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Zinc(II) coordinated lipid for effective and serum-tolerant gene delivery

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Abstract: In recent years, non-viral vectors, such as cationic polymers and liposomes, have attracted increasing attentions for their applications in gene delivery. However, reduced transfection efficiency inhibits their further use in the presence of serum. Therefore, it is necessary to develop serum-tolerant non-viral gene vectors. Herein, a novel Zn coordinated lipid is synthesized, which self-assembles with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine lipid (DOPE), forming cationic liposome (Zn-DTAc liposome). The experimental results demonstrate that Zn-DTAc liposomes can effectively condense pDNA into nanoparticles, which have high cellular uptake and effective endosomal escape and exhibit efficient gene transfection even in the presence of 50% fetal bovine serum.

Keywords: gene delivery; non-viral vector; Zn(II) coordinated lipid; serum-tolerance
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1 Introduction

The delivery of nucleic acids with transient activity into cells is a promising technology with potential applications in the treatment of diseases such as cancer[1], infectious diseases[2], and heritable disorders. Due to the negative charge and large size of nucleic acids, it is difficult for them to diffuse across the negatively charged plasma membrane. In addition, the naked nucleic acids are vulnerable to enzymes that widely exist in the biological systems. Thus, the intracellular delivery of nucleic acids is quite important for successful gene therapy[3]. Both the viral and non-viral vectors can deliver nucleic acids into cells. Despite viral vectors exhibited high transfection efficiency, they have many drawbacks, such as oncogenic[4, 5], mutagenic[6, 7], auto-immunogenic side effects[8, 9], and expensive large-scale production that limit their clinical application. Non-viral vectors such as cationic polymers[10] and liposomes[11] with low immunogenicity and easiness to be modified hold great promise for gene delivery[3, 12].

Generally, most of the therapeutic agents are delivered to the lesions via intravenous injection, thus the interactions between therapeutic reagents and blood components, such as salt and protein, will highly affect the gene delivery and transfection[10]. Previous studies have demonstrated that negatively charged serum proteins would strongly interact with positively charged carrier/nucleic acid complexes to form protein corona[13], resulting in dissociation of the formed complexes[14], size change[15], and nucleic acid degradation by serum nucleases[16], which will decrease the transfection efficiency of non-viral vectors, hampering their further applications. For example, the transfection efficiency of polyethyleneimine (PEI) is dramatically reduced in the presence of serum due to the dissociation of complexes, less internalization and endo/lysosomal escape[17]. Therefore, it would be very important to develop non-viral vectors with high transfection efficiency even in the presence of serum. Previous studies found that Zinc (II)-dipicolylamine unit (Zn-DPA) can strongly interact with nucleic acids due to its ability to bind anionic phosphate groups in the backbone of the nucleic acids[18]. The introduction of Zn-DPA on the cationic polymers[19, 20] could enhance serum stability. Herein, we synthesized a novel Zn-DPA based lipid, which could self-assemble into cationic liposomes (Zn-DTAc liposomes) in the presence of DOPE for effective plasmid transport in a serum-containing environment.

2 Materials and methods

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2.1 Materials

All the reaction reagents were purchased from Energy Chemical (Shanghai, China). DOPE was purchased from Aladdin (Shanghai, China). Branched polyethyleneimine (PEI_{25kDa}) was purchased from Sigma-Aldrich (Shanghai, China). Dulbecco's modified eagle medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from HyClone (USA). Green fluorescent protein plasmids (GFP pDNA) and Luciferase plasmids (Luci pDNA) were purchased from Aldevron (USA). The other reagents were purchased from Beyotime Biotechnology (Shanghai, China).

2.2 Synthesis

2.2.1 Synthesis of DPA-Tyramine

To synthesize DPA-Tyramine, DPA-Tyramine-^{t}BOC was synthesized first. Briefly, a mixture of dipicolylamine (5.98 g, 30 mmol), paraformaldehyde (0.94 g) and hydrochloric acid (catalytic amount) were added to the methanol (50 mL) and heated to reflux. When the mixture became a clear yellow solution through reflux, N-(tert-butoxycarbonyl) tyramine (9.10 g, 36 mmol) was added to the solution, and then the solution was refluxed for 4 d. The solvent was removed by rotary evaporation, and the crude product was purified by column chromatography on silica gel with ethyl acetate/methanol (10:1, v/v). The eluent was removed by rotary evaporation to give a colorless solid.

