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Article

α, ω-Cholesterol-Functionalized Low Molecular Weight Polyethylene Glycol as a Novel Modifier of Cationic Liposomes for Gene Delivery

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Abstract: Here, three novel cholesterol (Ch)/low molecular weight polyethylene glycol (PEG) conjugates, termed α, ω-cholesterol-functionalized PEG (Ch_2-PEG_n), were successfully synthesized using three kinds of PEG with different average molecular weight (PEG_{600}, PEG_{1000} and PEG_{2000}). The purpose of the study was to investigate the potential application of novel cationic liposomes (Ch_2-PEG_n-CLs) containing Ch_2-PEG_n in gene delivery. The introduction of Ch_2-PEG_n affected both the particle size and zeta potential of cationic liposomes. Ch_2-PEG_{2000} effectively compressed liposomal particles and Ch_2-PEG_{2000}-CLs were of the smallest size. Ch_2-PEG_{1000} and Ch_2-PEG_{2000} significantly decreased zeta potentials of Ch_2-PEG_n-CLs, while Ch_2-PEG_{600} did not alter the zeta potential due to the

short PEG chain. Moreover, the *in vitro* gene transfection efficiencies mediated by different Ch_2-PEG_n-CLs also differed, in which Ch_2-PEG_{600}-CLs achieved the strongest GFP expression than Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs in SKOV-3 cells. The gene delivery efficacy of Ch_2-PEG_n-CLs was further examined by addition of a targeting moiety (folate ligand) in both folate-receptor (FR) overexpressing SKOV-3 cells and A549 cells with low expression of FR. For Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs, higher molar ratios of folate ligand resulted in enhanced transfection efficacies, but Ch_2-PEG_{600}-CLs had no similar in contrast. Additionally, MTT assay proved the reduced cytotoxicities of cationic liposomes after modification by Ch_2-PEG_n. These findings provide important insights into the effects of Ch_2-PEG_n on cationic liposomes for delivering genes, which would be beneficial for the development of Ch_2-PEG_n-CLs-based gene delivery system.

Keywords: cholesterol; polyethylene glycol; cationic liposomes; gene delivery; folate ligand

1. Introduction

Cholesterol (Ch), an essential membrane component in higher eukaryotes, modulates functions of membrane proteins and participates in several membrane trafficking and transmembrane signaling processes [1]. Ch facilitates the formation of semi-permeable barriers between cellular compartments and regulates membrane fluidity [1,2]. Ch has been widely used for liposome preparation and other lipid-based drug delivery systems [3,4]. Furthermore, the hydroxyl group in Ch can be modified with other moieties [5,6]. Poly (ethylene glycol) (PEG) cholesterol conjugates (PEG-Ch) had been developed to enhance the stability and activity of liposomes and other lipid-based drug delivery systems [7–9]. PEG-Ch conjugates were further modified with targeting ligands or antibodies to increase the targeting efficacy of the delivery systems [10–12].

As a PEG-Ch conjugate, it has been reported that the hydrophobic groups in α, ω-Ch-modified PEG (Ch_2-PEG_n) were capable of inserting into the hydrophobic interior of lipid bilayers or membranes [13–15]. High molecular weight Ch_2-PEG_n (PEG_{30000}–PEG_{35000}) had been used to prepare liposome gels [16,17] and core-shell emulsion particles [18]. However, there is no report about applying Ch_2-PEG_n to gene delivery up to now.

For PEG conjugates, the molecular weight of PEG had a significant effect on the properties of the drug delivery systems containing them [7,19–21]. PEG_{2000} modified polyethylenimine (PEI) was more efficient than PEG_{5000} modified PEI as evaluated by *in vitro* gene transfer [22]. PEG_{400} significantly enhanced fractional laser-assisted drug delivery when compared with PEG_{2050} and PEG_{3350} [23].

Therefore, the purpose of the study was to explore the potential application of novel cationic liposomes (Ch_2-PEG_n-CLs) containing Ch_2-PEG_n using three kinds of PEG with different average molecular weight (PEG_{600}, PEG_{1000} and PEG_{2000}) for gene delivery. The impacts of variation in PEG molecular weight on the properties, toxicities and gene delivery efficacies were determined. In addition, the gene delivery efficacy of Ch_2-PEG_n-CLs was further investigated by introduction of the targeting moiety (folate ligand) in both folate-receptor (FR) overexpressing SKOV-3 cells and A549 cells with low expression of FR.

2. Results and Discussion

2.1. Synthesis and Identification of Ch_2-PEG_n

The successful synthesis of Ch_2-PEG_n was confirmed by ^{1}H-NMR and mass spectra. As shown in Scheme 1, Ch_2-PEG_n was synthesized by esterification of succinic anhydride-cholesterol (suc-Ch) with PEG using 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDCI) as catalysts. The ^{1}H-NMR spectra of Ch_2-PEG_n were shown in Figure 1A. The principal peaks of suc-Ch and poly (ethylene glycol) (PEG) moieties were observed [8,24]. The molecular weights of Ch_2-PEG_n were measured by the Quadrupole-Time of Flight (Q-TOF) mass spectra as shown in Figure 1B. For Ch_2-PEG_{600} and Ch_2-PEG_{1000}, the mass-to-charge ratio (*m*/*z*) spectrums showed dominant ions at *m*/*z* 1545 and 1896. Their *m*/*z* ions were singly charged [$(M + H)^+$]. Therefore, the measured molecular weight of Ch_2-PEG_{600} and Ch_2-PEG_{1000} were 1544 and 1895 Da respectively after subtracting H^+. For Ch_2-PEG_{2000}, the *m*/*z* values differed by 0.5 Da as z, so the number of charges was equal to 2. The *m*/*z* ions were doubly charged [$(M + 2H)^{2+}$]. The measured molecular weight of Ch_2-PEG_{2000} was 3012 Da [$(1508 - 2) \times 2$]. All the calculated molecular weights were consistent with the true molecular weights.

The melting points and appearances of Ch_2-PEG_n were summarized in Table 1. Three kinds of Ch_2-PEG_n had different melting points, which might impact the film-forming property of the mixed lipids and therefore influence the stability of cationic liposomes.

Scheme 1. Synthesis route of Ch_2-PEG_n conjugates.

