Synergistic effects in gene delivery—a structure–activity approach to the optimisation of hybrid dendritic–lipidic transfection agents†

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Received (in Cambridge, UK) 10th July 2008, Accepted 13th August 2008
First published as an Advance Article on the web 3rd September 2008
DOI: 10.1039/b811852c

Novel gene delivery agents based on combining cholesterol units with spermine-functionalised dendrons exhibit enhanced transfection ability—we report significant synergistic effects in mixed (hybrid) systems which combine aspects of both main classes of synthetic vectors, *i.e.*, cationic polymers and lipids.

Gene therapy could have a dramatic impact on diseases such as cystic fibrosis,[1] but requires the development of vectors that are capable of delivering genetic material safely and efficiently into cells.[2] To avoid adverse patient response from viral vectors,[3] increasing attention has focused on developing non-viral vectors.[4] These synthetic vectors are typically divided into two classes—cationic polymers[5] and cationic lipids.[6] Cationic polymers use multiple cationic sites to yield strong nucleic acid binding, whilst cationic lipids achieve this by assembling into highly charged aggregates. Dendrimers are an important class of cationic polymer for gene delivery,[7] with poly(amidoamine) (PAMAM) dendrimers,[8] and dendrimers based on L-lysine,[9] being widely investigated. The groups of Diederich, Florence and Safinya have developed dendrons attached *via* the focal point to hydrophobic units for gene delivery—such cationic lipid-like systems exhibit good transfection activities.[10]

We recently reported a dendritic system (**Z-G2**, Fig. 1) which showed high-affinity DNA binding at low nanomolar concentrations as a consequence of its multivalent, biologically-derived spermine ligands.[11] Disappointingly, these dendrons had poor transfection abilities—several orders of magnitude less effective than branched poly(ethyleneimine) (bPEI).[12] Effective transfection was only observed in the presence of chloroquine, which is known to disrupt endosomal membranes.[13] Given that escape from endosomes is a vital step in transfection, we reasoned this was limiting the activity of our dendrons. Our dendrons are synthetically flexible—the Z-protecting group can readily be removed by hydrogenolysis and replaced with other functional groups.[14] We reasoned that giving our dendrons more lipidic character may enhance transfection by promoting self-assembly. We also realised that we could generate systems with hybrid characteristics—containing aspects of both cationic polymers and lipids. A handful of previous reports have demonstrated that such a strategy can enhance gene delivery.[15] This communication reports some of our key findings about synergistic effects on gene delivery.

We synthesised dendrons in which the Z-group at the focal point was replaced with cholesterol (Fig. 1). Cholesterol has often been exploited as the hydrophobic unit in transfection agents,[16] and like our spermine surface groups, is a naturally occurring building block which should be well tolerated in biological systems. We reasoned that the hydrophobic unit could have two beneficial effects: (i) aid self-assembly of the dendrons and hence DNA binding/protection, (ii) disrupt endosomal membranes, a key step in effective transfection.[17] We therefore synthesised **Chol-G1** and **Chol-G2** and also compound **Chol$_2$-G1** with an additional cholesterol group. We reasoned that this small family of compounds would help us determine the optimum balance between lipophilic cholesterol units and hydrophilic polyvalent cationic spermine groups, and hence elucidate their effects on gene transfection.

Initially, we investigated the ability of these vectors to bind DNA using ethidium bromide (EthBr) displacement assays[18] (Table 1) and gel electrophoresis (Table 1 and ESI†). These methods provide N : P ratios, which reflect the amount of dendron required to effectively bind DNA—it should be noted that the N : P ratios from the two methods will not be equivalent as the EthBr assay reflects competitive binding, whereas electrophoresis measures direct dendron–DNA interactions. Surprisingly, we found that using both techniques, **Chol-G1** binds DNA at lower N : P ratios than **Chol-G2**, *i.e.*, less **Chol-G1** is required to bind DNA. This is a remarkable observation, as **Chol-G1** contains far fewer DNA binding spermine units than **Chol-G2**. In our previous studies on **Z-G2**, DNA binding correlated with dendritic generation through multivalency.[11] In terms of N : P ratio, **Chol-G1** is the most effective DNA binder we have synthesised thus far. Interestingly, **Chol$_2$-G1** bound DNA similarly to **Chol-G1** in the EthBr assay.

