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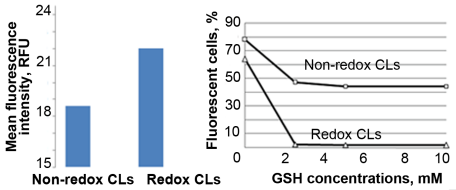

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ABSTRACT

A novel redox-sensitive polycationic amphiphile (**2S3**) with disulphide linkers for nucleic acid delivery was developed. Cationic liposomes formed by **2S3** and the helper lipid DOPE demonstrated effective DNA delivery into HEK293 cells with a maximal transfection activity that is superior than both nonredox-sensitive cationic liposomes and Lipofectamine® 2000 at an N/P ratio of 6/1. Redox-sensitivity was tested by experiments with extracellular glutathione which shown the ability of disulphide linker degradation. Our results suggest that polycationic amphiphile **2S3** is a promising candidate for nucleic acid delivery.

Gene therapy is a tool for the treatment of both inherited and acquired diseases by nucleic acid (NA) transfer. For this purpose, a special vehicle is needed for NA protection against nucleases and other damaging factors. For example, cationic liposomes (CLs) based on cationic amphiphiles (CAs) represent a safe and promising vehicle, which has advantages in terms of lower immunogenicity, relative ease of production, and high carrying capacity.[1] Nevertheless, it is known that CL/NA complexes can induce an inflammatory response involving the release of cytokines into the serum.[2,3]

Despite these benefits liposomes do not possess a high enough efficiency for clinical applications because of a number of biological barriers that reduce NA transfer into the target cells.[4–6] Extracellular barriers include nucleases, serum proteins and cell membrane while intracellular ones represent endosomal trafficking, cytosolic transport and transfer into the nucleus. There are different approaches to overcoming biological barriers, for example excess positive charge of CLs provides electrostatic interactions with negatively charged cell membranes contributing to cellular internalisation.

A typical CA consists of hydrophilic and hydrophobic domains, a linker connecting them, and a spacer that maintains the sterical arrangement of domains. There may be various combinations of the respective components.[7,5] Nevertheless, each CA element contributes to the resulting delivery efficiency. One approach to improve delivery efficiency is to modify the CA structure with labile stimuli-responsive groups, degradable after the internalization of CLs into the cells. Intracellular CA degradation may lead to NA release from liposomes/NA complexes and a more effective endosomal escape.[8]

The most common linker is an acetal-type linker, degradable under acidic pH during endosomal trafficking.[8–13] A disulphide redox-sensitive linker, degradable by intracellular reducing agents such as glutathione (GSH)[14] is another type of linker. Recently, Yang *et al.* demonstrated that disulphide cationic gemini amphiphile with two lysine polar heads possesed a high potential for NA delivery into HeLa cells.[15] Sheng *et al.* synthesised a number of disulphide CAs based on cholesterol which revealed a high transfection efficiency that was comparable with commercial agents. The most effective CAs against COS-7 cells were compounds with a polyamine cationic domain (L-lysine or triethylenetetramine).[16] Bajaj *et al.* synthesised three redox-sensitive gemini CAs based on thiocholesterol with tertiary amine groups and various spacers. Among them, CAs with flexible hydrophilic spacer and ether linkers were the most effective against hard-to-transfect HaCaT cells.[17] Disulphide groups were also introduced into cationic lipophosphoramidates, and NA delivery was shown to be effective compared with that of Lipofectamine®.[18] Disulphide linker insertion in CA molecules may not lead to an increase in efficiency but can substantially decrease CA toxicity, thus