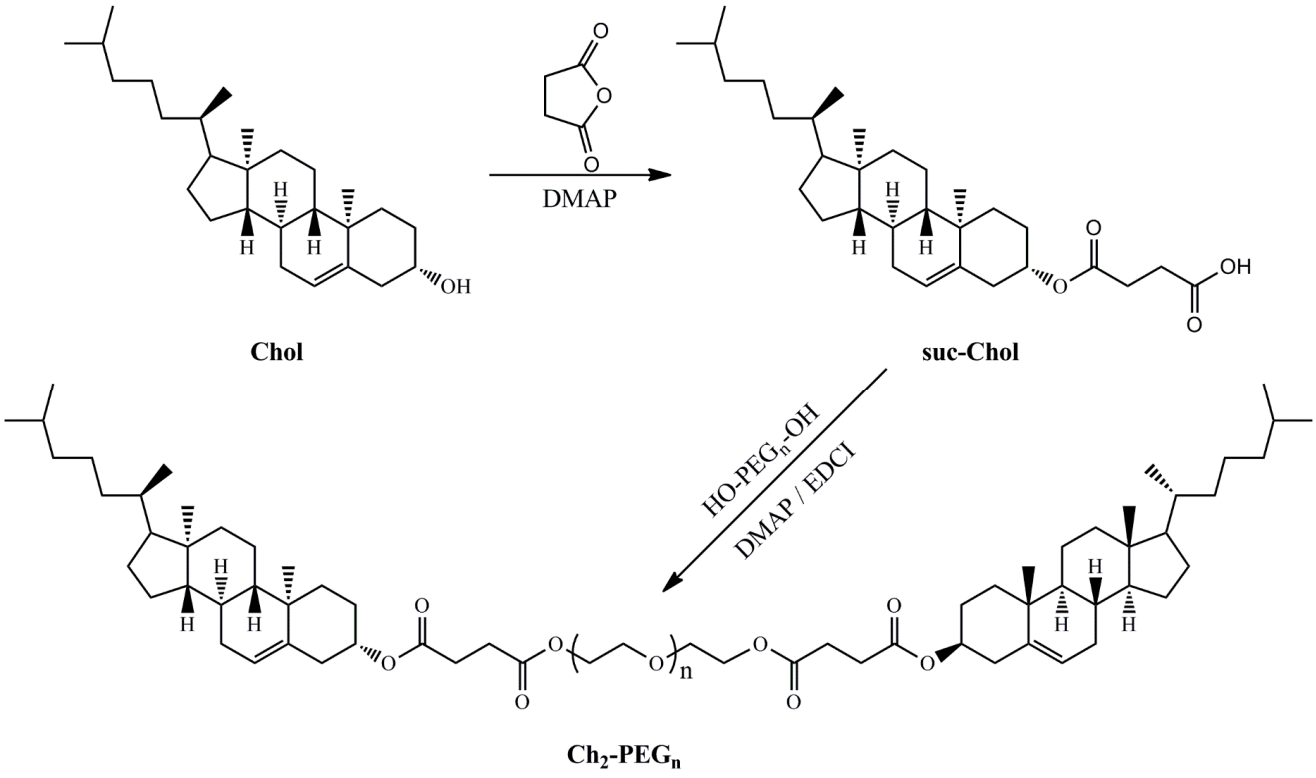


Figure 1. ^{1}H-NMR and mass spectra of Ch_2-PEG_n. (**A**) ^{1}H-NMR spectra (400 MHz) of Ch_2-PEG_n in $CDCl_3$; (**a**,**b**) 6- and 3-position protons in Chol; (**c**) protons of methylene in PEG and (**d**) methylene proton of succinyl group. The principal proton peaks of Chol-suc and PEG were found in Ch_2-PEG_n; (**B**) Mass spectra of Ch_2-PEG_n. The *m*/*z* ions of Ch_2-PEG_{600} and Ch_2-PEG_{1000} are singly charged molecular-related ions; the *m*/*z* ions of Ch_2-PEG_{2000} are doubly charged molecular-related ions. The measured molecular weight of Ch_2-PEG_{600}, Ch_2-PEG_{1000} and Ch_2-PEG_{2000} were 1544, 1895 and 3012 Da, respectively.

