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A Novel Disulfide-Containing Polycationic Amphiphile: 1,28-Di[(cholest-5-en-3β-yl)disulfanyl]-4,25-dioxo-3,8,12,17,21,26-hexaazaoctacosane Tetrahydrochloride

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Abstract: The absence of highly effective delivery systems is a major challenge for gene therapy. Our work was aimed at the development of novel cationic liposomes possessing high transfection efficiency. For this purpose, a novel disulfide polycationic amphiphile **2S4** was synthesized. Cationic liposomes based on **2S4** and a helper lipid DOPE were formed by the thin film hydration method and exhibited effective plasmid DNA delivery into the HEK293 cells, with a transfection activity superior to that of the commercial agent Lipofectamine® 2000. Our results suggest that the polycationic amphiphile **2S4** is a promising candidate for in vitro nucleic acid delivery.

Keywords: redox-sensitive; disulfide linker; gemini amphiphiles; cationic liposomes; gene therapy

1. Introduction

Gene therapy is an attractive tool for the treatment of both inherited [1,2] and acquired diseases [3,4] and is based on the delivery of therapeutic nucleic acid (NA) into cells. Moreover, DNA delivery has been used for effective induction of both cellular and humoral immunity against the encoded antigen [5]. Nucleic acid delivery requires a special vehicle to protect NA against nucleases and to help them pass through different intracellular and extracellular barriers. The most attractive and safe delivery vehicles are the non-viral ones, such as cationic liposomes (CLs). Despite their advantages, including safety, low cost, and the ability to be produced at scale, CLs have inadequate delivery (also called transfection) efficiency [6]. This fact induces the development of novel liposomal systems. CLs are formed from cationic amphiphiles (CAs) and the structure of CAs has a crucial influence on delivery efficiency. Typical CAs consist of hydrophobic and hydrophilic domains, a connecting linker, and a spacer that maintains the steric arrangement of domains [6,7]. In order to increase transfection efficiency, modification of the CA structure with labile stimuli-responsive groups may be performed. Intracellular stimuli cause CA degradation which, in turn, can enhance NA release from the liposomes/NA complexes and stimulate endosomal escape [8]. For example, a disulfide linker is degradable by intracellular reducing agents such as glutathione (GSH) [9].

Recently, Zhao et al. designed disulfide CAs based on a polar amino acid head and tocopherol, with the disulfide bond introduced as a cystamine moiety [10]. The transfection efficiency of these CAs with respect to HEK293 cells was comparable to that of the commercial agent Lipofectamine® 2000. High transfection efficiency both in vitro and in vivo was demonstrated by a disulfide CA based

on lysine and arginine [11]. A number of disulfide CAs based on cholesterol have been synthesized and have demonstrated delivery efficiency comparable with that of commercial agents. CAs with a polyamine cationic domain (lysine or triethylenetetramine) were the most effective ones with respect to COS-7 cells [12]. Redox-sensitive gemini CAs based on thiocholesterol with a flexible hydrophilic spacer and ether linkers were the most effective for hard-to-transfect HaCaT cells [13]. Cationic lipophosphoramidates were also modified by disulfide linkers and demonstrated more effective NA delivery compared to Lipofectamine® [14]. It is also known that the location of a disulfide bond location have a strong effect on the transfection efficiency [15].

Recently, we have developed a polycationic gemini amphiphile **2X3** with a hexamethylene spacer and a carbamoyl linker (Figure 1) and have demonstrated its remarkably high transfection efficiency, which is superior to that of Lipofectamine® 2000 [16]. Here we studied the influence of the disulfide groups incorporated into the CA molecule on the transfection efficiency of CLs containing such CA.

In this work, we designed and synthesized a novel polycationic gemini amphiphile **2S4** (Figure 1) with two disulfide linkers placed close to the hydrophobic domains of the CA. We believe that disulfide groups degradable in the presence of reducing agents should contribute to better NA release into the cytosol. We also evaluated the physicochemical properties and transfection efficiency of liposomes composed of **2S4** in comparison with non-redox-sensitive **2X3** CLs and Lipofectamine® 2000.