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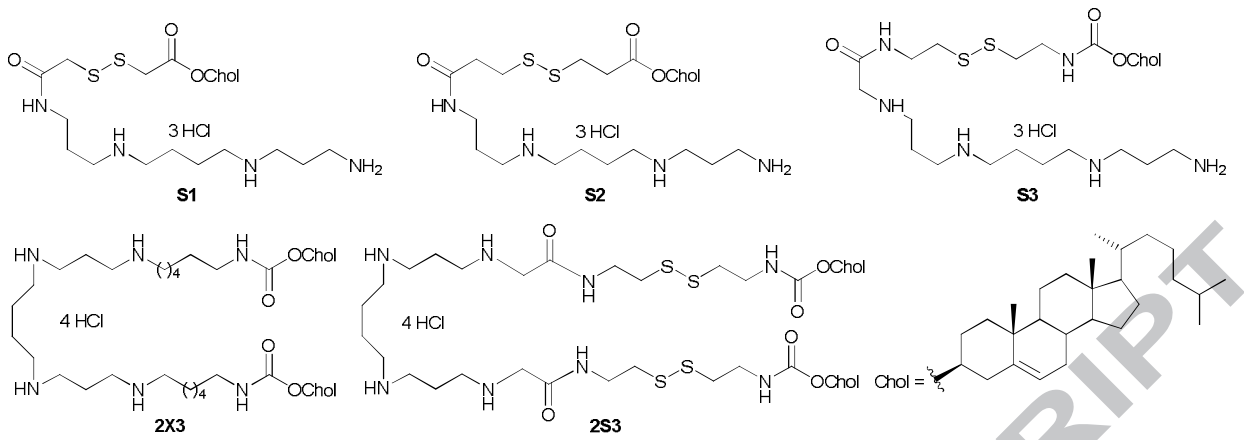

Figure 1. Structures of polycationic monomeric and gemini amphiphiles discussed.

allowing the use of higher NA-liposome complex concentrations.[19] Dauty *et al.* used thiol-to-disulphide conversion to form complexes with thiol-containing monomeric CAs and DNA. This approach allowed to obtain very small particles that favored nucleus entry of DNA.[20] Poly(disulphide)s may be also used in delivery of NA and other biologically active compounds but have different delivery mechanism.[21]

Gemini amphiphiles are molecules with two (at least) hydrophobic and two hydrophilic domains connected with various linkers and spacers. They may have symmetrical or unsymmetrical structure and possess a number of significantly enhanced surface properties as compared with their monomeric analogs. Their much lower CMC (critical micelle concentration) and ability to reduce surface tension make gemini amphiphiles an attractive tool for biological applications including gene therapy.[22,23] Recently, we developed a number of polycationic monomeric and gemini amphiphiles based on cholesterol and natural polyamine spermine. All of the CAs demonstrated high transfection efficiency on different cell lines.[24,25] However, gemini amphiphiles were transfected more effectively in comparison with their monomeric counterparts, with CAs bearing a hexamethylene spacer and a carbamoyl linker (**2X3**; Fig. 1) being the most active structures.[24] A disulphide linker was added to the structure of polycationic monomeric amphiphiles (**S1-S3**; Fig. 1) to improve their transfection efficiency. We found that disulphide monomeric amphiphiles **S1-S3** transfected a slightly lower percentage of the cells but provided a much greater transgene expression compared with analogs without similar degradable linkers.[25] Unfortunately, monomeric amphiphiles lost their transfection efficiency in the presence of serum unlike gemini amphiphiles which show better serum stability.[21] Therefore, in this work, we designed and synthesized polycationic gemini amphiphile **2S3** (Fig. 1) with a structure similar to the highly effective CA **2X3** but bearing disulphide groups. Disulphide groups degradable in the presence of reducing agents should contribute to better NA release into the cytosol. Synthetic protocol presented below (Fig. 2) permits protonation of all amino groups in spermine motif that increase CA charge and favor NA condensation. Moreover, we found appropriate reagents which provide an effective condensation of hydrophilic and hydrophobic domains. In addition, we evaluated the physiochemical properties and transfection efficiency of liposomes composed of **2S3**.