To obtain DPA-Tyramine, the generated DPA-Tyramine-^{t}BOC was dissolved in a mixture solution of CF_3COOH/CH_2Cl_2 (1:1, v/v), and stirred at room temperature for 12 h. The solvent was removed by rotary evaporation, then sodium hydroxide was applied to adjust the pH to 12. The solution was washed with dichloromethane, then the dichloromethane solution was collected and dried over anhydrous Na_2SO_4. Finally, the solvent was removed by rotary evaporation to give white solid. Yield: 7.31 g, 70%. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 8.76 (d, 2H; H_a), 8.10 (t, 2H; H_c), 7.73 (d, 2H; H_d), 7.63 (t, 2H; H_b), 7.14 (d, 2H; H_i), 6.88 (s, 1H; H_g), 6.82 (d, 1H; H_h), 4.58 (s, 4H; H_e), 4.37 (s, 2H; H_f).

2.2.2 Synthesis of DPA-Tyramine-Ac14

To synthesize DPA-Tyramine-Ac14, DPA-Tyramine (0.70 g, 2.0 mmol) and tetradecyl acrylate (1.21 g, 4.5 mmol) were dissolved in 2 mL DMSO, and 1.5 mL triethylamine was added. Then the mixture was put into a glass tube, and the oxygen in the tube was removed by a pump. Subsequently the tube was sealed. After stirring for 2 d at 90 °C, the solvent was removed by rotary evaporation, and the product was purified by column chromatography on silica gel with dichloromethane/methanol (20: 1 v/v) to give a yellow oil liquid. Yield: 0.67 g, 37.8%. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 8.55 (d, 2H; H_a), 7.61 (t, 2H; H_c), 7.33 (d, 2H; H_d), 7.14 (t, 2H; H_b), 7.97 (d, 1H; H_i), 6.85 (s, 1H; H_g), 6.81 (d, 1H; H_h), 4.03 (t, 4H; H_n), 3.85 (s, 4H; H_e), 3.75 (s, 2H; H_f), 1.63-1.56 (m, 4H; H_o), 0.86 (t, 6H; H_q). HRMS (ESI): $[M+H]^+$ calcd for $C_{55}H_{89}N_4O_5^+$, 886.2761; found, m/z 886.6968.

2.2.3 Synthesis of Zn-DTAc lipid

Zn-DTAc lipid was prepared by mixing DPA-Tyramine-Ac14 and 1.0 equivalent of $Zn(NO_3)_2 \cdot 6H_2O$ in acetone. After stirring for 4 h at room temperature, the solvent was removed by rotary evaporation and subsequently placed under high vacuum overnight to obtain a pale yellow solid. HRMS (ESI): $[M-3H]^+$ calcd for $C_{55}H_{85}N_4O_5Zn^+$, 947.6245; found, m/z 947.5340.

2.3 Preparation of Zn-DTAc liposomes

The Zn-DTAc liposomes were prepared via film dispersion method. Briefly, Zn-DTAc lipid (4.10 mg) and DOPE (5.60 mg) ($n_{lipid}/n_{DOPE} = 1/2$) were dissolved in chloroform in a vial. Then the solvent was removed by vacuum rotary evaporation to form a dry film, and the residual solvent was removed in vacuum drying oven for 3 h at room temperature. Finally, 2.5 mL ultra-pure water was added to the vial and the solution was sonicated in an ice bath for 30 min to afford the cationic liposomes.

2.4 Preparation and characterization of Zn-DTAc/pDNA complexes

Zn-DTAc/pDNA complexes were prepared by mixing appropriate volumes of Zn-DTAc liposomes with DNA (12.5 μg) in ultra-pure water and incubating for 30 min at room temperature. Then the complexes were diluted with ultra-pure water to a final volume of 2.5 mL. Size and zeta potential of the complexes at various Zn/P ratios (the Zn/P ratio was calculated as moral ratio of Zn-DTAc lipid to nucleotide residue) were analyzed with a dynamic light scattering system (Malvern Instruments, UK) at 25 °C. Transmission electron microscopy (TEM) was used to observe the morphology structures of the complexes, which was performed on a JEM-2100F field emission transmission electron microscope with an accelerating voltage of 100 kV.

2.5 Agarose gel electrophoresis

Zn-DTAc/pDNA complexes at different Zn/P ratios were prepared by adding required amounts of Zn-DTAc liposomes solution into 10 μL GFP pDNA (0.1 mg/mL). Then the mixture solution was diluted to a total volume of 26 μL. After incubating at room temperature for 30 min, 10 μL complexes solution was took out and used for DNA binding assay by agarose gel electrophoresis. The complexes were electrophoresed on a 0.9% (w/v) agarose gel containing GelRed at 100 V for 30 min, and then the electrophoresis gel was scanned on a UVP ED3 Imaging System.

2.6 Cell culture

Human embryonic kidney cell line 293T cells were purchased from ATCC (USA) and cultured in DMEM containing 10% FBS at 37 °C, 5% CO_2.

