Effects of structural modification on gene transfection and self-assembling properties of amphiphilic dendrimers†

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A library of novel amphiphilic, self-assembling dendrimers was designed and synthesised to evaluate the effects of structural changes on transfection efficiency.

A number of strategies are being employed to make the efficient delivery of foreign genetic material into a cell possible, *i.e.* gene transfection. While synthetic vectors are generally less efficient than their viral counterparts,[1] facile structural modifications allow for a systematic study of the factors governing transfection efficiency.[2] Cationic lipids, polymers and dendrimers are among the most promising synthetic vectors so far.[3,4] Combination of their essential features led to the realisation of small amphiphilic dendrimers for use as gene carriers.[5] Preliminary *in vitro* cell culture studies have validated this structural motif and identified compound **1** as the best candidate (Fig. 1). With this lead in hand, we examined the effects of systematically altering its chemical structure. We present here the first results in a comprehensive study of structure–activity relationships (SARs). Due to the diversity of the structural and biological parameters involved in the cellular delivery of plasmid DNA, an optimisation of transfection activity requires, in addition to exhaustive cell biological studies, correlations to physicochemical properties. For this reason, the basic ability of the vectors to self-assemble at the air–water interface was explored in Langmuir films.

Amphiphile **1** consists of two first generation dendrons, one lipophilic and one hydrophilic, connected by a rigid tolane (diphenylacetylene) core. The first variable of interest is the size and constitution of this linear spacer. Surprisingly, in many vector systems the spacer length has been found to exert a large impact on gene delivery efficacy.[6] Therefore, the tolane moiety was extended by one phenylene-ethynylene unit to provide structure **2**.

A common feature among both viral and cationic lipid gene carriers is their ability to self-assemble.[7] The lipophilic dendrons in our molecules are crucial as they direct this process, and the structures were thus varied as follows. Cholesterol has strong intermolecular packing ability[8] and is bio-compatable.[9] Hence, a cholesteryl ester was introduced to afford compound **3**. In another approach, the degree of lipophilicity in **1** was approximately maintained by the incorporation of *N*,*N*-dioctylamido branches to give vector **4**.[10] The main change involves an increase in the volume of the resulting dendron. The further exchange of the linear dodecylamido chains in lead compound **1** with rather exotic,

