DOI: 10.1002/cmdc.200800190

Guanidinylated Dendritic Molecular Transporters: Prospective Drug Delivery Systems and Application in Cell Transfection

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In the present review the crucial role of the guanidinium functional group in facilitating the transport of dendritic polymers through liposomal and cell membranes is discussed, along with other structural features of guanidinylated dendritic polymers that fine-tune their transport properties, and even determine their subcellular destinations. In this context, an ideal dendritic molecular transporter would need to possess a dendritic scaffold of the appropriate size and degree of guanidinylation, flexibility of the guanidinium moiety, and should exhibit a proper balance between hydrophilic and hydrophobic moieties located on the dendritic surface. All of the above are illustrated through selected paradigms from the relevant literature, which give a valuable insight into forging successful dendritic delivery systems for both drugs and genes. The main challenge for the future focus of the field is identified as the determination of the key structural and functional characteristics that will enhance cell internalisation, and secure localisation in specific subcellular organelles.

Introduction

Intracellular delivery of therapeutic molecules is an issue of significant scientific and practical interest, specifically in drug and gene delivery, and it has been addressed in many cases by the application of molecular transporters.[1–8] These encompass a diversity of molecular structures or aggregates that enable or enhance transport across biological membranes. The field originated from cell-penetrating peptides bearing guanidinium groups, and has extended to other molecular classes or aggregates such as dendrimers, nanotubes, nanoparticles, and liposomes, all sharing the cell-penetrating properties of peptides. In this context, the discovery that the uptake properties of a peptide are derived from the Tat protein, attributed to its array of guanidinium groups, led to the development of arginine-rich peptides,[9,10] peptoids,[11] oligocarbamates,[12] and guanidinylated dendritic polymers.[13] Although these compounds are structurally different, save for the common oligoguanidinium moiety, they all exhibit similar properties, including high water solubility and transport through cell membranes. Additionally, other types of molecular transporters have been investigated spanning a diverse range of molecular structures, such as sorbitol-based transporters,[14] which show high intracellular selectivity towards mitochondria, polyamines,[15] guanidinium–cholesterol-based lipids,[16] and nanotubes,[17] the latter being able to transport proteins into cells.

In the present review, we will address the transport of guanidinylated dendritic polymers through model liposomal and cell membranes, and also attempt to elucidate their transport mechanism. Moreover the prospective design and development of efficient dendritic drug and gene delivery systems will be illustrated through selected examples dealing with the transport of DNA-loaded guanidinylated dendrimers across cell membranes.

Arginine-Based Peptides and their Interactive Transport through Cell Membranes

To fully appreciate the role of the guanidinium group and other structural features of guanidinylated dendritic polymers in promoting their molecular transport across cell membranes, it is necessary to initially discuss the extent to which this functional group affects molecular transport of cell-penetrating peptides and analogous compounds. These peptides primarily consist of basic amino acids, L-arginine and/or L-lysine, indicating that there is a relationship between peptides with high basic amino acid content and the ability to transverse model liposomal or cell membranes. Relevant investigations proved that the translocation properties of these peptides are directly associated with the presence of L-arginine residues. In the case of Tat peptide, replacement of the L-arginine residues with less basic amino acids such as L-lysine, L-ornithine, or L-histidine led to reduced translocation ability.[9] Conversely, replacement of all non-L-arginine with L-arginine residues resulted in a several-fold enhancement of peptide internalisation.[11] To assess the effect of the L-arginine content on cellular uptake, Futaki et al.[18] employed a series of L-arginine homopolymers bearing 4–16 residues. From these experiments, the optimal number of L-arginine groups for maximum uptake was found to lie between 7–9, as these oligomers exhibited superior intracellular accumulation. In fact the L-arginine nonamer was found to be 20-fold more efficient than Tat peptide. The 7–9 L-arginine resi-

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due oligomers were localised in the cytosol and the nucleus, whereas their 4–6 L-arginine residue counterparts were not internalised by the cells. Nonetheless, oligomers with more than ten L-arginines were found to mostly localise in the plasma membrane. Analogous results were obtained by Wender et al.[11] regarding the cellular uptake of L-arginine oligomers bearing 5, 6, 7, 8, or 9 L-arginine residues.

To determine the structural requirements for cellular uptake of short, arginine-rich peptides, Wender et al.[10] investigated a series of L-arginine heptamers in which aminocaproic acid (aca) was introduced as a spacer between the L-arginine residues at different structural modes. The results showed the significance of the spacer for cellular uptake, the most efficient derivative being $R(acaR)_5acaR$. The increased distance between the L-arginine residues enhanced the conformational freedom of the peptide backbone that appeared to be necessary for all L-arginine residues to interact effectively with oppositely charged membrane moieties.

During the first stage of molecular transport, the guanidinium groups interact both electrostatically and through hydrogen bonding[19] with the phosphate, carboxylate, or sulfate groups located on the liposomal or cell surface because of the electronic and geometrical complementarity of these moieties. In this context, it was shown that this strong binding (for example, of guanidinium to phosphate groups) is progressively amplified as the organisation of the interacting substrate is enhanced, that is, as one progresses from isotropic conditions to liposomal and finally to macroscopic interfaces (Figure 1).[20] In addition, this strong binding is further enhanced by multivalent effects.[21,22] Thus, both organisational and multivalent effects act synergistically to enhance the binding of recognisable groups in close proximity on the relatively small external liposomal or cell surface. In this manner, effective association between the interacting parties is achieved.

Neutralisation of charge following interaction of guanidinium groups with the anionic membrane groups is not the only prerequisite for cellular entry. A transmembrane potential arising from the potassium ion concentration gradient was considered to play a crucial role in the transport of guanidinium-rich transporters. This hypothesis was set forth by Wender et al.[23] through experiments on cellular uptake of the L-arginine octamer (R8), performed in conditions where the membrane potential was decreased to almost zero by incubating cells with an isotonic buffer (K^+PBS). Uptake of R8 was reduced by up to 90% for all concentrations with respect to uptake when the cells were incubated with sodium PBS. Similar results were achieved when the cells were pretreated with gramicidin A, a pore-forming peptide, which also decreases the membrane potential. In contrast, hyperpolarisaton of the cell membrane with valinomysin, an antibiotic that selectively shuttles potassium ions across the membrane, resulted in a significant increase in transporter internalisation. From these experiments, it became clear that while flexibility and accessibility of transporter guanidinium groups followed by charge neutralisation is essential for effective internalisation, complex internalisation is also dependent on the transmembrane potential.