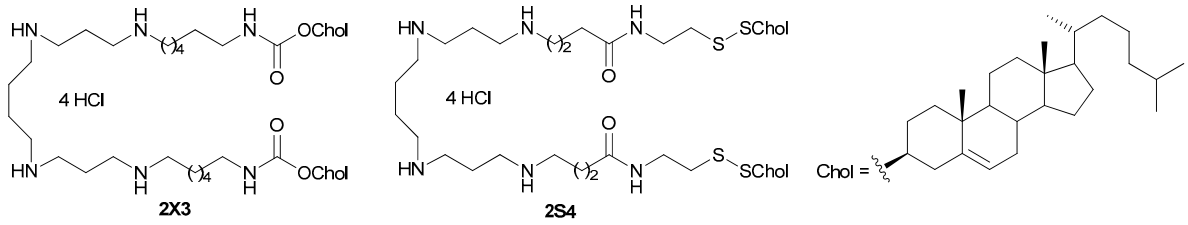


Figure 1. Polycationic gemini amphiphiles **2X3** and **2S4**.

2. Results and Discussion

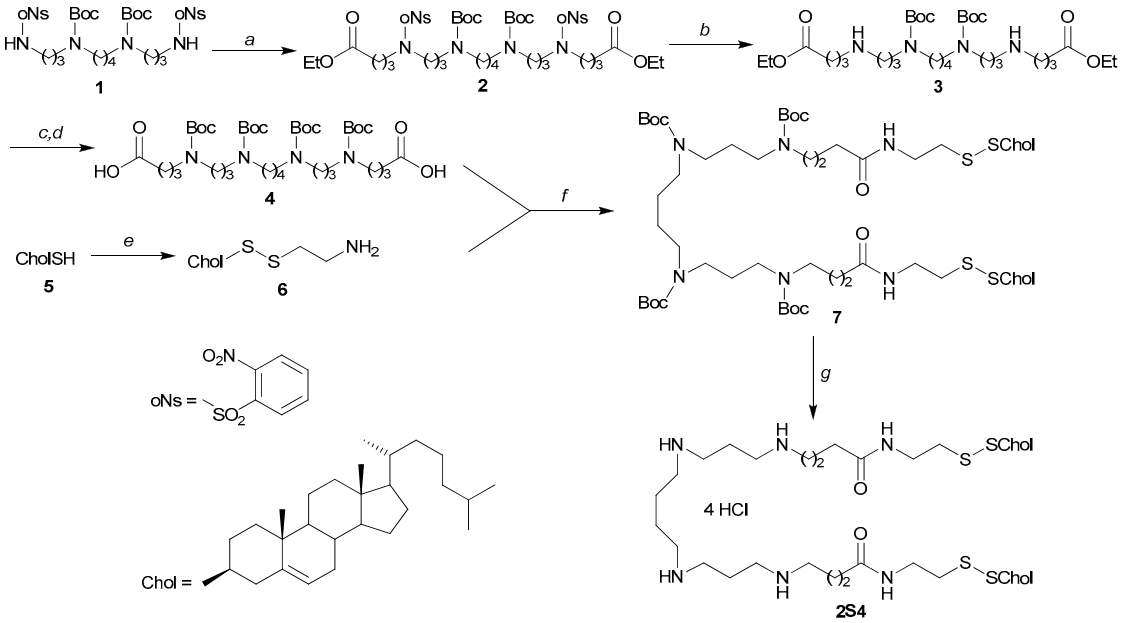
2.1. Synthesis of 2S4

To obtain the target amphiphile **2S4**, we synthesized a hydrophilic component (**4**), a hydrophobic component (**6**) containing disulfide linker, and then combined them into a single molecule (Scheme 1). The hydrophilic component is a dicarboxylic derivative of spermine (**4**), which was obtained through a number of synthetic steps. Firstly, a regioselectively protected derivative of spermine (**1**) was synthesized as described previously [17]. Alkylation of **1** was performed in the Fukuyama reaction conditions [18] with ethyl 4-bromobutyrate to give the compound **2**, which was isolated by column chromatography with 76% yield. Desulfonylation of **2** performed by the treatment with thiophenol in the presence of potassium carbonate afforded amine **3** in 18% yield. To avoid side reactions and to facilitate purification in the following steps, secondary amino groups of **3** were blocked with Boc_2O in the presence of TEA. The resulting fully protected diester was hydrolyzed with NaOH in the methanol-water solution. The desired dicarboxylic derivative of spermine **4** was isolated with 49% yield.