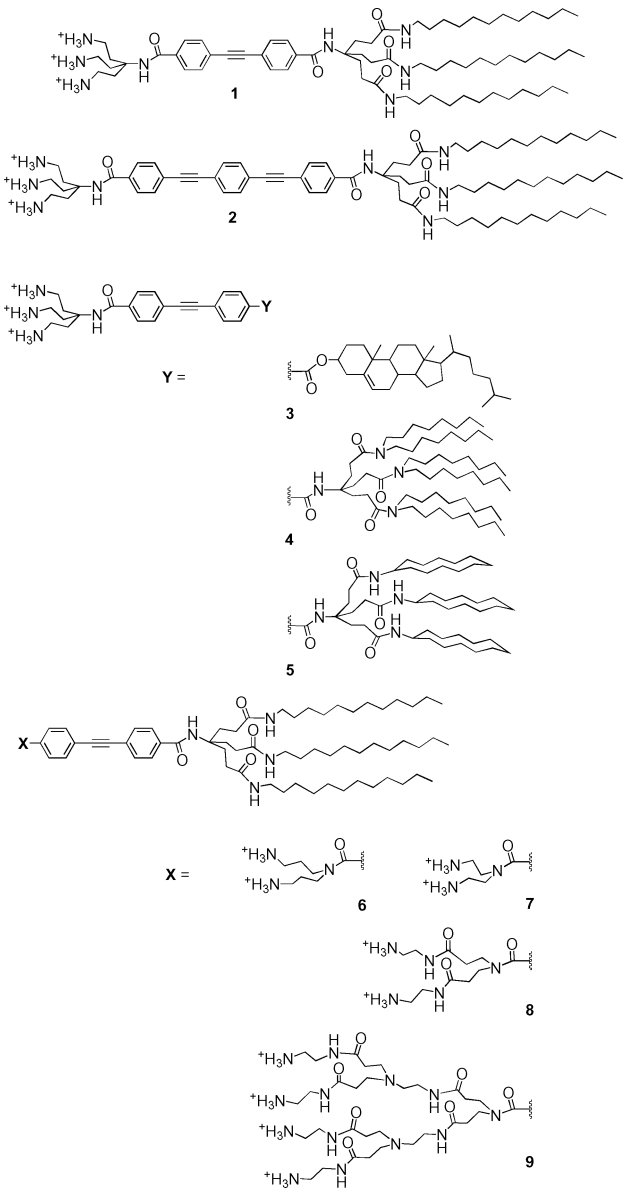

Fig. 1 Library of amphiphilic dendrimers for gene transfection. Counterions to the protonated amines are trifluoroacetates.

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large cyclododecylamido residues should lower the lipophilicity in derivative **5**.[11,12]

Careful design of the hydrophilic dendron is also required as the protonated ammonium centres must bind to plasmid DNA strongly enough for compaction and protection, allow interaction with the negatively charged extracellular matrix, elicit cellular uptake and then allow release into the cytosol and access to the nucleus prior to transfection.[13] In the case of cationic lipids, surface charge density has been identified as a determinant factor for the control of this process.[14] Structural motifs from commonly used poly(ethyleneimine) (PEI), poly(propyleneimine) (PPI), and poly(aminoamine) (PAMAM) dendrimers were therefore incorporated, providing first generation vectors **6–8**.[15]

Vector **9** incorporating a second generation PAMAM dendron was also synthesised. The DNA–cationic liposome complexes are usually taken up into the cell by an endocytotic pathway after which endosomal escape must occur. While the exact mechanism of the latter process is unknown for our amphiphilic dendrimers, a few theories in the field of gene transfection have been presented.[16] The internal, protonatable tertiary amine centres of **9** may result in swelling of the complex upon acidification of the endosome, invoking the so-called proton sponge effect. This mechanism may facilitate the release of DNA into the cytoplasm.

With this library in hand, we determined the biological activity and the self-assembling behaviour of the amphiphilic vectors. *In vitro* transfection assays were carried out using human cervical carcinoma cells (HeLa).‡ For compaction, plasmid DNA encoding for green fluorescent protein (GFP) was mixed with compounds **1–9** at various charge excess ratios (*CE*s)§ in the absence of serum, and was incubated with HeLa cell cultures. After 24 hours of incubation, the transfection efficiency (TE) was evaluated by fluorescence activated cell sorting (FACS). Toxicity of the compounds was determined in HeLa cells using a cell proliferation assay. As for Langmuir films, chloroform solutions of the vectors were deposited on the water surface and pressure–area (π–A) isotherms were then measured. The morphology of the amphiphilic monolayers was visualised using Brewster angle microscopy (BAM).

Results of the transfection experiments are shown in Fig. 2a for compounds **2–5** and in Fig. 2b for the hydrophilic library, in comparison to reference vector **1**. Generally, changes to the cationic dendron improve gene expression slightly, whereas modifications to the core and the lipophilic dendron have a negative effect on transfection. Connecting the dendrons of compound **1** to a slightly elongated core unexpectedly results in a marked decrease in the activity of compound **2** at CE = 2–4. A similar outcome was observed in preliminary studies when a much larger central scaffold was utilized and attempts at transfection failed.[17]

Equally unexpectedly, dendrimer **3** has almost no activity at low to medium charge excess ratios (CE = 2–8). Cationic lipids incorporating cholesterol units have been widely exploited for gene delivery but a helper lipid is usually required to destabilize the endosomal membrane and allow cytosolic access.[3,18,19] In our case, DNA condensation may be very tight and the resultant complex may have too low fluidity.