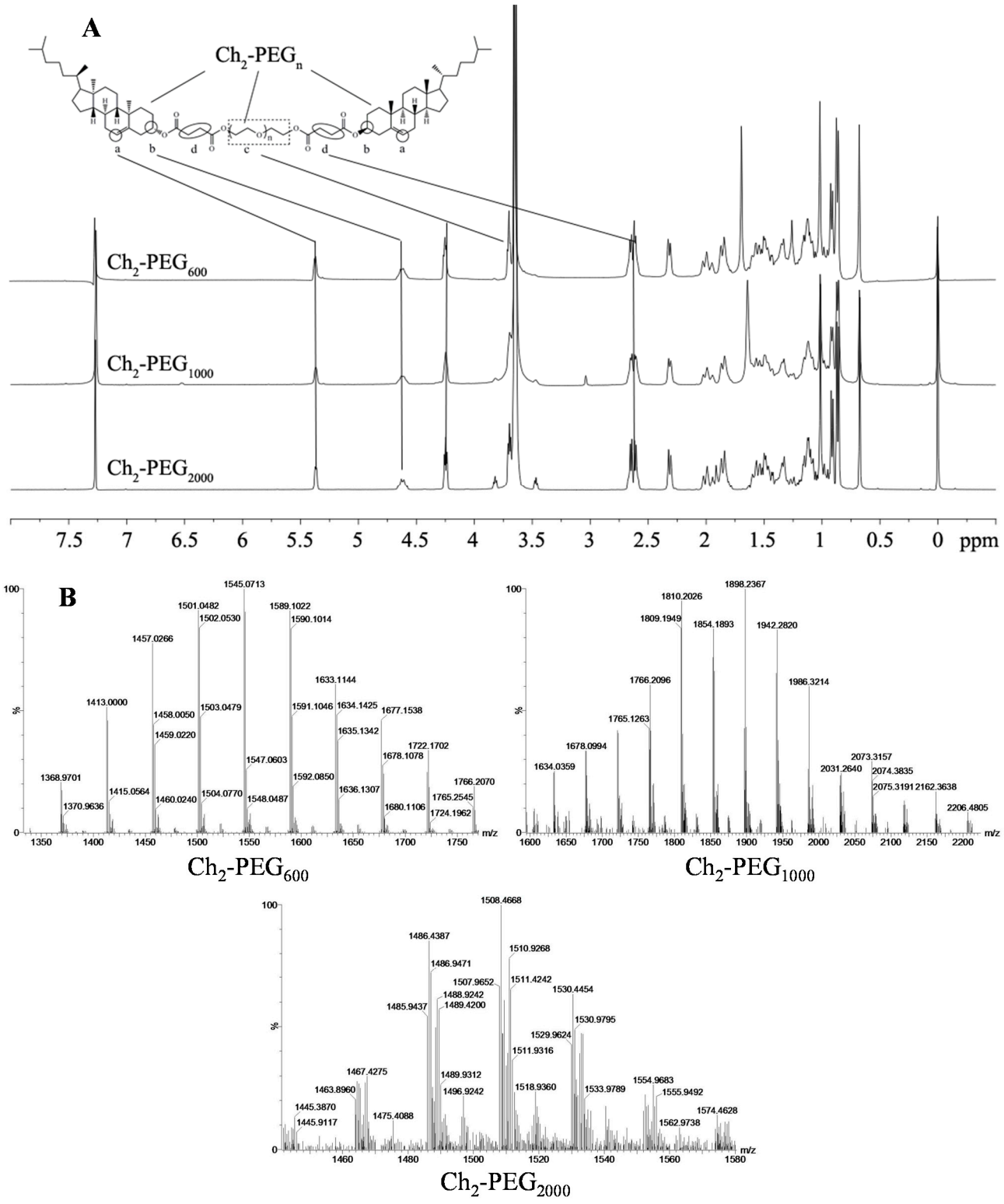


Table. 1. The melting points and appearances of Ch_2-PEG_n. The melting point was recorded as the midpoint value in the melting temperature range to facilitate comparison.

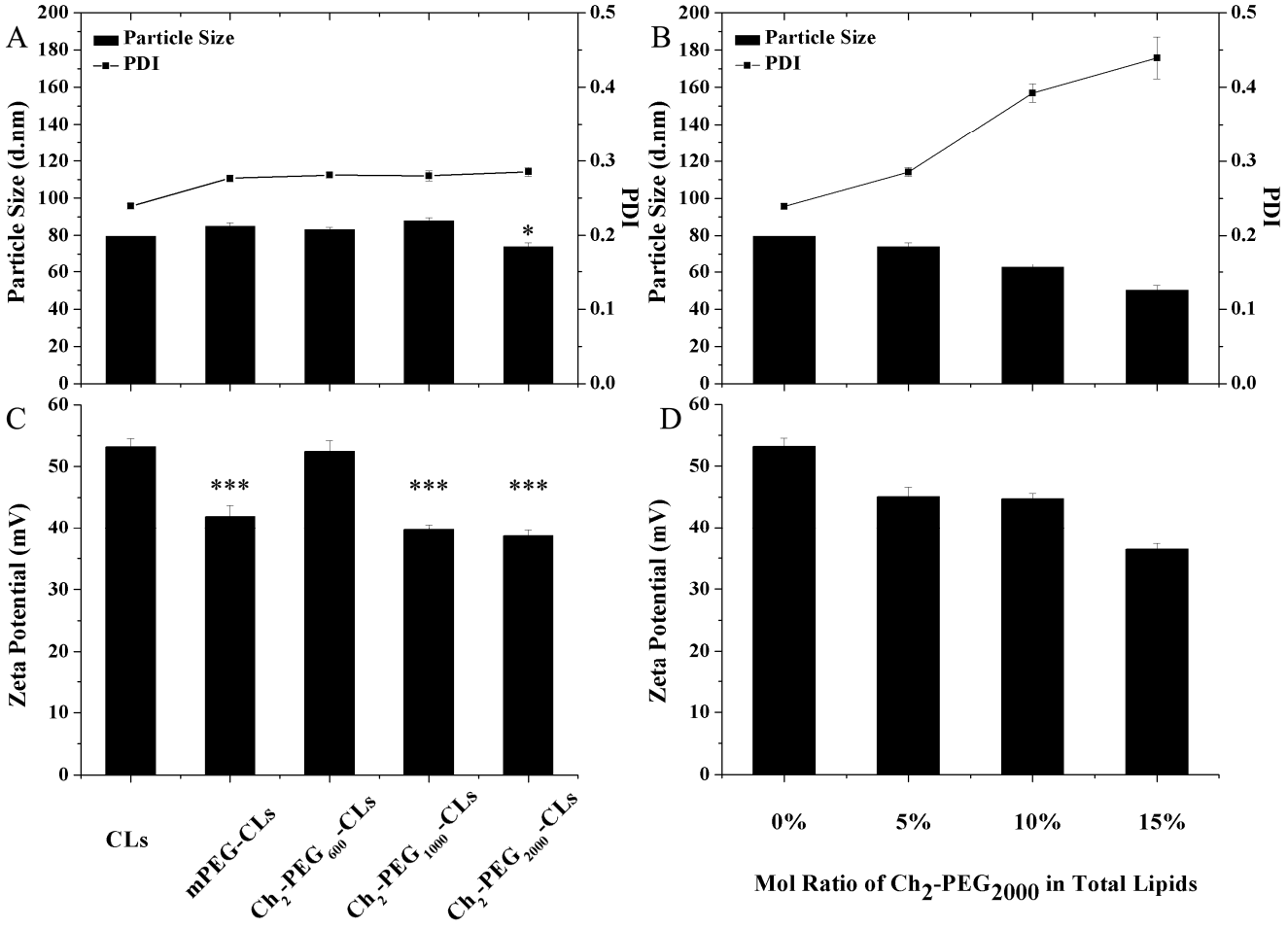
Samples	Melting Point (°C)	Appearance (25 °C)
PEG_{600} → Ch_2-PEG_{600}	20 → 33 (↑)	Clear liquid → Clear semi-solid
PEG_{1000} → Ch_2-PEG_{1000}	33 → 40 (↑)	White paste → Clear solid
PEG_{2000} → Ch_2-PEG_{2000}	52 → 47.5 (↓)	White flake → White powder

2.2. Physicochemical Properties of Ch_2-PEG_n-CLs

The particle size and polydispersity index (PDI) of Ch_2-PEG_n-CLs were shown in Figure 2A. There were no significant differences of the particle size and PDI when comparing Ch_2-PEG_{600}-CLs and Ch_2-PEG_{1000}-CLs with mPEG-CLs (CLs modified by $mPEG_{2000}$-suc-Ch). However, the particle size of Ch_2-PEG_{2000}-CLs (74 nm) was significantly smaller than those of Ch_2-PEG_{600}-CLs and Ch_2-PEG_{1000}-CLs ($p < 0.05$). It was considered that the size decrease of Ch_2-PEG_{2000}-CLs might be attributed to the introduction of Ch_2-PEG_{2000}, which anchored into the liposomal bilayer by Ch segments [25,26] and extended the PEG chain on the surface of the liposome. The PEG chain compressed the liposomal particle [22], and therefore reduced the particle size. Then the effect of Ch_2-PEG_{2000} on particle size was further studied. As shown in Figure 2B, the particle size of Ch_2-PEG_{2000}-CLs gradually decreased with a higher polydispersity when the molar ratio of Ch_2-PEG_{2000} increased. Due to the high curvature and micellar preference of the large hydrophilic PEG chain, it was challenging to stably integrate high molar ratio Ch_2-PEG_n into liposomal bilayer [27]. Therefore, 5 mol% Ch_2-PEG_{2000} was the optimal molar ratio.