Synthesis of the hydrophobic component with the disulfide linker was performed via a thiol-disulfide exchange reaction [19]. Direct thiol-disulfide exchange often proceeds with a low yield of the desired disulfide. Therefore, a two-step synthesis through an appropriate intermediate disulfide is preferable. To realize this synthetic approach, we synthesized a 2-[(cholest-5-en-3β-yl)disulfanyl]pyridine by reacting thiocholesterol (**5**) with 2,2′-dithiodipyridine [20]. However, a subsequent reaction of the disulfide obtained with 2-aminoethanethiol did not lead to the desired disulfide **6**. Intrigued by that, we performed a direct reaction between thiocholesterol (**5**) and cystamine dihydrochloride, which resulted in compound **6** with high yield. Thiol-disulfide exchange is a reverse reaction and the equilibrium

position appear to be depended from the nucleophilicity of starting and resulting thiols. We believe that the similar nucleophilicity of both 2-aminoethanethiol and pyridine-2-thiol in the first thiol-disulfide reaction did not favor the formation of the desire product **6**.

A key step in the synthesis was the condensation of hydrophilic (**4**) and hydrophobic (**6**) components. The reaction was carried out in the presence of a coupling reagent EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) and resulted in compound **7**, which was purified by column chromatography with 84% yield. The removal of Boc protecting groups by 3 M HCl in dioxane produced the target amphiphile **2S4** with 91% yield after recrystallization from ethanol and diethyl ether.



Scheme 1. Reagents and conditions of the synthesis: (a) $Br(CH_2)_3COOEt$, Cs_2CO_3, DMF, 65 °C, 36 h; (b) PhSH, K_2CO_3, DMF, 24 °C, 2 h; (c) Boc_2O, Et_3N, DCM, 24 °C, 48 h; (d) NaOH, MeOH, 24 °C, 56 h; (e) $NH_2(CH_2)_2SS(CH_2)_2NH_2$ * 2HCl, Et_3N, DMF, 24 °C, 72 h; (f) EEDQ, DIEA, DCM, 50 °C, 48 h; (g) 3 M HCl/dioxane, DCM, 22 °C, 24 h.

2.2. Cationic Liposomes and Their Transfection Efficiency

Based on the disulfide CA **2S4** and a zwitterionic helper lipid DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), CLs were prepared at lipid molar ratio of 1:1 by the thin film hydration method. Nonredox-sensitive **2X3** based CLs was prepared in the same manner and used as a positive control (here and thereafter referred as **2S4** and **2X3** CLs). The hydrodynamic diameter and the polydispersity index (PI) of **2S4** CLs determined by dynamic light scattering was 440 nm and 0.254, respectively, while **2X3** CLs had the diameter of 104 nm and PI 0.313. CLs obtained were used for the delivery of a pEGFP-C2 plasmid, which encodes enhanced green fluorescent protein (EGFP), into HEK293 cells at various N/P ratios (number of polycationic amino groups of CAs per phosphate group of nucleic acids). After the CL/NA complexation the hydrodynamic diameter of **2S4** CLs decreased to 250 nm and that of **2X3** CLs (N/P 6/1) increased slightly to 120 nm. Furthermore, all CLs and their complexes with NA had a positive zeta-potential.

To estimate the cytotoxic effect of liposomes, HEK 293 cells were incubated with the liposomes under serum-free conditions. IC_{50} values for cationic liposomes **2X3** and **2S4** lied within the range of 30–40 μM. Preliminary data showed, that 80% of HEK 293 cells were viable at N/P ratios used for intracellular delivery of pEGFP-C2 plasmid.

Transfection experiments were performed in the presence of 10% fetal bovine serum (FBS) to mimic the in vivo conditions. As shown by flow cytometry, transfection efficiency increased with increasing N/P ratios (Figure 2). Both **2X3** and **2S4** CLs showed better transfection efficiency than Lipofectamine® 2000. At lower N/P ratios (2/1 and 4/1), the **2S4** CLs mediated better percentage of transfected cells as well as higher mean fluorescence intensity than the **2X3** CLs. On the other hand, at higher N/P ratios, the percentage of transfected cells was similar for both **2X3** and **2S4** CLs, but mean fluorescence intensity was lower for **2S4** CLs.

