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ORIGINAL RESEARCH

High gene delivery efficiency of alkylated low-molecular-weight polyethylenimine through gemini surfactant-like effect

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Abstract: To our knowledge, the mechanism underlying the high transfection efficiency of alkylated low-molecular-weight polyethylenimine (PEI) is not yet well understood. In this work, we grafted branched PEI (molecular weight of 1,800 Da; bPEI1800) with lauryl chains (C_{12}), and found that bPEI1800-C_{12} was structurally similar to gemini surfactant and could similarly assemble into micelle-like particles. Stability, cellular uptake, and lysosome escape ability of bPEI1800-C_{12}/DNA polyplexes were all greatly enhanced after C_{12} grafting. bPEI1800-C_{12}/DNA polyplexes exhibited significantly higher transfection efficiency than Lipofectamine™ 2000 in the presence of serum. Bioluminescence imaging showed that systemic injection of bPEI1800-C_{12}/DNA polyplexes resulted in intensive luciferase expression in vivo and bioluminescence signals that could be detected even in the head. Altogether, the high transfection efficacy of bPEI1800-C_{12} was because bPEI1800-C_{12}, being an analog of gemini surfactant, facilitated lysosome escape and induced the coil–globule transition of DNA to assemble into a highly organized micelle-like structure that showed high stability.

Keywords: self-organization, alkylation, luciferase, bioluminescence imaging

Introduction

Gene therapy is essentially based on the development of efficient and safe gene carrier systems able to transfer plasmid DNA or small interfering RNA (siRNA) into cells.[1] Among the various nonviral systems, polyethyleneimine (PEI) is one of the most intensively studied systems due to its high transfection efficiency.[2,3] Unfortunately, the high transfer efficiency of PEI is usually accompanied by high toxicity, so various means of structure modification have been tried in order to obtain a good compromise between efficiency and toxicity for PEI, such as poly(lactic-co-glycolic acid)-linear PEI with a molecular weight of 22,000 Da (lPEI22K);[4] branched PEI (bPEI)-g-ovalbumin with a molecular weight of 600 Da (bPEI0.6K);[5] heparin-grafted bPEI with a molecular weight of 1,800 Da (bPEI1.8K);[6] hyaluronic acid-grafted bPEI with a molecular weight of 25,000 Da (bPEI25K);[7] polyethylene glycol-g-bPEI with a molecular weight of 25,000 Da (bPEI25K);[8] and hydrophobic grafting (cholesteryl, cholanic acid, fatty acid residues, and hydrophobic chains).[9] Among the many modification methods of PEI, grafting PEI with hydrophobic moieties has proved to be an effective way of obtaining a favorable tradeoff between efficacy and toxicity, by: 1) increasing the transfection efficiency by elevated lipophilicity, which enhances the affinity of PEI to biomembranes and facilitates the cellular entry;[10] 2) improving the stability of polyplexes against serum and DNAase;[11] and 3) lowering the toxicity by reduced charge density, which causes less cell damage.[12]

It has been demonstrated that hydrophobically modified low-molecular-weight (LMW) PEI is not only an efficient gene vector itself,[13,14] but can also act as an

intermediate in the fabrication of hybrid multifunctional carriers for gene delivery.[15–19] To our knowledge, however, the mechanism underlying the high transfection efficacy of hydrophobically modified LMW PEI is not yet well understood. The high transfection efficiency of PEI is widely considered to be due to its high charge density and the proton sponge effect, which triggers the escape of PEI/DNA polyplexes from lysosomes. It is believed that LMW PEIs, due to their low branching degree, cannot compact DNA efficiently, and such low compaction ability would become even lower were they conjugated with hydrophobic moieties. Likewise, it has been proved that LMW PEI itself could not evoke efficient endosomal escape by the proton sponge effect.[20] Thus, in the case of hydrophobically modified LMW PEI, it seems that neither positive charge density potential nor the proton sponge effect contributes to its high transfection efficiency.

It is widely assumed that escape of PEI/DNA polyplexes from lysosomes is triggered by the proton sponge effect. So far, however, the proton sponge hypothesis has not been proved by experiments, and this hypothesis has been recently challenged.[21] Besides the proton sponge effect, other effects, such as surfactant activity,[22] are believed to contribute to the lysosomal membrane permeabilization capacity of PEI[23] and cause lysosome release of polyplexes. It was found that cationic surfactant could complex with DNA, resulting in a micelle-like structure that precluded the aggregation of polyplexes, and such cationic surfactant could also destabilize the endosomal membrane and trigger DNA release into the cytoplasm.[24] Gemini surfactant, a recently extensively investigated gene vector,[25] was also found to be efficient at facilitating biomembrane penetration and endosomal escape.[26] Hydrophobic grafting of bPEI would give rise to cationic amphiphile bPEI derivatives, which are structurally similar to cationic surfactant or gemini surfactant, all of which possess cationic head groups and hydrophobic tails. Moreover, it was found that micelle-forming structures played a key role in enhancing transfection of lipid-modified bPEI (molecular weight: 1,800 Da [bPEI1800]),[27] so we speculated that the structure of hydrophobically modified LMW PEI might be highly related to its transfection efficiency.

In light of the aforementioned information, we aimed to investigate the structure/activity relationship of alkylated LMW PEI. Hydrocarbon chains (C_{12}) were used to graft LMW PEI (bPEI1800) and a series of cationic amphiphile PEI derivatives (bPEI1800-C_{12}) were obtained. The structure, physicochemical characteristics, interactions with cells, in vitro transfection, and, especially, the in vivo gene delivery efficacy of bPEI1800-C_{12} were fully investigated.

The obtained results suggested that bPEI1800-C_{12} could assemble into highly stable micelle-like particles and that they triggered the coil–globule transition of DNA similarly to cationic surfactant and possessed high gene delivery efficacy in vitro and in vivo.

Materials and methods
Materials and cells
All reagents and solvents were used as received without further purification. bPEI1800 was purchased from Alfa Aesar (Ward Hill, MA, USA). 1-bromododecane, heparin sodium salt (150 USP [United States Pharmacopeia] units/mg), N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid (HEPES), fluorescein isothiocyanate (FITC) isomer, ethidium bromide, chlorpromazine, methyl-β-cyclodextrin (M-β-CD), and sodium azide were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Trypsin, fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), and Roswell Park Memorial Institute (RPMI) 1640 medium were all provided by GE Healthcare UK Ltd (Little Chalfont, UK). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from AMRESCO LLC (Solon, OH, USA). LysoTracker® Red dye, Hoechst 33258, apoptosis detection kit (C1062), and Bradford protein assay kit (P0006) were all from Beyotime Institute of Biotechnology (Shanghai, People's Republic of China). Luciferase assay kit (E1500) was from Promega Corporation (Fitchburg, WI, USA). Other chemicals and reagents were of analytical grade. Mouse mammary tumor cell line 4T1 was provided by the Department of Pathology in Institute of Medicinal Biotechnology in Peking Union Medical College (Beijing, People's Republic of China) and cultured in RPMI 1640 containing 10% FBS.

Synthesis of bPEI1800-C_{12} derivatives
Grafting bPEI1800 with hydrophobic C_{12} chains was carried out as reported previously.[28] Briefly, 1-bromododecane was reacted with a solution of bPEI1800 (1 g, 0.56 mmol) in dichloromethane (150 mL) at room temperature (RT) for 24 hours. The resulting solvent was concentrated under reduced pressure and further dialyzed, first against an ethanol/water (1:1) solution (4 L five times), then against double-distilled water for 1 day. The mixture was concentrated and lyophilized. Proton nuclear magnetic resonance (^{1}H NMR) spectra of bPEI1800 and its derivatives were recorded on a 400 MHz Mercury plus instrument (Varian Inc., Palo Alto, CA, USA), and substitution degree was also calculated based on the integration of ^{1}H NMR. FITC fluorescent labeling of the polymers was performed as reported previously.[29]

pH titration

pH titrations were performed as reported previously.[30] In short, 2 mL of each PEI solution (113 mM in $-CH_2CH_2NH-$ units) were adjusted to pH 12 with NaOH. Five μL amounts of 1 M HCl were added sequentially, and the pH after each addition was measured; a solution of NaCl (113 mM) was titrated similarly as a control.

Gel retardation assay

Agarose gel retardation assays were conducted following standard procedure. Varying concentrations of PEI or PEI derivatives and equal volume (10 μL) of plasmid DNA solutions (concentration kept at 20 ng/μL) were mixed in HEPES-buffered saline ([HBS] 10 mM HEPES, 150 mM NaCl, pH 7.4), respectively, incubated at RT for 30 minutes, then mixed with 4 μL of 6× loading buffer, loaded into 1.0% agarose gel (in Tris-acetate-ethylenediaminetetraacetic acid [TAE] buffer), and run for 60 minutes at 80 V. After staining of the agarose gel with 0.5 μg/mL ethidium bromide for 30 minutes, the plasmid DNA bands were visualized at 254 nm with an ultraviolet gel imaging system (BIO- BEST 135A; SIM International Group Co., Ltd, Los Angeles, CA, USA).

Morphology

The morphology of bPEI/DNA polyplexes was visualized by transmission electron microscopy (TEM). Briefly, 20 μL of 20 ng/μL DNA was mixed and incubated with an equal volume of bPEI solution in HBS (pH 7.4) for 30 minutes at RT and diluted with 80 μL HBS (pH 7.4). Ten microliters of the diluted polyplexes solution was dropped onto a copper grid coated with carbon membrane, then the copper grid was air-dried and negatively stained with 1% sodium phosphotungstate. Subsequently, the morphology of polyplexes was observed using a transmission electron microscope (Hitachi H-7650; Hitachi Ltd., Tokyo, Japan) with an acceleration voltage of 80 kV.