2.7 In vitro gene transfection

To evaluate the gene transfection ability of Zn-DTAc liposomes in vitro, the transfection efficiencies of Zn-DTAc liposomes were tested in 293T cells by using GFP pDNA and Luci pDNA as reporter genes.

For the expression of the GFP gene, 293T cells were seeded in 48-well cell culture plates at a density of 40000 cells/well and cultured to reach 80% confluence before the transfection experiment. Various amounts of Zn-DTAc liposomes and GFP pDNA (1 μg DNA per well) were diluted softly in equal volume DMEM medium, respectively, and were mixed at room temperature to form complexes with different Zn/P ratios. Then culture medium containing 10% FBS was added to the complexes (a final volume of 200 μL). The culture medium of 293T cells was replaced with the medium containing complexes. After 4 h of incubation, the medium was removed and the cells were cultured for further 44 h with fresh DMEM containing 10% FBS. The microscopy images were obtained with an inverted fluorescent microscopy (Olympus), and the transfection efficiencies were quantitatively measured by using flow cytometry (Beckman CytoFLEX, USA). PEI_{25kDa} was used as a positive control according to the manufacturer's protocol.

For the Luciferase assay, 293T cells were seeded in 96-well culture plates at a density of 8000 cells/well, and cultured to reach 80% confluence prior to transfection. Liposomes/DNA complexes (0.5 μg DNA per well) at different Zn/P ratios were prepared as described above. Then the complexes were diluted with 100 μL culture medium containing 10% FBS. After 4 h of incubation, the mixtures were removed, and the cells were cultured for an additional 44 h in fresh DMEM containing 10% FBS. After 48 h of incubation, luciferase plasmids transfection efficiency was performed according to the manufacturer's protocols(Beyotime Biotechnology). The results were expressed as the relative fluorescence intensity (RLU) per milligram of cellular protein by BCA assay kit (Beyotime Biotechnology). PEI_{25kDa} was used to transfect 293T cells according to the manufacturer's protocol as positive control groups.

The antiserum transfection was similar to the ransfection described above. The only difference was that the complexes were diluted with culture medium containing various FBS contents (10%, 30%, 50%) before being added to 293T cells in antiserum transfection assay.

2.8 MTT cytotoxicity assay

The cytotoxicity of complexes was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 293T cells were seeded in a 96-well culture plate at a density of 10000/well. After 24 h, the culture medium was removed and replaced with 100 μL fresh medium containing 10% FBS and different Zn/P ratios of complexes (0.5 μg DNA per well). After 24 h of incubation, the medium was replaced with 100 μL DMEM containing 10 μL MTT stock solution (5.0 mg/mL) and incubated for 4 h at 37 °C. Then the solution was removed and 100 μL DMSO was added to dissolve the generated formazan crystals and the absorbance was measured in an ELISA plate reader (SpectraMax M2E, Molecular Device Inc. USA) at a wavelength of 570 nm. The cell viabilities were calculated by following formula: cell viability (%) = $(OD_{treated}/OD_{control}) \times 100$, where $OD_{treated}$ and $OD_{control}$ represented the optical density of sample and the optical density of blank control, respectively.

2.9 Cellular uptake

The cellular uptake of the complexes was obtained in the presence of 10%, 30%, 50% FBS by using flow cytometry. DNA was marked by Cy5 dye according to the manufacturer's instruction. 293T cells were seeded in a 48-well plate at a density of 40000 cells and cultured overnight. The culture medium was replaced with 100 μL DMEM containing different concentration FBS and Zn-DTAc/Cy5-DNA complexes (Zn/P ratio was 8 and 0.5 μg DNA was used per well). After 4 h of incubation, the medium was removed and the cells were harvested with 0.25% Trypsin/EDTA. Subsequently, the cells were washed with 1 × PBS three times by centrifugal and resuspended in DMEM. Mean fluorescence intensity was analyzed by using a flow cytometer.

