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1 *Short Note*

2 1,28-Di[(cholest-5-en-3β-yl)disulfanyl]-4,25-dioxo-3,8, 3 12,17,21,26-hexaazaoctacosane tetrahydrochloride

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

18 **Keywords:** redox-sensitive; disulfide linker; gemini amphiphiles; gene therapy.
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20 1. Introduction

21 Gene therapy is an attractive tool for the treatment of both inherited and acquired diseases and
22 is based on delivery of therapeutic nucleic acid (NA) into cells. It requires a special vehicle to protect
23 NA against nucleases and to help them pass through different intracellular and extracellular
24 barriers. The most attractive and safe delivery vehicles are the non-viral ones, such as cationic
25 liposomes (CLs). Despite their advantages, including safety, low cost, and the ability to be produced
26 at scale, CLs have inadequate delivery (also called transfection) efficiency [1]. This fact induces the
27 development of novel liposomal systems. CLs are formed from cationic amphiphiles (CAs) and the
28 structure of CAs has a crucial influence on delivery efficiency. Typical CAs consist of hydrophobic
29 and hydrophilic domains, a connecting linker, and a spacer that maintains the steric arrangement of
30 domains [1,2]. In order to increase transfection efficiency, modification of the CA structure with
31 labile stimuli-responsive groups may be performed. Intracellular stimuli cause CA degradation
32 which, in turn, can enhance NA release from the liposomes/NA complexes and stimulate endosomal
33 escape [3]. For example, a disulfide linker is degradable by intracellular reducing agents such as
34 glutathione (GSH) [4].
35 Recently, Zhao et al. designed disulfide CAs based on a polar amino acid head and tocopherol,
36 with the disulfide bond introduced as a cystamine moiety [5]. The transfection efficiency of these
37 CAs with respect to HEK293 cells was comparable to that of the commercial agent Lipofectamine®
38 2000. High transfection efficiency both *in vitro* and *in vivo* was demonstrated by a disulfide CA based
39 on lysine and arginine [6]. A number of disulfide CAs based on cholesterol have been synthesized
40 and have demonstrated delivery efficiency comparable with that of commercial agents. CAs with a
41 polyamine cationic domain (lysine or triethylenetetramine) were the most effective ones with respect
42 to COS-7 cells [7]. Redox-sensitive gemini CAs based on thiocholesterol with a flexible hydrophilic
43 spacer and ether linkers were the most effective for hard-to-transfect HaCaT cells [8]. Cationic
44 lipophosphoramidates were also modified by disulfide linkers and demonstrated more effective NA
45 delivery compared to Lipofectamine® [9]. It is also known that the location of a disulfide bond
46 location have a strong effect on the transfection efficiency [10].

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Recently, we have developed a polycationic gemini amphiphile **2X3** with a hexamethylene spacer and a carbamoyl linker (Fig. 1) and have demonstrated its remarkably high transfection efficiency, which is superior to that of Lipofectamine® 2000 [11]. 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** (Fig. 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 **c**omparison with non-redox-sensitive **2X3** CLs and Lipofectamine® 2000.

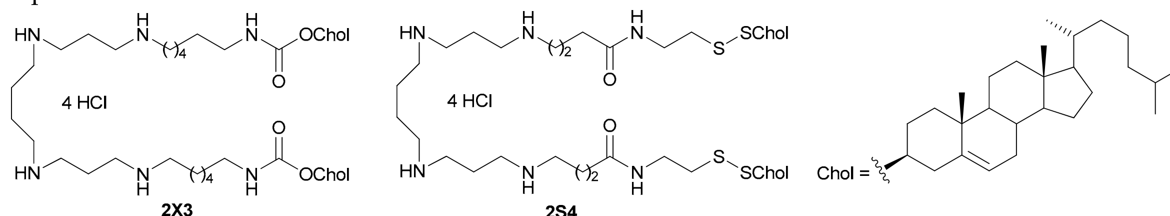

Figure 1. Polycationic gemini amphiphiles 2X3 and 2S4.

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. 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 [12]. Alkylation of **1** was performed in the Fukuyama reaction conditions [13] 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 [14]. 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 [15]. 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.

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.

