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Non-viral Charge Reversal Vectors for pDNA Delivery

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Abstract—**A synthetic vector that transform from a cationic to an anionic lipid intracellularly is described. This charge-reversal lipid was synthesized and characterized, including the supramolecular complex it forms with DNA. Enhanced gene transfection was observed using this synthetic vector compared to current cationic lipids.**

I. Introduction

One of the critical steps limiting the efficiency of non-viral gene delivery is the intracellular release of DNA from the vector complex. The complex formed between the lipid and DNA should be stable enough to prevent DNA degradation but also dissociate once inside the cell for subsequent transcription. Degradation of the synthetic vector is also required to reduce cytotoxicity and increase the life-time of transfected cells.[1-4] To facilitate the escape of DNA from the DNA-lipid complex in the cell, and thus improve transfection efficiency, we have developed esterase sensitive cationic lipids.[2] These lipids form a strong electrostatic complex with DNA. Once in the cell, esterases hydrolyze the terminal ester linkage of the lipid affecting a change in overall lipid charge from cationic to anionic. This *charge reversal effect* releases the DNA from the lipid carrier. A series of charge-reversal lipids were synthesized and characterized which possess different chain terminal functionalities. The complexation and dissociation of the lipid to DNA was monitored using an ethidium bromide displacement assay. The supramolecular lipid/DNA complexes were also characterized by DLS, TEM and X-ray diffraction. Enhanced gene transfection was observed with this new class of lipids.[5]

II. Experimental Methods

A. Synthesis

Benzyl formate was added to an octane solution of dodecanoic diacid in the presence of Dowex 50W-X2. The resulting compound was next coupled to 3-amino-propane-1,2-diol in the presence of DCC and DMAP to afford the desire compound. Finally, this compound was reacted with MeI in a CH_2Cl_2 solution to yield the cationic lipid **1**. The others compounds were prepared in a similar manner.

B. DNA Binding

DNA binding studies [6] were carried out by a competitive displacement fluorometric assay using ethidium bromide, EtBr. This assay involves the addition of the compound to a 3 mL solution of EthBr (1.3 μM) and calf thymus DNA (3 μM) in buffer (100 mM NaCl, 100 mM Tris, pH 7.4) with the decrease of fluorescence (λ_{exc}=546 nm, λ_{em}=600 nm; 1 cm path length glass cuvette, slit width 3 nm) recorded five minutes after each addition of lipid.

C. Transfection Experiments

Transfections were performed at 80% conflency of the cells. Briefly, plasmid DNA coding for a reporter gene, β-galactosidase was first mixed with lipids at room temperature. Depending on the experimental design, the ratio of DNA and lilpids was varied. The mixture was incubated for 15 minutes at room temperature before adding to the cells. The amount of DNA used was the same as used in naked DNA control and positive control (commercially available transfection reagents). After incubation at 37 °C and 5% CO_2 for 1 h, medium containing the mixtures was gently removed and fresh growth medium was added. Transfection efficiencies were assessed at 24 h to 48 h post transfection depending on the experimental design. Negative controls were medium alone and naked DNA controls contained medium with reporter gene. Positive controls were performed according to the manufacturer's protocol.

D. Cytotoxicity experiments

Cytotoxicity was determinated using a conventional tetrazolium based assay. In this assay, a modified tetrazolium compound, MTS (Owen's reagent) is bioreduced by viable cells to form a soluble colored Formazan product (490 nm). Blank control were used as a reference. The percentage of viability was calculated in relation to untreated controls. The cells transfected with the control vector and expressing β-galactosidase were visualized by microscopy. The cells appear blue following fixation and incubation with the substrate X-gal. Cells that have been not transfected with a β-galactosidase control vector were used as control in order to determine the level of background activity due to the β-galactosidase activity or its isoezymes.

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III. Discussion and Results

The charge-reversal lipids were designed to perform two roles: first the lipids must bind and then release DNA, and second, as anionic multi-charged species, the lipids must destabilize bilayers. This approach which uses a change in electrostatic interactions to promote DNA delivery and alter membrane properties is different than previous functional vectors. For the lipids to perform these roles, the lipids require three distinct structural entities: a cationic head group, hydrophobic chains, and terminal ester linkages. As shown in Figure 1, lipid **1**, has a cationic head group to bind DNA, lipophilic acyl chains to form a bilayer, and ester linkages at the acyl chains for enzymatic hydrolysis. We prepared the series of compounds **2** – **4,** shown in Figure 1, to assess the role of each structural component on DNA binding/release, supramolecular structure, and gene delivery efficacy.

