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Syntheses of Polycationic Dendrimers on Lipophilic Peptide Core for Complexation and Transport of Oligonucleotides

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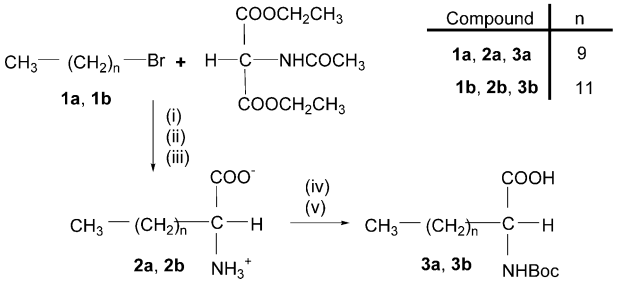
Abstract—Synthesis of novel polycationic lipophilic peptide core(s) was accomplished and these agents successfully transfected human retinal pigment epithelium cells with ODN1 upon complexation with the oligonucleotide. The level of transfection was indirectly measured by the decreased production of the protein hVEGF (human vascular endothelial growth factor) in comparison to the transfection agent cytofectin GSV™.

The lipoamino acids (Laas) and their homo-oligomers, the lipopeptides, represent a class of compound that combines the structural features of lipids with those of amino acids and peptides.[1] The Laas possess a high degree of membrane-like character, which facilitates their crossing of biological membranes, and they have the additional effect of protecting labile DNA from nuclease digestion.[2,3] The poly-lysine dendrimer head was attached to the linear peptide by a divergent approach.[4] As nonviral gene delivery systems[5–8] we designed lipid-lysine dendrimers with poly-cationic surfaces in order to deliver oligodeoxynucleotides (ODN) into the nuclei of human cells. These amphiphilic dendrimers contain a lipophilic tail of Laas and a poly-cationic head of poly-lysine. An important feature of these novel conjugates was that the lipo-dendrimer acted as a lipid solubilizer. The ion pair association of the cationic dendrimer with the anionic ODN was responsible for the improvement of membrane partitioning.[9] Herein we report the application of these dendrimers to produce stable and soluble ionic complexes with the oligodeoxynucleotide ODN1[10–15] that contains phosphodiester rather than phosphorothioate[16] linkages. This oligodeoxynucleotide is a sense sequence homologous to a portion of the 5′ untranslated region (5′ UTR) of the human vascular endothelial growth factor (hVEGF) operon and is capable of inhibiting its expression.



Scheme 1. Reagents and conditions: (i) Na/EtOH, reflux; (ii) concd HCl, reflux; (iii) NaOH, pH 7; (iv) $(BOC)_2O/H_2O/t$-butanol/NaOH; (v) citric acid, pH 3.

2-Aminododecanoic acid **2a** and 2-aminotetradecanoic acid **2b** and their Boc-protected intermediates (**3a**, and **3b**) were synthesised by literature methods (Scheme 1).[1]

Dendrimers **4**–**7** (Fig. 1, Table 1) varying the length and number of the lipid residues and the number of free amino functions on the polylysines were synthesised using standard solid-phase synthetic methods.[17,22,23]

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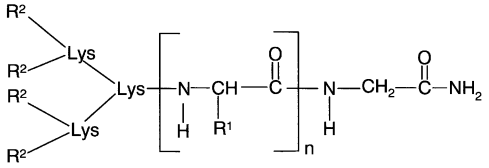

Figure 1. Synthesised dendrimers **4**–**7**. **4**: $R^1 = C_{10}H_{21}$, $R^2 = NH_2$, $n = 2$; **5**; $R^1 = C_{10}H_{21}$, $R^2 = \text{Lys-}(NH_2)_2$, $n = 2$; **6**: $R^1 = C_{12}H_{25}$, $R^2 = \text{Lys-}(NH_2)_2$, $n = 2$; **7**: $R^1 = C_{10}H_{21}$, $R^2 = \text{Lys-}(NH_2)_2$, $n = 3$.

All dendrimer constructs were complexed to ODN1 in a molar charge ratio of 5:1 (+/−) (dendrimer/ODN1).[18,19]

Complex formation was achieved by using four, eight, or sixteen amino substituents on the polylysine dendrimers, but compounds with 16 amino functions were significantly more toxic than dendrimers with four or eight amino functions.[18,24]

Isothermal titration calorimetry (ITC) experiments[20,21] were performed to determine the optimal ratio of the cationic dendrimers **4**–**7** and ODN1.