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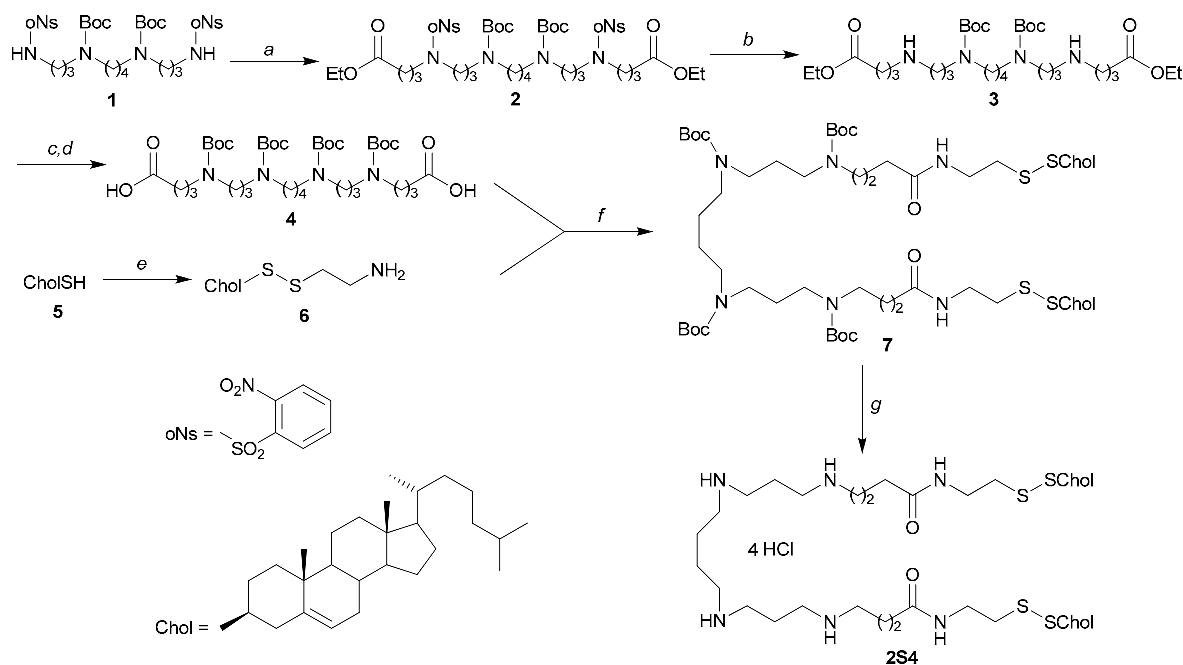

Scheme 1. Reagents and conditions of the synthesis: a) $Br(CH_2)_3COOEt$, Cs_2CO_3, DMF, 65 °C, 36 h; b) PhSH, K_2CO_3, DMF, 24 °C, 2 h; c) Boc_2O, Et_3N, DCM, 24 °C, 48 h; d) NaOH, MeOH, 24 °C, 56 h; e) $NH_2(CH_2)_2SS(CH_2)_2NH_2$ * 2HCl, Et_3N, DMF, 24 °C, 72 h; f) EEDQ, DIEA, DCM, 50 °C, 48 h; g) 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 of 2S4 CLs determined by dynamic light scattering was about 440 nm, while 2X3 CLs had the diameter of about 100 nm. 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. 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 (Fig. 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.

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

In this study, a novel disulfide polycationic amphiphile 2S4 was synthesized for pDNA 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