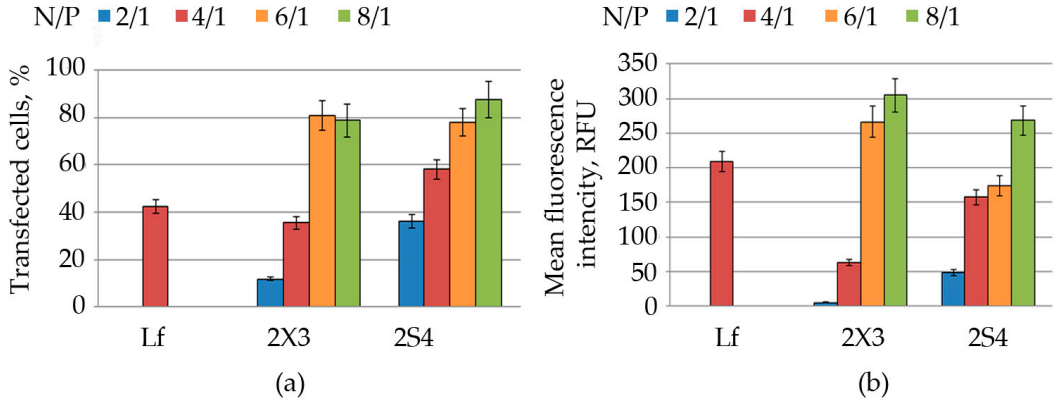


Figure 2. Transfection efficacy of CL/pEGFP-C2 complexes formed at different N/P ratios: (**a**) Percentage of transfected cells; (**b**) Mean fluorescence intensity of the cell population. Lf (Lipofectamine® 2000) and **2X3** cationic liposomes (CLs) were used as positive control. Experiments were performed in the presence of 10% FBS, each in triplicate. Standard deviation did not exceed 7–9%.

From the physicochemical point of view, the differences between the **2X3** and **2S4** CLs may be explained by the fact that CL/pEGFP-C2 complexes of **2S4** were larger (250 nm versus 120 nm) and more heterogeneous (PIs 0.369 and 0.143, respectively) as compared with CL/pEGFP-C2 complexes of **2X3** at N/P ratio of 6/1.

In this study, a novel disulfide polycationic amphiphile **2S4** was synthesized for plasmid DNA delivery. Transfection efficiency was evaluated by measuring the level of transgene expression in HEK293 cells. We demonstrated that the transfection efficiency of **2S4** CLs was higher than that of Lipofectamine® 2000 and similar to that of nonredox-sensitive **2X3** CLs. Nevertheless, **2S4** CLs have a potential for in vitro transfection at low N/P ratios. In addition, we plan to continue the study of the redox-sensitive delivery vehicles and the influence of disulfide bond location within CA on transfection efficiency.

3. Materials and Methods

DIEA, DMF, EEDQ, TEA, cystamine dihydrochloride, 2,2′-dithiodipyridine, ethyl 4-bromobutyrate, spermine, thiocholesterol were obtained from Sigma-Aldrich (St. Louis, MO, USA); Boc_2O, cesium carbonate were obtained from Fluka (Buchs, Switzerland); thiophenol was obtained from Merck (Darmstadt, Germany). Other solvents and reagents were purchased from Russian companies.

CH_2Cl_2, TEA, DIEA were refluxed with CaH_2 and distilled prior to the reaction. EtOH was refluxed with magnesium turnings and iodine and distilled prior to the reaction. MeOH and DMF were kept over calcined molecular sieves 3 Å and 4 Å, respectively.

Column chromatography was carried out on silica gel Kieselgel 60 (0.040–0.063 mm, Merck). ^{1}H-and ^{13}C-NMR spectra were recorded on a Avance DPX-300 and Avance DRX-500 pulse Fourier transform spectrometers (Bruker, Karlsruhe, Germany) in $CDCl_3$ unless otherwise stated. Chemical shifts were recorded in ppm on the δ scale relative to $CHCl_3$ solvent residual peak (7.26 ppm for ^{1}H and 77.0 ppm for ^{13}C-NMR spectra). Coupling constants (*J*) are absolute values and recorded in Hz.

Mass spectra were run on a Thermo Finnigan MAT 900XL-TRAP mass spectrometer and on a Orbitrap Fusion mass spectrometer (Thermo Scientific, Pittsburgh, PA, USA) with electrospray ionization (ESI). Melting points were determined on a IA9100 digital melting point apparatus (Electrothermal, Stone, Great Britain).