Molecular Transporters based on Guanidinylated Dendritic Polymers

The idea of using guanidinylated dendritic polymers as molecular transporters resulted from the facile conversion of amino group terminated dendritic polymers to their guanidinylated counterparts.[24] Specifically, primary amino groups were guanidinylated with 1*H*-pyrazole-1-carboxamidine hydrochloride employing a method analogous to the one reported in the literature.[25] Spherical nanoparticles with flexible external guanidinium groups were formed preferentially as depicted by the general structure of Figure 2. In addition to having the possibility to bear a varying number of guanidinium moieties, guanidinylated dendritic polymers also contain nanocavities, in which drugs may be noncovalently encapsulated. Furthermore, dendritic polymers exhibit the so-called adaptive solubility behaviour,[26,27] that is, the ability to become either hydrophobic or hydrophilic depending on the environment. This is a general property of dendritic polymers independent of the anion effect which tunes hydrophobicity in guanidinium bearing peptides.[28,29] In the latter case the hydrophobicity or hydrophilicity of the peptides is determined by the hydrophobic or hydrophilic character of the counterion.

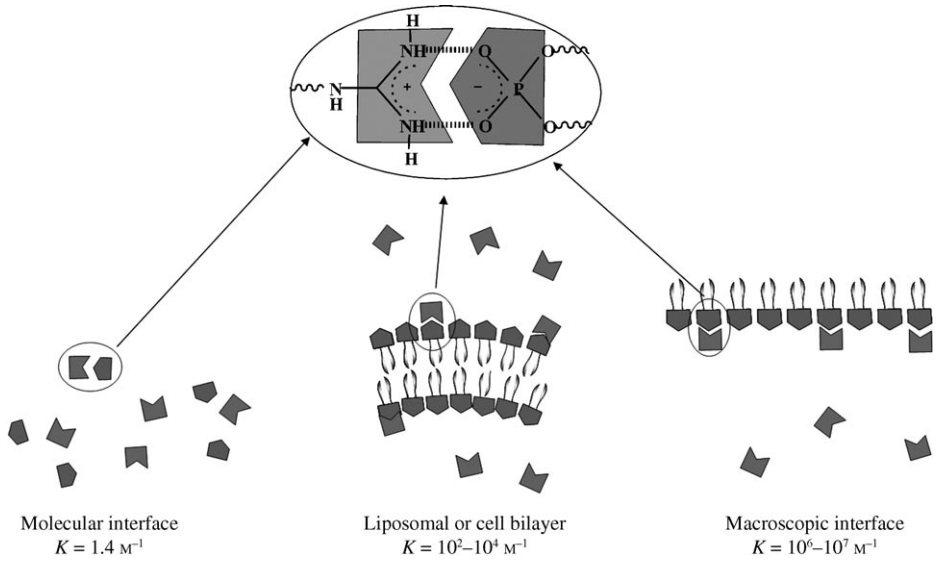


Figure 1. Binding constants of guanidinium and phosphate complex formation at isotropic, liposomal and macroscopic interfaces through combined electrostatic forces and hydrogen bonding enhanced by organisational and multivalent effects.

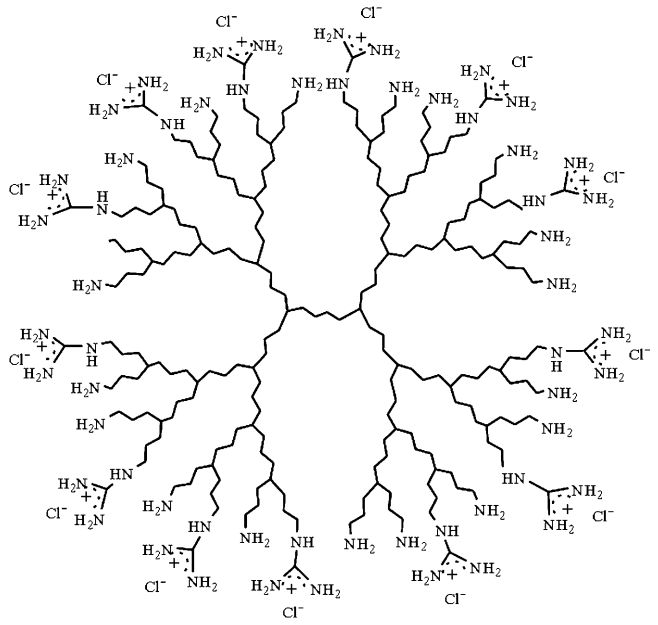
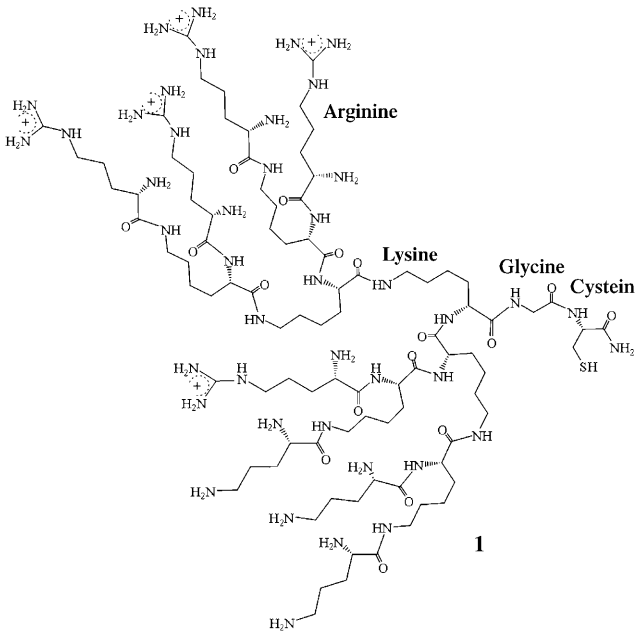


Figure 2. Structure of a dendrimeric molecule bearing guanidinium groups at its external surface.