Heparin displacement assay

Ten microliters of polyplex solution in HBS (pH 7.4), prepared according to the method described in the Gel retardation assay section, were exposed to 5 μL of HBS (pH 7.4) containing serial concentrations of heparin sodium salt for 1 hour at RT. Then, the mixture was run in 1% agarose gel as described.

Hemolysis assay

Rat erythrocytes were collected from heparin-treated blood and washed with 0.01 M isotonic phosphate-buffered saline ([PBS] pH 7.4) four times (700 g for 10 minutes at 4°C). The erythrocyte pellet was diluted tenfold with 0.01 M isotonic PBS (pH 7.4) to a concentration of 10^9/mL.

A 75 μL aliquot was added to a 96-well plate containing 75 μL serial solutions of bPEI1800 or its derivatives in the corresponding buffer. The final concentrations of bPEI1800 and its derivatives varied from 6 μg/mL to 100 μg/mL. After incubation for 1 hour at 37°C with constant shaking, unlysed erythrocytes were removed by centrifugation (700 g for 10 minutes at 4°C), then 80 μL supernatant was transferred to a new 96-well plate and hemoglobin absorption was measured at 450 nm using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices LLC, Sunnyvale, CA, USA). Triton™ X-100 (1%) (Sigma-Aldrich Co.) was used as the positive control (100% lysis). Experiments were performed in triplicate.

Zeta potential measurement

Polyplexes were prepared at ratios from 1:1 to 20:1 (polymer to DNA, weight [w/w]) using the method described in the Gel retardation assay section, then diluted with HBS (pH 7.4), and the final DNA concentration was 0.5 μg/mL. The zeta potential of each sample was measured by a Nicomp 380 ZLS analyzer (Particle Sizing Systems, Port Richey, FL, USA). Experiments were performed in triplicate.

Cytotoxicity

Cytotoxicity of bPEI1800-C_{12} was evaluated by MTT assay as previously reported.[31] Briefly, 4T1 cells were seeded onto 96-well plates at a density of 4×10^3/well. After incubation overnight for adherence, the previous medium was replaced with FBS-supplemented medium containing various concentrations of bPEI1800 or bPEI1800-C_{12}. After 20 or 44 hours of incubation, the medium was removed and 100 μL fresh medium containing 0.5 mg/mL MTT was added to each well. Cells were further incubated for 4 hours, and medium was replaced with 150 μL dimethyl sulfoxide to solubilize the converted formazan. Absorbance was measured at 570 nm with the SpectraMax 190 Absorbance Microplate Reader. Cells without exposure to the polymers were used to represent 100% cell viability. Experiments were performed in triplicate.

Cellular uptake studies

For confocal laser scanning microscope visualization, 4T1 cells were seeded onto a 24-well plate with sterile glass slides at a density of 4×10^4 cells/well and incubated overnight for attachment. Then 4T1 cells were rinsed thrice with serum-free medium and incubated with polyplexes prepared with FITC-labeled polymers for 4 or 24 hours at 37°C: the first 4-hour incubation was in serum-free medium; for longer incubation, medium with serum was supplemented. At the determined time point, cells were washed thrice with PBS and the cell nuclei were stained with Hoechst 33258 before

observation with a confocal scanning laser microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany).

Endocytosis pathway

For endocytosis pathway studies, 4T1 cells were seeded onto 12-well plates until grown to 85% confluence, rinsed thrice with serum-free medium, and preincubated in serum-free medium containing 10 µg/mL chlorpromazine, 5 mM M-β-CD, or 10 mM sodium azide for 1 hour. Then, the previous medium was removed and supplemented with fresh serum-free medium that contained chemical pathway inhibitors (10 µg/mL chlorpromazine, 5 mM M-β-CD, or 10 mM sodium azide) and polyplexes prepared with FITC-labeled polymers. Incubation was continued for 4 hours. Then, cells were washed thrice with PBS, detached by trypsin, centrifuged for 5 minutes, resuspended, and analyzed with a FACSCaliber™ flow cytometer (BD, Franklin Lakes, NJ, USA). The intracellular amount of polyplexes was represented by the relative fluorescence intensity, and the group without treatment, in which mean fluorescence intensity was determined to be 100%, was set as the control.[32]

Intracellular trafficking

Intracellular trafficking studies were performed similarly to cellular uptake studies. 4T1 cells were similarly seeded, attached, treated with FITC-labeled polyplexes, and visualized, except that, after PBS washing, cells were further treated with LysoTracker Red and Hoechst according to the manufacturer's protocol.

In vitro transfection

In vitro transfection studies were performed with plasmid GL 4.50 (pGL 4.50) and plasmid EGFP (pEGFP), which encoded luciferase and green fluorescence protein, respectively. 4T1 cells were seeded onto a 96-well plate at a density of 4×10^3 cells/well and grown for 24 hours. Then, medium was replaced with 40 µL serum-containing medium, and 10 µL of freshly prepared polyplexes in HBS containing 100 ng DNA was added to each well. lPEI22K and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) were used as the controls. The transfection of lPEI22K was carried out similarly to that of the bPEI1800, except that the weight ratio was set at 5:1 (polymer to DNA), and the transfection experiments with Lipofectamine 2000 were performed according to the manufacturer's instructions. After 4 hours of transfection, 125 µL of 10% FBS medium was added to each well, and the cells were incubated for an additional 20 hours. Finally, cells were washed twice with PBS, luciferase activity was detected according to standard protocol for the luciferase assay system, and the protein concentration of each well was measured using the Bradford protein assay kit.

In vivo gene delivery

Animal experiments (BALB/c mouse, female, 6-8 weeks old) adhered to the Principles of Laboratory Animal Ethics Committee of the Institute of Materia Medica in Peking Union Medical College, and were conducted with necessary humane care. Five hundred microliters of 1,000 ng/µL bPEI1800 and bPEI1800-C_{12}-19.72 (bPEI1800-C_{12} where the substitution degree of C_{12} was 19.72) in HBS were separately mixed with an equal volume of 200 ng/µL pGL 4.50 solutions, and the resulting polyplexes were incubated at RT for 30 minutes before being injected into mice through the tail vein (200 µL polyplexes solution containing 20 µg plasmid DNA for each mouse). Naked plasmid DNA solution was used as the control. Twenty-four hours post-injection, mice were anesthetized with isoflurane, and imaging began 8 minutes after administration of D-luciferin. Bioluminescence imaging was performed with an IVIS® Spectrum in vivo imaging system (IVISSPE; Perkin-Elmer, Inc., Waltham, MA, U.S.) and the images were analyzed by Living Image software (version 4.3.1; PerkinElmer, Inc.). Anesthesia was maintained during imaging and images were acquired for 2 minutes. After bioluminescence imaging, the mice were quickly killed by cervical dislocation; their livers, hearts, spleens, lungs, kidneys, and brains were collected, washed with normal saline, and suspended in 800 µL (liver, brain) or 400 µL (heart, spleen, lung, kidney) lysis buffer consisting of passive lysis buffer, phenylmethylsulfonyl fluoride, and protease inhibitor mixture,[30] homogenized by probe sonication for 30 seconds, and centrifuged (4°C, $20,000 \times g$, 10 minutes). Luciferase activity of the supernatants was detected according to the standard protocol for the luciferase assay system and the protein concentration was measured by the Bradford protein assay kit.

Statistical analysis

Results were expressed as mean ± standard deviation of at least three independent experiments. Student's *t*-test was used to evaluate the difference between two groups. One-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used to compare the statistical significance among the groups, and $P < 0.05$ was considered significant.

Results

Synthesis of bPEI1800-C_{12} derivatives

1-bromododecane was chosen as C_{12} chain donor for the hydrophobic grafting of bPEI1800. Characteristic chemical shift (δ) of 0.92 (−CH_3) and 1.32 (−$(CH_2)_{11}$−) in the

^{1}H NMR spectrum of bPEI1800-C$_{12}$ indicated the successful grafting of C$_{12}$ (Figure S1). By varying the weight ratios of 1-bromododecane and bPEI1800, we obtained bPEI1800 derivatives of different substitution degrees which were calculated by integration of ^{1}H NMR. Since the molecular formula of bPEI1800-C$_{12}$ is $(C_2H_4N)_{42}(C_{12}H_{25})_{42}n$, the degree of C$_{12}$ substitution was calculated by the following equation:

$$25n/4=(\text{peak area of } -C_{12}H_{25})/(\text{peak area of } -C_2H_4N-) \quad (1)$$

where n is the substitution degree of C$_{12}$. The calculated substitution degrees (6.08, 13.44, 17.12, and 22.72) were quite close to their theoretical values (5.28, 12.5, 19.72, and 25), so the bPEI1800 derivatives were abbreviated to bPEI1800-C$_{12}$-5.28, bPEI1800-C$_{12}$-12.5, bPEI1800-C$_{12}$-19.72, and bPEI1800-C$_{12}$-25, respectively.