Diethyl N^9,N^{14}-Di(tert-butyloxycarbonyl)-N^5,N^{18}-bis(2-nitrobenzenesulfonyl)-5,8,14,18- tetraazadocosane-1,18-dioate (**2**). Cesium carbonate (0.84 g, 2.57 mmol) and ethyl 4-bromobutyrate (0.50 mL, 3.59 mmol) were added to a solution of compound **1** (0.80 g, 1.04 mmol) in anhydrous DMF (9 mL). The reaction mixture was stirred at 65 °C for 36 h, filtered on Celite® 545. The filtrate was evaporated to dryness, the residue was chromatographed on a silica gel column eluted with DCM-MeOH (120:1). The product **2** was obtained as a pale yellow oil (0.78 g, 76%). ^{1}H-NMR (300 MHz): 1.18 (t, 6 H, 2 $CH_2C\underline{H}_3$, J = 7.1), 1.42 (br. s, 22 H, 2 CMe_3, $NCH_2(C\underline{H}_2)_2CH_2N$), 1.76–1.81 (m, 4 H, 2 $NCH_2C\underline{H}_2CH_2N$), 3.12–3.18 (m, 8 H, 2 $NC\underline{H}_2CH_2CH_2N$, $NC\underline{H}_2(CH_2)_2C\underline{H}_2N$), 3.37–3.42 (m, 4 H, 2 $NCH_2CH_2C\underline{H}_2N$), 4.09 (q, 4 H, 2 $C\underline{H}_2CH_3$, J = 7.1), 4.18 (s, 4 H, 2 CH_2COO), 7.58–7.63 (m, 2 H), 7.67–7.72 (m, 4 H) and 8.04–8.07 (m, 2 H, 2 C_6H_4). ^{13}C NMR (75 MHz): 14.33, 23.46, 27.48, 27.87, 28.58, 31.06, 31.25, 44.63, 45.48, 46.86, 60.66, 79.65, 124.31, 130.94, 131.81, 133.50, 133.65, 148.22, 155.54, 172.76. Anal. Calcd for $C_{44}H_{68}N_6O_{16}S_2$: C, 52.79; H, 6.85; N, 8.39. Found: C, 52.55; H, 6.68; N, 8.11.

Diethyl N^9,N^{14}-Di(tert-butyloxycarbonyl)-5,8,14,18-tetraazadocosane-1,22-dioate (**3**). Potassium carbonate K_2CO_3 (1.13 g, 8.14 mmol) was added to a solution of compound **2** (0.78 g, 0.79 mmol) in anhydrous DMF (10 mL), and the reaction mixture was stirred at 24 °C for 10 min. Then thiophenol (0.80 mL, 7.80 mmol) was added and the reaction mixture was additionally stirred at 24 °C for 2 h, filtered on Celite® 545. The filtrate was evaporated to dryness, the residue was chromatographed on a silica gel column eluted with DCM-MeOH-25% aq. ammonia (from 8:1:0.1 to 6:1:0.1). The product **3** was obtained as yellow oil (0.089 g, 18%). ^{1}H-NMR (300 MHz): 1.24 (t, 6 H, J = 7.1, 2 CH_3), 1.44 (br. s, 18 H, 2 Boc), 1.50–1.62 (m, 4 H, 2 $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 2.07–2.26 (m, 8 H, 4 $CH_2C\underline{H}_2CH_2$), 2.46 (t, 4 H, J = 7.2, 2 $C\underline{H}_2COOEt$), 2.85–3.07 (m, 8 H, 4 $C\underline{H}_2NH$), 3.20–3.45 (m, 8 H, 4 $C\underline{H}_2N$), 4.10 (q, 4 H, J = 7.1, 2 $C\underline{H}_2CH_3$). HRMS (ESI), *m/z*: 631.4641 $[M + H]^+$. Calculated for $C_{32}H_{62}N_4O_8$: 631.4646 $[M + H]^+$.