We reasoned that **Chol-G1** and **Chol$_2$-G1** must behave as cationic lipids, and self-assemble into an aggregate in which the cholesterol units become hydrophobically packed. In this way, multiple spermine units are displayed on the surface of the aggregate, thus giving rise to a strong multivalency effect. On the other hand, for **Chol-G2**, the hydrophilic–lipophilic balance[19] is biased towards the hydrophilic spermine groups, and the cholesterol unit will have less ability to direct self-assembly. Although a full study of the phase behaviour of these compounds was beyond the scope of this initial study, dynamic light scattering (DLS) studies on the dendron–DNA

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† Electronic supplementary information (ESI) available: Full characterisation of new vectors, methods for biological assays, gel electrophoresis data, confocal microscopy images, toxicity data and further transfection data. See DOI: 10.1039/b811852c

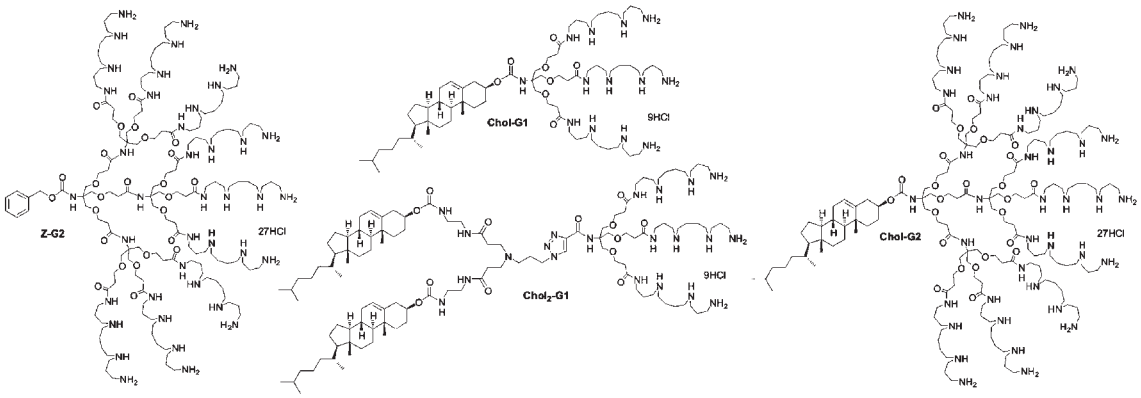

Fig. 1 Compounds used in this paper for gene delivery: **Z-G2**, **Chol-G1**, **Chol-G2** and **Chol$_2$-G1**.

Table 1 N : P ratios for DNA binding determined from: (a) EthBr displacement assays (50% displacement) and (b) gel electrophoresis (complete retention in loading well)

Compound	N : P ratio from EthBr displacement assay	N : P ratio from gel electrophoresis
Chol-G1	0.52	0.34
Chol-G2	1.35	0.51
Chol$_2$-G1	0.49	0.85

complexes (Table 2) showed that the complexes formed with DNA by **Chol-G1** were larger than those formed by **Chol-G2**, indicating aggregation. Aggregate size in both cases increased with increasing dendron loading. The complexes formed between **Chol$_2$-G1** and DNA were much smaller, better defined, and less dependent on the N : P ratio—demonstrating that the additional hydrophobic cholesterol group also has a direct influence on the mode of self-assembly.

We then made a preliminary assessment of the transfection potential of these vectors using a luciferase assay with HEK293 cells. Disappointingly, although **Chol-G1** is a highly effective DNA binder, it was unable to transfect HEK293 cells (Fig. 2). In the presence of chloroquine, some transfection was observed (up to 23% of bPEI positive control—see ESI†). It is possible that the large **Chol-G1** : DNA complexes are too big for entry into cells, but confocal microscopy using DNA labelled with YOYO dye indicated that cellular uptake had taken place (ESI†), with the cell interiors (but not the nuclei) becoming stained. It therefore seems likely that endosomal escape/release is limiting transfection.

Pleasingly, **Chol-G2** was able to transfect HEK293 cells to a certain extent (up to 10% of bPEI, Fig. 2) in the absence of chloroquine. Clearly changing from first to second generation dendron assists the transfection process. There are two possible reasons for this: (i) **Chol-G2** is a weaker DNA binder than **Chol-G1** and may release the DNA more efficiently after cellular uptake; (ii) **Chol-G2** has a greater number of amines and capacity to buffer endosomal pH (well-known to assist endosomal escape for cationic polyamines).[20] As such, we argue that **Chol-G2** behaves somewhat more like a cationic polymer, with the polyvalent spermine units significantly aiding transfection. Interestingly, the transfection observed with **Chol-G2** was an improvement over that observed for **Z-G2**, which could only achieve up to 4% transfection at higher loading levels (Fig. 2). This indicates that in addition to the key role of the dendritic spermine array, the cholesterol unit also plays an active role in assisting transfection. This is probably because the cholesterol unit can disrupt endosomal membranes.[17]

Most excitingly, **Chol$_2$-G1** showed significantly improved transfection into HEK293 cells—up to 62% of bPEI (Fig. 2). Clearly the introduction of the second cholesterol unit significantly enhances transfection. We argue that the second cholesterol unit endows **Chol$_2$-G1** with additional hydrophobicity (lipid character), assisting endosome disruption.[17]

We therefore had two vectors capable of moderate gene delivery. Compound **Chol-G2** has more 'cationic polymer' characteristics—'polymer-like' buffering ability of the multivalent