The translocation of branched-chain arginine peptides through cell membranes was investigated by Futaki et al.[30] In particular, branched-chain arginine peptides with varying numbers of L-arginine residues (from 4 to 24) were synthesised for this purpose. These dendritic molecules were internalised by cells, and most efficiently by the derivative bearing eight arginine moieties, **1**. The time course of internalisation of this peptide is shown in Figure 3. Interestingly enough, the introduction of a three glycine spacer on the dendritic polymer surface rendered the 24 arginine group derivative an efficient transporter. It can therefore be deduced that the characteristics that



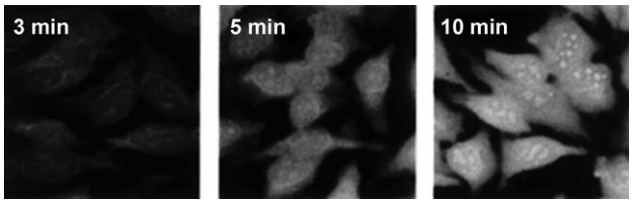


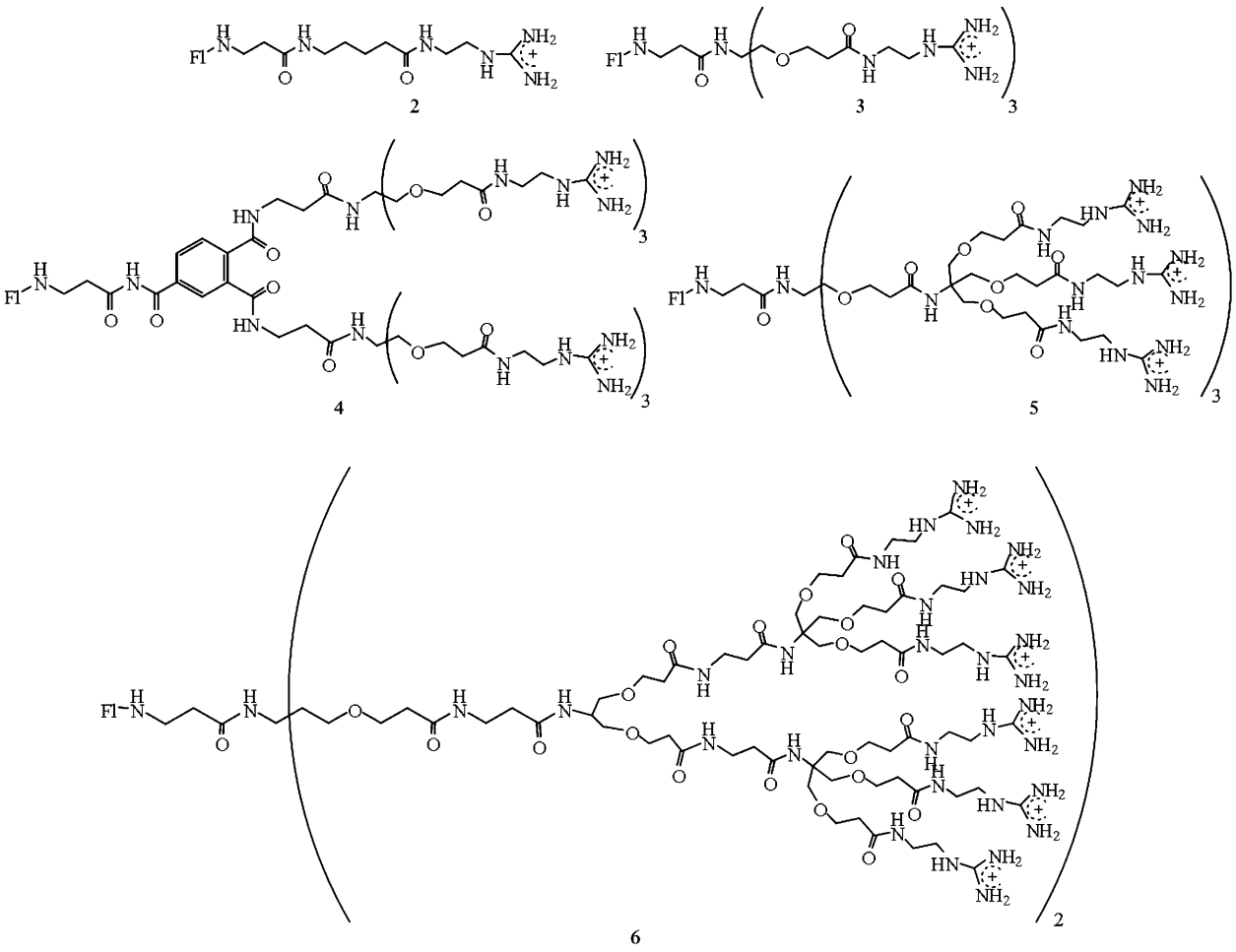
Figure 3. Internalisation of a branched-chain peptide bearing eight arginine moieties into HeLa cells over a 10 min time course, as shown by confocal fluorescence microscopy.

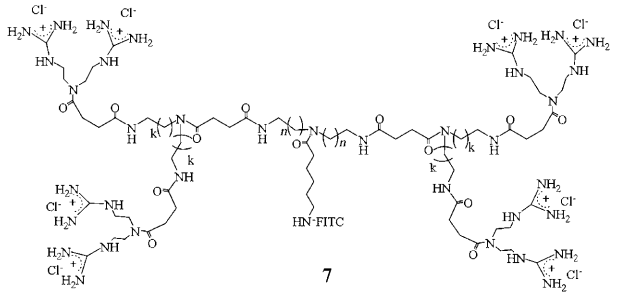
make the linear guanidinium oligomers effective transporters also apply to dendritic derivatives.