To synthesise amphiphile **2S3**, we obtained the spermine

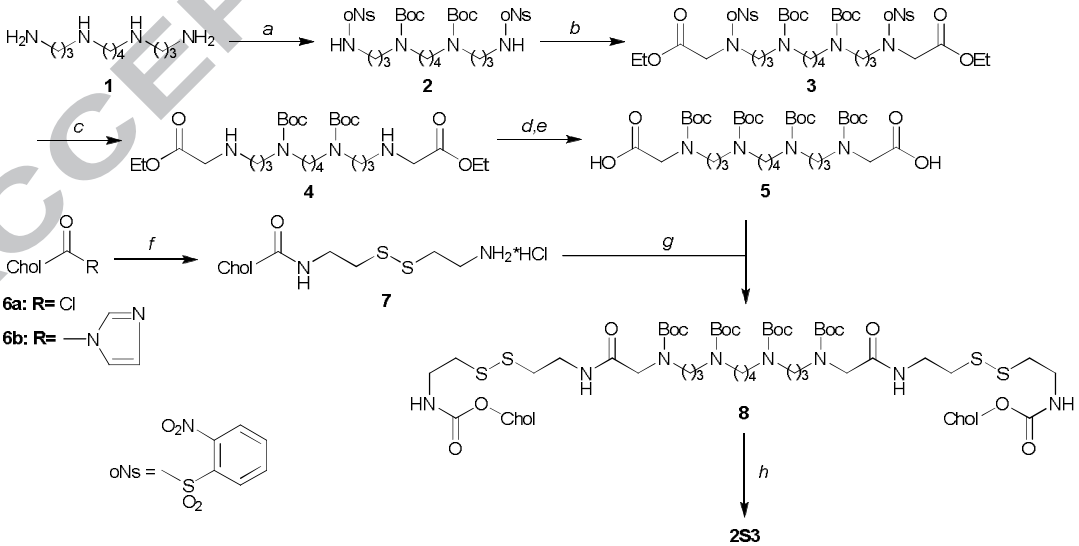

Figure 2. Synthetic route of **2S3**. a) CF_3COOEt, MeOH, 70 °C, 5 h, next Boc_2O, Et_3N, 24 °C, 96 h, next NaOH, MeOH, 24 °C, 48 h, next 2-$NO_2C_6H_4SO_2Cl$, Et_3N, DCM, 24 °C, 5 h;[23] b) $BrCH_2COOEt$, Cs_2CO_3, DMF, 65 °C, 30 h; c) PhSH, K_2CO_3, DMF, 24 °C, 1 h; d) Boc_2O, Et_3N, DCM, 24 °C, 48 h; e) NaOH, MeOH, 24 °C, 96 h; f) $NH_2(CH_2)_2SS(CH_2)_2NH_2$, Et_3N, DCM, for **6a** 22 °C, 2 h, for **6b** 40 °C, 45 h; g) 5, EEDQ, DIEA, DCM, 50 °C, 48 h; h) 3 M HCl/dioxane, DCM, 22 °C, 24 h.

derivative (**2**) by regioselective protection of spermine (**1**), previously described.[26]

The alkylation of **2** with ethyl bromoacetate in the presence of cesium carbonate in Fukuyama reaction conditions[27,28] produced a good yield of compound **3** (90 %). The removal of 2-nitrobenzenesulfonyl protecting group by treatment with thiophenol in the presence of potassium carbonate gave amine **4**, which was isolated by column chromatography with 63 % yield. Secondary amino groups were blocked with Boc_2O in the presence of TEA at 0 °C, the resulting fully protected spermine derivative was treated with NaOH in methanol-water solution to give compound **5** with two-step yield of 60 %.

There are different approaches to introduce a disulphide group into a target molecule, such as the conversion of hydroxyl or amino groups into thiols, followed by a mild oxidation or using of bifunctional reagents.[29] We used an approach based on the reaction between cystamine (2,2′-diaminodiethyl disulfide) and activated target cholesterol precursor. The activation may be performed by various reagents, for example *N*-hydroxysuccinimide[30] or *N,N'*-disuccinimidyl carbonate.[31,32] We used two activated derivatives, namely cholesterol chloroformate **6a** and (cholest-5-en-3β-yl) imidazole-1-carboxylate **6b**[33] (Fig. 2). Commercially available cystamine dihydrochloride was transformed into a free base by treatment with aqueous NaOH to improve its solubility in organic solvents. The use of the more active cholesterol chloroformate (**6a**) led to a rapid and complete conversion under mild conditions. In this case, disulphide lipophilic derivative **7** was obtained in 66 % yield, which exceeded that of reaction with (cholest-5-en-3β-yl) imidazole-1-carboxylate **6b** (55 %), as well as literature data (40-50 %).[30–32]