Table 2 Dynamic light scattering data for the diameters (nm) of complexes between vectors and DNA at different w/w ratios assuming spherical combination. Errors: ±10% for **Chol-G2** and **Chol$_2$-G1**, ±20% for **Chol-G1**

	Diameters of complexes (nm) at various w/w ratios of dendron : DNA					
Dendron	1 : 1	5 : 1	10 : 1	20 : 1	30 : 1	40 : 1
Chol-G1	331	447	551	690	723	771
Chol-G2	257	329	350	428	427	530
Chol$_2$-G1	242	315	290	265	255	257

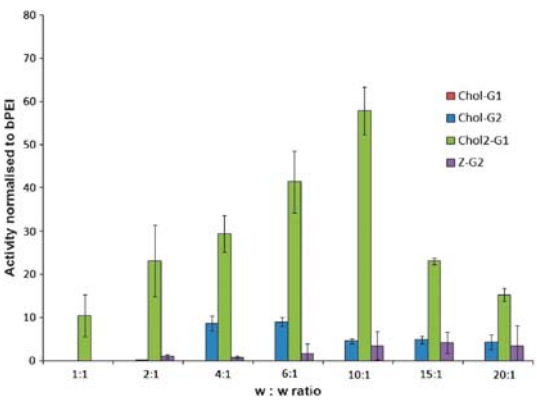

Fig. 2 Transfection efficiency of dendritic vectors in HEK293 cells. Data for luciferase expression were calculated in RLU per μg of protein and quoted as percentages of the transfection efficiency of bPEI.‡ (N = 6, error bars represent standard deviation).

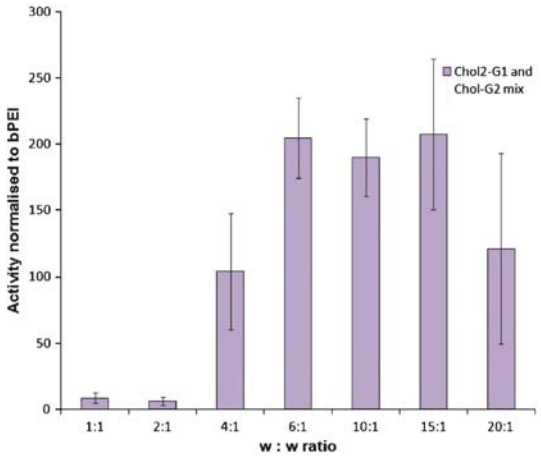

Fig. 3 Transfection efficiency of synergistic mixture of **Chol-G2** : DNA (4 : 1) and **$Chol_2$-G1** (in w/w ratio given) in HEK293 cells. Data for luciferase expression were calculated in RLU per μg of protein and are quoted as percentages of the transfection efficiency of bPEI.‡ ($N = 6$, error bars represent standard deviation).

spermine array assisted by the hydrophobic cholesterol unit. Compound **$Chol_2$-G1**, however, has more 'cationic lipid' character—the second cholesterol potentially enhances transfection *via* disruption of endosomal membranes. We reasoned that combining these two vectors may be beneficial, as it could combine beneficial aspects of the two different classes of non-viral vector.[15]

We found remarkable synergistic effects when carrying out transfections with mixed vectors containing 4 : 1 **Chol-G2** : DNA and varying amounts of **$Chol_2$-G1** (Fig. 3). Indeed, transfection levels *ca.* 200% of bPEI control were observed. This is much more than the sum of the individual contributions from each vector, demonstrating that this hybrid mixed approach has considerable promise for the development of new transfection agents. We investigated other mixtures of these vectors, but other combinations did not show the same type of synergistic effects on gene delivery.

We determined the toxicity of these vectors using a cell titre blue assay. Neither **Chol-G2** nor **$Chol_2$-G1** showed significant toxicity at the levels required for transfection. We observed $>90\%$ cell viability at concentrations up to 9 μg mL^{-1} for **Chol-G2** and 17 μg mL^{-1} for **$Chol_2$-G1** (see ESI†).

In summary, this paper demonstrates a structure–activity optimisation of gene delivery and development of hybrid vectors with aspects of both cationic polymers and lipids. Synergistic effects were observed in gene delivery. Current work is focusing on applying vectors such as this in more challenging transfection conditions and a wider range of cell lines.§ We are continuing to develop and optimise structural features in order to maximise gene protection, delivery and biocompatibility, whilst minimising toxicity and immunogenicity.

Notes and references

‡ Transfection data were normalised to bPEI in order to enable comparability between different batches of cells. Typical transfection levels were >1000 RLU per μg protein.

§ Preliminary data indicate that synergistic effects of mixing **Chol-G2** and **$Chol_2$-G1** were also observed in MDA-MB231 cells, although absolute levels of transfection were lower. Synergistic effects were also observed when using HEK293 cells in 10% serum, although the absolute levels of transfection were reduced.

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