The zeta potentials of Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs were significantly lower than that of Ch_2-PEG_{600}-CLs as seen in Figure 2C ($p < 0.001$). It suggested that Ch_2-PEG_{1000} and Ch_2-PEG_{2000} could shield the electric charge of cationic liposomes. Therefore, they were good candidates like $mPEG_{2000}$-suc-Ch for preparing long-circulating or stealth liposomes. The zeta potential of Ch_2-PEG_{600}-CLs was close to CLs (Figure 2C), which was consistent with the previous report that low molecular weight PEG did not effectively shield the positive charge of cationic particles [19]. The potential impact of molar ratio of Ch_2-PEG_{2000} on zeta potentials was also studied. As shown in Figure 2D, 15 mol % was the most efficient ratio in reducing zeta potential. Similar zeta potential values were found with 5 and 10 mol % Ch_2-PEG_{2000}.

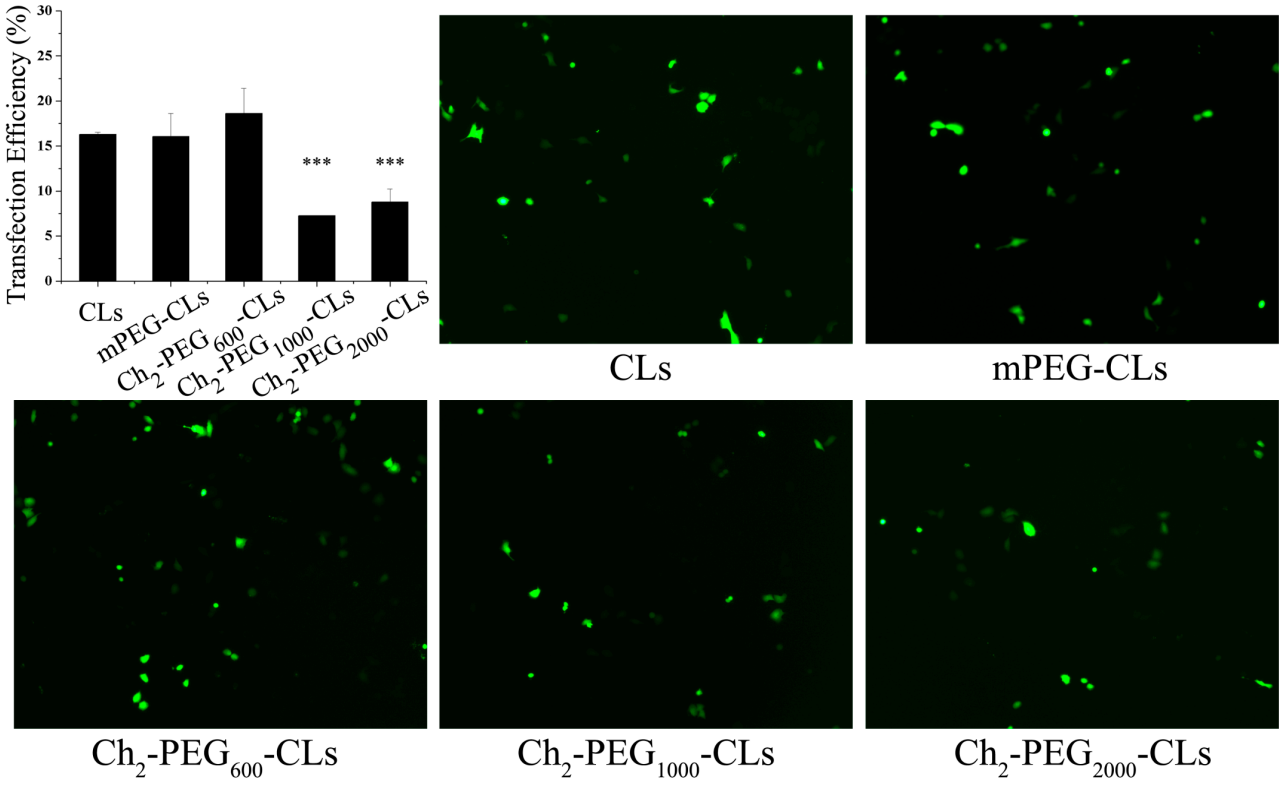
Figure 2. Particle size, PDI and zeta potential of Ch_2-PEG_n-CLs (Mean ± SD, n = 3, * $p < 0.05$, *** $p < 0.001$). (**A**,**C**) Particle size, PDI, and zeta potential of Ch_2-PEG_n-CLs, CLs (normal cationic liposomes without PEG introduction) and mPEG-CLs; (**B**,**D**) The effect of molar ratio of Ch_2-PEG_{2000} on the particle size, PDI and zeta potential of Ch_2-PEG_{2000}-CLs.