N^5,N^9,N^{14},N^{18}-Tetra(tert-butyloxycarbonyl)-5,9,14,18-tetraazadocosane-1,22-dioic acid (**4**). A solution of compound **3** (0.089 g, 0.141 mmol) and anhydrous TEA (0.10 mL, 0.705 mmol) in anhydrous DCM (4 mL) was cooled to 0 °C. Boc_2O (0.092 g, 0.423 mmol) in anhydrous DCM (0.50 mL) was added and the reaction mixture was stirred at 24 °C for 48 h, diluted with DCM (20 mL), washed with 3% aq. HCl (1 × 10 mL) and water (3 × 10 mL). The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. The residue was dissolved in MeOH (4 mL) and was added into a solution of NaOH (0.013 g, 0.33 mmol) in MeOH-H_2O (1.1 mL, 10:1 v/v). The reaction mixture was stirred at 24 °C for 56 h, then 0.5 M aq. HCl was added dropwise until pH 4, and the reaction mixture was evaporated to dryness in vacuo. The residue was chromatographed on a silica gel column eluted with DCM-MeOH-1% aq. AcOH (from 15:1:0.1 to 5:1:0.1). The product **4** was obtained as beige amorphous solid (0.025 g, 49%). ^{1}H-NMR ($CDCl_3$:CD_3OD = 1:1, 300 MHz): 1.43 (br. s, 36 H, 4 Boc), 1.46–1.53 (m, 4, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 1.67–1.90 (m, 8 H, 4 $CH_2C\underline{H}_2CH_2$), 2.26 (t, 4 H, J = 7.2, 2 $C\underline{H}_2COOH$), 3.08–3.27 (m, 16 H, 8 $C\underline{H}_2N$). ^{13}C NMR (75 MHz): 23.59, 25.66, 27.40, 27.93, 27.98, 29.36, 31.06, 44.88, 46.37, 46.82, 79.69, 79.83, 155.84, 175.34. MS (ESI), *m/z*: 774.08 $[M]^+$, 797.49 $[M + Na]^+$. Calculated for $C_{38}H_{70}N_4O_{12}$ 774.50 $[M]^+$, for $C_{38}H_{70}N_4NaO_{12}$ 797.72 $[M + Na]^+$.

2-[(Cholest-5-en-3β-yl)disulphanyl]ethanamine (**6**). Thiocholesterol (**5**) (0.18 g, 0.44 mmol) and anhydrous TEA (0.25 mL, 1.77 mmol) were added to a solution of cystamine dihydrochloride (0.050 g, 0.22 mmol) in DMF (10 mL) under argon atmosphere. The reaction mixture was further purged with argon for 5 min and stirred at 24 °C for 72 h, then evaporated to dryness in vacuo. The residue was chromatographed on a silica gel column eluted with DCM-MeOH-25% aq. ammonia (40:1:1). The product **8** was obtained as beige amorphous solid (0.092 g, 86%). ^{1}H-NMR (300 MHz): 0.67 (s, 3 H, C(13)Me), 0.85 (d, 3 H, J = 6.6, C(25)Me), 0.87 (d, 3 H, J = 6.6, C(25)Me), 0.90 (d, 3 H, J = 6.2, C(20)Me), 1.00 (s, 3 H, C(10)Me),

1.03–1.67 (m, 21 H, Chol), 1.65–2.07 (m, 5 H, Chol), 2.17–2.40 (m, 2 H, H_2C(4) Chol), 2.57–2.71 (m, 1 H, H(3) Chol), 2.75 (t, 2 H, *J* = 6.2, CH_2S), 2.90–3.06 (m, 2 H, $C\underline{H}_2NH_2$), 5.32–5.40 (m, 1 H, H(6) Chol). HRMS (ESI), *m/z*: 478.3535 $[M + H]^+$. Calculated for $C_{29}H_{52}NS_2$ 478.3541 $[M + H]^+$.