Buffering capacity of bPEI1800-C$_{12}$

Since the high buffering capacity of PEI is widely considered as a guarantee for efficient endosomal escape of polyplexes and high transfection efficiency, we investigated the influence of C$_{12}$ grafting on the buffering capacity of bPEI via pH titration. Buffering capacity of bPEI1800-C$_{12}$ derivatives was markedly reduced compared with that of bPEI1800 (Figure 1A). After C$_{12}$ grafting, partial primary amines of PEI turned into secondary amines and thus led to reduced protonation ability. Additionally, grafting bPEI1800 with C$_{12}$ caused strong hydrophobic repulsion in bPEI1800-C$_{12}$ molecules, and such hydrophobic repulsion rearranged amine groups of bPEI1800-C$_{12}$, resulting in decreased amine spacing, which led to retarded protonation and reduced buffering capability.[33]
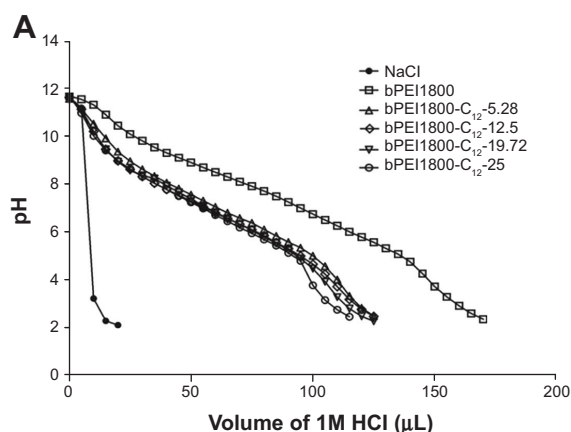
DNA condensation of bPEI1800-C$_{12}$ via retardation assay

As shown in Figure 1B, bPEI1800 could completely condense DNA at a weight ratio of 0.4:1 (polymer to DNA), while, in the case of bPEI1800-C$_{12}$ derivatives, with the increase of C$_{12}$ substitution degree, weight ratios for complete DNA condensation were 0.6:1, 0.8:1, or 1:1 (polymer to DNA, w/w), suggesting that C$_{12}$ grafting reduced the binding ability of bPEI1800-C$_{12}$ to DNA, and the binding ability of bPEI1800-C$_{12}$ decreased with the increment of substitution degree. The reasons that bPEI1800-C$_{12}$ exhibited less efficient binding to DNA compared with the parent bPEI1800 is that electrostatic interaction between PEI and DNA is the driving force for compaction and that C$_{12}$ grafting retarded the protonation of bPEI1800-C$_{12}$ (Figure 1A).

Morphology of bPEI1800-C$_{12}$/DNA polyplexes

bPEI1800-C$_{12}$ polymers at a concentration of 4 mg/mL showed the appearance of light blue opalescence (Figure S2), indicating that bPEI1800-C$_{12}$ self-organized into nano-sized particles in water solution. TEM visualization showed that bPEI1800-C$_{12}$-19.72/DNA polyplexes exhibited a micelle-like structure at pH 7.4 (Figure 2B), which consisted of a hard inner core and outer shell (about 300 nm in diameter). bPEI1800/DNA polyplexes displayed a plain solid spherical structure ([Figure 2A] about 180 nm in diameter) at pH 7.4. With the synthesis route of the present study, C$_{12}$ chains were mostly grafted to the primary amino groups of bPEI1800, and such primarily grafted C$_{12}$ chains were believed to locate around the periphery of bPEI1800.[13] In order to gain thermodynamic stability, bPEI1800-C$_{12}$ should rationally

Figure 1 pH titration profiles and gel retardation assays of bPEI polymers.
Notes: (**A**) pH titration profiles of bPEI1800 and bPEI1800-C$_{12}$. (**B**) Gel retardation assays of bPEI1800 (a), bPEI1800-C$_{12}$-5.28 (b), bPEI1800-C$_{12}$-12.5 (c), bPEI1800-C$_{12}$-19.72 (d), and bPEI1800-C$_{12}$-25 (e) (1% agarose, TAE buffer). 5.28, 12.5, 19.72, and 25 represent the substitution degree of C$_{12}$. Weight ratios (w/w) are expressed as the ratio of polymer to DNA.
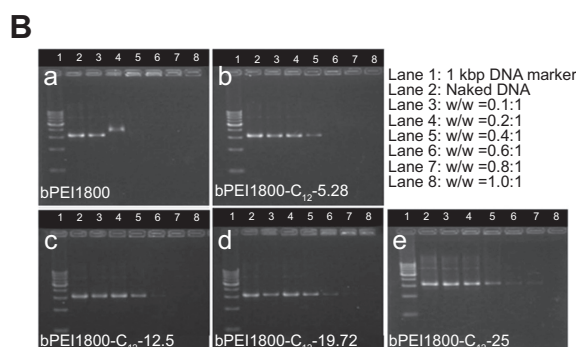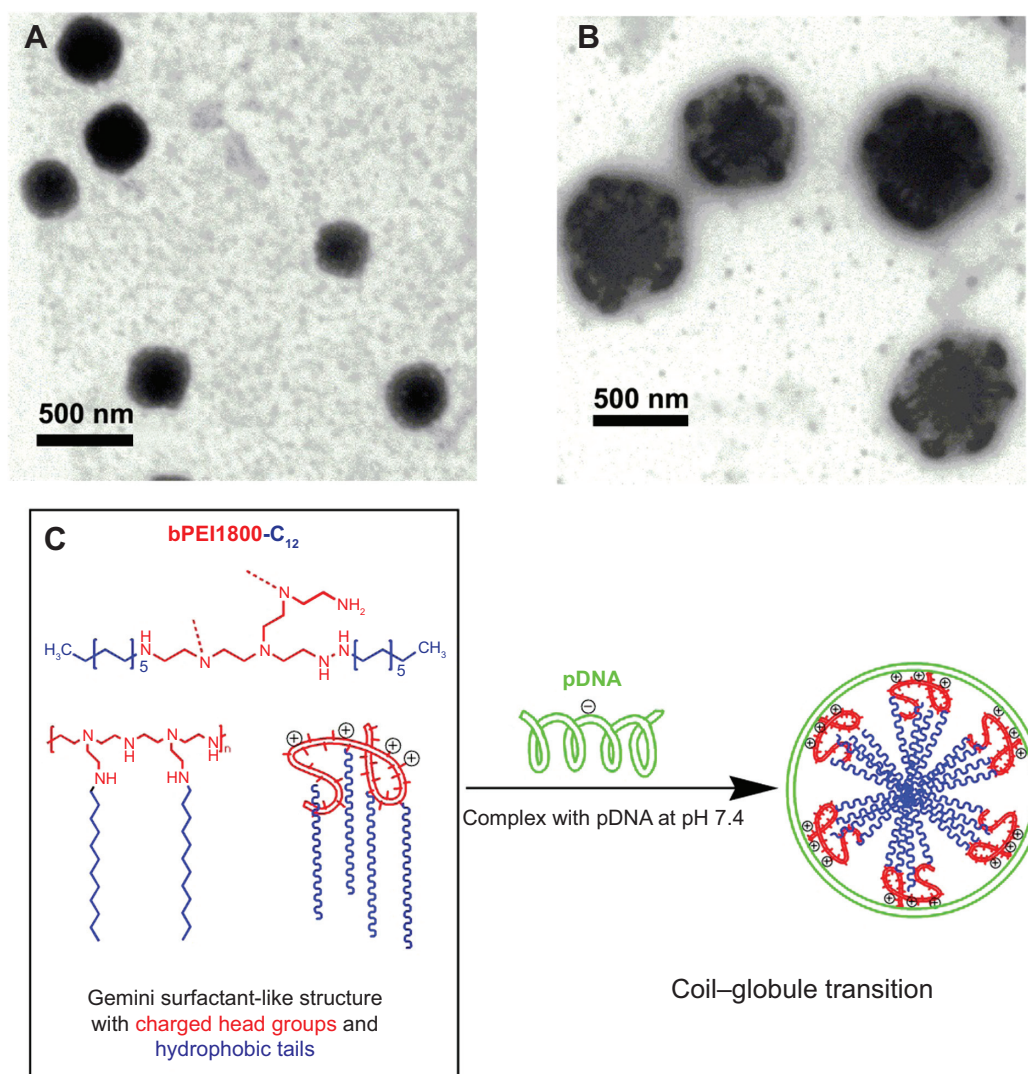**Abbreviations:** bPEI, branched polyethylenimine; bPEI1800, bPEI with molecular weight 1,800 Da; bPEI1800-C$_{12}$, bPEI1800 grafted with hydrophobic C$_{12}$ chains; kbp, kilobase pair; TAE, Tris-acetate-ethylenediaminetetraacetic acid; w, weight.

Figure 2 Morphology and schematic illustration of self-assembly process of bPEI1800-C$_{12}$/DNA polyplexes.
Notes: Transmission electron microscope images of bPEI1800/DNA polyplexes (**A**) and bPEI1800-C$_{12}$/DNA polyplexes (**B**) at a weight ratio of 3:1 (polymer to DNA) at pH 7.4. Schematic illustration of the self-assembly process of bPEI1800-C$_{12}$/DNA polyplexes (**C**).
Abbreviations: bPEI1800, branched polyethylenimine with molecular weight 1,800 Da; bPEI1800-C$_{12}$, bPEI1800 grafted with hydrophobic C$_{12}$ chains; pDNA, plasmid DNA.

self-assemble into a micelle-like structure in aqueous media, with C$_{12}$ chains forming the hydrophobic inner core and bPEI moiety composing the outer shell (Figure 2C). When complexing DNA with bPEI1800-C$_{12}$ at pH 7.4, DNA underwent coil–globule transition[34] and was bound to the exterior bPEI moiety of the bPEI1800-C$_{12}$ micelle (Figure 2C), forming multi-hydrophobic domains[24] along the DNA chain.

