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Effect of Pluronic on cellular uptake of cationic liposomes- mediated antisense oligonucleotides

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Cationic liposomes modified by different Pluronic® block copolymers were prepared. The influence of Pluronic on the cellular uptake of antisense oligonucleotides (ODN) based on cationic 3β[*N*-(*N*′, *N*′-dimethylaminoethan)-carbamoyl] cholesterol (DC-Chol) liposomes was studied by flow cytometric analysis. It showed that DC-Chol liposomes containing Pluronic gave 1.7–2.3 times higher capacity of cellular uptake of ODN, despite the diminution of ODN loading efficiency. The level of improvement by Pluronic is related to the hydrophobic propylene oxide (PO) units Pluronic contains as well as the lipophile/hydrophile value of the molecule. This preliminary study indicated that modifying liposomes with another excipient is a useful pharmaceutical technique to improve ODN delivery.

1. Introduction

The ability to disrupt gene expression efficiently has become an important experimental approach for studying the biological function of a target gene at the cell level (Miller and Das 1988). In particular, agents causing down-regulation of expression of genes that relate to disease states might be of therapeutic value. Selective inhibition can be achieved by the binding of appropriate single-strand oligodeoxyribonucleotides, which define antisense strategies. The field of antisense aligonncleotides (ODN) has made remarkable advances over the past two decades (Tamm et al. 2001). Development of ODN delivery systems has a significant impact on successful clinical application (Akhtar et al. 2000). Cationic liposomes are one of the most promising non-viral vectors. However, there still exist many problems to be resolved or improved, such as low transfection efficiency, inhibition by serum, expression in non-target organs, etc (Tseng and Huang 1998; Tomlinson and Rolland 1996; Pouton and Seymour 2001).

Many papers have demonstrated the positive role of modifying the surface of liposomes (Chung et al. 2001; Oku et al. 2001). The aim of this study was to investigate the influence of liposomes containing Pluronic block copolymers on the cellular uptake of ODN. Firstly, a series of Pluronic eopolymers were incorporated into cationic liposomes consisting of 3β[N-(*N*′, *N*′-dimethylaminoethan)-carbamoyl] cholesterol (DC-Chol) and soy bean phosphatidylcholine (SPC). The properties of the liposome/ODN complexes were investigated. Finally, we used the fluorescence labeled ODN to evaluate the cellular uptake of different liposome-mediated ODN.

Table 1: Size of liposomes and liposome/ODN complexes with or without Pluronic (nm)

Pluronic	Liposomes	Liposomes/ODN complexes
–	214.0 (0.25)	1244.2 (0.32)
F68	153.4 (0.18)	863.2 (0.25)
F87	159.2 (0.21)	879.1 (0.28)
F88	152.9 (0.20)	1052.8 (0.27)
F108	154.0 (0.19)	856.9 (0.31)
P85	164.6 (0.18)	804.4 (0.22)

Liposome/ODN complexes with +/− charge ratio of 2 : 1. Particle size expressed by intensity values. Data represented by mean values (n = 10) and polydispersity index (PI) shown in parentheses.

2. Investigations, results and discussion

2.1. Size of liposomes and liposomes/ODN complexes

Table 1 shows that smaller particles and a narrower size distribution are obtained in the presence of Pluronic. When the pre-formed liposomes were mixed with ODN, particle sizes were greatly increased, which contributed to the formation of liposome/ODN complexes. The particle sizes of complexes with Pluronic were also smaller than those without Pluronic. The size of pre-lipsomes or complexes with different Pluronic eopolymers were closely similar.

2.2. ODN loading efficiency

A Nanosep centrifuge tube with 30kD molecular cut-off could separate the unbound ODN efficiently. After centrifugation, the unbound ODN passed through a filter membrane while the complexes were removed. We verified that centrifugation three times was enough to recover all the unbound ODN because A_{260} in the second filtrate was negligible.