A key step of the synthesis was the condensation of spermine (**5**) and cholesterol (**7**) derivatives. It is known that disulfide compounds may be obtained with moderate overall yields.[18,34] Possible explanations may include their lower solubility in organic solvents as compared with non-disulfide molecules or eventual disulfide bond degradation under some conditions. Nevertheless, disulphide amphiphile syntheses with high yields were also described,[16,35] demonstrating the importance of optimizing reaction conditions. We used three condensation agents: TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) and EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline). TBTU is commonly used in the peptide synthesis as well as disulfide amphiphile synthesis;[36] however, it provided a very low yield of **8** (Table 1, run 1) because of heterogeneous reaction conditions caused by a limited range of appropriate solvents. The other reagent using in amide and ester syntheses is DMTMM.[37] A disadvantage of DMTMM is its decomposition in chlorinated and some other solvents. We carried out reactions with DMTMM under several conditions, and the best yield was 30 % (Table 1, runs 2-4). According to reaction mechanism, the presence of NMM normally liberated from DMTMM during reaction pass leads to rapid and selective amide formation.[38] Considering this, we used NMM as a base. As expected, the additional amount of NMM increased the reaction yield (Table 1, run 4). EEDQ is another condensing reagent, which can be used with chlorinated solvents including CH_2Cl_2 that made reaction conditions completely homogeneous. Employment of EEDQ produced compound **8** with maximal yield (Table 1, run 5). The removal of Boc protecting groups by 3 M HCl in dioxane produced the target CA **2S3** with 91 % yield after recrystallisation from ethanol and ether.

Table 1. Optimisation of the synthesis of **8**.

	Reagents	Solvent	Yield[a] of 8, %
1	**5**, TBTU, DIEA	DMF	9
2	**5**, DMTMM, DIEA	DMF	n.d.[b]
3	**5**, DMTMM, DIEA	Et_2O	22
4	**5**, DMTMM, NMM	MeOH:THF=5:1	30
5	**5**, EEDQ, DIEA	CH_2Cl_2	69

[a]Isolated yield;
[b]TLC indicates no product forming.

Based on the redox-sensitive CA **2S3** and zwitter-ionic helper lipid DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) **2S3** CLs were prepared at lipid molar ratio of 1:1 by the thin film hydration method. The average diameter of CLs studied by dynamic light scattering was about 90 nm (Table 2).

2S3 CLs 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). CL/pDNA complexes formed at an N/P ratio of 2/1 had diameters above 300 nm and a negative ξ-potential (Table 2). Increasing the amount of CLs in the complexes led to a decrease in average hydrodynamic diameter and polydispersity index as well as changing the ξ-potential from negative to positive. These changes indicated better complex stabilisation due to more complete DNA condensation at the surface of the CLs through N/P increasing.[39,40]

The cytotoxicity of cationic liposomes was evaluated by a MTT assay in HEK293 cells. Cells were incubated with cationic liposomes at concentrations ranging from 1 to 80 μM for 24 h in the presence of serum. The results was presented as IC_{50} values (concentration of the liposomes providing 50% cell viability), the CLs of **2X3** and **2S3** had IC_{50} 37 μM and 66 μM, respectively. We suggest that the lower cytotoxicity of **2S3** CLs can be explained by better biodegradability caused by the presence of disulphide groups.. IC_{50} values for Lipofectamine® 2000 (Lf 2000) was determined in μL since the chemical composition of this proprietary formulation is not disclosed. IC_{50} for Lf 2000 was 8.55 μL as shown previously.[24]

The results of plasmid DNA (pDNA) delivery experiments monitored by flow cytometry are summarised in Table 3. Nonredox-sensitive **2X3** CLs and Lf 2000 were used as a positive

Table 2. Physicochemical characterisation of CLs.

CL/ pEGFP-C2	2X3			2S3		
	Diameter, mean, nm	PI[a]	ξ-potential, mV	Diameter, mean, nm	PI[a]	ξ-potential, mV
1/0	92.4	0.374	22.1	91.5	0.332	27.9
2/1	377.4	0.355	-26.2	350.9	1.0	-6.5
4/1	171.6	0.200	7.0	98.1	0.247	9.4
6/1	130.8	0.250	15.4	82.5	0.253	18.7

[a]PI: polydispersity index.