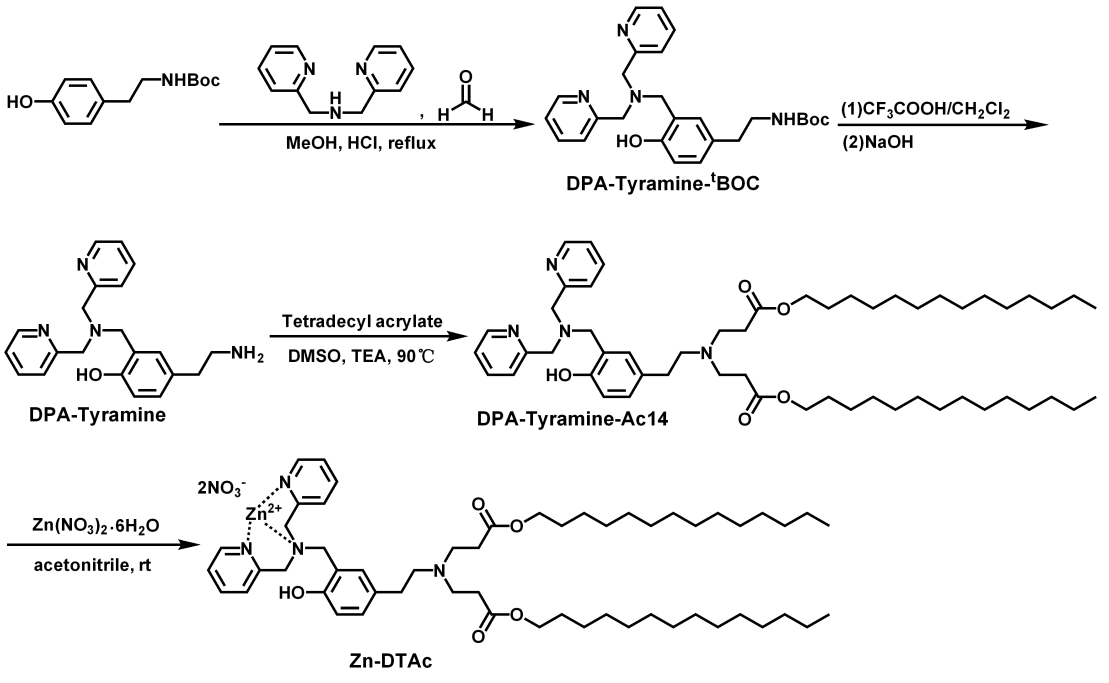
2.10 Confocal imaging

293T cells were seeded in 35 mm × 10 mm confocal dishes at a density of 20000 cells per well and cultured for 24 h. The culture medium was replaced with 200 μL DMEM containing different concentrations FBS and Zn-DTAc/Cy5-DNA complexes(Zn/P ratio was 8 and 1 μg DNA was used per well). After incubating for 4 h, the medium was removed and the cells were washed with 1 × PBS three times. Subsequently, 200 μL Lyso-Tracker Green (6 μM) was added and incubated at 37 °C for 40 min to mark the endosome. Then the Lyso-Tracker Green was removed and 200 μL DAPI was used to stain the nucleus at 37 °C for 10 min. 1 × PBS buffer was used to wash the cells three times. Finally, the cells were fixed with 4% paraformaldehyde for 20 min at 37 °C. Then paraformaldehyde was removed and the cells were washed with 1 × PBS one time. The images were obtained by using a confocal laser scanning microscope (CLSM, Zeiss, Germany) with a 63× objective.

3 Results and discussions

3.1 Synthesis and characterization of materials

The synthesis of Zn-DTAc was shown in Scheme 1. The synthetic compounds were characterized by ^{1}H NMR



Scheme 1 Synthesis route of Zn-DTAc lipid.

(400 MHz, $CDCl_3$) and mass spectrometry (Fig. 1). The results have shown that Zn-DTAc lipid was successfully synthesized.

Particle size and zeta potential are essential factors for colloidal stability, internalization and endocytosis. The mean particle sizes and zeta potentials of Zn-DTAc/pDNA complexes at different Zn/P ratios were shown in Fig. 2(a) and 2(b). As shown in Fig. 2(a), the average hydrodynamic diameters were getting smaller as the Zn/P ratios increasing, indicating that Zn-DTAc can condense pDNA to form complexes. When the Zn/P ratios were beyond 4, the complexes had particle sizes in the range of 100 ～ 200 nm, which was proper for effective endocytosis. Representative TEM image (Fig. 2 (c)) showed that the complexes had a spherical shape with a diameter around 130 nm, which was consistent with the DLS result. The zeta potentials changed from negative to positive value at the Zn/P ratio of 1.5, which suggested that Zn-DTAc liposomes could bind DNA at low Zn/P ratio. And all the positive values were about 20 ～ 30 mV, which could keep the particles from aggregation and facilitate their internalization. The DNA binding ability of Zn-DTAc liposomes was quantified by agarose gel electrophoresis assay. As shown in Fig. 2(d), Zn-DTAc liposomes could almost completely bind DNA at Zn/P ratios of 1, which demonstrated the strong DNA binding ability.