DC-Chol/SPC liposomes showed a high adsorption capacity for ODN. When the +/− charge ratio exceeded 2 : 1, a loading efficiency of nearly 90% was achieved. Below this value, the adsorption profile was found to be dependent on

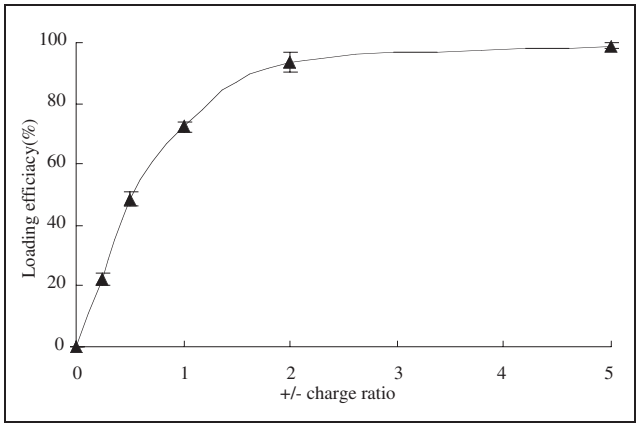

Fig. 1: ODN loading efficiency of DC-Chol/SPC liposomes as a function of +/− charge ratio. Each point represents the mean ± S.D. of four experiments

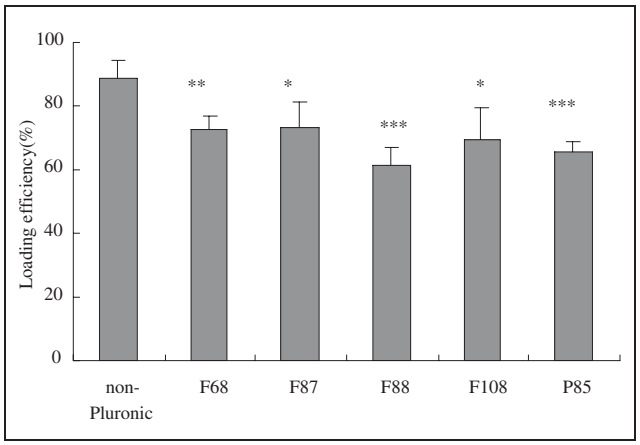

Fig. 2: Effect of various pluroniccopolymers on ODN loading efficiency. The +/− charge of liposomes/ODN was kept at 2 : 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Vs non-Pluronic group using Student's t-test. Each point represents the mean ± S.D. of four experiments

the +/− charge ratio (Fig. 1). Obviously, the high drug loading efficiency was the result of electrostatic interaction between the positively charged lipid and the negatively charged ODN (Stuart and Allen 2000). However, DC-Chol/SPC liposomes incorporating Pluronic showed decreased ODN loading efficiency with the same 2 : 1 +/− charge ratio (Fig. 2). A possible reason is that some part of the hydrophilic ethylene oxide (EO) chain in Pluronic is distributed at the outer surface of the lipid membrane interfering with adsorption of ODN (Chandaroy et al. 2001). There is only a slight difference in ODN loading efficiency between liposomes modified by different Pluronic copolymers.

2.3. Cellular uptake of ODN

When 6-carboxy-fluorescene (FAM)-labeled ODN was kept at a constant concentration, cellular uptake of ODN based on DC-Chol/SPC liposomes was largely dependent on the +/− charge ratio. The total fluorescence intensity (TFI) in cytoplasm was increased with higher +/− charge ratio (Fig. 3). It is reasonably believed that the high +/− charge ratio not only adsorbs ODN efficiently, but also facilitates the interaction of negatively charged cell membranes and positively charged liposomes. Thus, cells can easily take in complexes via endocytosis (Zelphati and Szoka 1996; Sakurai et al. 2000). However, cytotoxicity occurred at high +/− charge ratio (for 5 : 1 and 8 : 1, less than 90% and 70% viable cells).