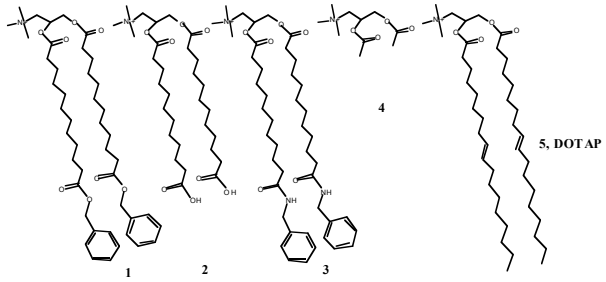
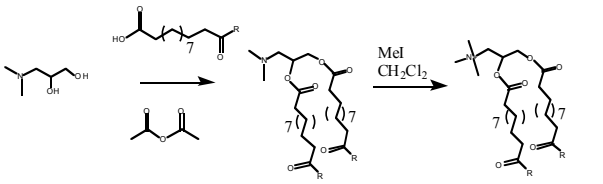


Fig. 1. Lipids under investigation for gene delivery.

The synthetic route to the lipids is shown in Scheme 1. The benzyl ester fatty acid was prepared from the dodecanoic diacid with benzyl formate in the presence of Dowex 50W-X2 in octane at 80 ºC. Next, the fatty acid was coupled to the 3-dimethylamino-propane-diol in the presence of DCC, DMAP in dichloromethane. The acetate analog was prepared using acetic anhydride in pyridine. Finally, the lipid was reacted with MeI in dichloromethane to quaternize the tertiary amine in quantitative yield to give the final product.



1: R = OBn
2: R = OH
3: R = NHBn
4: acetate derivative

Scheme 1. Synthesis of the charge reversible lipids

To determine whether the lipids bind DNA, we performed a standard ethidium bromide-DNA fluorescence assay. DNA

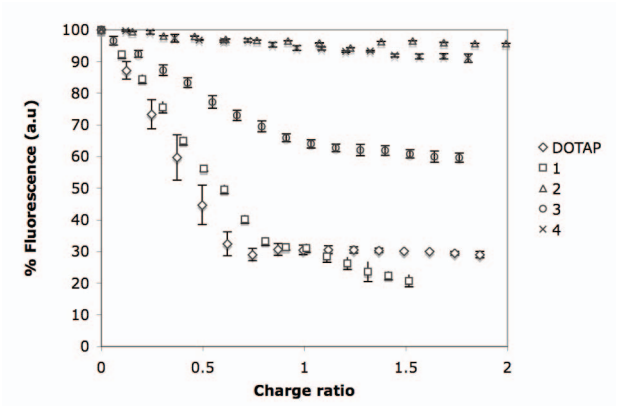


Fig. 2. Ethidium bromide displacement assay showing the fluorescence intensity as a function of synthetic vector/DNA charge ration for compound **1-4** and DOTAP.

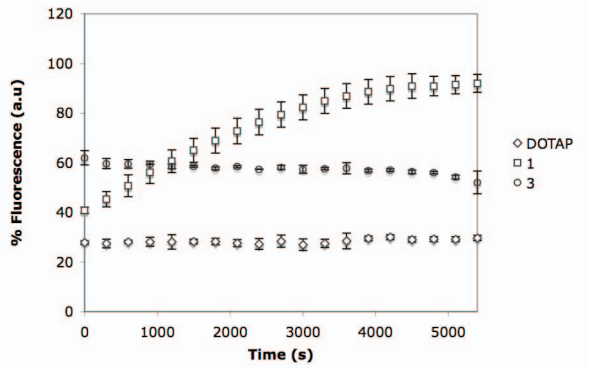


Fig. 3. Ethidium bromide displacement assay showing the fluorescence intensity as a function of the time in the presence of a porcine liver esterase (1000 units/mL).

binding is observed for all of the compounds except **2** and **4** (Figure 2).

Next the DNA-EtBr-lipid solution was incubated with an esterase. The fluorescence intensity increases over time with lipid **1**. This result is consistent with the hydrolysis of the terminal esters and disruption of the DNA-lipid supramolecular complex followed by re-intercalation of EtBr in DNA. No increase in fluorescence over time is observed with the others lipids, which possess a terminal amide linkage or methyl group (Figure 3).

The fluorescence data indicate: release of DNA from the supramolecular assembly does not occur with lipids lacking a terminal hydrolysable ester linkage and the ester linkages near the cationic head group are less accessible to enzymatic hydrolysis.

The supramolecular structures formed by the different lipids were investigated by dynamic light scattering (DLS), transmittance electronic microscopy (TEM) and by x-ray diffraction.

The mean diameter of the structures formed by the different lipids and by the complexes lipid/DNA were

measured by dynamic light scattering. Compound **1** formed liposomes with a mean diameter of 110 nm, and when in presence of the DNA the diameter increases to 562 nm. Compounds **2**, **3**, and **4** do not form stable complexes with DNA. After the preparation, a precipitate is often formed.