Along these lines, Goodman et al.[5] synthesised a series of guanidinylated dendritic polymers **2**–**6**, that readily enter cells. These dendritic derivatives were either conjugated to fluorescein (Fl) or to a green fluorescent protein (GFP) mutant. Both the small molecule and the protein conjugates translocated efficiently into HeLa S3 cells as quantified by FACS analysis. Conjugates **5** and **6**, bearing nine (G9) and 12 (G12) guanidinium groups, respectively, exhibit better uptake than analogues **3** and **4**, with three and six guanidinium groups, correspondingly. This suggests that nine guanidinium groups are optimal. The localisation of the conjugates was also investigated in HeLa S3 cells treated with G9-Fl, G9-GFP, and Tat(49–57)-GFP. The cells were subsequently fixed and visualised by deconvolution microscopy. The resultant images of G9-Fl and G9-GFP indicate that these complexes enter cells and localise in the nucleus and cytoplasm.

To elucidate the effect of branching on cellular uptake, Wender et al.[31] prepared a series of dendrimeric molecules (**7**) all bearing eight guanidinium groups on their external surfaces. The flexibility of these derivatives was adjusted by the introduction of various chain-length spacers between the branching points. The hexyl–hexyl ($n = k = 5$) and hexyl–propyl ($n = 5$, $k = 2$) dendrimers were most effectively internalised whereas the ethyl–ethyl ($n = k = 1$) dendrimer was not intracellularly detectable during the course of the experiment. The most effective of these dendrimers outperform the nonarginine reference system.

To simulate the cell membrane, a model liposomal membrane consisting of phosphatidylcholine (PC), cholesterol (Chol), and dihexadecyl phosphate (DHP), at a PC/Chol/DHP 19:9.5:1 molar ratio, was employed.[27] DHP molecules bore the phosphate groups necessary for interaction with the complementary guanidinium groups of guanidinylated poly(propylene imine) dendrimers (DAB). The effect of the degree of surface guanidinylation on membrane crossing was evaluated by employing a fourth generation poly(propylene imine) dendrimer with 32 surface amino groups (DAB32). Five dendrimeric derivatives were prepared, all labelled by fluorescein isothiocyanate (FITC), bearing 6, 12, 18, 26, and 32 guanidinium groups. The remaining toxic amino groups of the partially guanidinylated dendrimers were modified through interaction with propylene oxide, forming the corresponding hydroxylated derivatives for subsequent in vitro cell experiments (Figure 4). The derivatives bearing six or 12 guanidinium groups demonstrated enhanced





translocation ability across the liposomal bilayer in its liquid crystalline phase.

The transport ability of guanidinylated poly(propylene imine) dendrimers, varying in generation and surface functionalisation, was further investigated[32] in model liposomes and A549 human lung carcinoma cells. Third and fourth generation poly(propylene imine) dendrimers (DAB16 and DAB32, respectively) with the same degree of surface guanidinylation were studied comparatively. Initially, the surface amino groups of the dendrimers were made more hydrophobic through partial acetylation, whereas the remaining amino groups were subsequently guanidinylated, leading to derivatives of varying hydrophobicity (Figure 5a). Furthermore, fully guanidinylated

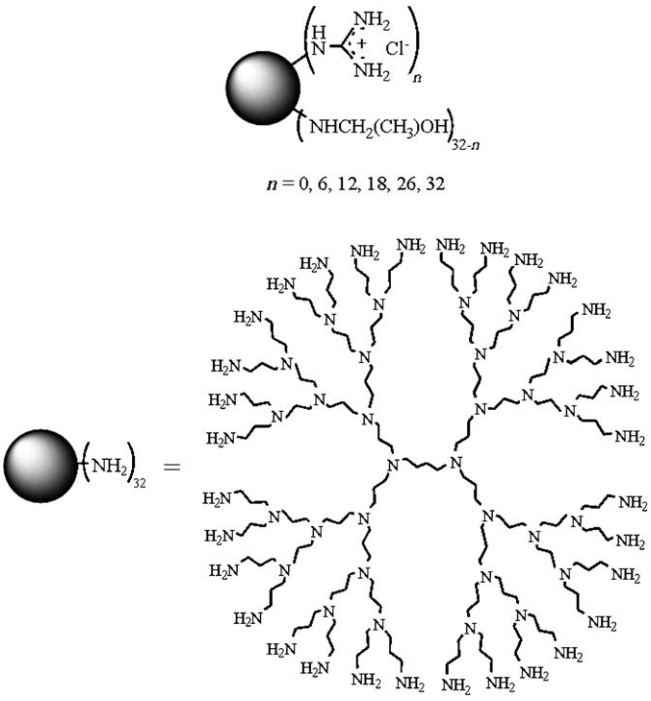


Figure 4. Poly(propylene imine) dendrimers bearing 6, 12, 18, 26, and 32 guanidinium groups.

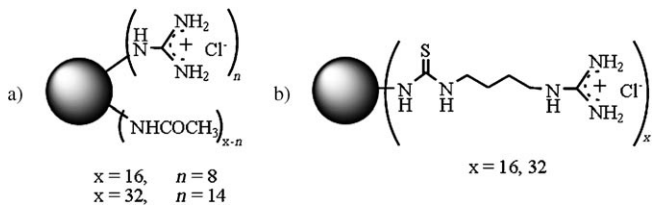


Figure 5. Guanidinylated third and fourth generation poly(propylene imine) dendrimers with a) partial acetylation and b) completely guanidinylated derivatives bearing a spacer between the guanidinium and the surface amino group.

dendrimeric derivatives bearing a spacer between the guanidinium group and the surface amino group were prepared (Figure 5 b), to investigate the effect of this spacer on the ability of the dendrimers to cross the liposomal bilayer. The application of acetylated and guanidinylated dendrimers eliminated the effects attributed to dendrimer size with regard to their ability to cross the lipid bilayers of small unilamellar liposomes. With respect to the dendrimer derivatives bearing the spacer, only the third generation dendrimer exhibited efficient translocation. The relatively small size of the third generation dendrimeric derivative along with the spacer flexibility guaranteed the formation of the appropriate nonpolar complex, which subsequently crossed the liposomal bilayer. Conversely, the spacer flexibility was unable to compensate for the increased size of the fourth generation dendrimeric derivative, leading to profoundly reduced translocational ability.