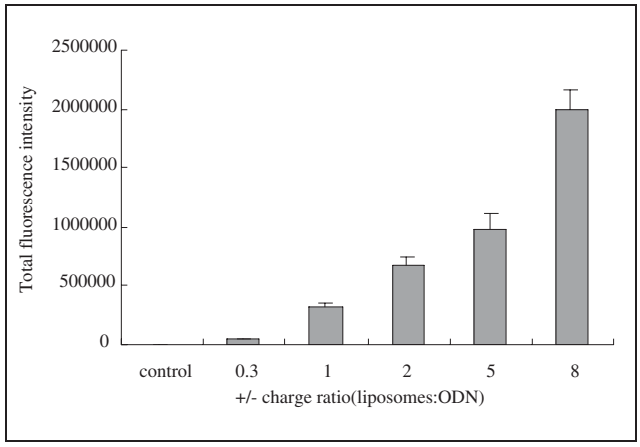

Fig. 3: Effect of +/− charge ratio on the cellular uptake of ODN by HeLa cell mediated by cationic Chol/SPC liposome. Each point represents the mean ± S.D. of three experiments

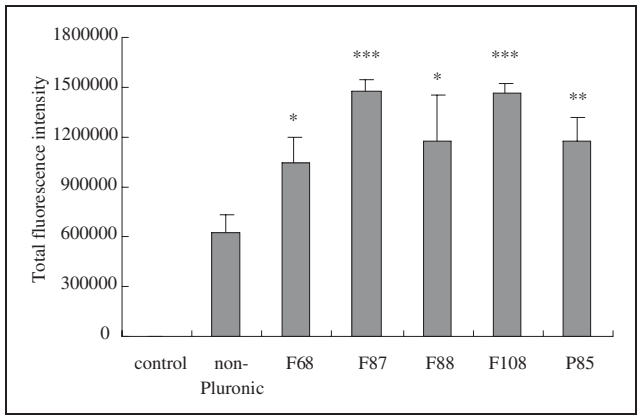

Fig. 4: Effect of different pluroniccopolymers incorporated into DC-Chol/SPC liposomes on the cellular uptake of ODN by HeLa cells. The +/− charge of liposome/ODN complexes was kept at 2 : 1. * $p < 0.05$, * $p < 0.01$, *** $p < 0.001$ Vs non-Pluronic group using Student's t-test. Each point represents the mean ± S.D. of three experiments

Astafieva et al. (1996) and Cho et al. (2000, 2001) have demonstrated that DNA uptake and transgene expression based on cationic polymers such as poly(*N*-ethyl-4-vinylpyridinium bromide)polyethylenimine and poly(L-lysine) can be increased by mixing or grafting with Pluronic block copolymers. In our experiments, we investigated whether the improvement could be achieved with cationic liposomes. Although Pluronic reduced the ODN loading efficiency, cellular uptake of ODN could be further enhanced by introducing Pluronic into liposomes, as shown in Fig. 4. The cellular uptake of ODN from Pluronic-based-liposomes was 1.7–2.3 times higher than from DC-Chol/SPC liposomes while no significant cell viability was observed in these tested formulations. It is well-known that the targeted site of pharmacology of ODN is in cytoplasm. So the advantage of incorporating Pluronic into liposomes is not only increased ODN uptake, but also a reduction in the cytotoxicity caused by cationic lipids if the same concentration in cytoplasm is necessary for therapy. Pluronic block copolymers consist of EO and PO blocks arranged in a triblock structure. It has been reported that Pluronic promotes permeation of cell membranes (Nakamura et al. 1996; Erukova et al. 2000). The possible mechanism may involve their disturbance of bilayer lipids. A reasonable explanation is that the hydrophobic nature of PO in Pluronic facilitates the interaction of the membrane bilayer and liposomes. Table 2 lists the

Table 2: Structure of Pluronic

Type	Formula	L/H value
F68	$(PEO)_{80}-(PPO)_{30}-(PEO)_{80}$	0.1875
F87	$(PEO)_{64}-(PPO)_{40}-(PEO)_{64}$	0.3125
F88	$(PEO)_{103}-(PPO)_{39}-(PEO)_{103}$	0.1093
F108	$(PEO)_{129}-(PPO)_{56}-(PEO)_{129}$	0.2170
P85	$(PEO)_{25}-(PPO)_{40}-(PEO)_{25}$	0.8000

L/H: the ratio value of lipophile/hydrophile

molecular structure and lipophile/hydrophile values of Pluronic used in our experiments. Among these, F108 has the most PO units while F68 contains least, and the TFI in cytoplasm delivered by F108 liposomes is more intense than that given by F68 liposomes. We suggest that Pluronic copolymers containing more hydrophobic PO units will cause stronger effects on the cell membrane structure than hydrophilic ones, which allows more liposome/ODN complexes to enter the cytosol. On the other hand, the PO units of F87, F88 and P85 are the same or similar, but the TFI values mediated by these three liposomes are nevertheless different. Thus, we speculate that the lipophile/hydrophile value of the Pluronic molecule might be another important parameter. F87 might have a more suitable lipophile/hydrophile value than the other two, thus giving the highest TFI in the cytoplasm. However, details of the mechanism will be investigated further.