N^8,N^{12},N^{17},N^{21}-Tetra(tert-butyloxycarbonyl)-1,28-di[(cholest-5-en-3β-yl)disulphanyl]-4,25-dioxo-3,8,12,17,21,26-hexaazaoctacosane (**7**). Anhydrous DIEA (27 μL, 0.155 mmol) was added to a solution of compound 6 (0.037 g, 0.077 mmol) in anhydrous DCM (4 mL) and stirred for 10 min. Solutions of compound 4 (0.024 g, 0.031 mmol) in anhydrous DCM (3 mL) and EEDQ (0.026 g, 0.077 mmol) in anhydrous DCM (2 mL) were successively added to the stirring reaction mixture. After 48 h at 50 °C, the reaction mixture was cooled to 24 °C, diluted with DCM (30 mL), then washed with saturated aq. Na_2CO_3 (1 × 10 mL), water (1 × 10 mL), 0.2 M aq. HCl (1 × 10 mL), water (2 × 10 mL). The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. The residue was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (from 80:1 to 60:1). The product 7 was obtained as pale yellow amorphous solid (0.037 g, 84%). ^{1}H-NMR (300 MHz): 0.67 (s, 6 H, 2 C(13)Me), 0.84 (d, 6 H, *J* = 6.6, 2 C(25)Me), 0.86 (d, 3 H, *J* = 6.6, 2 C(25)Me), 0.89 (d, 6 H, *J* = 6.2, 2 C(20)Me), 1.00 (s, 6 H, 2 C(10)Me), 1.05–1.62 (m, 46 H, Chol, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 1.44 (br. s, 36 H, 4 Boc), 1.66–2.06 (m, 18 H, 4 $CH_2C\underline{H}_2CH_2$, Chol), 2.16 (t, 4 H, *J* = 7.2, 2 $C\underline{H}_2CONH$), 2.17–2.40 (m, 4 H, 2 H_2C(4) Chol), 2.57–2.71 (m, 2 H, 2 H(3) Chol), 2.79 (t, 4 H, *J* = 6.2, 2 CH_2S), 3.04–3.30 (m, 16 H, 8 NCH_2), 3.46–3.62 (m, 4 H, 2 $C\underline{H}_2NHCO$), 5.31–5.39 (m, 2 H, 2 H(6) Chol). ^{13}C NMR (125 MHz): 12.00, 18.86, 19.45, 21.09, 22.68, 22.93, 23.97, 24.41, 24.61, 26.02, 28.14, 28.34, 28.63, 29.16, 31.98, 32.02, 33.67, 35.91, 36.33, 36.90, 38.44, 39.08, 39.16, 39.66, 39.90, 42.46, 45.09, 46.18, 47.00, 50.23, 50.39, 53.53, 56.32, 56.90, 79.48, 79.80, 121.58, 141.50, 155.58, 172.69. HRMS (ESI), m/z: 1694.1792 $[M + H]^+$, 847.5927 $[M + 2H]^{2+}$. Calculated for $C_{96}H_{169}N_6O_{10}S_4$: 1694.1783 $[M + H]^+$, for $C_{96}H_{170}N_6O_{10}S_4$: 847.5931 $[M + 2H]^{2+}$.

1,28-Di[(cholest-5-en-3β-yl)disulphanyl]-4,25-dioxo-3,8,12,17,21,26-hexaazaoctacosane tetrahydrochloride (**2S4**). A solution of 3 N HCl in anhydrous dioxane (6 mL) was added to a cooled (0 °C) solution of compound **7** (0.090 g, 0. 050 mmol) in anhydrous DCM (10 mL), and the reaction mixture was stirred at 24 °C for 24 h, then evaporated to dryness in vacuo. The residue was recrystallized successively from ethanol (5 mL) and diethyl ether (5 mL). The product **2S4** was obtained as white crystals (0.070 g, 91%), decompose without melting above 185 °C. ^{1}H-NMR ($CDCl_3$:CD_3OD = 3:1, 500 MHz): 0.67 (s, 6 H, 2 C(13)Me), 0.84 (d, 6 H, *J* = 6.6, 2 C(25)Me), 0.85 (d, 3 H, *J* = 6.6, 2 C(25)Me), 0.90 (d, 6 H, *J* = 6.2, 2 C(20)Me), 0.99 (s, 6 H, 2 C(10)Me), 1.03–1.62 (m, 50 H, Chol, $CH_2C\underline{H}_2C\underline{H}_2CH_2$, 2 $CH_2C\underline{H}_2CH_2$), 1.66–2.06 (m, 14 H, 2 $CH_2C\underline{H}_2CH_2CO$, Chol), 2.12–2.17 (m, 4 H, 2 $C\underline{H}_2CONH$), 2.19–2.27 (m, 4 H, 2 CH_2S), 2.30–2.38 (m, 4 H, 2 H_2C(4) Chol), 2.55–2.63 (m, 2 H, 2 H(3) Chol), 3.30–3.38 (m, 16 H, 8 NCH_2), 3.56–3.65 (m, 4 H, 2 $C\underline{H}_2NHCO$), 5.33–5.37 (m, 2 H, 2 H(6) Chol). HRMS (ESI), *m/z*: 1293.9682 $[M + H]^+$, 647.4882 $[M + 2H]^{2+}$. Calculated for $C_{76}H_{137}N_6O_2S_4$: 1293.9686 $[M + H]^+$, for $C_{76}H_{138}N_6O_2S_4$: 647.4882 $[M + 2H]^{2+}$.