It was further established[32] that these guanidinylated derivatives were efficiently internalised by A549 cells, localising in their nuclei, nuclear membranes and, to a lesser extent, cytoplasm, as shown by fluorescence time-course experiments and fluorescence microscopy in the presence and absence of FBS (Figure 6). In the presence of FBS, cell internalisation was significantly diminished most probably due to complexation of the dendrimers with serum proteins. The guanidinylated dendrimers were not, in principle, toxic to the cells as shown by standard XTT (mitochondrial redox function) assays, with the exception of increased concentrations of G14 ($>50\ \mu M$). In this case, the toxicity was attributed to repeat entry and concomitant inelastic membrane joint dislocation as revealed by the trypan blue (membrane integrity) assay.

Two generations of lysine dendrigrafts (DGL2, **8** and DGL3, **9**) and their partially guanidinylated derivatives (DGLG2, **10** and DGLG3, **11**) were investigated for their interaction and translocation across model liposomal membranes and A549 human lung carcinoma cells (Figure 7).[33] Interaction with liposomes demonstrated that the fusogenic properties of these compounds were dependent on dendrimer generation and

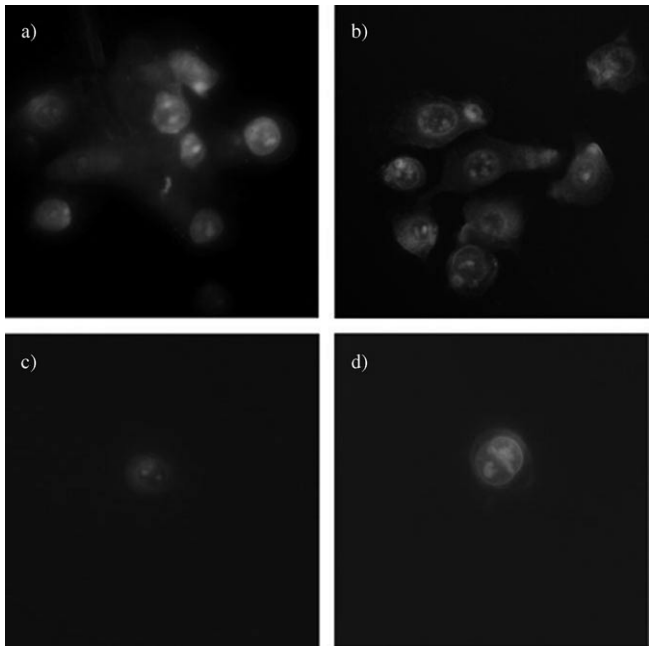


Figure 6. Fluorescence microscopy images of A549 cells incubated for 4 h at 20 mM dendrimer concentration: a) DAB16(Ac)8G8; b) DAB32(Ac)18G14; c) DAB16(Ac)8G8, FBS (10%); d) DAB32(Ac)18G14, FBS (10%).

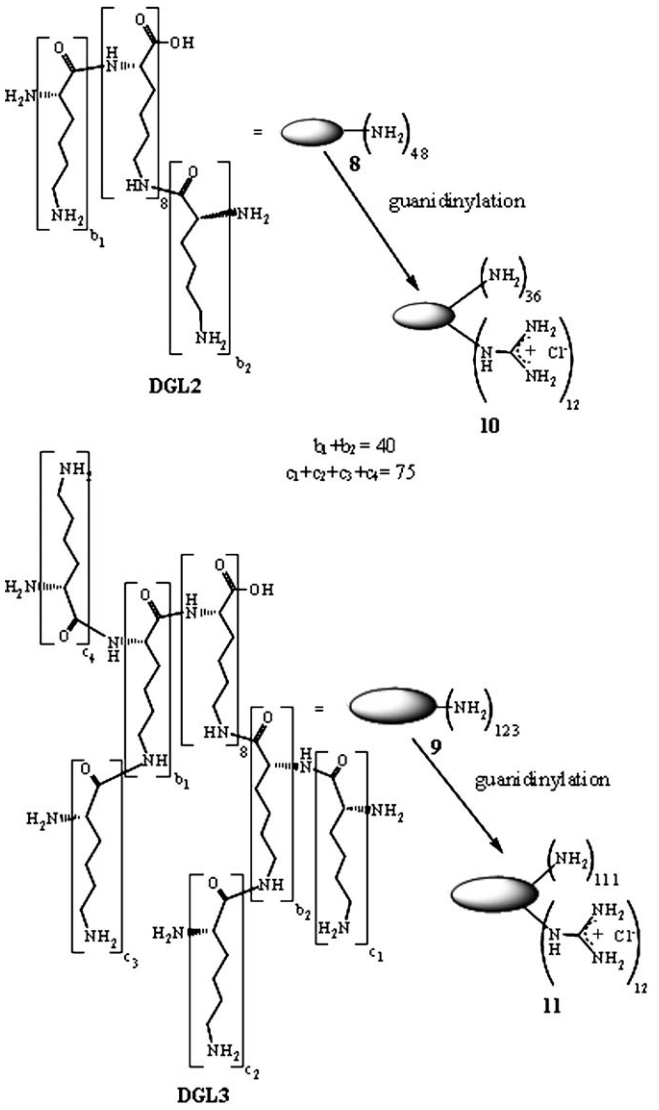


Figure 7. Structures of second and third generation Lysine DendriGrafts (DGL2 **8** and DGL3 **9**, respectively) and their partially guanidinylated derivatives **10** and **11.**

phase organisation of the lipid bilayer. The translocational ability of the second generation dendrigrafts was higher compared with that of the third generation, likely due to fewer size-imposed constraints, as well as the charge and the flexibility of the branches. This ability was also dependent on the lipid bilayer phase, and was significantly enhanced when the bilayer was in the liquid crystalline phase. Cellular uptake by A549 cells revealed efficient dendrimer internalisation. Specifically, the dendrigrafts were preferentially localised in the nucleus and nuclear membrane as revealed by fluorescence microscopy of fluorescein-labelled dendrigrafts (Figure 8). It has to be noted that the third generation nonguanidinylated dendrigraft proved quite toxic to the cells, however, its second generation counterpart was well tolerated for incubation concentrations up to 50 μM. Guanidinylation of the dendrigrafts increased the toxicity because of profoundly enhanced (>fourfold) cell penetration. The findings of this work suggest that the guanidinylated second generation dendrigraft is an ideal candidate as a drug and gene delivery system, at subtoxic levels (≤20 μM); the biodegradable character of this dendrigraft renders the above candidacy even more promising.