3.2 Gene transfection in vitro

To study the gene transfection ability of Zn-DTAc liposomes in vitro, the transfection efficiencies of Zn-DTAc/pDNA complexes at various Zn/P ratios were determined in 293T cells by using GFP plasmids and Luciferase plasmids as reporter gene. PEI_{25kDa} was used as a control. As shown in Fig. 3(a), the brightest green fluorescence of GFP in Zn-DTAc group was achieved at Zn/P ratio of 8, which was significantly brighter than that of PEI_{25kDa}. Furthermore, The GFP positive cells in each group were quantitatively measured by flow cytometry, and as shown in Fig. 3(b), the transfection efficiency of PEI_{25kDa} (N/P = 10) reached a quantitative value of 65%, while the transfection efficiency of Zn-DTAc liposomes reached a quantitative value of 76% at Zn/P ratio of 8. For the expression of Luciferase plasmids in 293T cells, the transfection efficiency of Zn-DTAc liposomes at Zn/P ratio of 8 was also higher than that of PEI_{25kDa} (Fig 3(c)). These encouraging results revealed that Zn-DTAc liposomes showed superior transfection efficiency than PEI_{25kDa}, thus, Zn-DTAc liposomes have potential application as a non-viral vector for effective gene delivery.

Subsequently, the serum resistance of Zn-DTAc liposomes was investigated by transfection in medium containing 10%, 30%, 50% FBS. As shown in Fig. 4(a) and 4(b), it could be found that Zn-DTAc liposomes preserved high transfection efficiency in the presence of different serum contents. In contrast, the transfection efficiency of PEI_{25kDa} dropped obviously when serum existed because of its poor serum-tolerance. In addition, in the presence of 50% serum, the transfection efficiency of Zn-DTAc liposome was 15 times higher than that of PEI_{25kDa}.These results demonstrated that Zn-DTAc/pDNA complexes were stable and could retain