2.3. Gene Transfection Efficiencies of Ch_2-PEG_n-CLs

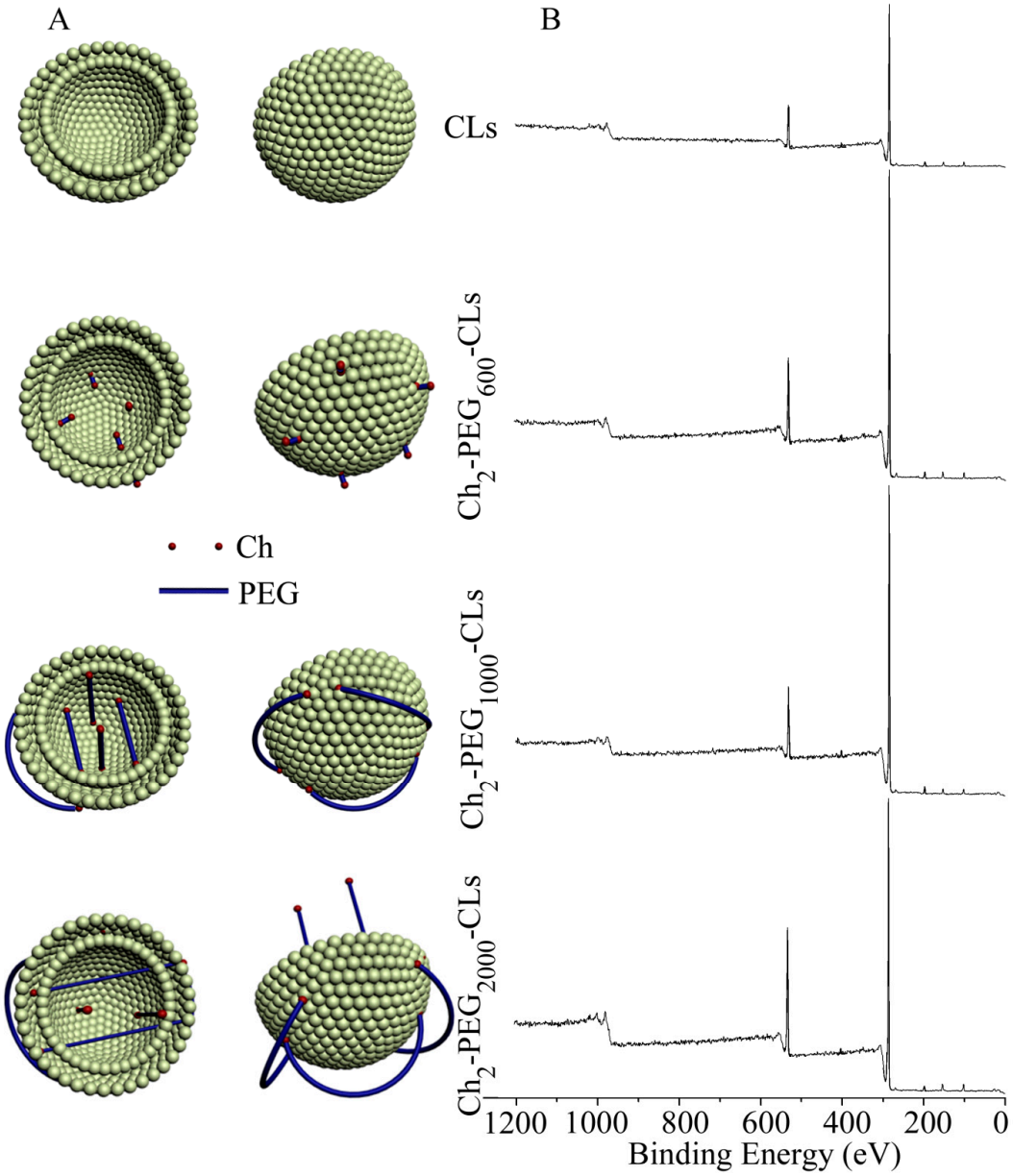
Different types of cationic liposomes were evaluated in gene transfection efficiencies by comparison. As shown in Figure 3, Ch_2-PEG_{600}-CLs had slightly higher transfection efficiency than CLs and mPEG-CLs, but there were no significant differences. In contrast, the transfection efficiencies of Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs were significantly lower than that of Ch_2-PEG_{600}-CLs ($p < 0.001$). The higher zeta potential of Ch_2-PEG_{600}-CLs (Figure 2C) than the other Ch_2-PEG_n-CLs might be due to its high transfection efficiency. Therefore, zeta potential is an important parameter when applying Ch_2-PEG_n to gene delivery. Ch_2-PEG_{2000}-CLs, with the same zeta potential but the smaller particle size (Figure 2A), achieved a bit higher transfection efficiency than Ch_2-PEG_{1000}-CLs.

Figure 3. Transfection efficiency and fluorescence images (200×) of Ch_2-PEG_n-CLs (mean ± SD, $n = 3$, *** $p < 0.001$).



To further explain the differences in zeta potential and transfection efficacies of several cationic liposomes consisting of Ch_2-PEG_n with different molecular weights, XPS analysis was carried out of various Ch_2-PEG_n-CLs and the schematic diagrams are shown in Figure 4. More oxygen atoms from ethylenedioxy groups of PEG were found on the surface of CLs with the increase of PEG molecular weight as shown in Figure 4B. The presence of PEG chains outside of CLs had the potential to shield the electronic charge, and decrease the zeta potential as shown in Figure 4A. However, Ch_2-PEG_{600}-CLs, with the shorter PEG chain, which could not shield the surface charge of liposomes, had a significant higher zeta potential than Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs, which resulted in the increased transfection efficiency. The zeta potentials of Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs were comparable and might be due to the saturation of the shielding effect. Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs also showed comparable transfection efficiency due to similar zeta potential.

Figure 4. Schematic diagram and XPS analysis of Ch_2-PEG_n-CLs. (**A**) Ch segments of Ch_2-PEG_n anchored into the lipid bilayer of CLs. Due to the short PEG chain, Ch_2-PEG_{600} did not effectively shield the charge of CLs. Ch_2-PEG_{1000} and Ch_2-PEG_{2000} decreased the positive charge by covering the surface of CLs. With longer PEG chains, Ch_2-PEG_{2000} compressed the liposomal particle and therefore Ch_2-PEG_{2000}-CLs showed a smaller particle size; (**B**) XPS analysis demonstrated that more PEG segments were located on the surface of liposomes when PEG molecular weight increased.



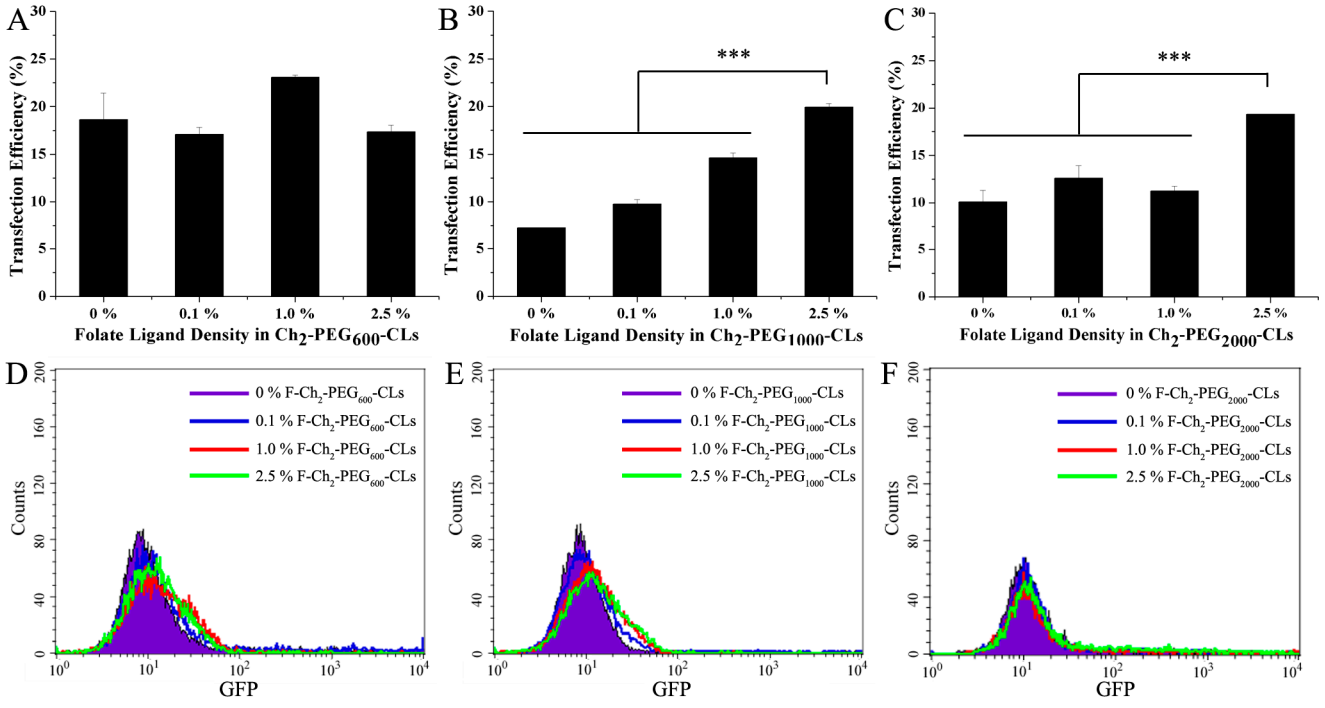
2.4. Gene Transfection Efficiencies of F-Ch_2-PEG_n-CLs with Introduction of a Folate Ligand