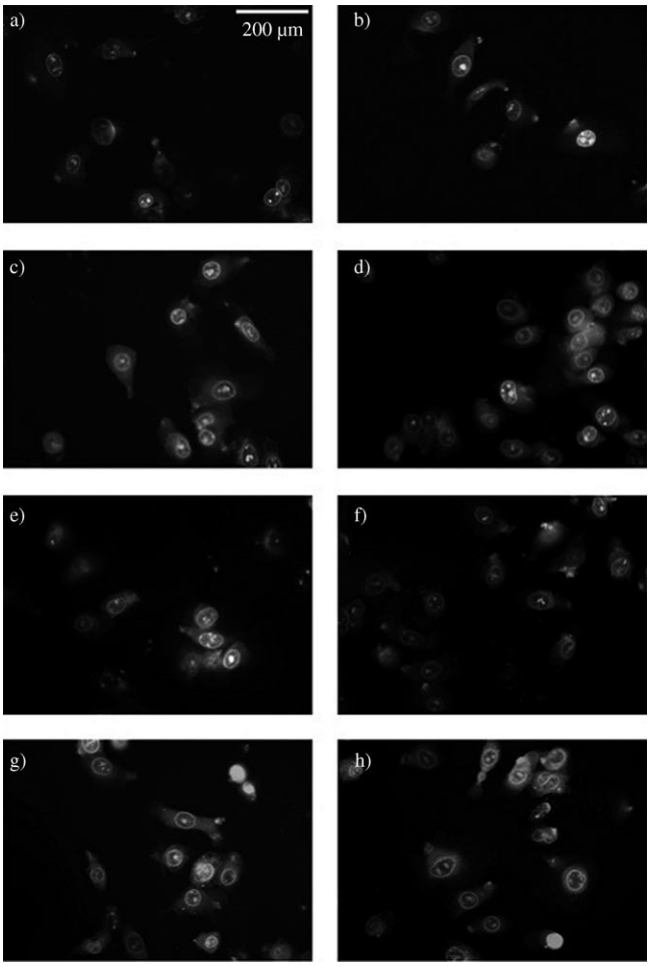
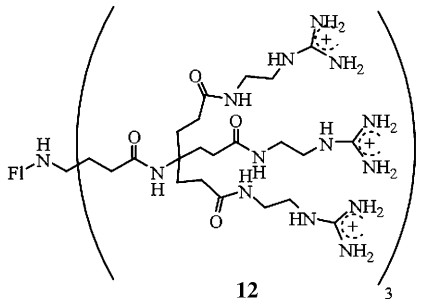
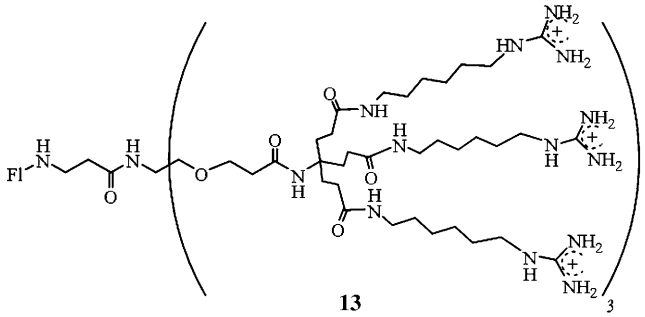


Figure 8. Fluorescence imaging of A549 cells following incubation with fluorescein-labelled dendrigrafts: a) FDGL2, 1.5 h, 10% FBS; b) FDGL2, 1.5 h; c) FDGLG2, 1.5 h, 10% FBS; d) FDGLG2, 1.5 h; e) FDGL2, 3 h, 10% FBS; f) FDGL2, 3 h; g) FDGLG2, 3 h, 10% FBS; h) FDGLG2, 3 h.

In a recent publication[34] fluorescein-labelled, guanidinylated dendrimers, **12** and **13**, which exhibit structure-dependent subcellular localisation, have been synthesised. Derivative **12**





preferentially accumulated in the nucleus whereas **13** accumulated in the cytosol. The experiments were performed on NIH-3T3 fibroblasts and human microvascular endothelial cells with similar results. In Figure 9 the cell internalisation time course of the two derivatives is shown by confocal microscopy. The internalisation increased in a time-dependent manner. The different localisation sites of the two molecular transporters were successfully controlled by variation of the spacer length at the dendrimer terminus. These molecular transporters not only enhanced cell internalisation but also targeted specific subcellular regions.

Therefore, it becomes evident that several structural characteristics affecting the penetrability of the linear guanidinylated architectures also apply to the dendritic analogues. In particular, the degree of guanidinylation although a crucial structural feature is not the sole factor in determining transport across liposomal and cell membranes. The size of the dendritic scaffold, the flexibility of the guanidinium moiety, and the appropriate balance between hydrophilic and hydrophobic groups on the dendritic surface also affect their transport properties and subcellular destinations. It has to be noted that the adaptive solubility behaviour exhibited by dendritic polymers is not only attributable to anion neutralisation[28–29] and hydrophobic groups introduced on the dendrimer surface,[32] but also to the intrinsic properties of the dendritic polymers themselves.[13]

Transport Mechanism for Guanidinylated Dendrimers

Based on the internalisation mechanisms proposed for other guanidinium-rich transporters,[6,13,35] but also taking into con-