Vectors **4** and **5** have similar transfection capabilities but overall are less efficient than **1**. From literature studies, a ranking of lipophilicity in surfactants may be made.[10–12] If this ranking were

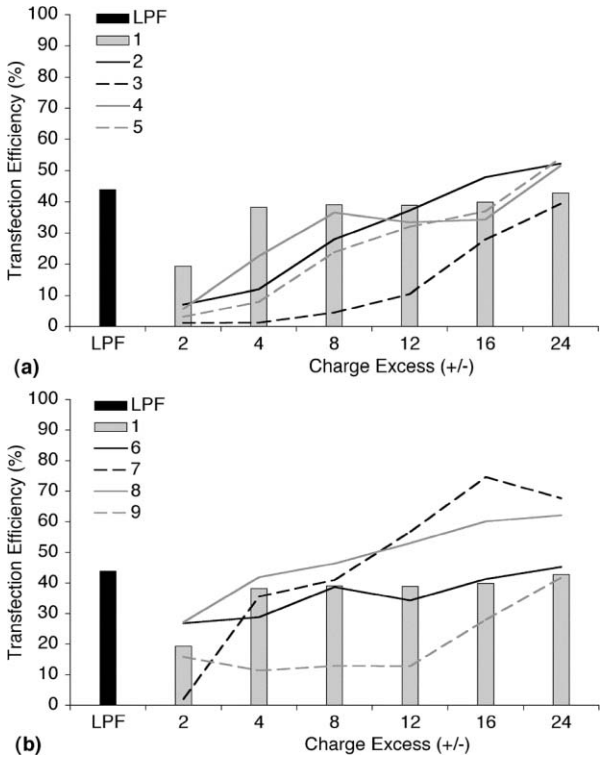

Fig. 2 Transfection efficiency of new dendrimers in HeLa cells in comparison with reference compound **1**, measured as percentage of living cells transfected and normalized to Lipofectamine™ 2000 (LPF): (a) Compounds **2–5**, core extension and lipophilic library. (b) Compounds **6–9**, hydrophilic library.

extendable to our dendrimeric system, the lipophilicity of the vectors should be as follows, **1** ≈ **4** > **5**. However, this ranking is not reflected by the transfection results. Direct correlations between increased lipophilicity in solution and increased gene transfer efficiency in a biological system cannot be necessarily postulated, with each specific carrier motif having its own rationale.[20] Given that the new dendrons in **4** and **5** have in common shorter, bulkier branches than **1**, it is possible that steric hindrance could disturb the self-assembling process.[11]

All vectors **6–8** bearing two positive charges have a similar to slightly better activity than the triply charged reference compound **1** at $CE \geq 4$ (Fig. 2b). Therefore, within the first generation, changes to the number, length and constitution of the branches have little to no effect at these concentrations. The only difference in activity is observed at CE = 2 between the PPI derivative **6** and the PEI derivative **7**. The branches of **6** and **7** differ only by a single methylene unit, but the activity of the shorter molecule **7** is completely abolished at this concentration.

In spite of the expected proton sponge effect, a large decrease in activity is observed between the first and the second generation PAMAM derivatives **8** and **9**. Different generations of globular PAMAM dendrimers have the same charge density although in vector **9** only one dendron has been incorporated, allowing spreading of the charge due to electrostatic repulsion between the protonated amines. The resultant decrease in surface charge density may in turn hinder escape from the endosome and explain the low activity of **9**.

The toxicity of synthetic gene carriers is a major concern. Cell death was evaluated at the same concentrations as were used in the transfection assays for compounds **2–9** (Fig. 3). Structural changes have a high impact on cell viability. The modification of the core as in compound **2**, results in a dramatic increase in toxicity compared to reference vector **1** (Fig. 3a). On the contrary, variation of the lipophilic dendron generally leads to a decrease in cellular toxicity. Vectors **4** to **5** are slightly less toxic than **1**. Compound **3** is harmless to the cells even at the concentrations where some activity is detectable, which may be linked to the biodegradability of the cholesterol sub-unit.[9,18]