The gene delivery efficacy of Ch_2-PEG_n-CLs was further examined by addition of a targeting moiety (folate ligand) in both folate-receptor (FR) overexpressing SKOV-3 cells and A549 cells with low expression of FR.

We previously demonstrated that SKOV-3 cells overexpress the folate receptor alpha [24]. The effects of folate ligand and its density on the transfection efficiencies of Ch_2-PEG_n-CLs in

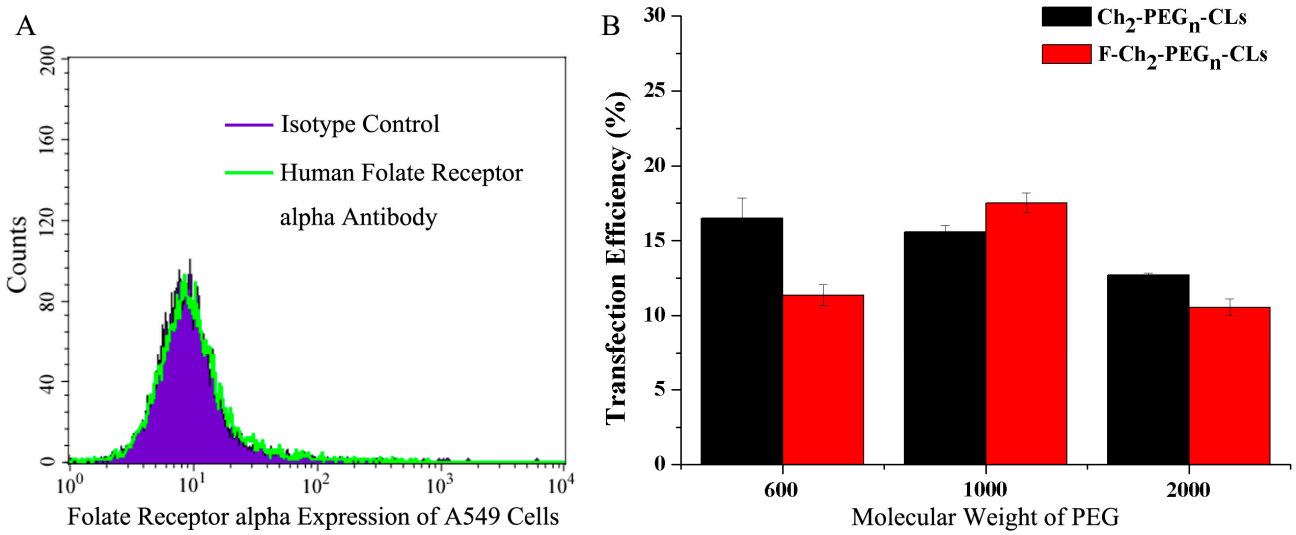
SKOV-3 cells are shown in Figure 5. For Ch_2-PEG_{600}-CLs, the transfection efficacy was close to 19% without folate ligand modification. There was no special trend with the change of folate ligand ratio for F-Ch_2-PEG_{600}-CLs. Their transfection efficacies were all around 20% (Figure 5A,D). In contrast, transfection efficiencies of F-Ch_2-PEG_{1000}-CLs were significantly elevated with the increase of folate ligand density ($p < 0.001$, shown in Figure 5B,E). About 20% cells were transfected with 2.5% F-Ch_2-PEG_{1000}-CLs, which was almost 3-fold of that for Ch_2-PEG_{1000}-CLs without folate modification. Similarly, 2.5% F-Ch_2-PEG_{2000}-CLs had higher transfection efficiency than other Ch_2-PEG_{1000}-CLs as shown in Figure 5C,F. Therefore, the optimal ligand density should be screened when various PEG with different molecular weight was employed for preparing CLs in the future.

Figure 5. The effect of folate ligand densities in F-Ch_2-PEG_n-CLs on transfection efficiencies (mean ± SD, $n = 3$, *** $p < 0.001$). (**A**–**C**) The transfection efficiencies of F-Ch_2-PEG_{600}-CLs, F-Ch_2-PEG_{1000}-CLs and F-Ch_2-PEG_{2000}-CLs. 0%: Ch_2-PEG_n-CLs without folate ligand modification; 0.1%, 1.0% and 2.5%: Ch_2-PEG_n-CLs modified by 0.1, 1.0 and 2.5 mol % F-suc-PEG_{2000}-Chol in total lipids, respectively; (**D**–**F**) The flow cytometry results of transfection by F-Ch_2-PEG_n-CLs.



As shown in Figure 6A, folate receptor alpha is not expressed on A549 cells by folate receptor assay as previously reported [24]. When Ch_2-PEG_n-CLs and F-Ch_2-PEG_n-CLs were used to transfect A549 cells, F-Ch_2-PEG_n-CLs did not significantly increase the transfection efficiency of Ch_2-PEG_n-CLs as shown in Figure 6B.

Figure 6. Folate receptor alpha expression and transfection efficiency on A549 cells. (**A**) Folate receptor alpha expression on A549 cells by flow cytometry and (**B**) The transfection efficiencies of Ch_2-PEG_n-CLs and F-Ch_2-PEG_n-CLs on A549 cells.