In vitro stability of bPEI1800-C$_{12}$/DNA polyplexes

Heparin displacement assay was conducted to test the stability of polyplexes. bPEI1800/DNA and bPEI1800-C$_{12}$-19.72/DNA polyplexes prepared at a weight ratio of 3:1 (polymer to DNA) were separately exposed to heparin in

HBS (pH 7.4) for 1 hour at RT. Results from the subsequent gel electrophoresis (Figure 3A) indicated that DNA in bPEI1800/DNA polyplexes was completely displaced by heparin at a concentration of 0.25 mg/mL, but bPEI1800-C$_{12}$-19.72/DNA polyplexes were quite robust even when the concentration of heparin was up to 4 mg/mL, suggesting that C$_{12}$ grafting offered bPEI1800-C$_{12}$/DNA polyplexes high stability against anion displacement. Since there are many polyanions in serum that are apt to cause instability of polyplexes, the robustness of bPEI1800-C$_{12}$/DNA polyplexes against anion displacement suggested its potential for in vivo application. Additionally, zeta potentials of all polyplexes prepared with bPEI1800-C$_{12}$ were much higher than those of the polyplexes prepared with bPEI1800 (Figure S3) at
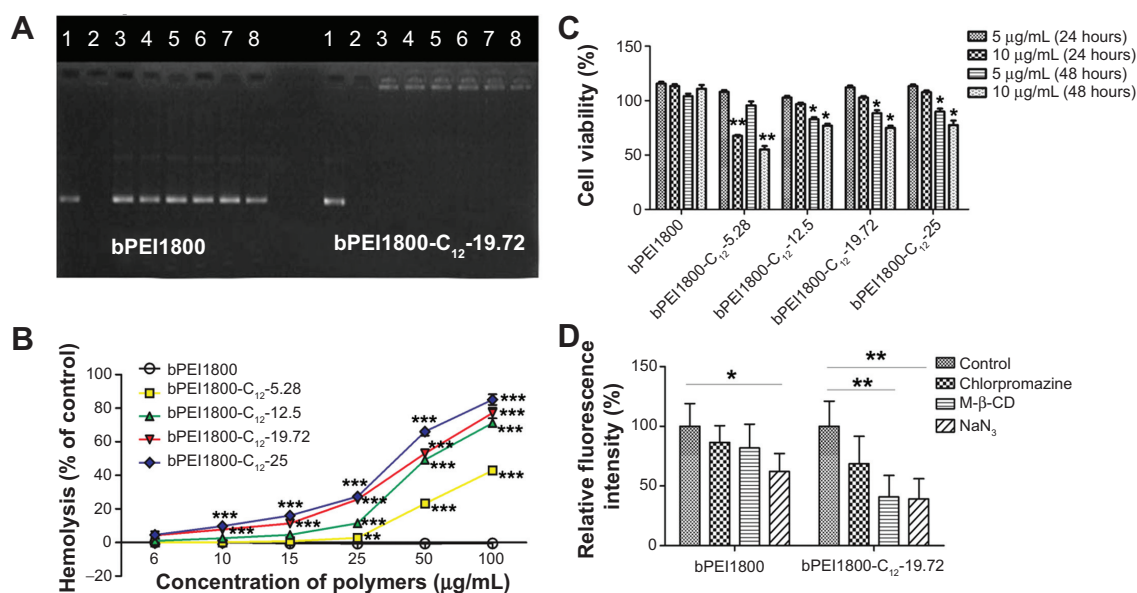
Figure 3 Characterization of bPEI1800-C$_{12}$ and bPEI1800-C$_{12}$/DNA polyplexes.
Notes: (**A**) Heparin displacement assay of bPEI1800/DNA polyplexes and bPEI1800-C$_{12}$-19.72/DNA polyplexes. Both of the polyplexes were prepared at a weight ratio of 3:1 (polymer to DNA) and exposed to various concentrations of heparin in HBS (pH 7.4) for 1 hour at room temperature. Lane 1: naked DNA; lane 2: 0.01 mg/mL heparin; lane 3: 0.25 mg/mL heparin; lane 4: 0.5 mg/mL heparin; lane 5: 1.0 mg/mL heparin; lane 6: 2.0 mg/mL heparin; lane 7: 3.0 mg/mL heparin; lane 8: 4.0 mg/mL heparin. (**B**) Hemolysis assay of bPEI polymers at pH 7.4, n=3, **$P<0.01$, ***$P<0.001$ versus bPEI1800 of the same concentration. (**C**) Cytotoxicity of polymers on 4T1 cells after 24 and 48 hours' incubation, n=3, *$P<0.05$, **$P<0.01$ versus bPEI1800 of the same concentration and incubation time. (**D**) Effects of chemical endocytosis pathway inhibitors on the internalization of fluorescein isothiocyanate-labeled bPEI1800/DNA and bPEI1800-C$_{12}$-19.72/DNA polyplexes in 4T1 cells after 4 hours' incubation, n=3, *$P<0.05$, **$P<0.01$. 5.28, 12.5, 19.72, and 25 represent the substitution degree of C$_{12}$.
Abbreviations: bPEI, branched polyethylenimine; bPEI1800, bPEI with molecular weight 1,800 Da; bPEI1800-C$_{12}$, bPEI1800 grafted with hydrophobic C$_{12}$ chains; HBS, N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid-buffered saline; M-β-CD, methyl-β-cyclodextrin.

the same weight ratio (polymer to DNA), suggesting that bPEI1800-C$_{12}$/DNA complexes maintained much higher stability in aqueous solution than bPEI1800/DNA complexes due to their highly organized structure.

Membrane destabilization activity of bPEI1800-C$_{12}$

It has been reported that hydrocarbon chain-grafted PEI showed much stronger interaction with phospholipids than unmodified PEI,[13] so we performed a hemolysis assay to study the interaction between biomembranes and bPEI1800-C$_{12}$. bPEI1800-C$_{12}$ polymers were incubated with erythrocytes at pH 7.4 at 37°C, and the released amount of hemoglobin was measured to represent membrane destabilization activity. As depicted in Figure 3B, bPEI1800 showed no membrane destabilization activity at concentrations up to 100 μg/mL,[35] while bPEI1800-C$_{12}$ imposed strong destabilization activity, and such destabilization activity increased with the increasing of both C$_{12}$ substitution and concentration of bPEI1800-C$_{12}$. The results obtained here indicate that bPEI1800-C$_{12}$ showed great affinity to biomembranes and possessed fairly good membrane destabilization activity, which could help penetrate biomembranes and facilitate lysosome escape.

Cytotoxicity of bPEI1800-C$_{12}$ on 4T1 cells

The cytotoxicity of bPEI1800-C$_{12}$ on 4T1 cells was studied by MTT assay. As illustrated in Figure 3C, bPEI1800 showed no cytotoxicity on 4T1 cells after 24 and 48 hours' incubation; 24-hour incubation of cells with 5 or 10 μg/mL bPEI1800-C$_{12}$ polymers exerted no cytotoxicity; 5 or 10 μg/mL bPEI1800-C$_{12}$ polymers were moderately toxic to 4T1 cells after 48 hours of treatment. The cytotoxicity of bPEI1800-C$_{12}$ was probably due to membrane destabilization activity[23] caused by membrane insertion of the free bPEI1800-C$_{12}$, as demonstrated by hemolysis assay (Figure 3B).

Cellular uptake and endocytosis of bPEI1800-C$_{12}$/DNA polyplexes

In order to investigate the cellular uptake of bPEI1800-C$_{12}$/DNA polyplexes, FITC-labeled bPEI1800 and bPEI1800-C$_{12}$ were complexed with DNA at a weight ratio of 3:1 (polymer to DNA), and their cellular uptake was visualized with confocal laser scanning microscopy. As depicted in Figure 4A, cellular uptake of both bPEI1800/DNA and bPEI1800-C$_{12}$/DNA polyplexes was time-dependent, and cellular uptake of

bPEI1800-C_{12}/DNA polyplexes was dramatically higher than that of bPEI1800/DNA at the same time point.

Since bPEI1800-C_{12}/DNA and bPEI1800/DNA polyplexes showed discriminated cellular uptake, we further studied their endocytosis using three kinds of chemical inhibitors of endocytosis:[36] 1) chlorpromazine, an inhibitor of clathrin-mediated endocytosis pathway; 2) M-β-CD, a cholesterol depletor for the inhibition of caveolae-mediated endocytosis; and 3) NaN_3, which is used to inhibit the energy-dependent endocytosis pathway.