Table 3. Transfection efficacy of CL/pDNA complexes.

	Lf 2000	2X3			2S3		
		N/P					
		2/1	4/1	6/1	2/1	4/1	6/1
Fluorescent cells, %	44.5	17.6	52.3	78.3	16.0	52.4	64.2
Mean fluorescence intensity, RFU	14.5	4.0	17.6	18.6	4.0	11.9	22.0

*The standard deviation did not exceed 7%–9%.

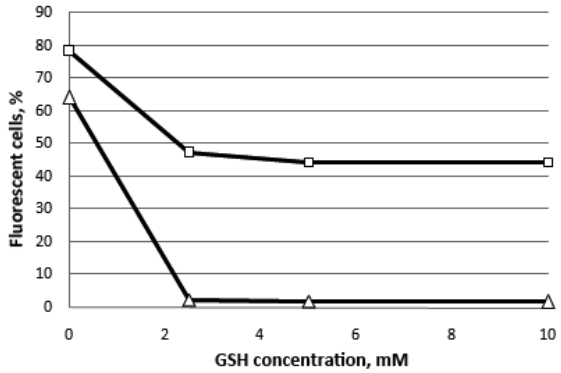

Figure 3. Influence of extracellular glutathione (GSH) on transfection activity of CL/DNA complexes formed by pEGFP and cationic liposomes **2X3** (open squares) or **2S3** (open triangles) at N/P = 6/1 in the presence of 10% FBS.

control. The data are presented as a percentage of fluorescent cells and a mean fluorescence intensity of the cell population in a sample. Cells were incubated with CL/pDNA complexes in the presence of 10% FBS to mimic *in vivo* conditions. Results showed that the transfection activities of the CLs of **2X3** and **2S3** exceeded that of Lf 2000 at N/P ratios of 4/1 and 6/1, respectively (Table 3). The transfection efficiency of **2S3**/pDNA complexes was similar to that of **2X3**/pDNA complexes at an N/P ratio of 4/1. The highest number of EGFP-positive cells in population samples of **2X3** and **2S3** was 78.3 % and 64.2 %, respectively. However, the average fluorescence intensity of individual cells in the case of nonredox-sensitive **2X3** was lower than that of those of redox-sensitive **2S3** (18.6 RFU and 22.0 RFU, respectively) at an N/P ratio of 6/1 (Table 3). Similar correlations were previously demonstrated for disulphide monomeric amphiphiles **S1-S3**.[25] The higher levels of EGFP expression mediated by **2S3** resulted from i) the better pDNA release from complexes with CLs (probably due to disulphide linker degradation) and ii) smaller average hydrodynamic diameter at N/P ratio of 6/1 (Table 2,3).

To demonstrate the ability of **2S3** CLs in releasing pDNA via disulphide linker degradation, HEK293 cells were transfected with CL/pDNA complexes pre-incubated with GSH. **2X3**/pDNA complexes without disulphide linker were used as the negative control in this experiment. Exposure of redox-sensitive **2S3**/pDNA complexes to GSH completely abolished pDNA delivery mediated by **2S3** CLs (100 % transfection inhibition was observed at 2.5 mM GSH) and only slightly affected pDNA delivery by **2X3** CLs in the whole range of GSH concentrations (Fig. 3). Thus, extracellular GSH can affect complex structures of disulphide amphiphiles and leads to their reduced transfection efficiency *ex vivo*. Results obtained are in a good agreement with literature data.[41]

In this study, a novel redox-sensitive polycationic gemini amphiphile **2S3** was synthesised for DNA delivery, and synthetic procedures were optimised. The transfection activity of **2S3** CLs was high in comparison with that of both nonredox-sensitive **2X3** CLs and the commercial transfectant Lipofectamine® 2000. The redox sensitivity of **2S3** was demonstrated by GSH treatment. Therefore, **2S3** CLs are a potential vehicle for DNA delivery. DNA release may be triggered by reducing agents (GSH) that degrade disulphide bonds.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at

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