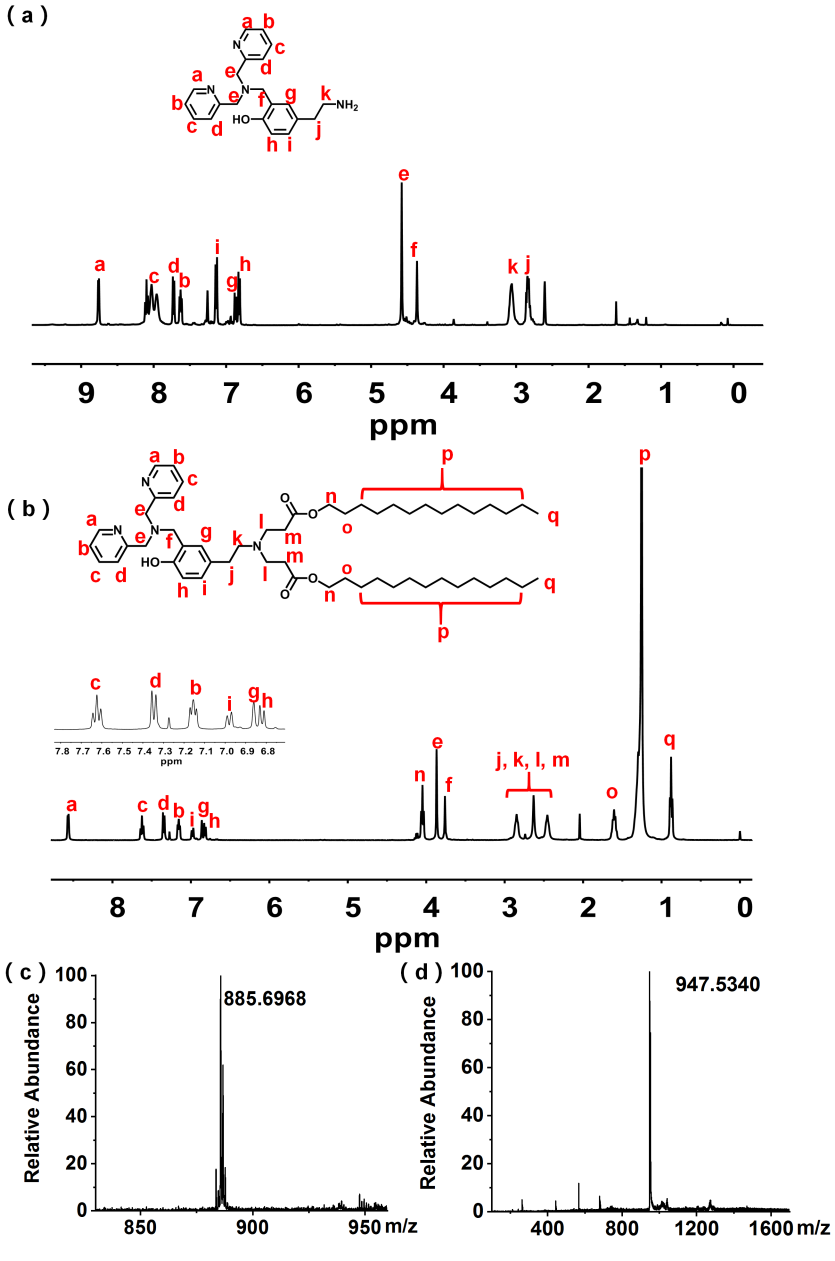


Fig. 1 ^{1}H NMR spectra of (a) DPA- Tyramine and (b) DPA-Tyramine-Ac14 in $CDCl_3$. ESI-MS of (c) DPA-Tyramine-Ac14 and (d) Zn-DTAc lipid.

high transfection efficiency even in the presence of a large amount of serum, which demonstrated their excellent serum tolerance ability. Previous study[21] has demonstrated that the serum adsorption ability of Zn (II) coordinated polycations was much lower than that of PEI, the serum resistance of Zn-DTAc/pDNA complexes may be due to its low serum adsorption in the presence of Zn ion. In addition, the high binding affinity of Zn-DTAc liposomes to DNA also made the complexes stable in serum.

It is known that the serum has influence on cellular uptake and endosomal escape of vector/DNA complexes, which may affect the transfection efficiency of non-viral vectors[17, 22].Thus, we further investigate whether serum will inhibit the cellular uptake and endosomal escape of Zn-DTAc/pDNA complexes. In the cellular uptake experiment, Zn-DTAc/pDNA complexes were prepared by using Cy5-labelled DNA, then 293T cells were incubated with Zn-DTAc/pDNA complexes in various serum contents for 4 h, and the cellular uptake of the complexes was quantitatively measured by using flow cytometry. As shown in Fig. 5(a), the mean Cy5 fluorescence intensities in each group were not significantly different, elucidating the fact that serum did not inhibit the internalization of Zn-DTAc/pDNA complexes. For the intracellular fate, we investigated the distribution and localization of Zn-DTAc/pDNA complexes in 293T cells by CLSM. Endosomes and

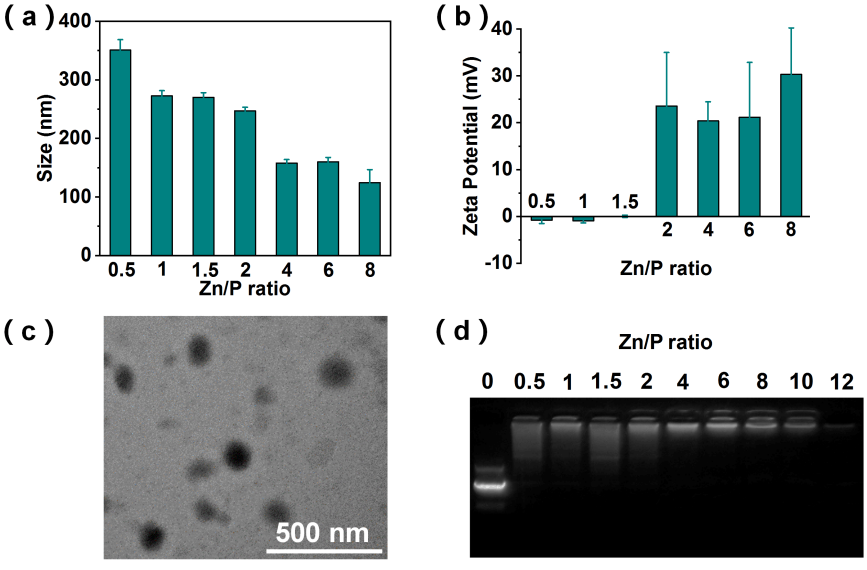


Fig. 2 (a) Mean particle sizes and (b) zeta potentials of Zn-DTAc/pDNA complexes at various Zn/P ratios. (c) Representative TEM image of Zn-DTAc/pDNA complexes (Zn/P = 6). (d) Gel retardation assay of Zn-DTAc/pDNA complexes at various Zn/P ratios. Data represent mean ± SD (n = 5).