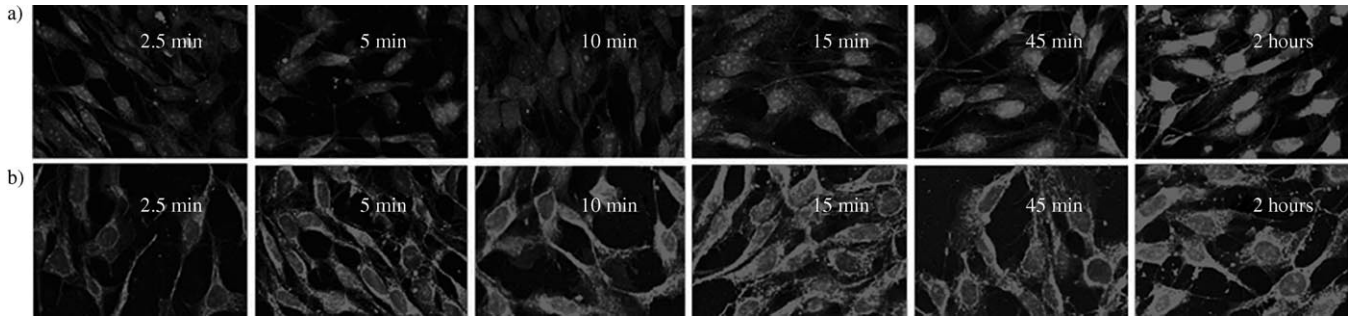


Figure 9. Internalisation of dendritic polymers by NIH-3T3 fibroblasts over 2 h as shown by confocal microscopy at 37 °C (following cell fixation): a) polymer **12** (10 μM); b) polymer **13** (10 μM).

sideration the structural features of dendritic polymers and the results discussed above, the mechanism proposed for a model liposomal system (Figure 10),[13] is also, in principle, applicable to cells. The guanidinium groups adhere to the membrane following their interaction with the negatively charged groups (e.g. phosphate, sulphate, carboxylate), and strong binding is induced by multivalent effects[13,21,22] due to the target membrane structure (step 1). Following charge neutralisation, the adhered guanidinylated dendrimers become less polar and therefore prone to entering the hydrophobic bilayer. The adaptive solubility behaviour of dendrimers, that is, their "chameleon behaviour" as coined by Paleos et al.,[27] results in a change of conformation leading to exposure of the dendrimers hydrophobic interior to the bilayer medium (step 2). As a result, the guanidinylated derivatives become appreciably hydrophobic and consequently appropriate for transport through the hydrophobic liposomal membrane (step 3). Finally the dendrimers are translocated to the liposomal interior (step 4). Notably, endocytosis plays a major role in cellular systems, where internalisation will additionally be dependent on the membrane potential, as discussed above.

Cell Transfection by Guanidinylated Dendrimers

Dendritic polymers have been extensively employed as vectors for delivering bioactive compounds into cells.[36–43] In addition, cationisation of dendritic polymers and subsequent interaction with genetic material (DNA, plasmids) leads to the formation of complexes, which can be applied for cell transfection. It has to be noted, however, that these synthetic, nonviral gene delivery systems exhibit lower efficiency in comparison to viral vectors.[44–47] Structural modifications have been applied to dendritic polymers to increase their transfection efficiency. Among the most effective of these modifications is the introduction of cell-penetrating moieties to the carrier for enhanced intracellular delivery of the genes. The efficacy of this strategy will be illustrated through two typical paradigms employing guanidinylated dendrimers as gene delivery systems.

A poly(amidoamine) dendrimer functionalised with arginine (PAMAM-Arg) has been synthesised,[48] in which the PAMAM surface was covered with L-arginine moieties (Figure 11). The introduction of arginine on the PAMAM surface greatly en-

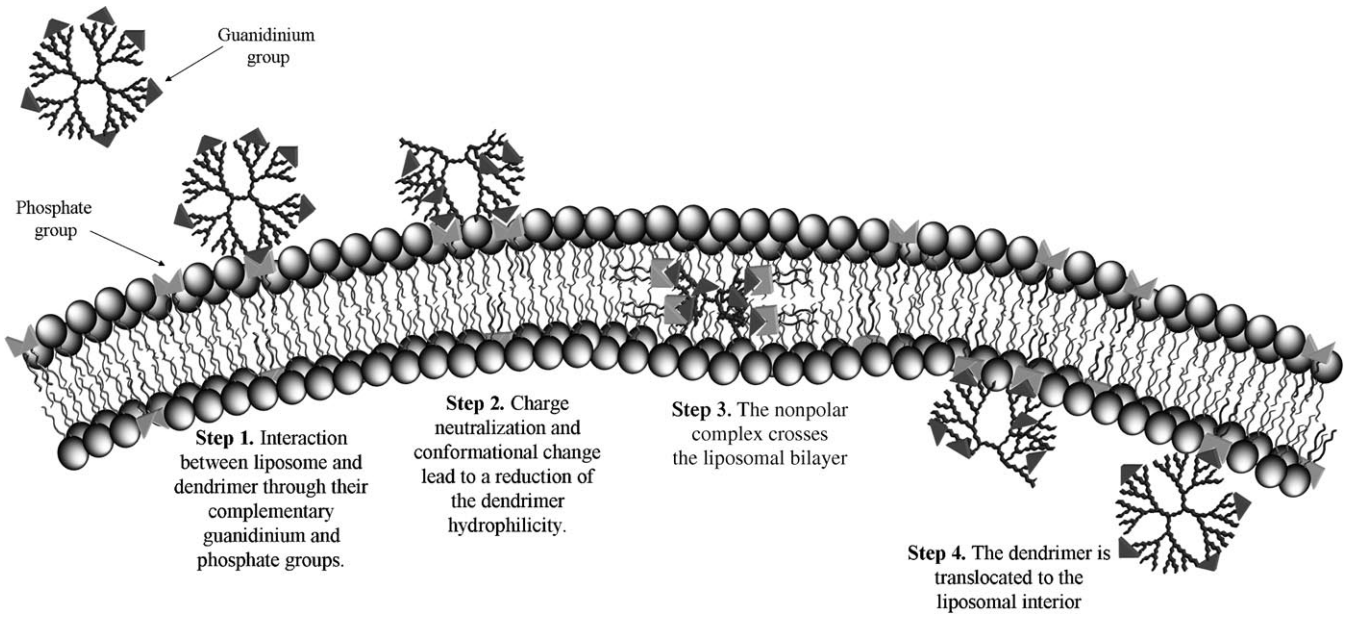


Figure 10. Proposed mechanism for the transport of a guanidinylated dendrimer through a model liposomal membrane.