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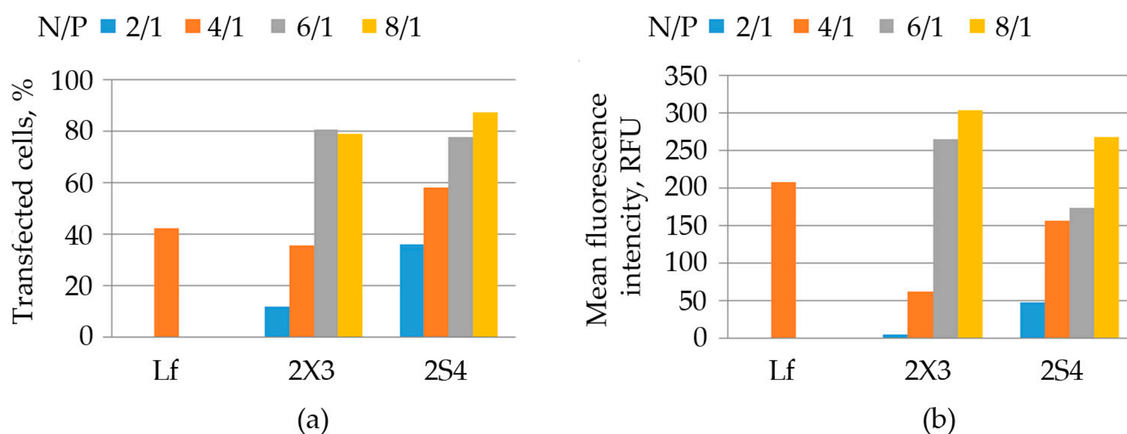

Figure 2. Transfection efficacy of CL/pDNA complexes formed at different N/P ratios: (**a**) Percentage of transfected cells; (**b**) Mean fluorescence intensity of the cell population. Lf (Lipofectamine® 2000) and 2X3 CLs were used as positive control. Experiments were performed in the presence of 10%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

122 redox-sensitive delivery vehicles and the influence of disulfide bond location within CA on
123 transfection efficiency.

124 **4. Materials and Methods**

125 DIEA, DMF, EEDQ, TEA, cystamine dihydrochloride, 2,2′-dithiodipyridine, ethyl
126 4-bromobutyrate, thiocholesterol were obtained from Aldrich; Boc_2O, cesium carbonate were
127 obtained from Fluka; spermine was obtained from Sigma; thiophenol was obtained from Merck.
128 Other solvents and reagents were purchased from Russian companies.

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

132 Column chromatography was carried out on silica gel Kieselgel 60 (0.040 – 0.063 mm, Merck).
133 ^{1}H and ^{13}C NMR spectra were recorded on a Avance DPX-300 and Avance DRX-500 pulse Fourier
134 transform spectrometers (Bruker, Germany) in $CDCl_3$ unless otherwise stated. Chemical shifts were
135 recorded in ppm on the δ scale relative to $CHCl_3$ solvent residual peak (7.26 ppm for ^{1}H and
136 77.0 ppm for ^{13}C NMR spectra). Coupling constants (*J*) are absolute values and recorded in Hz. Mass
137 spectra were run on a Ultraflex time-of-flight mass spectrometer (Bruker, Germany) with matrix
138 assisted laser desorbtion/ionization (MALDI) and on a Finnigan MAT 900XL-TRAP mass
139 spectrometer (San Jose, CA) with electrospray ionization (ESI). Melting points were determined on a
140 IA9100 digital melting point apparatus (Electrothermal, Great Britain).
141

142 **Diethyl** N^9,N^{14}**-di(*tert*-butyloxycarbonyl)-**N^5,N^{18}**-bis(2-nitrobenzenesulfonyl)-5,8,14,18-**
143 **tetraazadocosane-1,18-dioate (2).** Cesium carbonate (0.84 g, 2.57 mmol) and ethyl 4-bromobutyrate
144 (0.50 mL, 3.59 mmol) were added to a solution of compound **1** (0.80 g, 1.04 mmol) in anhydrous
145 DMF (9 mL). The reaction mixture was stirred at 65 ºC for 36 h, filtered on Celite®545. The filtrate
146 was evaporated to dryness, the residue was chromatographed on a silica gel column eluted with
147 DCM – MeOH (120:1). The product **2** was obtained as a pale yellow oil (0.78 g, 76 %). ^{1}H NMR (300
148 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,
149 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,
150 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),
151 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,
152 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,
153 172.76.