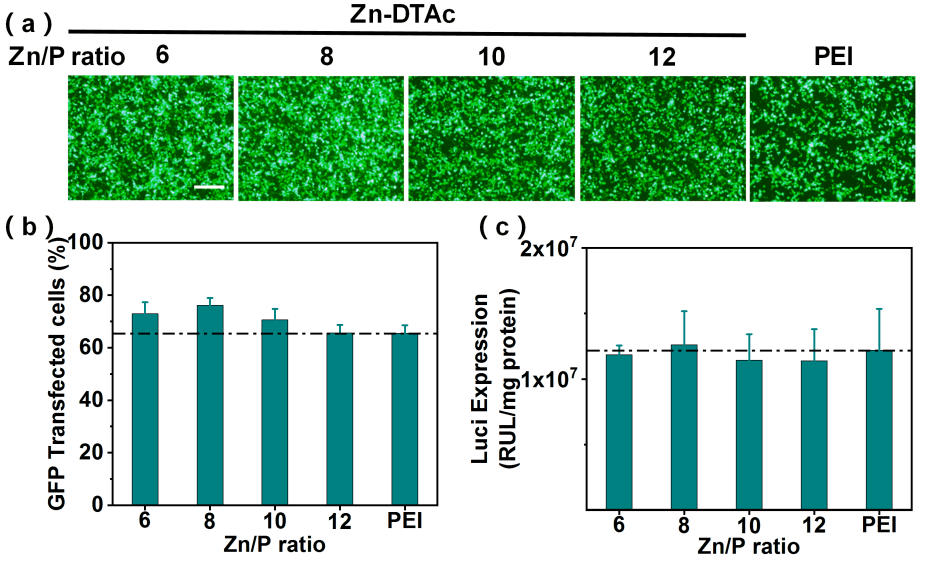


Fig. 3 Gene transfection efficiency. (a) Fluorescence images of GFP transfection and (b) GFP-positive cells calculated using a flow cytometer at various Zn/P ratios in 293T cells. (c) Luciferase transfection efficiency at various Zn/P ratios in 293T cells. PEI_{25kDa} (N/P = 10) was used as control. Scale bars, 50 mm. Data represent mean ± SD (n = 3).

nucleus were marked by Lyso-Tracker Green (green fluorescence) and DAPI (blue fluorescence) respectively. As shown in Fig. 5(b), it could be seen that part of red fluorescence overlapped with green fluorescence after incubation for 4 h in all groups, suggesting that a portion of Zn-DTAc/pDNA complexes were captured by lysosomes. Nevertheless, it could be found that a considerable amount of red fluorescence co-localized with blue fluorescence in all groups (yellow arrows), indicating that part of Cy5-labelled DNA had escaped from the lysosomes and entered the nucleus, which revealed that serum could hardly inhibit the endosomal escape of Zn-DTAc/pDNA complexes. All these results indicated that Zn-DTAc/pDNA complexes could break down the barriers and traps of cell membranes and lysosomes in the presence of serum. These may be the reasons that Zn-DTAc/pDNA complexes could achieve successful transfection in the presence of serum.

3.3 Cytotoxicity evaluation

Only the gene vectors with low cytotoxicity can be

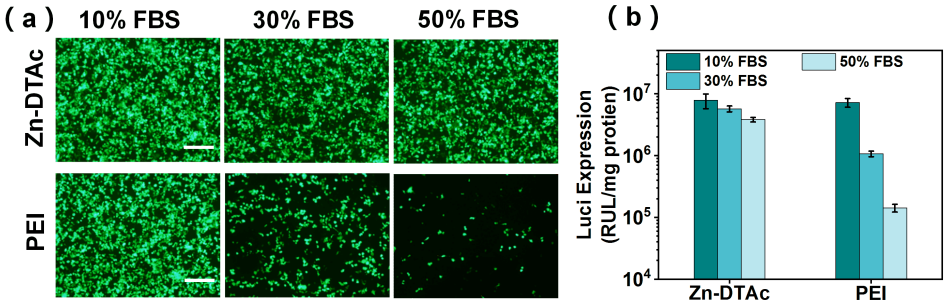


Fig. 4 (a) Fluorescence images of GFP transfection and (b) Luciferase transfection efficiencies in 293T cells with 10%, 30%, and 50% FBS medium (Zn/P＝8). PEI_{25kDa}(N/P＝10) was used as controls. Scale bars, 50 mm. Data represent mean ±SD (n＝3).