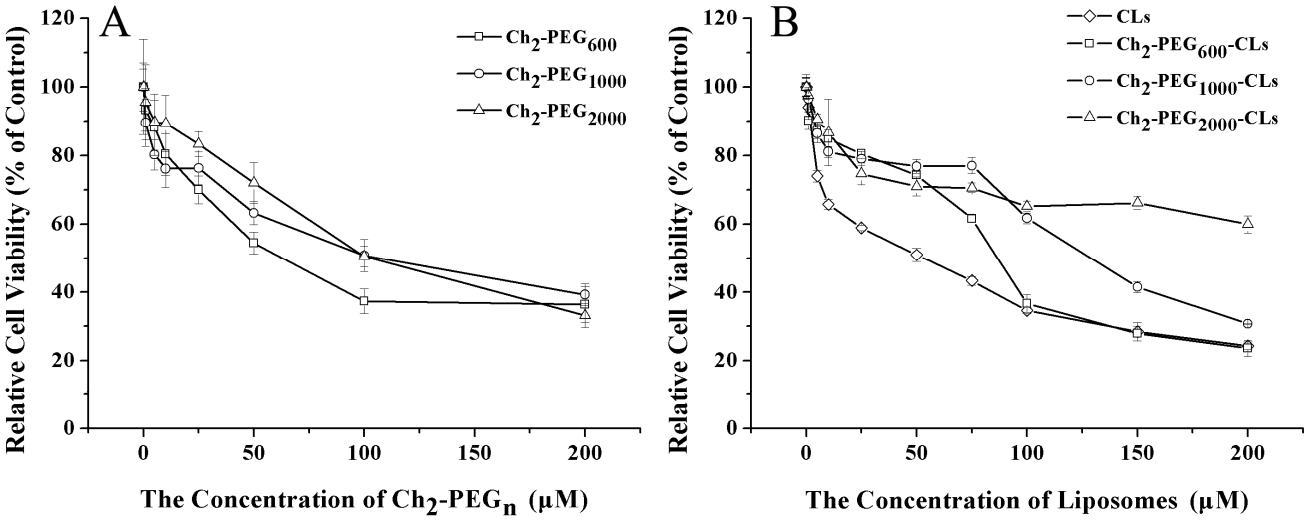


2.5 Cytotoxicity Evaluation

The cytotoxicities of Ch_2-PEG_n on SKOV-3 cells were shown in Figure 7A. The toxicities were concentration-dependent. After treatment for 24 h, the half maximal inhibitory concentration (IC_{50}) for Ch_2-PEG_{600}, Ch_2-PEG_{1000} and Ch_2-PEG_{2000} on SKOV-3 cells were about 78, 145 and 127 μM, respectively. Ch_2-PEG_{600} was slightly more toxic to SKOV-3 cells than the other. However, even for Ch_2-PEG_{600}, the IC_{50} value was higher than other PEG-lipid conjugates that have been extensively used for drug and gene delivery [24,28]. Therefore, Ch_2-PEG_n may be one kind of safe material for gene delivery.

The cytotoxicities of Ch_2-PEG_n-CLs on SKOV-3 cells were shown in Figure 7B. The cytotoxicities gradually decreased with the increase of PEG molecular weight. The IC_{50} values for CLs, Ch_2-PEG_{600}-CLs, Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs were about 38, 100, 143 and 302 μM, respectively. 5 mol % Ch_2-PEG_n significantly enhanced the safety of cationic liposomes when compared with CLs ($p < 0.001$). Therefore, employing Ch_2-PEG_n in preparation was an effective way to reduce the cytotoxicity of cationic liposomes.

Figure 7. Cytotoxicity of Ch_2-PEG_n and Ch_2-PEG_n-CLs on SKOV-3 cells by the MTT assay (Mean ± SD, n = 4–6). (**A**) IC_{50} values for Ch_2-PEG_{600}, Ch_2-PEG_{1000} and Ch_2-PEG_{2000} were about 78, 145 and 127 μM, respectively; (**B**) IC_{50} values for CLs, Ch_2-PEG_{600}-CLs, Ch_2-PEG_{1000}-CLs and Ch_2-PEG_{2000}-CLs were about 38, 100, 143 and 302 μM, respectively. The cytotoxicity of CLs was reduced by introducing Ch_2-PEG_n into Ch_2-PEG_n-CLs.



3. Experimental Section

3.1. Materials

Cholesterol (Ch) was obtained from Bio Life Science & Technology Co., Ltd. (Shanghai, China). Poly (ethylene glycol) (PEG) [molecular weight 600 (PEG_{600}), molecular weight 1000 (PEG_{1000}) and molecular weight 2000 (PEG_{2000})] and 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) was provided by Accela ChemBio Co., Ltd. (Shanghai, China). 4-dimethylaminopyridine (DMAP) was obtained from AstaTech Pharma. Co., Ltd. (Chengdu, Sichuan, China). 1, 2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Folate-PEG-succinyl-cholesterol conjugate (F-PEG_{2000}-suc-Ch) and mPEG-succinyl-cholesterol conjugate ($mPEG_{2000}$-suc-Ch) were synthesized and purified in the same procedures as those recorded in our previous publications [8,24]. Green fluorescent protein plasmid DNA (pDNA) was extracted according to the EndoFree Plasmid Purification Handbook (QIAGEN, Hilden, Germany). All the other reagents and solvents were of analytical grade and were used without further purification except for chloroform used for cationic liposomes preparation.

3.2. Synthesis and Identification of Ch_2-PEG_n

3.2.1. Synthesis of Ch_2-PEG_n

α, ω-Ch-modified PEGs (Ch_2-PEG_n) were synthesized according to scheme 1. Firstly, Ch succinic anhydride ester (suc-Ch) was synthesized as described before [8]. In brief, Ch, succinic anhydride and

DMAP were dissolved in dichlormethane and stirred for 48 h at room temperature. After removing the solvent, the crude product was washed by acetic acid. White suc-Ch was obtained. Secondly, PEG (PEG_{600}, PEG_{1000}, or PEG_{2000}), suc-Ch, DMAP and EDCI were dissolved in chloroform. The mixture was refluxed for 72 h, concentrated under vacuum, and purified on a silica-gel column eluting with dichlormethane and methanol. Ch_2-PEG_{600}, Ch_2-PEG_{1000} and Ch_2-PEG_{2000} were obtained.

3.2.2. ^{1}H-NMR and Mass Spectra of Ch_2-PEG_n

^{1}H-NMR spectra of Ch_2-PEG_n were recorded on a Bruker ADVANCE^{III} spectrometer (400MHz) (Billerica, MA, USA) at room temperature. Ch_2-PEG_{600}, Ch_2-PEG_{1000} and Ch_2-PEG_{2000} were dissolved in $CDCl_3$ with tetramethylsilane as the internal standard. The mass spectra of Ch_2-PEG_n were measured using a Waters Q-TOF Premier (Milford, MA, USA) equipped with ion spray source and N_2 as nebulization gas.