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154 **Diethyl** ***N*⁹,*N*¹⁴-di(*tert*-butyloxycarbonyl)-5,8,14,18-tetraazadocosane-1,22-dioate (3).**
155 Potassium carbonate K_2CO_3 (1.13 g, 8.14 mmol) was added to a solution of compound **2** (0.78 g, 0.79
156 mmol) in anhydrous DMF (10 mL), and the reaction mixture was stirred at 24 ºC for 10 minutes.
157 Then thiophenol (0.80 mL, 7.80 mmol) was added and the reaction mixture was additionally stirred
158 at 24 ºC for 2 h, filtered on Celite®545. The filtrate was evaporated to dryness, the residue was
159 chromatographed on a silica gel column eluted with DCM – MeOH – 25% aq. ammonia (from 8:1:0.1
160 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,
161 *J* = 7.1, 2 CH_3), 1.44 (br. s, 18 H, 2 Boc), 1.50 – 1.62 (m, 4 H, 2 $CH_2\underline{CH_2}\underline{CH_2}CH_2$), 2.07 – 2.26 (m, 8 H,
162 4 $CH_2\underline{CH_2}CH_2$), 2.46 (t, 4 H, *J* = 7.2, 2 $\underline{CH_2}$COOEt), 2.85 – 3.07 (m, 8 H, 4 $\underline{CH_2}$NH), 3.20 – 3.45 (m, 8 H,
163 4 $\underline{CH_2}$N) ,4.10 (q, 4 H, *J* = 7.1, 2 $\underline{CH_2}CH_3$).
164 **Diethyl *N*⁵,*N*⁹,*N*¹⁴,*N*¹⁸-tetra(*tert*-butyloxycarbonyl)-5,8,14,18-tetraazadocosane-1,22-dioate.** A
165 solution of compound **3** (0.089 g, 0.141 mmol) and anhydrous TEA (0.10 mL, 0.705 mmol) in
166 anhydrous DCM (4 mL) was cooled to 0 °C. Boc_2O (0.092 g, 0.423 mmol) in anhydrous DCM
167 (0.50 mL) was added and the reaction mixture was stirred at 24 ºC for 48 h, diluted with DCM
168 (20 mL), then washed with 3% aq. HCl (1 × 10 mL) and water (3 × 10 mL). The organic phase was
169 dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. The residue was chromatographed
170 on a silica gel column eluted with toluene – acetone (from 12:1 to 2:1). The product was obtained as
171 colorless amorphous solid (0.055 g, 47 %). ^{1}H NMR (300 MHz): 1.23 (t, 6 H, *J* = 7.1, 2 CH_3), 1.42 (br. s,
172 40 H, 4 Boc, $CH_2\underline{CH_2}\underline{CH_2}CH_2$), 1.64 – 1.76 (m, 4 H, 2 $NCH_2\underline{CH_2}CH_2N$), 1.76 – 1.88 (m, 4 H,
173 2 $\underline{CH_2}CH_2$COOEt), 2.26 (t, 4 H, *J* = 7.2, 2 $\underline{CH_2}$COOEt), 3.02 – 3.25 (m, 16 H, 8 $\underline{CH_2}$N), 4.10 (q, 4 H,
174 *J* = 7.1, 2 $\underline{CH_2}CH_3$). ^{13}C NMR (75 MHz): 14.33, 23.75, 25.91, 28.57, 28.59, 29.78, 31.59, 32.01, 44.94, 46.37,
175 46.92, 60.43, 79.37, 79.53, 155.56, 173.17. MS (MALDI), *m/z*: 853.539 $[M + Na]^+$, 869.497 $[M + K]^+$.
176 Calculated for $C_{42}H_{78}N_4NaO_{12}$: 853.551 $[M + Na]^+$, for $C_{42}H_{78}KN_4O_{12}$: 869.525 $[M + K]^+$.
177 ***N*⁵,*N*⁹,*N*¹⁴,*N*¹⁸-tetra(*tert*-butyloxycarbonyl)-5,9,14,18-tetraazadocosane-1,22-dioic acid (4).** A
178 solution of NaOH (0.013 g, 0.33 mmol) in MeOH – H_2O (1.1 mL, 10:1 v/v) was added to a solution of
179 diethyl *N*⁵,*N*⁹,*N*¹⁴,*N*¹⁸-tetra(*tert*-butoxycarbonyl)-5,8,14,18-tetraazadocosane-1,22-dioate (0.055 g,
180 0.066 mmol) in MeOH (4 mL). The reaction mixture was stirred at 24 ºC for 56 h, then 0.5 M aq. HCl
181 was added dropwise until pH 4, and the reaction mixture was evaporated to dryness in vacuo. The
182 residue was chromatographed on a silica gel column eluted with DCM – MeOH – 1% aq. AcOH
183 (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
184 NMR ($CDCl_3$:CD_3OD=1:1, 300 MHz): 1.43 (br. s, 36 H, 4 Boc), 1.46 – 1.53 (m, 4 , $CH_2\underline{CH_2}\underline{CH_2}CH_2$),
185 1.67 – 1.90 (m, 8 H, 4 $CH_2\underline{CH_2}CH_2$), 2.26 (t, 4 H, *J* = 7.2, 2 $\underline{CH_2}$COOH), 3.08 – 3.27 (m, 16 H, 8 $\underline{CH_2}$N).
186 ^{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,
187 155.84, 175.34. MS (ESI), *m/z*: 774.08 $[M]^+$, 797.49 $[M + Na]^+$. Calculated for $C_{38}H_{70}N_4O_{12}$ 774.50 $[M]^+$,
188 for $C_{38}H_{70}N_4NaO_{12}$ 797.72 $[M + Na]^+$.
189 **2-[(Cholest-5-en-3β-yl)disulphanyl]ethanamine (6).** Thiocholesterol (**5**) (0.18 g, 0.44 mmol) and
190 anhydrous TEA (0.25 mL, 1.77 mmol) were added to a solution of cystamine dihydrochloride
191 (0.050 g, 0.22 mmol) in DMF (10 mL) under argon atmosphere. The reaction mixture was further
192 purged with argon for 5 min and stirred at 24 ºC for 72 h, then evaporated to dryness in vacuo. The
193 residue was chromatographed on a silica gel column eluted with DCM – MeOH – 25% aq. ammonia
194 (40:1:1). The product **8** was obtained as beige amorphous solid (0.092 g, 86 %). ^{1}H NMR (300 MHz):
195 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,
196 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,
197 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,
198 $\underline{CH_2}NH_2$), 5.32–5.40 (m, 1 H, H(6) Chol).
199 ***N*⁸,*N*¹²,*N*¹⁷,*N*²¹-tetra(*tert*-butyloxycarbonyl)-1,28-di[(cholest-5-en-3β-yl)disulphanyl]-4,25-diox
200 o-3,8,12,17,21,26-hexaazaoctacosane (7).** Anhydrous DIEA (27 μL, 0.155 mmol) was added to a
201 solution of compound **6** (0.037 g, 0.077 mmol) in anhydrous DCM (4 mL) and stirred for 10 minutes.
202 Solutions of compound **4** (0.024 g, 0.031 mmol) in anhydrous DCM (3 mL) and EEDQ (0.026 g,
203 0.077 mmol) in anhydrous DCM (2 mL) were successively added to the stirring reaction mixture.
204 After 48 h at 50 ºC, the reaction mixture was cooled to 24 ºC, diluted with DCM (30 mL), then
205 washed successively with saturated aq. Na_2CO_3 (1 × 10 mL), water (1 × 10мл), 0.2 M aq. HCl