The final concentrations of chlorpromazine, M-β-CD, and NaN_3 used here were 10 μg/mL, 5 mM, and 10 mM, respectively, none of which showed cytotoxicity to 4T1 cells (Figure S4). As shown in Figure 3D, NaN_3 treatment reduced the cellular uptake of bPEI1800/DNA polyplexes and bPEI1800-C_{12}-19.72/DNA polyplexes by 37.8% and 61%, respectively, implying that both polyplexes entered cells by the combined processes of endocytosis and non-endocytosis. Only a small portion of bPEI1800/DNA polyplexes entered cells by energy-dependent pathways (37.8%), but most
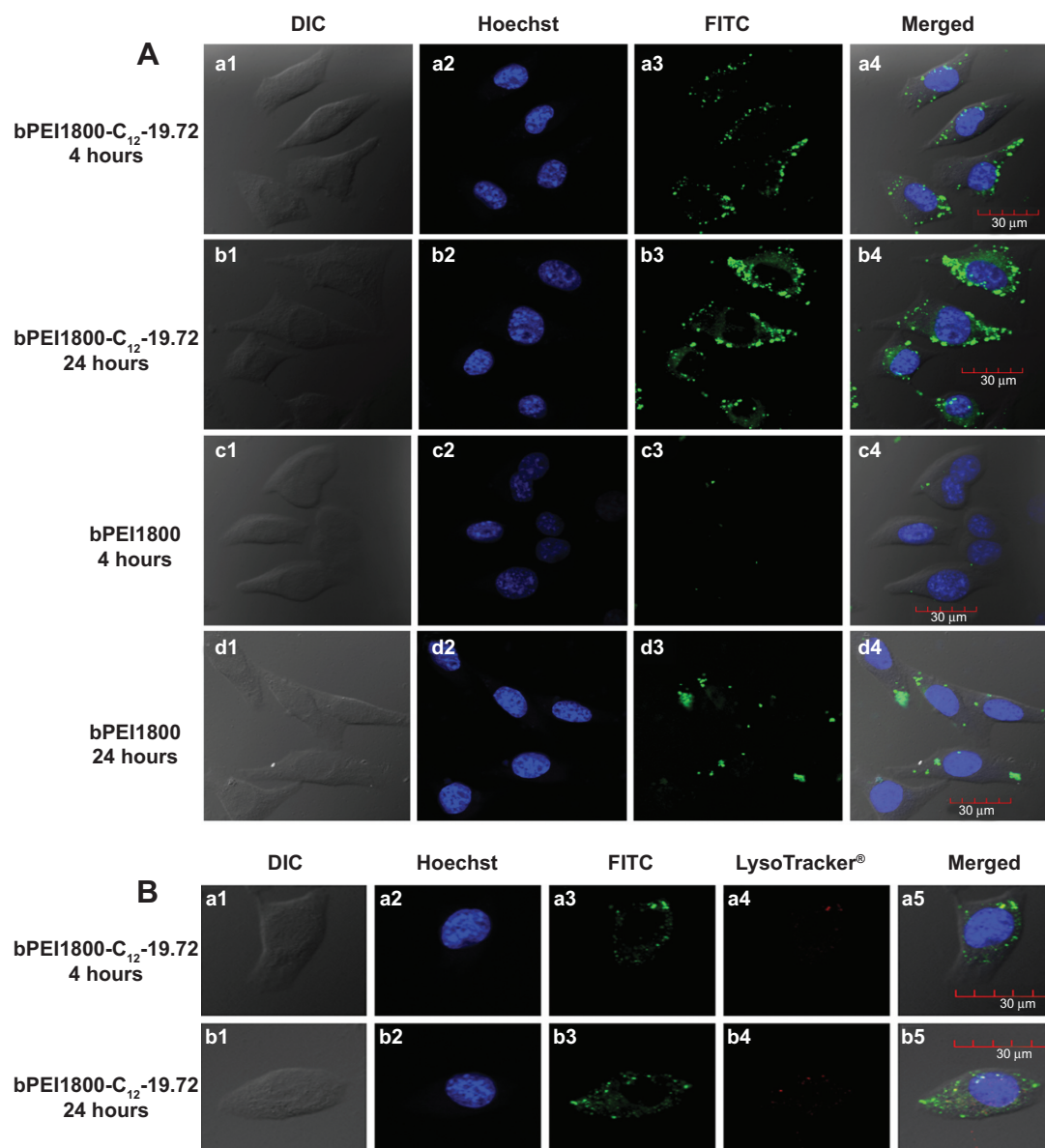

Figure 4 Cellular uptake studies of FITC-labeled bPEI1800-C_{12}-19.72/DNA polyplexes on 4T1 cells.
Notes: (**A**) Typical CLSM images of 4T1 cells after 4 and 24 hours' incubation with FITC-labeled bPEI1800/DNA polyplexes and FITC-labeled bPEI1800-C_{12}-19.72/DNA polyplexes. (**B**) Representative images of intracellular trafficking of FITC-labeled bPEI1800-C_{12}-19.72/DNA polyplexes by CLSM on 4T1 cells. bPEI1800 and bPEI1800-C_{12} were labeled with FITC (green), cell nuclei were stained with Hoechst 33258 (blue), and lysosomes were stained with LysoTracker® Red dye (red; purchased from Beyotime Institute of Biotechnology, Shanghai, People's Republic of China).
Abbreviations: bPEI1800, branched polyethylenimine with molecular weight 1,800 Da; bPEI1800-C_{12}-19.72, bPEI1800 grafted with hydrophobic C_{12} chains with a substitution degree of 19.72; CLSM, confocal laser scanning microscopy; DIC, differential interference contrast; FITC, fluorescein isothiocyanate.

of the bPEI1800-C_{12}-19.72/DNA polyplexes (61%) were internalized into cells through energy-dependent endocytosis. When treated with M-β-CD, cellular uptake of bPEI1800-C_{12}-19.72/DNA polyplexes dropped by nearly 59.2%, which was close to the cellular uptake reduction rate of those treated with NaN_3 (61%), suggesting that bPEI1800-C_{12}-19.72/DNA polyplexes entered cells predominantly by caveolae-mediated endocytosis. Additionally, a previous report showed that particles larger than 200 nm in size were more likely to be internalized into cells through caveolae-mediated pathway.[37] Hence, bPEI1800-C_{12}/DNA polyplexes with bigger size and highly organized structure were more favorable to caveolae-mediated endocytosis. Additionally, the clathrin-mediated endocytic pathway also contributed to the endocytosis of bPEI1800-C_{12}-19.72/DNA polyplexes, as proved by the 31.3% drop in cellular uptake caused by chlorpromazine.

Along with cellular uptake, intracellular trafficking is also an important issue for efficient delivery of DNA. Figure 4B illustrates the intracellular trafficking of bPEI1800-C_{12}-19.72/DNA polyplexes after 4 and 24 hours' incubation. We can see that only a few FITC-labeled bPEI1800-C_{12}-19.72/DNA polyplexes (green fluorescence) were co-located in the lysosomes (red fluorescence), generating a yellow appearance due to the merge of green and red color (Figure 4Ba5 and b5), while most of the polyplexes were not situated in lysosomes but distributed throughout the cytoplasm, giving only green fluorescence (Figure 4Ba5 and b5). We have proved that most of the bPEI1800-C_{12}-19.72/DNA polyplexes entered cells through caveolae-mediated endocytosis, which has been reported to be able to bypass lysosomes,[36] so most of the bPEI1800-C_{12}-19.72/DNA polyplexes could move directly to the nucleus without significant cellular obstacles.[38] Moreover, although partial polyplexes could be trapped in the endosomes, some of them would have been released into the cytoplasm due to the membrane-destabilizing activity of the free bPEI1800-C_{12}. As a result, only a few polyplexes that are not released into cytoplasm in time will end up being trapped in the lysosomes. Based on the above results, we concluded that C_{12} grafting not only greatly improved the cellular uptake of bPEI1800-C_{12}-19.72/DNA polyplexes, but also altered their endocytosis pathways, leading to cellular trafficking without the obstacle of lysosomes, which was beneficial for the transfection of DNA.
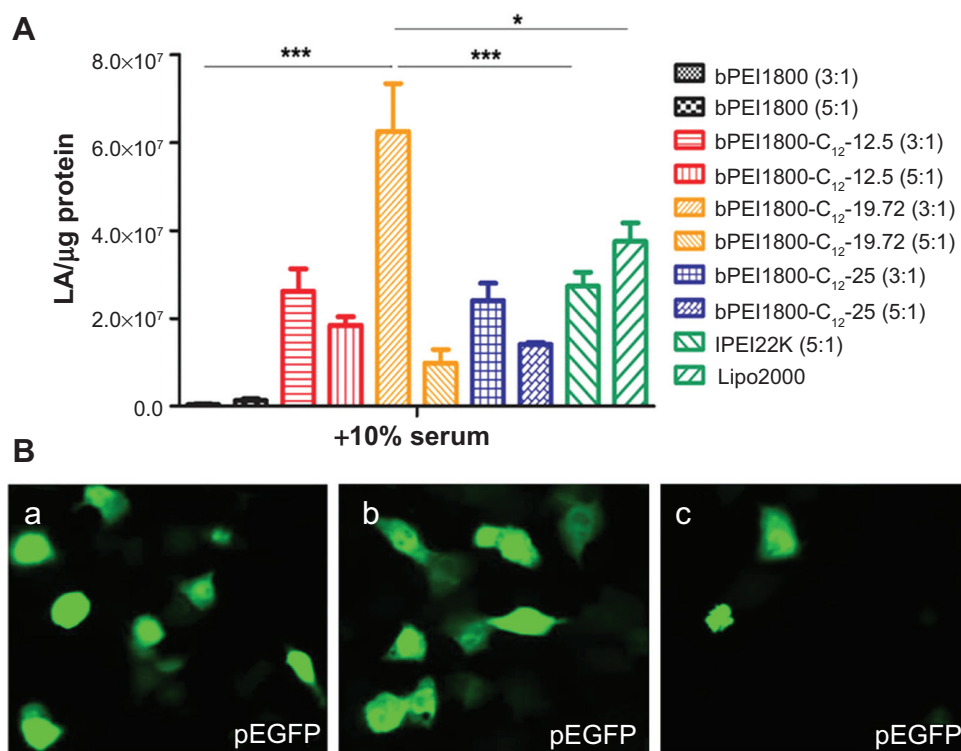