In conclusion, Pluronic block copolymers can further enhance cellular uptake of ODN based on the DC-Chol/SPC liposome. The effect may be related to the structure of Pluronic, as well as to its lipophile/hydrophile value. This study indicates that modifying the liposomes is a useful pharmaceutical technique for improving ODN delivery.

3. Experimental

3.1. Materials

18-mer phosphorothioate antisense oligonucleotides with the sequence 5′-CTC, AGT, TAG, GGT, TAG, ACA-3′ and part of the 5′-end labeled using 6-carboxy-fluorescence (FAM) were synthesized by Boya Biotech (Shanghai, China). DC-Chol was synthesized as previously reported (Gao and Huang 1991). SPC was provided by Lipoid (Ludwigshafen, Germany). Pluronic® copolymers were obtained from BASF Corporation (USA). HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. All the cell-culture supplements were purchased from Gibco-BRL (Sango, Shanghai, China). Other chemicals were of analytical grade.

3.2. Preparation of liposomes

Liposomes were prepared by a reverse phase evaporation procedure. Briefly, 15 ml chloroform solution (in which 10 mg DC-Chol and 10 mg SPC were dissolved) and 6 ml phosphate buffered saline (PBS, pH 7.4) were sonicated in a bath-type sonicator to form a homogeneous w/o emulsion. The organic phase was evaporated under nitrogen flow. The raw suspension was filtered through 200 nm millipore filtering membranes. To modify the liposomes, 10 mg of different Pluronic copolymers were pre-dissolved in PBS. The final volume of each liposome was 10 ml.

ODN loading to the liposomes was achieved by mixing the appropriate concentration of liposomes with ODN solution (dissolved in PBS) at room-temperature. In order to obtain the desired +/− charge ratio, the theoretical charge ratio of liposomes/ ODN was calculated as a molar ratio of a nucleotide unit to DC-Chol (monovalent) (Kawakami et al. 2000).

3.3. Characterization of liposomes

The particle sizes were determined by photon correlation spectroscopy (PCS) using a Zetasizer 3000HS (Malvern Instruments, UK). The liposomes and liposome/ODN complexes with +/− charge ratio of 2 : 1 were diluted in PBS.

ODN loading efficiency was determined by seperating the unbound ODN using a Nanosep® OD030C33 centrifuge tube (30kD cut-off, Pall Biosciences Lab, USA). Liposome/ODN complexes with a suitable +/− charge ratio were prepared in 100 μl total volume of PBS in a Nanosep tube. After filtration and centrifugation, an addtional 100 μl PBS was added, this procedure being repreated three times. The filtrate was combined and A_{260} was measured by UV spectrophotometer (Pharmacia Biotech, Gene Quant II). The amount of unbound ODN was calculated using a calibration curve. ODN loading efficiency was calculated from this value with respect to the initial ODN (3 μg).

3.4. Cellular uptake

HeLa cells were seeded in 24-well plates at a density of 2×10^5 cells per well and preincubated overnight. After approximately 80% confluency, culture media were replaced with serum-free RPMI 1640. Then 100 μl serum-free RPMI 1640 media containing different liposomes and FAM-ODN (3 μg/well) complexes were added. After 4 h, cells were harvested and analyzed by Facscan flow cytometry (Coulter, USA). Data were acquired on viable and random 10^4 cells. The percentage of positive fluorescence cells (α) and mean fluorescence values (MFI) were determined and cellular uptake efficiency was evaluated by calculating TFI as follows: $TFI = 10^4 \times \alpha \times MFI$. Each time point was performed in triplicate and the values were expressed as mean ± S.D.

3.5. Statistics

Statistical analyses were performed using Student's t-test.

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