ITC data suggested that this ratio between dendrimers and ODN1 was 6:1 (Fig. 2).

Figure 2 shows the sequential release of heat (μcal/s) during the dendrimer–ODN1 complex formation. At a molar charge ratio of 6:1 (dendrimer:ODN1) the complex formation has been finalised, since no additional release of heat could be measured. According to previous studies[18] the dendrimer concentration was set at 5:1, since at higher ratio the complex became increasingly cytotoxic.

The effectiveness of the dendrimer–ODN1 complexes versus a cytofectin–ODN1 complex (which is a standard method of nonviral DNA introduction into cells) were tested and compared in their ability to transfect retinal

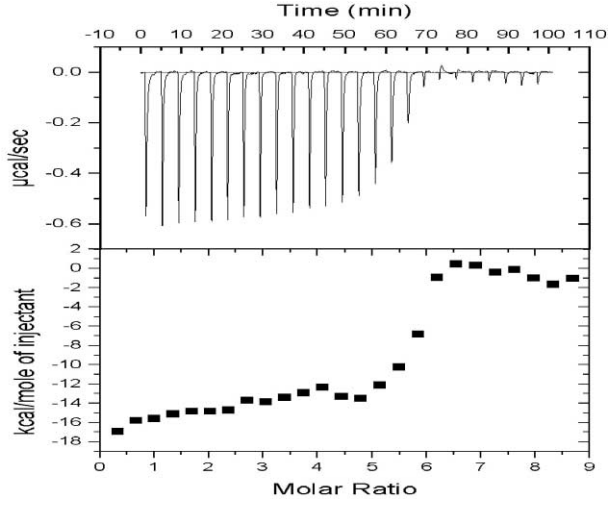

Figure 2. Isothermal titration calorimetry curve of the complex formation between dendrimer **7** and ODN1.

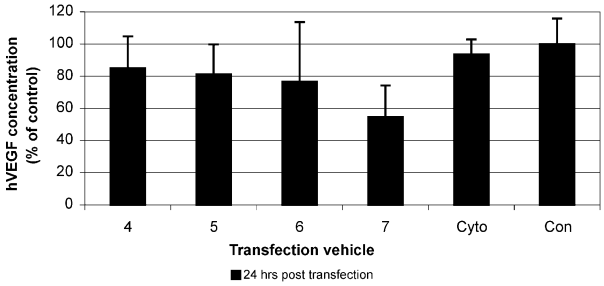

Figure 3. ELISA for hVEGF concentration after 24 h in conditioned media of transfection vehicles (dendrimer–ODN1 complexes **4**–**7**, cytofectin–ODN1 complex) and control; means ±SD.

pigment endothelial cells (RPE), using a control which did not contain any ODN1 complexes. The transfection efficacy was indirectly measured by the reduction of hVEGF expression, due to the presence of ODN1, by a well-established ELISA-system (Cytelisa™ Human VEGF).[10,25] Cytofectin is a commercially available lipoplex transfection agent containing a 'helper-lipid' such as dioleylphosphatidylethanolamine (DOPE), and it can deliver both ODNs and plasmids.

The results of the ELISA-assay (Fig. 3) exhibited an observed reduction of hVEGF levels (up to 39%) for all dendrimers tested, compared to cytofectin GSV™. However, statistical analysis using one way ANOVA with a post hoc Tukey test with a 95% confidence limit revealed that only dendrimer **7** possessed a significant difference ($P = 0.035$).

In conclusion we have synthesised a series of polycationic lipophilic peptide-dendrimers by solid-phase methods. Oligonucleotide ODN1 complexes were formed with these dendrimers. The complexes were highly efficient in transfecting human cells (D-407). All four complexes reduced significantly the hVEGF-levels after 24 h compared to a control. More significantly ODN1 complex with dendrimer **7** (8 amino groups; 3 lipids) showed much higher reduction of the hVEGF-level than the commercially available standard cytofectin GSV™.

