	123.7 ± 19.0
Zeta/mV	44.5 ± 2.8	38.7 ± 2.3	43.4 ± 1.1	47.6 ± 2.5

surfactant can destabilize the liquid crystal structures of membranes (Almgren 2000), which could facilitate microparticles entering cells. This niosomal system could serve as a potential non-viral carrier for gene delivery.

Experimental

1. Materials

Sorbitan monoesters surfactants including Span® 80, 60, 40 and 20 were obtained from Shanghai Chemical Reagent Co., China. Soybean phospholipids was purchased from Lipoid GmbH. DC-Chol was synthesized as described by Gao and Huang (Gao and Huang 1991). 15-mer phosphorothioate oligonucleotides with the sequence 5′-CTC, AGT, TAG, GGT, TAG-3′ were randomly synthesized by Shanghai Sangon Bio-engineering Technology Co and 5′ end was labeled with 5-carboxyfluorescein. All other reagents were of analytical grade kindly supplied by Huadong Medical Co., China.
COS-7 cell line was kindly provided by Sir Run Run Shaw Hospital, Zhejiang University and cultured with Dulbecco's Modification of Eagle's Medium (Gibco) with 10% Fetal Bovine Serum (FBS) (Hangzhou Sijiqing Bio-engineering Material Co. China).

2. Procedures

2.1. Preparation of cationic niosomes of spans®

Niosomes composed of span® and DC-Chol in equimolar amounts were prepared by the film hydration method with sonication. In brief, 20 μmol of lipids mixture was dissolved in chloroform in a pear-shaped flask, subsequently attached to a rotary evaporator to dry the organic solvent. The dried lipid film was hydrated with 5 ml double distilled water and rotated in a water bath at 60 °C for 20 min. The niosome dispersions were then sonicated with 200 W for 3 min by ultrasound probe (JY92, Ningbo Scientz Biotechnology Co., LTD). The final dispersion was filtered through a 0.2 μm filter membrane.

2.2. Measurements of particle size and zeta potential

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

2.3. Cytotoxicity study (MTT method)

COS-7 cells (5×10^3 cells/well) were seeded in 96-well plates and cultured to 80% confluence at 37 °C in a 5% CO_2 humidified incubator. After replacing the culture medium with FBS-free medium, niosomes dispersions were added to the wells with final concentrations of lipids as 20, 40, 60, 80, 120 μM, and incubated for 4 h. The medium was again replaced with 10% FBS-culture medium and the cells were cultured for 24 h. The cytotoxicity was determined by comparing the amount of MTT reduced by the cells treated with niosomes to that reduced by control cells. Briefly, MTT was added (100 μg per well), and the plate was incubated at 37 °C for 4 h. Then the medium was carefully aspirated. A 150 μL DMSO (per well) was added, and the absorbance at 490 nm was measured for each well on Microplate Reader (BIO-RAD, USA).

2.4. Gel electrophoresis

OND/niosomes complexes were prepared by mixing equal volumes of the niosomes and OND at the lipid positive charge/nucleotide negative charge ratio as 1 : 1, 1 : 2, 1 : 4, 1 : 6 and 1 : 8, respectively. The OND solution was rapidly injected into the cationic niosomes suspension to prepare complexes with an excess positive charge ratio or at the charge equivalence point and incubated for 10 min at room temperature. Agarose gel (0.8%) was prepared. The complexes were carefully added at a volume representing 2 μg of OND per well. Free OND was also added as the control.

2.5. Cellular uptake in vitro

COS-7 cells (2×10^5 cells/well) were seeded in 24-well plates and cultured to 80% confluence before cellular uptake experiment. The complexes with 10 μL of niosomes and 1 μg of OND were prepared as described above (the lipid concentration was 40 μM). The complexes were added to cells right after replacing the culture medium with FBS-free medium. Cells were incubated for 4 h. The culture medium was then removed. Cells were washed twice with PBS and collected by trypsinization, centrifuged ($1200 \times g$, 3 min), washed twice with PBS. Then cells were resuspended in PBS and analyzed by flow cytometry (Beckman Coulter, USA). The percentage of positive fluorescence cells (α) and mean of fluorescent intensity (MFI) were determined. Cellular uptake efficiencies were evaluated by calculated total fluorescent intensity (TFI) according to the following formula:

$$\mathrm{TFI} = \alpha \times \mathrm{MFI} \times 10^4 \quad (1)$$

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