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Spacer structure and hydrophobicity influences transfection activity of novel polycationic gemini amphiphiles

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ABSTRACT

Three novel polycationic gemini amphiphiles with different spacers were developed and evaluated in terms of their physiochemical properties and transfection efficiencies. Cationic liposomes formed by these amphiphiles and the helper lipid DOPE were able to successfully condense DNA, as shown by gel mobility shift and ethidium bromide intercalation assays. Transfection activity of the liposomes was superior to Lipofectamine® 2000 and was dependent on spacer structure, hydrophobicity, and nucleic acid type (pDNA or siRNA). We demonstrated that the cationic liposomes 2X6/DOPE and 2X7/DOPE are potential non-toxic vehicles for gene delivery.

Among the different gene delivery systems, non-viral systems such as cationic liposomes (CLs) and cationic polymers remain the most safe and attractive carriers. CLs are formed by cationic amphiphiles (CAs) that are cheap, easy to manufacture, and scalable, but still not particularly effective.[1] The transfection efficiency of CLs is highly dependent on the structure of the CA forming CLs. Typical CAs consist of hydrophilic and hydrophobic domains, a linker connecting them, and a spacer that maintains the steric arrangement of the domains.[1,2] Each CL component plays a key role in liposome structure maintenance and influences the resulting efficiency of gene delivery.

To date, a large number of CAs utilised for gene delivery were based on various polyamines.[3] Foremost is the natural polyamine spermine, which has the unique ability to efficiently condense nucleic acids.[4] CLs that included an amphiphile as a core component were shown to be promising gene delivery vectors.[5–9] Gemini amphiphiles (including those that are polyamine-based) have also been found suitable for nucleic acid delivery. The advantages of these amphiphiles are a much lower critical micelle concentration (CMC) and an ability to reduce surface tension, which favours improved cell membrane permeability.[10,11] We recently developed the polycationic gemini amphiphile **2X3**, which is based on cholesterol and spermine (Fig. 1).[12] CLs containing this amphiphile exhibited high transfection efficiency, significantly exceeding the transfection efficiency of both Lipofectamine® 2000 and monomeric counterparts.[13,14]

As mentioned above, each CA component plays a role in the biological activity of CA, with the spacer possibly being one of the key domains that should be optimised to improve gene delivery activity. It has been shown that spacer length affects DNA condensation and the physicochemical properties of CL-DNA complexes.[15] Gemini cholesterol-based CAs with different spacer lengths (3–12 carbon atoms) were tested, and the highest transfection efficiency (TE) observed for CAs with spacer lengths of five or six carbon atoms.[16] Similar results were observed for amino acid-based CAs, for which the optimal spacer length was shown to be seven carbon atoms.[17] Our own investigations also showed that gemini CAs with a spacer length of six carbon atoms mediated improved gene delivery compared to analogous CAs with a spacer length of four carbon atoms.[14] In addition to the hydrocarbon spacers, which are hydrophobic, hydrophilic oxyethylene-type spacers have also been used.[17–19] Bajaj et al. demonstrated that introduction of an oxyethylene unit had a positive effect on gene delivery.[18] By contrast, gene delivery mediated by amino acid-based CAs with an oxyethylene-type spacer was poor.[17] This discrepancy in transfection efficiency is likely linked with differences in the cell lines used as well as the influence of other CA structural components. Supporting evidence for this hypothesis was provided by an investigation of the structure-activity relationships of gemini CAs bearing hydrophobic

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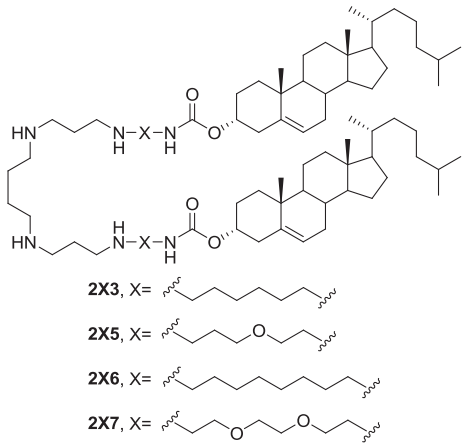

Fig. 1. Structures of the polycationic gemini amphiphiles.

or hydrophilic spacers.[20] CAs with hydrophobic spacers were shown to be effective in HeLa cells, whereas CAs with hydrophilic spacers were more effective in HT1080 cells. Introduction of hydroxyl groups into the spacer chain also did not lead to appreciable changes in transfection efficiency.[19] Further investigations are therefore required to better understand the influence of spacer structure on TE.

In this study, we designed, synthesised, and performed a detailed analysis of the transfection activity of three novel polycationic gemini amphiphiles (**2X5**, **2X6**, **2X7**; Fig. 1). These new CAs consisted of cholesterol and the natural polyamine spermine as the lipophilic and cationic domains and varied in spacer structures and hydrophobicity. CLs formed by the novel CAs and the helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were tested in terms of physicochemical properties, toxicity, and transfection efficiency. Moreover, we compared the transfection activities of the novel amphiphiles with the previously studied CA **2X3**[14] which has a similar structure, and the commercial transfectant Lipofectamine® 2000.

Several approaches have been used to connect polyamine and hydrophobic domains upon synthesis of polycationic amphiphiles. Most approaches are primarily based on coupling of the spacer to the polyamine, followed by reaction with the activated hydrophobic domain.[21–23] We used an alternative approach and first coupled the spacer with the hydrophobic domain, followed by condensation of the intermediate with the spermine derivative.