Figure 5 In vitro transfection of bPEI/DNA polyplexes encoding luciferase and GFP on 4T1 cells.
Notes: (**A**) LA (per μg protein) in 4T1 cells 24 hours post-transfection with bPEI1800/DNA polyplexes and bPEI1800-C_{12}/DNA polyplexes encoding LA. IPEI22K at a weight ratio of 5:1 and Lipo2000 were used as controls. n=4, *$P<0.05$, ***$P<0.001$. (**B**) Typical images of transfection efficacy of bPEI1800-C_{12}-19.72/DNA polyplexes (a), Lipo2000/DNA lipoplexes (b), and bPEI1800/DNA polyplexes (c) 48 hours post-transfection on 4T1 cells with the presence of serum using the GFP reporter gene (plasmid EGFP, pEGFP). 12.5, 19.72, and 25 represent the substitution degree of C_{12}. Ratios are expressed as the ratio of polymer to DNA (w/w). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA, USA).
Abbreviations: bPEI, branched polyethylenimine; bPEI1800, bPEI with molecular weight 1,800 Da; bPEI1800-C_{12}, bPEI1800 grafted with hydrophobic C_{12} chains; EGFP, enhanced green fluorescent protein; GFP, green fluorescence protein; LA, luciferase activity; Lipo2000, Lipofectamine™ 2000; IPEI, linear PEI; IPEI22K, IPEI with a molecular weight of 22,000 Da; w, weight.

In vitro transfection

We also studied the transfection efficiency of bPEI1800-C$_{12}$/DNA polyplexes on 4T1 cells using a luciferase reporter gene (pGL 4.50). The results depicted in Figure 5A indicate that all bPEI1800-C$_{12}$/DNA polyplexes prepared at a weight ratio of 3:1 (polymer to DNA) showed higher luciferase activity than those prepared at a weight ratio of 5:1. Importantly, bPEI1800-C$_{12}$-19.72/DNA at a weight ratio of 3:1 possessed the highest luciferase activity in serum-containing medium, which was 160-fold higher than that of bPEI1800 and significantly higher than that of lPEI22K ($P<0.001$) and Lipofectamine 2000 ($P<0.05$) under the same condition. Moreover, transfection efficacy of bPEI1800-C$_{12}$-19.72 was also assessed using a green fluorescence protein reporter gene (pEGFP) on 4T1 cells in the presence of serum. Under the above optimized transfection condition (w/w =3, polymer to DNA), 4T1 cells exhibited strong green fluorescence 48 hours post-transfection with bPEI1800-C$_{12}$-19.72/pEGFP polyplexes (Figure 5Ba), which was comparable to the green fluorescence of cells transfected with Lipofectamine 2000 (Figure 5Bb) and dramatically higher than that of cells transfected with bPEI1800 (Figure 5Bc). Such results were in line with the results of using

pGL 4.50 as a reporter gene, suggesting that bPEI1800-C$_{12}$-19.72 could deliver a variety of plasmid DNA types into cells and guarantee their successful expression as well.

In vivo transfection

The fact that bPEI1800-C$_{12}$-19.72/DNA polyplexes showed high stability against heparin and maintained higher in vitro transfection efficiency than Lipofectamine 2000 in the presence of serum indicated their great promise for in vivo application, so we studied the in vivo gene delivery efficiency of bPEI1800-C$_{12}$-19.72 by bioluminescence imaging, and the in vivo distribution of bPEI1800-C$_{12}$-19.72/DNA polyplexes was also investigated via luciferase activity measurement. Naked DNA, because of their low stability, cannot circulate into the systemic circulation and can only express locally at the injection site of the tail (Figure 6Aa). Compared to bPEI1800/DNA polyplexes (Figure 6Ab), bPEI1800-C$_{12}$-19.72/DNA polyplexes showed dramatically stronger luminescence intensity in vivo (Figure 6Ac). Amazingly, luminescence signal could even be detected in the head after injection of bPEI1800-C$_{12}$-19.72/DNA polyplexes (Figure 6Ac). Unfortunately, when the freshly dissected scalps and skulls of mice 24 hours post-injection with bPEI1800-C$_{12}$-19.72/DNA
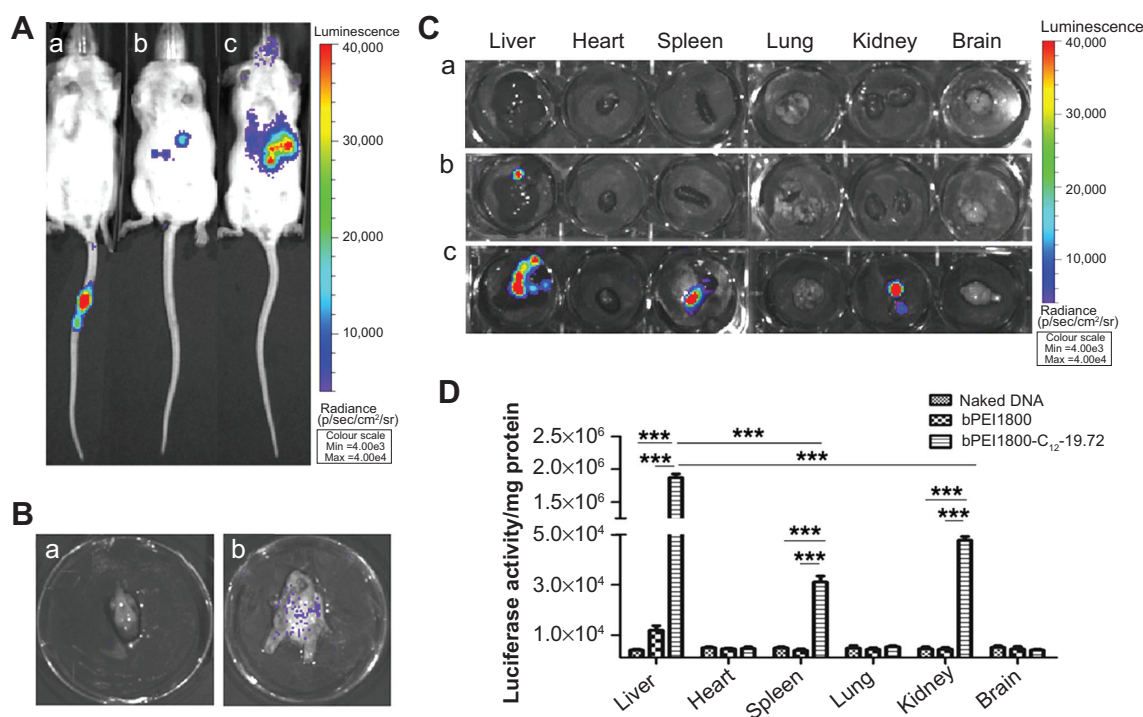

Figure 6 In vivo gene delivery of bPEI/DNA polyplexes encoding luciferase in mice.
Notes: (**A**) Typical bioluminescence images of mice 24 hours post-injection with naked DNA (a), bPEI1800/DNA polyplexes (b), and bPEI1800-C$_{12}$-19.72/DNA polyplexes (c) via tails. (**B**) Bioluminescence imaging of the dissected skull (a) and scalp (b) of mice 24 hours post-injection with bPEI1800-C$_{12}$-19.72/DNA polyplexes. (**C**) Bioluminescence imaging of organs dissected from mice 24 hours post-injection with naked DNA (a), bPEI1800/DNA polyplexes (b), and bPEI1800-C$_{12}$-19.72/DNA polyplexes (c) via tails. (**D**) Luciferase activity of organs dissected from mice 24 hours post-injection with naked DNA and bPEI/DNA polyplexes via tails. n=3, ***$P<0.001$. Color was used to signify the degree of luminescence, from a minimum value of 4,000 to a maximum of 40,000.
Abbreviations: bPEI, branched polyethylenimine; bPEI1800, bPEI with molecular weight 1,800 Da; bPEI1800-C$_{12}$-19.72, bPEI1800 grafted with hydrophobic C$_{12}$ chains with a substitution degree of 19.72.

polyplexes were imaged simultaneously, we found that luminescence signal in the head was actually located in the scalp rather than the skull (Figure 6B); this phenomenon was also confirmed by the result that neither luminescence signal (Figure 6Cc) nor luciferase expression (Figure 6D) were detected in the resected brain.

In vivo distribution studies revealed that naked DNA showed no luciferase expression in any of the detected organs (Figure 6Ca) and bPEI1800/DNA polyplexes expressed only in liver (Figure 6Cb), whereas bPEI1800-C_{12}-19.72/DNA polyplexes possessed significantly higher expression in liver ($P<0.001$), spleen ($P<0.001$), and kidneys ($P<0.001$) (Figure 6Cc). The corresponding luciferase activity of each organ, shown in Figure 6D, was in accordance with the results of bioluminescence imaging (Figure 6C). As illustrated in Figure 6D, 24 hours after injection of bPEI1800-C_{12}-19.72/DNA polyplexes, luciferase activity in liver was significantly higher than in spleen ($P<0.001$) and kidneys ($P<0.001$), and luciferase activity of all three organs (liver, spleen, kidneys) was significantly higher than in the corresponding organs of mice injected with bPEI1800/DNA polyplexes and naked DNA ($P<0.001$).