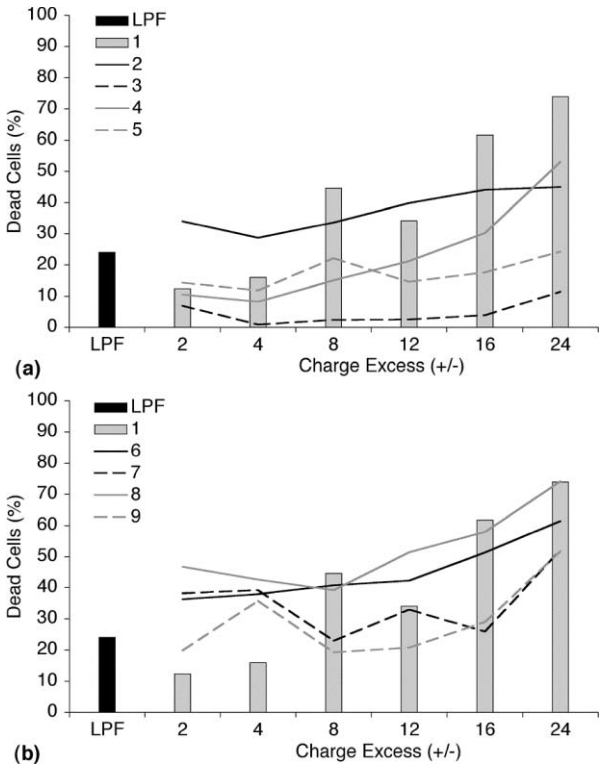

Fig. 3 Toxicity of new dendrimers in comparison with reference compound **1** and normalized to Lipofectamine™ 2000 (LPF): (a) Compounds **2–5**, core extension and lipophilic library. (b) Compounds **6–9**, hydrophilic library.

The most efficient gene carriers, compounds **6** to **8**, are also found to be the most toxic ones in the whole library (Fig. 3b). Contrasting these results is the lower toxicity of second generation vector **9**. Generally, an increase in dendrimer generation corresponds to increased toxicity.[21] In this case, the low cell death is most likely due to the low transfection activity of **9**.[22]

While study of the Langmuir films offers some insight into the biological results, strict correlations are naturally difficult to establish. One may nevertheless observe some trends. The most active vectors in this library (namely **6** to **8**) are the compounds that behave similarly to reference molecule **1**: their isotherms reveal a liquid-like behaviour, with no first-order phase transition and final molecular areas ranging between 85 Å^2 (molecule **1**) (Fig. 4a) and 95 Å^2 (molecule **6**). These observations and the value of the final molecular areas indicate that the molecules most probably form monomolecular films within which the intermolecular interactions are dominantly van der Waals (alkyl chains) and/or electrostatic