3.2.3. Melting Point and Appearances

Melting points of Ch_2-PEG_n, PEG_{1000}, PEG_{2000}, $mPEG_{2000}$-suc-Ch, suc-Ch and Ch were determined using SGW X-4 melting point apparatus (Shanghai Precision & Scientific Instrument CO., LTD., Shanghai, China). The appearances of the materials were recorded.

3.3. Preparation and Characterization of Cationic Liposomes

3.3.1. Preparation of Liposomes

Ch_2-PEG_n-CLs were prepared by film dispersion method as described before [29]. In brief, DOTAP, Ch and Ch_2-PEG_n (Ch_2-PEG_{600}, Ch_2-PEG_{1000} or Ch_2-PEG_{2000}) at different molar ratios were dissolved in chloroform. Then the organic solvent was removed from the lipids solution using a Büchi rotary evaporator. A thin film was formed and further dried under high vacuum for 6 h at room temperature. The lipid film was hydrated with 5% (*w*/*v*) glucose solution and sonicated by a VCX130 Vibra-Cell (Sonics & Materials Inc., Newtown, CT, USA) until a translucent lipid suspension was obtained. Ch_2-PEG_n-CLs were formed. They were passed through a 0.22 μm Millipore microporous membrane and stored at 4 °C until use.

CLs and mPEG-CLs (served as controls), folate modified Ch_2-PEG_n-CLs (F-Ch_2-PEG_n-CLs, active targeted CLs) were prepared in the same way. CLs were made of DOTAP and Ch. mPEG-CLs were composed of DOTAP, Ch and $mPEG_{2000}$-suc-Ch. F-Ch_2-PEG_n-CLs were consisted of F-PEG_{2000}-suc-Ch, DOTAP, Ch and Ch_2-PEG_n.

3.3.2. Size and Zeta Potential Determination

The mean particle size and zeta potential of the liposomes were measured by a Zetasizer Nano ZS ZEN 3600 (Malvern Instruments, Ltd., Malvern, Worcestershire, UK). The mean particle size was determined at a fixed angle of 173°. The zeta potential of 5 mg/mL liposome at pH 6.0 was automatically calculated from the electrophoretic mobility at 25 °C. All the experiments were performed in triplicate.

3.4. Cell Culture

Human ovarian carcinoma SKOV-3 cell line and human lung carcinoma A549 cell line were obtained from American Type Culture Collection (ATCC). Cells were cultured as a monolayer in Dulbecco's Modified Eagles's Medium (DMEM, Gibco, Carlsbad, CA, USA) or Roswell Park Memorial Institute medium (RPMI)-1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2 at 37 °C.

3.5. In Vitro Transfection Experiments

SKOV-3 or A549 cells were seeded on Costar 6-well plates (Corning Inc., Corning, NY, USA) at a density of 1.5×10^5 cells per well and cultured in DMEM medium or RPMI-1640 as described before [24]. 30 min prior to transfection, the culture medium was replaced by 800 μL serum-free DMEM or RPMI-1640 in each well. Then Ch_2-PEG_n-CLs/pEGFP, CL/pEGFP, mPEG-CLs/pEGFP or F-Ch_2-PEG_n-CLs/pEGFP complexes (200 μL, containing 1 μg pDNA) was added to the wells respectively. Three wells were used for each lipoplex. After incubating for 5–6 h, cell culture medium was changed to DMEM or RPMI-1640 with serum and the cells were incubated for another 42–43 h. The transfected cells were observed under an inverted research microscope, Eclipse T*i* (Nikon Corporation, Tokyo, Japan). Then they were trypsinized with 0.25% trypsin-EDTA, centrifuged and resuspended with PBS. The cell suspensions were analyzed by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) to determine the transfection efficiency of the complexes.

3.6. Cytotoxicity of Ch_2-PEG_n and Ch_2-PEG_n-CLs

Cytotoxicity of Ch_2-PEG_n was evaluated in the SKOV-3 cell line by MTT assay as described previously [30]. Briefly, cells were seeded on 96-well plates (Corning Inc., Corning, NY, USA) in 100 μL medium at a density of 5×10^3 cells per well. After overnight incubation, another 100 μL Ch_2-PEG_n solutions at various concentrations (ranged from 1 to 200 μM) were added to the wells. The cells were incubated for another 24 h. Then 20 μL MTT solution (5 mg/mL in saline) was added to each well. After culturing at 37 °C for 4 h, the medium was removed. 160 μL DMSO was added to each well to dissolve formazan crystals. The absorbance was measured at 570 nm on a Multiskan MK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Untreated cells were used as controls. The relative cell viability compared with control was calculated based on the following equation: Relative cell viability (%) = $(A_{treated}/A_{control}) \times 100$.

3.7. Statistical Analysis

Statistical analysis was performed using Student's Independent-Samples *t*-Test on SPSS (V 19.0, IBM Corp., Armonk, NY, USA). All the statistical tests were two-sided. $p < 0.05$ was considered as statistical significant difference.

4. Conclusions

In this manuscript, a series of Ch_2-PEG_n at different PEG molecular weights (600, 1000 and 2000) were successfully synthesized. Ch_2-PEG_n-CLs containing various Ch_2-PEG_n presented different particle size, zeta potential and *in vivo* transfection efficacy, and Ch_2-PEG_{600}-CLs exhibited the strongest GFP expression in SKOV3 cells due to its highest zeta potential. However, Ch_2-PEG_{600}-CLs also had the highest *in vitro* cytotoxicity. After introduction of a folate ligand, the targeting efficacies and optimized ligand densities of F-Ch_2-PEG_n-CLs still depended on the molecular weights of PEG. In sum, Ch_2-PEG_n-CLs are promising carriers for gene delivery. The current work demonstrates the possibility of utilizing Ch_2-PEG_n for gene delivery, and a corresponding systematic investigation of this study would benefit the future development of Ch_2-PEG_n-CLs-based gene delivery systems.

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Author Contributions

Cui-Cui Ma and Zhi-Yao He performed the experiments, analyzed the data and wrote the paper; Shan Xia, Li-Wei Hui and Han-Xiao Qin performed the experiments; Ke Ren and Jun Zeng wrote the paper; Ming-Hai Tang contributed reagents/materials/analysis tools; Xiang-Rong Song conceived and designed the experiments.

Conflicts of Interest

The authors declare no conflict of interest.

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