Discussion

As previously reported, grafting bPEI25K with acyl chains reduced the buffering capacity of PEI;[10] herein, results from both pH titration and gel retardation assay led to the same conclusion, that C_{12} grafting lowered the compaction efficiency of bPEI1800-C_{12} to DNA. There is widespread agreement that polymers with strong DNA-binding capacity do not always guarantee high transfection efficiency.[26] There is believed to be an optimal level of binding strength, which should be strong enough to compact DNA outside the cells, but not so strong that DNA can be released efficiently inside the cells.[26] Moderate C_{12} grafting could reduce the DNA-binding strength of bPEI1800-C_{12} to a level that is beneficial to its transfection efficiency. Using central composite design, bPEI1800-C_{12} with C_{12} substitution degree of 19.72 was determined to be the optimal bPEI1800-C_{12} derivative for DNA delivery (S Liu, unpublished data, 2014).

In comparison with alkylated high-molecular-weight PEI, alkylated LMW PEI underwent a different self-assembly process. It was reported that alkylated bPEI25K could assemble into a liposome-like structure at pH 7.0, and this "polycationic liposomes" phenomenon is thought to be common for hydrophobic derivatives of bPEI25K.[28] Due to the huge difference in the chain length between bPEI25K and hydrocarbons, it might be impossible for alkylated bPEI25K to form a micelle-like structure. But in the case of LMW

PEI (such as bPEI1800), whose chain length is comparable to that of medium-chain fatty acids and hydrocarbons, the micelle-like assembly of bPEI1800-C_{12} might occur more easily. Additionally, phospholipid-conjugated bPEI1800 was found to assemble into micelle-like nanoparticles by PEI moiety complexing with DNA, resulting in the inner PEI/DNA core and phospholipid moiety along with specially added helper lipids forming the outer lipid monolayer.[18] Taken together, alkylated high-molecular-weight PEI is apt to form a liposome-like structure, and alkylated LMW PEI is likely to assemble into a micelle-like structure, but when LMW PEI was conjugated with amphiphilic moieties (such as phospholipid), the assembly process was different from that of PEI grafted with pure hydrophobic moieties (such as hydrocarbon chains).

Interestingly, we found that the TEM result of bPEI1800-C_{12}/DNA polyplexes (Figure 2B) was quite similar to the assumed structure of C_{14}-CO dimer/DNA complexes[24] (Figure S5), and bPEI1800-C_{12} had a similar molecular structure to gemini/twinned surfactant, as illustrated in Figure S5. Thus, bPEI1800-C_{12} could be seen as an analog of gemini surfactant with bPEI1800 moiety representing the charged/polar head groups with an inherent inner spacer, and C_{12} chains being the hydrophobic tails, as illustrated in Figure 2C. In accordance with gemini/twinned surfactant, the assembly process of bPEI1800-C_{12}/DNA complexes was also driven by dual forces: 1) the outside, electrostatic interaction between bPEI1800 moiety and DNA; and 2) the inside, cooperative hydrophobic assembly of C_{12} chains. During the assembly process of bPEI1800-C_{12}/DNA complexes, DNA undergoes coil–globule transition, forming hydrophobic domains along the DNA chain (Figure 2C) in the same manner as cationic surfactant.[24,39] To our knowledge, no such analogy or assembly process of alkylated LMW PEI has been reported in the literature so far.

Stability of bPEI/DNA polyplexes is of prime importance for efficient in vivo gene delivery. bPEI1800-C_{12}/DNA polyplexes were found to be quite robust against anion displacement (Figure 3A) and exhibited high in vitro transfection efficiency in the presence of serum (Figure 5). The molecular formula of bPEI1800-C_{12} is $(C_2H_4N)_{42}$ $(C_{12}H_{25})_{42}n$, where n is the C_{12} substitution degree,[28] so it was thought there would be 8.3 C_{12} chains in every molecule of bPEI1800-C_{12}-19.72. It has been reported that cationic surfactant with more than one protonation center and hydrophobic chain showed much higher stability than their corresponding monovalent (single hydrophobic chain and single head group).[24,26] As such, the high stability of bPEI1800-C_{12}/DNA polyplexes was likely due to the same

"chelate effect"[26] as gemini surfactant: multi-protonation centers (DNA compacted more tightly) and multiple hydrophobic chains that could assemble into a highly organized micelle-like structure. Moreover, as depicted in Figure 2C, when complexing DNA with bPEI1800-C$_{12}$, the individual duplex DNA exhibited a coil–globule transition and formed multi-hydrophobic domains[24] along the DNA chain, thus the DNA chain was likely "fixed" to the micelle-like particles. This might be another reason for the high stability of bPEI1800-C$_{12}$/DNA polyplexes.

As shown in Figure 6C and D, most of the bPEI1800-C$_{12}$/DNA polyplexes accumulated in reticuloendothelial system organs, but it should be noted that they still exhibited efficient gene expression in these reticuloendothelial system organs after systemic delivery. Moreover, it was surprising that luciferase gene expression could be detected even in the head, suggesting the high in vivo stability of these bPEI1800-C$_{12}$/DNA polyplexes. Given that the bPEI1800-C$_{12}$/DNA polyplexes developed in this study were rather simple, in order to improve their in vivo biodistribution, one could fabricate functional nanoparticles based on these bPEI1800-C$_{12}$ derivatives, such as further conjugating with PEG[18] or targeting peptide moieties (cRGD, NGR, etc), and one could also develop co-delivery platforms, such as solid lipid-PEI hybrid nanocarrier.[19] In particular, the innate brain accumulation property of bPEI1800-C$_{12}$ could be utilized to develop brain-targeting nanoparticles with further modification. The interesting phenomenon, that bPEI1800-C$_{12}$/DNA polyplexes partly accumulated in the head/brain after systemic injection, was likely because bPEI1800-C$_{12}$, being an analog of gemini surfactant, possessed surfactant activity to help passage through the blood–brain barrier to enter the brain, but further work is needed to prove this assumption and to elucidate why bPEI1800-C$_{12}$/DNA polyplexes accumulated in the scalp rather than the skull.

Conclusion

We successfully grafted bPEI1800 with lauryl chains (C$_{12}$) and obtained bPEI1800-C$_{12}$ which could assemble into a micelle-like structure similarly to gemini surfactant. After C$_{12}$ grafting, stability and cellular uptake of bPEI1800-C$_{12}$/DNA polyplexes were greatly improved, and bPEI1800-C$_{12}$/DNA polyplexes showed superior lysosome escape ability, all leading to high in vitro and in vivo transfection efficiency. In the future, we will focus on the fabrication of nanoparticles with favorable in vivo behaviors based on bPEI1800-C$_{12}$ derivatives. More studies are required to elucidate the brain (head) accumulation property of bPEI1800-C$_{12}$. The results of

this work suggest that hydrophobic modification of LMW bPEI may give rise to a kind of gemini surfactant-like bPEI derivative with totally altered properties and fantastic benefits.

Disclosure

The authors report no conflicts of interest in this work.

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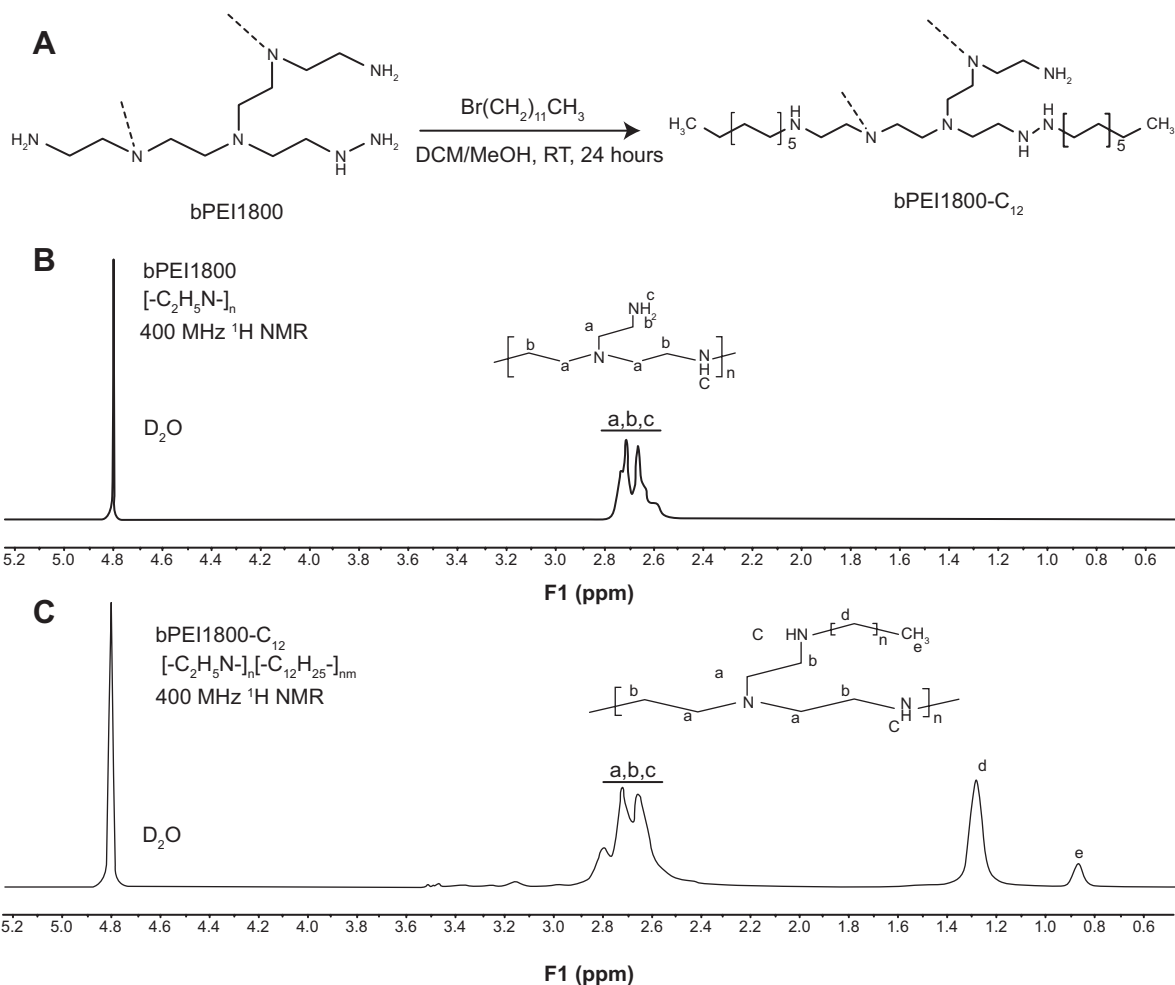
Supplementary materials