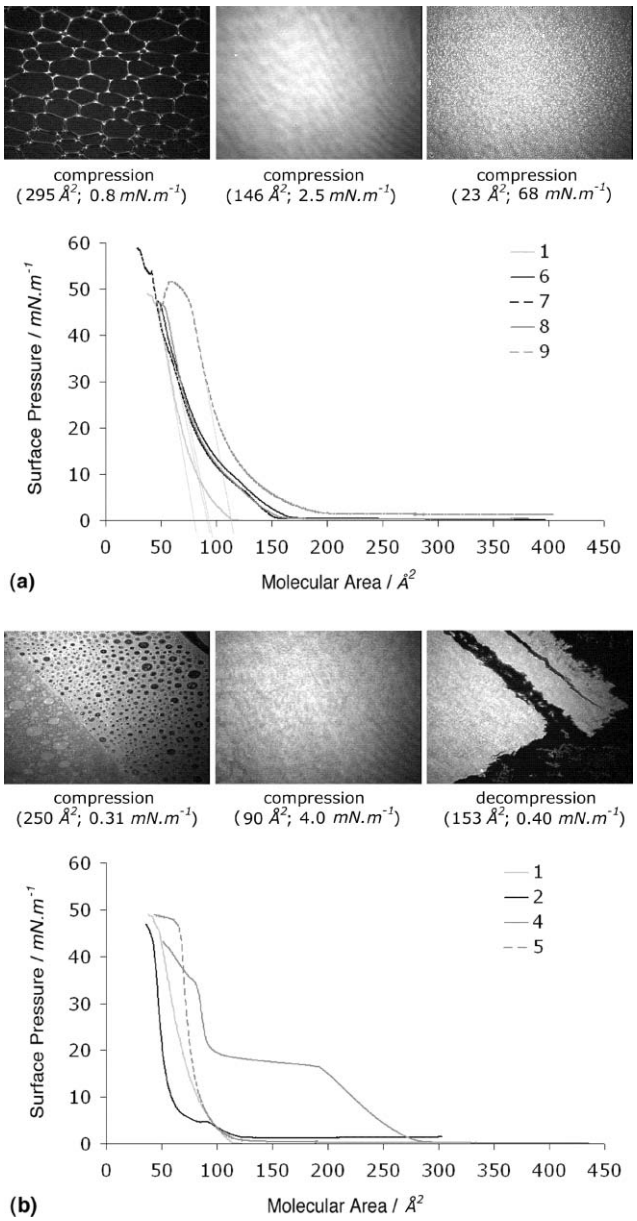

Fig. 4 *π–A* Isotherms recorded on pure water subphase and Brewster-angle microscopy images: (a) Isotherms of vectors **6–9** in comparison with reference compound **1** and BAM images of the film of **6**. (b) Isotherm of vectors **2–5** in comparison with reference compound **1** and BAM images of the film of **2**.

(protonated ammonium groups). The cross-section of the tolane (*ca.* 25 Å^2) is somewhat smaller than the cross-section of the chains or polar heads and hence creates a void space. In order to compensate for this void space, the cores are either strongly tilted (70°) or, more probably, the molecules dimerise through core–core interaction (π–π interactions). These dimers then behave as a single "supermolecule" with a critical packing factor of ~1 (as defined in ref. 10). Brewster-angle microscopy (BAM) observations confirm that these compounds (and also **4** and **9**) indeed form homogeneous monolayers. Molecules with such a packing factor are usually capable of forming bilayers or vesicles, hence the ability to mimic organic structures such as membranes or liposomes.

On the contrary, the compounds with lower transfection efficiency show markedly different behaviours on the Langmuir trough, *e.g.* solid-like behaviour (**2** and **5**) and first-order phase transitions (**2** and **4**) (Fig. 4b).[23] The longer core of **2** and the larger lipophilic heads of **4** and **5** make it impossible for a dimerisation to occur. These molecules therefore have a critical packing factor greater than **1**, which does not favour the formation of films or membranes. Indeed, BAM observations reveal the presence of three-dimensional supramolecular architectures in the "films" of compounds **2**, **3** and **5**. Compound **4** has a transition to some undetermined supramolecular organisation that is *not* a bilayer. BAM can only hint at such supramolecular architectures through the observation of uneven or optically heterogeneous films and getting a definitive proof would require additional experiments such as cryo-transmission electron microscopy (TEM) or small angle X-ray scattering. Still, a reasonable hypothesis based on our observations and on the molecular architecture would be that compounds **2–5** form micelles.

In conclusion, we have presented the effect of rational structural modifications on the transfection activity of small amphiphilic dendrimers. A clear sensitivity to steric requirements of the lipophilic dendron was observed. Within this first library, every change that has an effect on the packing ability clearly lowers the transfection efficiency. On the other hand, small alterations to the cationic dendron structure have limited impact on the biological activity in comparison to lead compound **1**.

Acknowledgements

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Notes and references

‡ In comparison to the original studies,[5] the HeLa cell line was used instead of HEK293 cells and the transfection efficiency was evaluated using FACS. Consequently, the absolute values for transfection efficiency of reference vector **1** are higher in the present study.

§ The unitless *CE* ratio is defined as the number of positive charges on the dendrimers divided by the number of negative charges present on the plasmid DNA. Based on an average molecular weight of 666 g mol^{-1} per base pair (bp), 1 μg of DNA is assumed to carry 3 nmol of negative charges.

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23 Compound **3** is poorly soluble in chloroform, very soluble in water and could not give a reproducible isotherm.