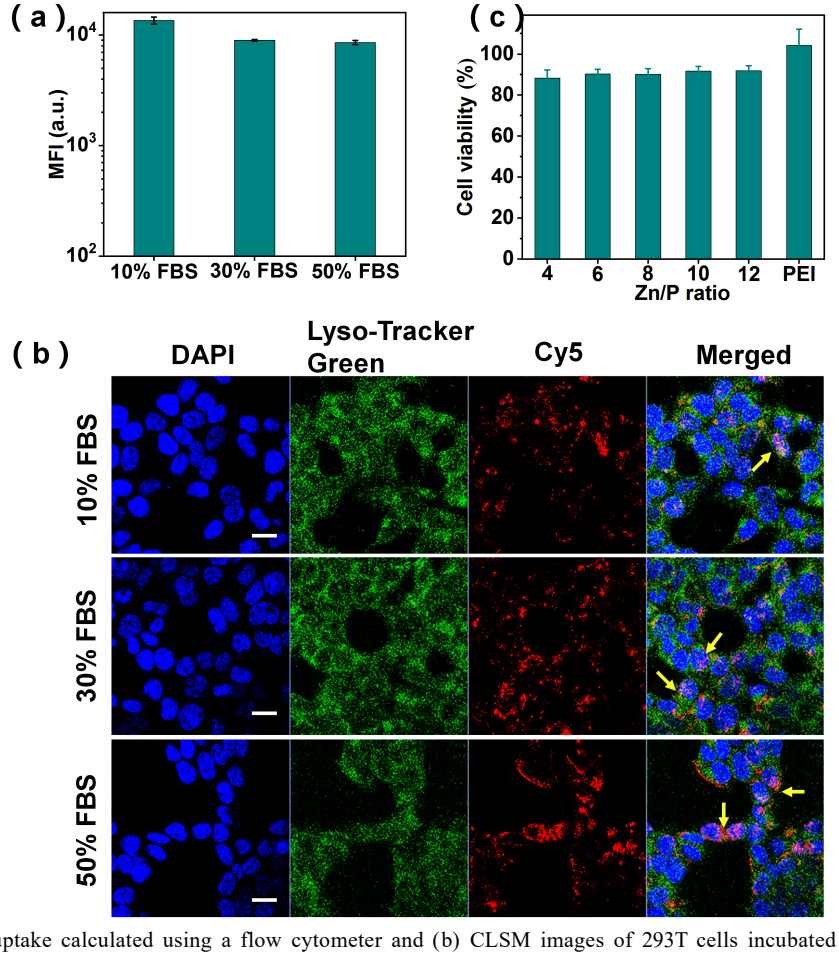


Fig. 5 (a) Cellular uptake calculated using a flow cytometer and (b) CLSM images of 293T cells incubated with Zn-DTAc/Cy5-DNA complexes (Zn/P＝8) in different concentrations FBS. For CLSM images, cell nucleus, lysosomes and pDNA were labeled by DAPI (blue fluorescence), Lyso-Tracker Green (green fluorescence) and Cy5 (red fluorescence) respectively. (c) Cytotoxicity of Zn-DTAc/pDNA complexes at different Zn/P ratios toward 293T cells. Scale bars, 20 mm. Data represent mean ±SD (n＝3).

further used in clinic applications. Thus, we evaluated the cytotoxicity of Zn-DTAc/pDNA complexes toward 293T cells by MTT assay. As shown in Fig. 5(c), the cell viabilities of Zn-DTAc/pDNA complexes at various Zn/P ratios (4～12) were around 90%, which demonstrated negligible toxicity at optimal transfection ratio. Therefore, Zn-DTAc liposome could be used as a promising gene carrier.

4 Conclusions

In this work, we designed a novel Zn containing liposome, which could be used as a serum-tolerant gene delivery platform. The results showed that Zn-DTAc liposomes could effectively condense pDNA into nanoparticles. In vitro experiments suggested that Zn-DTAc/pDNA complexes possessed significant

endocytosis efficiency, excellent endosomal escape ability and showed efficient transfection in the presence of high concentration serum. In addition, cytotoxicity assay demonstrated that Zn-DTAc/pDNA complexes were non-toxic at the optimal transfection ratio. Collectively, Zn-DTAc liposome can be applied as a non-viral vector for effective and serum-tolerant gene delivery, which holds great promise for DNA-based gene therapies.

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Conflict of interest

The authors declare no conflict of interest.

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Zn(II)配位脂质体用于高效且血清耐受的基因转染

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摘要: 近年来,阳离子聚合物、阳离子脂质体等非病毒载体在基因递送中的应用越来越受到人们的关注.然而,血清的存在会降低它们的转染效率,从而阻碍它们在临床中的使用.因此,非常有必要开发能抵抗血清干扰的非病毒基因载体.本工作合成了一种新型的锌离子配位的脂质,其与 1, 2-二油酰-SN-甘油-3-磷酰乙醇胺脂质(DOPE)共自组装形成阳离子脂质体(Zn-DTAc 脂质体).实验结果表明,Zn-DTAc 脂质体可以有效地压缩质粒形成纳米复合物.即使在 50% 胎牛血清存在的情况下,该复合物也能进行有效的细胞内化和内涵体逃逸,并实现高效的基因转染.

关键词: 基因递送;非病毒载体;Zn(II)配位脂质体;抗血清