A

Br(CH₂)₁₁CH₃
DCM/MeOH, RT, 24 hours

bPEI1800 → bPEI1800-C₁₂

B

bPEI1800
[-C₂H₅N-]ₙ
400 MHz ¹H NMR

D₂O

a,b,c

C

bPEI1800-C₁₂
[-C₂H₅N-]ₙ[-C₁₂H₂₅-]ₙₘ
400 MHz ¹H NMR

D₂O

a,b,c   d   e

Figure S1 Synthesis route and structure confirmation of bPEI1800 and bPEI1800-C₁₂.
Notes: (**A**) Synthesis route of bPEI1800-C₁₂. (**B**) ¹H NMR spectrum of bPEI1800. (**C**) ¹H NMR spectrum of bPEI1800-C₁₂.
Abbreviations: bPEI1800, branched polyethylenimine with molecular weight 1,800 Da; bPEI1800-C₁₂, bPEI1800 grafted with hydrophobic C₁₂ chains; DCM, dichloromethane; F1, frequency; ¹H NMR, proton nuclear magnetic resonance; MeOH, methanol; RT, room temperature.
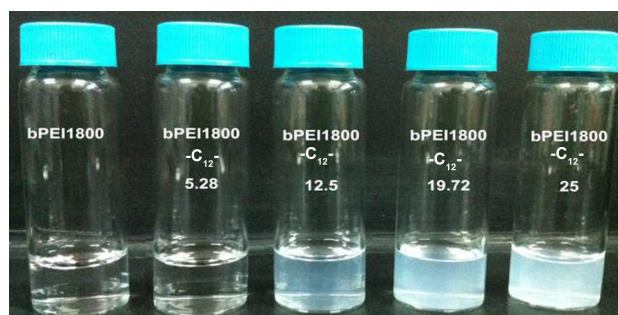
Figure S2 Appearance of bPEI1800 and its C₁₂ grafting derivatives at a concentration of 4 mg/mL in double-distilled water.
Note: 5.28, 12.5, 19.72, and 25 represent the substitution degree of C₁₂.
Abbreviations: bPEI1800, branched polyethylenimine with molecular weight 1,800 Da; bPEI1800-C₁₂, bPEI1800 grafted with hydrophobic C₁₂ chains.
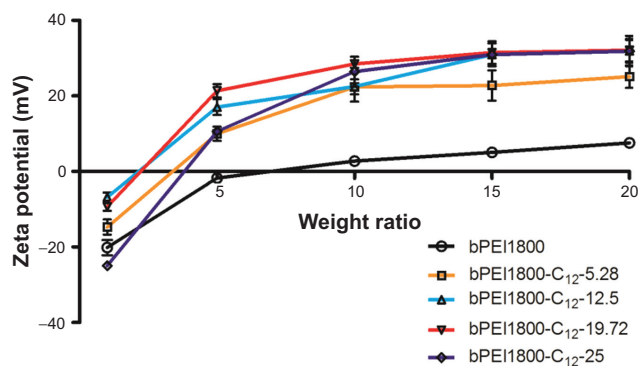
Figure S3 Zeta potentials of bPEI/DNA polyplexes at various weight ratios.
Notes: 5.28, 12.5, 19.72, and 25 represent the substitution degree of C₁₂. Weight ratios are presented as the ratio of polymer to DNA.
Abbreviations: bPEI, branched polyethylenimine; bPEI1800, bPEI with molecular weight 1,800 Da; bPEI1800-C₁₂, bPEI1800 grafted with hydrophobic C₁₂ chains.
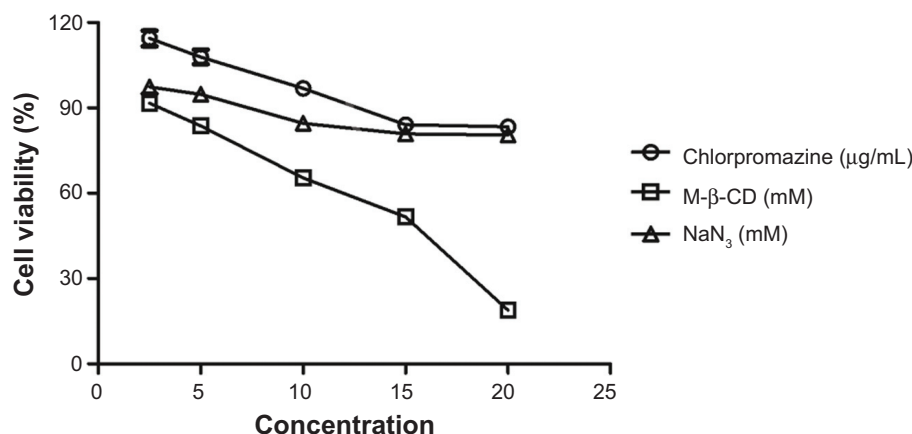
Figure S4 Cytotoxicity of chlorpromazine, M-β-CD, and NaN₃ in 4T1 cells determined by MTT assays.
Notes: Chlorpromazine, M-β-CD, and NaN₃ were incubated with 4T1 cells for 5 hours in serum-free medium, and their cytotoxicity was determined by regular MTT assays. Based on the results of the MTT assays, the final concentrations of chlorpromazine, M-β-CD, and NaN₃ chosen for use were 10 μg/mL, 5 mM, and 10 mM, respectively.
Abbreviations: M-β-CD, methyl-β-cyclodextrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
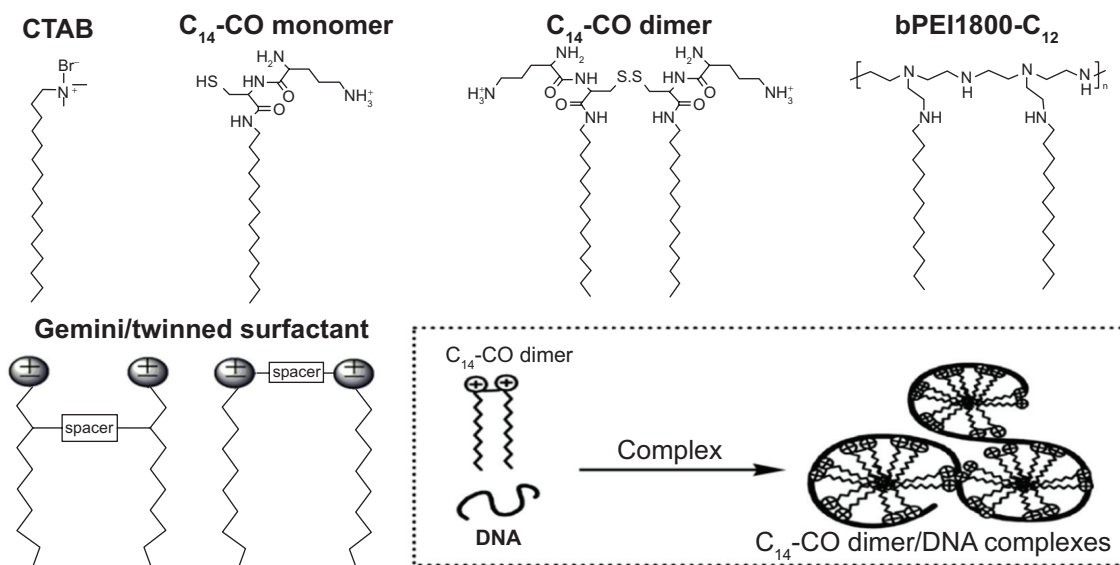
Figure S5 Chemical structures of CTAB; reduced ornithinyl-cysteinyl-tetradecylamide (C_{14}-CO monomer); oxidized ornithinyl-cysteinyl-tetradecylamide (C_{14}-CO dimer); bPEI1800-C_{12}; gemini/twinned surfactant; and C_{14}-CO dimer/DNA complexes.
Note: Reprinted from *Chemistry and Physics of Lipids*, 111(1), Lleres D, Dauty E, Behr JP, Mély Y, Duportail G, DNA condensation by an oxidizable cationic detergent. Interactions with lipid vesicles, 59–71, Copyright © (2001), with permission from Elsevier.[1]
Abbreviations: bPEI1800-C_{12}, branched polyethylenimine with molecular weight 1,800 Da grafted with hydrophobic C_{12} chains; CTAB, hexadecyltrimethylammonium bromide.

Reference

1. Lleres D, Dauty E, Behr JP, Mély Y, Duportail G. DNA condensation by an oxidizable cationic detergent. Interactions with lipid vesicles. *Chem Phys Lipids*. 2001;111(1):59–71.