These supramolecular structures were also evaluated by TEM. Only compound **1,** in the presence of calf thymus DNA, forms vesicular assemblies. The TEM micrograph of **1** shows multilamellar structures. The average size of the vesicles was 200 nm. To obtain further information on the supramolecular assembly SAXS experiments were performed. The lipid alone exhibits three diffraction orders of a lamellar repeat period of 5.22 ± 0.03 nm with a sharp wide-angle spacing of 0.46 ± 0.01nm. In presence of DNA, the lamellar repeat period is 5.31 ± 0.14 nm with a wide-angle spacing of 0.46 ± 0.01 nm.

Transfection experiments using the reporter gene, β-galactosidase (β-gal, pVax-LacZ1, invitrogen) were performed with Chinese hamster ovarian cells (CHO). The cationic lipid **1** was the most effective vector for transfection. The other compounds showed minimal transfection activity comparable to the negative control and naked DNA, except for DOTAP.

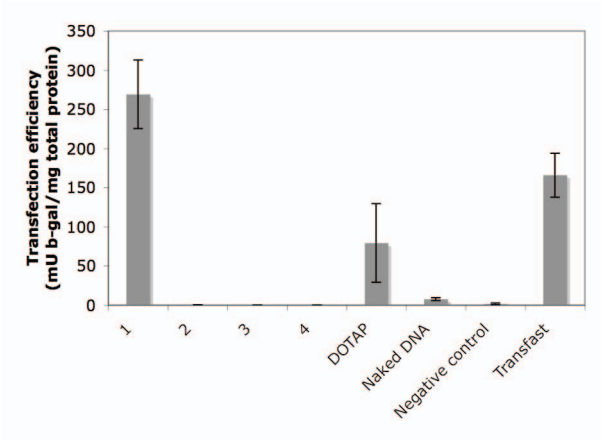


Fig. 4. Transfection results after 48 h in CHO cells using the reporter

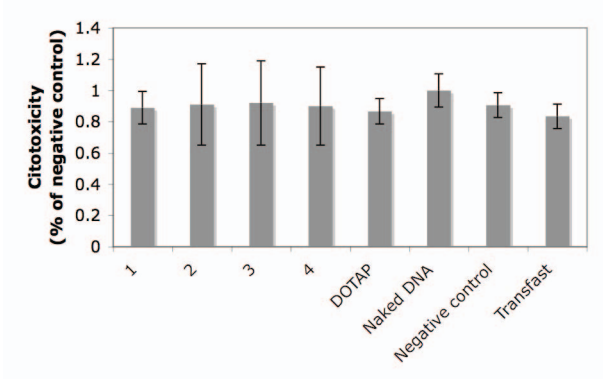


Fig. 5. Cytotoxicity results after 48 h in CHO cells.

Cytotoxicity experiments were performed with CHO cells using both a formazan-based proliferation assay and a total protein assay. The cells were seeded on to a 96-multiwell microliter plate with an appropriate density of $1x10^4$ cells per well. The compounds under investigation were added to the cells 24 hours later. After an additional 24 h, cell proliferation was determined and expressed as a percentage of non-treated cells. None of the lipids showed a significant cytotoxicity, with values similar to the negative control (i.e., non treated cells).

With these encouraging results, we next evaluated the transfection activity of lipid **1** in two other cells lines. Transfection experiments using human embryonic kidney (HEK293) and erythroleukemic (K562) cell lines were performed with compound **1**. As shown in Figure 6, good transfection activity was observed in both cell lines.

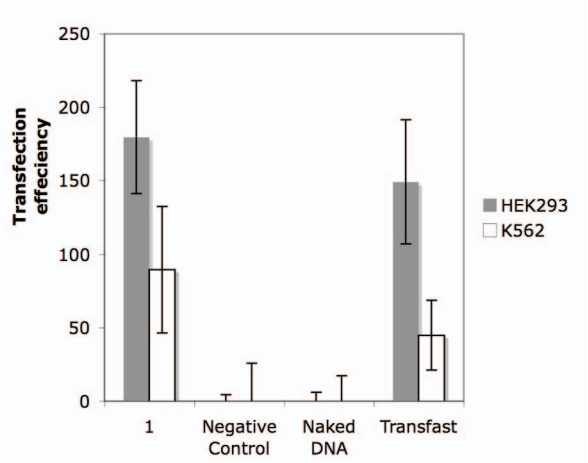


Fig. 6. Transfection results after 48 h in HEK 293 and K562 cell lines.

IV. Conclusions

In summary, a lipid vector for DNA delivery is described which was designed to change its electrostatic interactions with DNA once inside the cell to improve overall gene transfection efficiency.

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