Preparation of cationic liposomes (CLs). CLs were prepared by hydrating of thin lipid films. Briefly, polycationic amphiphile and lipid helper 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, Alabaster, AL, USA) were dissolved at a molar ratio of 1:1 in a mixture of $CHCl_3$ and CH_3OH. Organic solvents were removed in vacuo. The lipid film obtained was dried for 4 h at 0.1 Torr to remove residual organic solvents. The dried lipid film was hydrated using deionized MilliQ water at 4 °C overnight, the resulting liposomal dispersion was sonicated for 15 min at 70–75 °C in a bath-type sonicator (Bandelin Sonorex Digitec DT 52H, Berlin, Germany), filtrated through a 0.45 μm pore polycarbonate membrane, flushed with argon and stored at 4 °C. The final polycationic amphiphile concentrations were 1 mM.

Preparation of CL/NA complexes. Prior to their use, the complexes of the CLs and NA were formed in a serum-free Opti-MEM medium (Invitrogen, Waltham, MA, USA) by vigorous mixing of nucleic acid (0.5 μg pEGFP-C2) and liposome suspensions taken at concentrations corresponding to

the appropriate N/P (nitrogen to phosphate) ratio; the resulting mixtures were incubated for 20 min at 24 °C. 1 μg of DNA corresponds to 3.1×10^{-9} mol of phosphates.

Liposome and CL/NA complexes sizes and zeta potentials. The particle size and zeta potential were measured using a dynamic light scattering method by a Malvern Zetasizer Nano (Malvern Instruments Ltd., Malvern, UK) at 25 °C. For CL/NA complexes characterisation, 25 μL of nucleic acid solution prepared in MilliQ water was mixed with 25 μL of liposomes solution at N/P ratio 6/1. After incubation for 20 min at room temperature, 900 μL of water was added and the complexes were analysed using a 1-mL cuvette.

Cell lines and growth conditions. HEK 293 (human embryo kidney) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA), 100 μg/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The cells were plated in 24-well culture plates (at a density of 1.2×10^5 cells/well) and allowed to adhere overnight.

Cell transfection. HEK 293 (1.2×10^5 cells/well) cells were seeded in 24-well plates and grown as described above. On the day of the experiment, the culture medium of cells was replaced by 200 μL of fresh medium supplemented with 10% FBS. The CL/NA complexes at various N/P ratios (as described above) were added to the cells and incubated for 4 h. After the incubation, the cells were washed twice with PBS and then preserved in the DMEM medium (500 mL) with 10% FBS. The expression levels of EGFP were measured 48 h post transfection. All the experiments were performed in triplicate.

FACS analysis. Flow cytometry was used to characterize the transfection efficiency of cationic liposomes. Prior to analysis, cells were rinsed twice with PBS and detached from the plate by trypsin treatment (0.5 mg/mL in PBS) at 37 °C for 2 min. Trypsinized cells were then resuspended in the culture medium and collected by centrifugation (Contron T42K centrifuge, Centricon Instruments) at 1000 rpm for 10 min at 4 °C. The medium was removed, and the cells were washed with PBS and fixed with 4% formaldehyde in PBS. The resulting samples were assayed by flow cytometry using NovoCyte 3000 (Biosciences Inc., Allentown, PA, USA). A total of 2×10^4 cells were analyzed from each sample. All experimental points were prepared in triplicate for statistical analysis. The standard deviation did not exceed 7–9%.

Cell viability test. The relative amount of living cells after the incubation with liposomes or lipoplexes was determined by the MTT test. HEK 293 cells plated as described above in 96-well plates were incubated with cationic liposomes (final concentration in the well 1–80 μM) or lipoplexes (appropriate N/P ratio) for 4 h under serum-free conditions, then serum was added to each well and cells were additionally incubated for 20 h. After 24 h of incubation, a solution of MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrasolium bromide] (Sigma, St. Louis, MO, USA) was added to a final concentration of 0.5 mg/mL, and cells were incubated for an additional 3 h. Then the culture medium was removed, formosan crystals were solubilized in DMSO, and the differences in absorbance at 570 and 620 nm were measured spectrophotometrically using Multiscan RC (Labsystems, Cergy-Pontoise, France). Results were expressed as mean values of measurements for three wells ± S.D.

Supplementary Materials: NMR and mass spectra are available online at www.mdpi.com/1422-8599/2018/1/M981/s1.

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