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(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.

1,28-Di[(cholest-5-en-3β-yl)disulphanyl]-4,25-dioxo-3,8,12,17,21,26-hexaazaoctacosane tetrahydrochloride (2S4). A solution of 3N 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 ºC 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). MS (MALDI), *m/z*: 1294.118 $[M+2H]^+$. Calculated for $C_{76}H_{136}N_6O_2S_4$: 1292.961 $[M]^+$.

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) 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 water (MilliQ) at 4°C overnight, the resulting liposomal dispersion was sonicated for 15 min at 70°C–75°C in a bath-type sonicator (Bandelin Sonorex Digitec DT 52H, 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, USA) by vigorous mixing of nucleic acid (0.5 μg pDNA) 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, 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) (Gibco BRL, Germany), 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

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258 washed twice with PBS and then preserved in the DMEM medium (500 mL) with 10% FBS. The
259 expression levels of EGFP were measured 48 h post transfection. All the experiments were
260 performed in triplicate.

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

270 **Supplementary Materials:** NMR and mass spectra are available online at www.mdpi.com/link.

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277 performed the synthesis; E.V.S. performed the physicochemical and biological experiments; M.A.Z.
278 contributed reagents for transfection experiments; P.A.P. wrote the paper; M.A.M., N.G.M. and M.A.Z.
279 corrected the draft.

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