Table 1. Structural-, mass spectral data and yields for the dendrimers **4**–**7**

Dendr.	Formula	Mass	MS (m/z)	Postive charges	Yield (%)
4	$C_{44}H_{89}O_6N_{10}$	854.20	853.8 ($z=1$) 427.4 ($z=2$) 285.4 ($z=3$)	4	81
5	$C_{68}H_{137}O_{10}N_{18}$	1366.08	683.8 ($z=2$) 456.2 ($z=3$) 342.4 ($z=4$)	8	75
6	$C_{72}H_{145}O_{10}N_{18}$	1422.14	711.8 ($z=2$) 474.8 ($z=3$) 356.4 ($z=4$)	8	76
7	$C_{80}H_{160}O_{11}N_{19}$	1563.25	782.6 ($z=2$) 522.0 ($z=3$) 391.8 ($z=4$)	8	70

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22. General experimental procedure for the synthesis of compounds **4**–**7** in detail: MBHA resin (4-methyl benzhydrylamine, substitution ratio, 0.62 mmol/g, 1000 mg) was swelled in dimethylformamide in a sintered glass peptide synthesis vessel for 90 min. An activation mixture consisting of Boc-amino acid (3 equiv per mol amino-group), HBTU (2-(1H benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluoro phosphate, 0.5 M in DMF, 3 equiv) and DIEA (0.442 mL, 4 equiv) was shaken with the resin for 12 min. Negative ninhydrin reaction (5 min) showed nearly quantitative coupling ($\geq$99.98%) and the Boc protecting group was subsequently removed using 100% TFA. Between all manipulations the resin was washed intensively with DMF. Coupling and deblocking of Boc protecting groups were done in an analogous manner for peptides **4**–**7**. Upon completion of the synthesis and removal of the terminal Boc groups, the resin was washed with DMF, methanol and DCM. The resin was dried to constant weight over KOH in vacuum. The peptides were cleaved from the resin using a high HF method and *p*-cresol as scavenger. The cleaved peptide was precipitated using diethyl ether, redissolved in 2.5% aqueous acetic acid and lyophilised to afford an amorphous powder.
23. Analytical RP-HPLC was performed on a Shimadzu instrument (LC-10AT liquid chromatograph, SCL-10A system controller, SPD-6A UV detector, a SIL-6B auto injector with a SCL-6B system controller, and columns C4, C18; 25 cm Vydac C4, C18 column with 5 nm pore size and 4.6 mm internal diameter) in order to optimize the appropriate gradient for the preparative HPLC and to identify the synthesised peptide. The amount of 100 mg of each crude peptide (**4**–**7**) was preparative separated on a Waters HPLC system (Model 600 controller, 490E UV detector, F pump, and TSK-GEL C4/C18 columns with 10 nm pore size and 2.5 cm internal diameter) using a acetonitrile/water gradient and characterised by electrospray-MS (Perkin Elmer API 3000 instrument). The resulting peptides were used as diastereomeric mixtures.
24. Complex formation: to manufacture complexes with the required molar charge ratio, oligodeoxynucleotide ODN1 (0.5 mg/mL) was added to each dendrimer (1.5 mg/mL) mixed for 15 min, diluted with 250 μL sterile water and lyophilised. The optimal molar charge ratio (+/−) for the complex was adjusted at 5:1 (dendrimer/ODN1).[16] ITC experiments were performed in a MicroCal VP-ITC microcalorimeter. The oligonucleotide solution (2 μM) was placed in the sample cell and the dendrimer (243 μM) was placed in the syringe. The cell temperature was maintained at 30 °C and the dendrimer was added to the solution of oligonucleotide using 25 × 4 μL injections, each injection was 4 min apart.
25. In vitro assay Cells of human RPE origin, D407 were grown to 80% confluency in a 24-well plate and transfected with 1 μM (final concentration) of ODN1 in quadruplet sets using either cytofectin GSV™ (as per manufactures instructions) or the dendrimer/ODN1–complexes. The cells were grown under hypoxic conditions (5% CO_2/2% O_2) for 24 h after which the media was removed for analysis. 500 μL of media from each of the sample was placed in a microcon-30™ concentrator and centrifuged to a volume of 10 μL. 500 μL of phosphate buffer (pH 7.2) was then applied to wash the sample which was centrifuged to a final volume of 100 μL. The samples were then used in an sandwich ELISA assay as per the manufactures instructions (Cytelisa™ Human VEGF kit, CYTIMMUNE Sciences Inc., Maryland, USA).