Spacers were synthesised from available commercial reagents. To obtain the hydrophobic spacer **4**, the amino group of 2-aminoethanol (**1**) was routinely protected with the Boc group (Fig. 2). Introduction of the terminal allyl group was carried out using 3-bromo-1-propene in the presence of tetrabutylammonium bromide (TBAB) in aqueous NaOH. Then, 9–borabicyclo[3.3.1]nonane (9-BBN) was used for the conversion of alkene **3** into corresponding alcohol. 9-BBN is an organoborane compound with a high regioselectivity in the hydroboration of terminal alkenes.[24] Subsequent oxidative cleavage of boron derivatives with H_2O_2 in an aqueous alkali base gives terminal alcohols with high yields. After the removal of the Boc protecting group by treatment with 3 M HCl in dioxane, compound **4** was isolated by column chromatography with a three-step yield of 81%. The hydrophobic and hydrophilic spacers **7a**,**b**, each with a chain length of eight atoms, were synthesised in two steps. The azides **6a**,**b** were obtained from the chlorinated alcohols **5a**,**b** by treatment with sodium azide (Fig. 2). Catalytic hydrogenation in the presence of 5% palladium on carbon subsequently produced the amines **7a**,**b** in 80% yields.

An activated hydrophobic motif needed for coupling with spacers **4** and **7a**,**b** was next obtained. We specifically used two activated cholesterol derivatives, namely cholest-5-en-3β-yl imidazole-1-carboxylate (**8a**)[25] and commercially available cholesterol chloroformate (**8b**) (Fig. 3). Imidazolide **8a** did not react with **7a** since its corresponding alcohol was not detected. We thus used cholesterol chloroformate instead. The alcohols obtained were subsequently brominated by tetrabromomethane in the presence of triphenylphosphine. The product **9b** was crystallised from diethyl ether with a two-step yield of 64%. The bromo derivatives **9a**,**c** were isolated by column chromatography with 43–56% yields.

To synthesise amphiphiles **2X5**, **2X6**, and **2X7**, 4,9-di(*tert*-butoxycarbonyl)-1,12-bis(2-nitrobenzenesulfonylamino)-4,9-diazadodecane[12] was treated with the corresponding bromo derivatives **9a**–**c**, in the presence of cesium carbonate under Fukuyama reaction conditions,[26] and gave compounds **10a**–**c** with 51–66% yields (Fig. 3). Removal of the 2-nitrobenzenesulfonyl protecting group by treatment with thiophenol in the presence of potassium carbonate, followed by *N*-Boc deprotection with 3 M HCl in dioxane, produced CAs **2X5**, **2X6**, and **2X7** with two-step yields of 47–65%. The synthesised compounds were characterised by ^{1}H NMR, ^{13}C NMR, and mass spectrometry (See Supplementary materials). The critical micelle concentration values (25–30 μM) were also determined by dynamic light scattering and shown to be independent on spacer structure.

Based on the CAs **2X3**,[12] **2X5**, **2X6**, **2X7**, and the zwitter-ionic helper lipid DOPE, the CLs 2X3/DOPE, 2X5/DOPE, 2X6/DOPE, and 2X7/DOPE were prepared at a lipid molar ratio of 1:1 using the thin film hydration method. The hydrodynamic diameter of each CL was determined by dynamic light scattering and found to be between 40 and 60 nm; however, 2X5/DOPE had an additional minor fraction of particles with an approximate diameter of 200 nm (Table 1). All CLs were positively charged.

The ability of CLs to bind and condense nucleic acids was studied using the gel mobility shift assay.[27] CLs and plasmid DNA (pDNA) complexes were prepared at various N/P ratios (number of polycationic amino groups of CAs per phosphate groups of nucleic acids) and the resulting mixtures were resolved on an ethidium bromide (EB) containing agarose gel, under natural conditions, to detect the presence of non-bound pDNA (Fig. 4A). Low N/P ratios (1/1 and 2/1) resulted in pDNA not being fully bound to CLs since free pDNA remained visible in the gel. On increasing the N/P ratio to 4/1 (or to 2/1, in the case of 2X6/DOPE and 2X7/DOPE, both with spacer lengths of eight atoms), all pDNA appeared to be complexed with CLs. Analysis of the physicochemical characteristics of CL/pDNA complexes formed at N/P 6/1 (Table 1) showed that compact particles are formed under these conditions; the smallest was formed with pDNA and 2X7/DOPE,

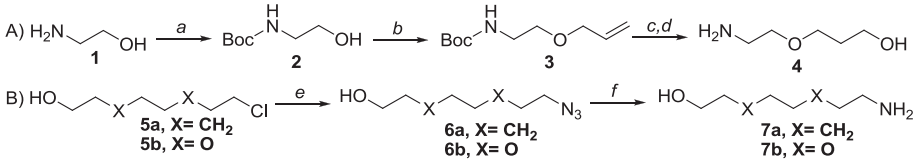

Fig. 2. Synthesis of spacers **4** (A) and **7a**,**b** (B). (a) Boc_2O, NaOH, THF, 24 °C, 5 h; (b) 3-bromo-1-propene, TBAB, NaOH, 24 °C, 19 h; (c) 9-BBN, THF, 9 °C, 4 h, then 30% aq. H_2O_2, NaOH, 5 °C, 1 h; (d) 3 M HCl/dioxane, DCM, 20 °C, 1.5 h; (e) NaN_3, DMF, 70 °C, 27–74 h; (f) H_2, 5% Pd/C, MeOH, 24 °C, 2–10 h.