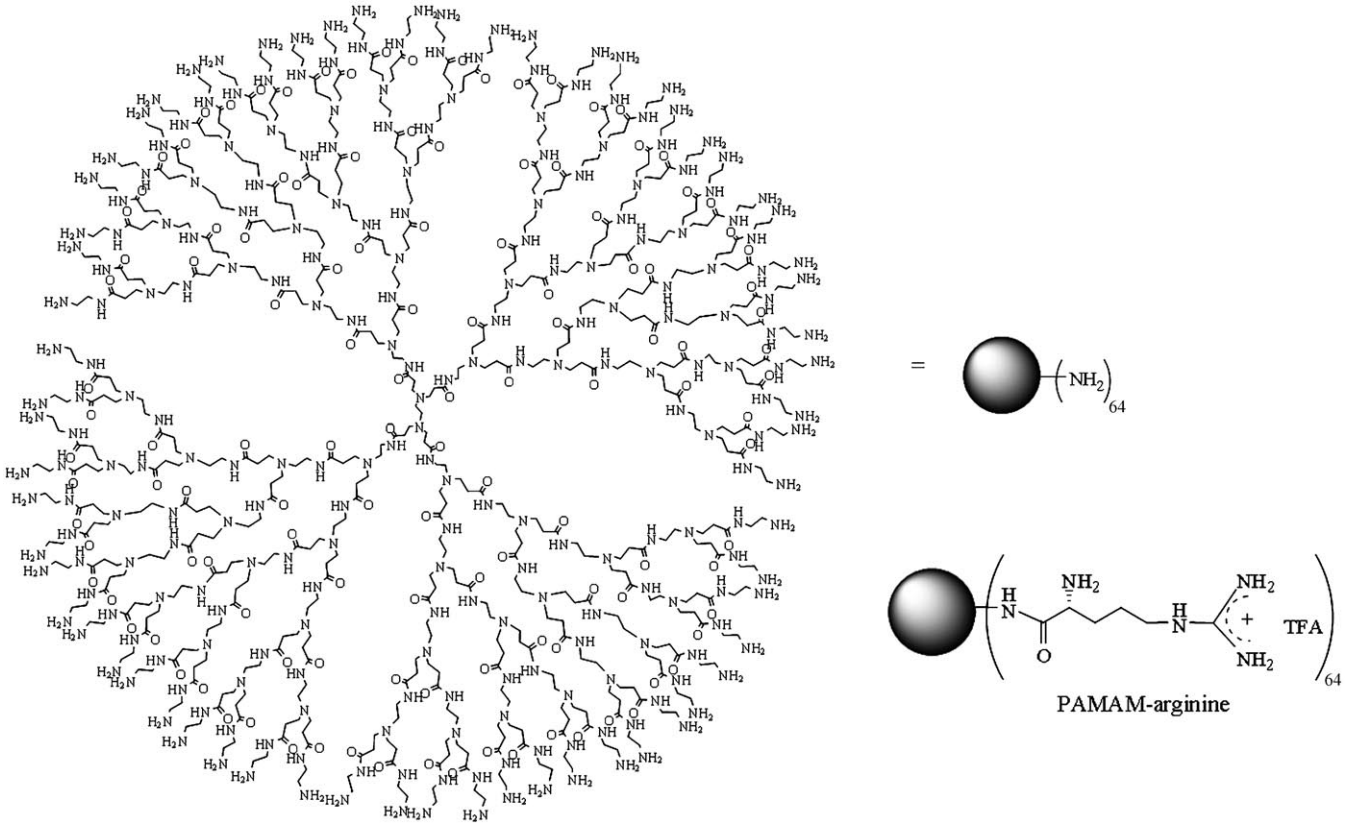


Figure 11. Arginine functionalised PAMAM dendrimers.

hanced cell transfection efficiency following complexation with plasmid DNA when compared with the unmodified PAMAM dendrimer. The transfection efficiency of the guanidinylated complex was comparable to that of poly(ethylene imine) hyperbranched polymer (PEI) in HepG2 and primary rat vascular smooth muscle cells, while it was greater than the transfection efficiency of both PEI and lipofectamine in neuro 2 A cells. L-Lysine-grafted PAMAM (PAMAM-Lys) was used as a control, showing slightly better transfection efficiency than PAMAM in HepG2 cells, though no notable difference between the two was observed in primary cells. Arginine functionalised PAMAM can be conveniently synthesised and possesses high transfection efficiency with relatively low cytotoxicity. These properties render PAMAM–Arg a promising nonviral transfection vehicle. Equally effective and nontoxic is the arginine-conjugated poly(propylene imine) dendrimer gene vector as recently reported.[49]

In a related study,[50] fourth generation DAB was completely or partially functionalised with guanidinium groups. The remaining toxic primary amino groups of the partially guanidinylated dendrimers were reacted with propylene oxide affording the corresponding hydroxylated derivatives. Five derivatives were investigated, four bearing 6, 12, 24, or 32 guanidinium groups and one nonguanidinylated (Figure 4). These guanidinylated dendrimers formed complexes with plasmid DNA affording the corresponding dendriplexes. Transfection efficiency was assessed employing the HEK 293 and COS-7 cell lines, and the serum effect was studied in HEK 293 cells. It was found that full replacement of the primary amino groups with the hydroxylated moieties resulted in complete loss of transfection efficacy. On the contrary, guanidinylation of the parent dendrimer resulted in significant enhancement of the transfection efficiency. This enhancement depended on the number of guanidinium groups per dendrimer, the cell line used, and the presence or absence of FBS. The fully guanidinylated dendrimer exhibited the best transfection efficiency under all the conditions investigated. This may be attributed to the fact that the phosphate groups of DNA compete with the cell membrane phosphates for neutralisation of the dendrimer guanidinium groups, and therefore complete or significant guanidinylation is required for effective transfection. It was also found that the derivative with 12 guanidinium groups exhibited the lowest toxicity, however this may be due to decreased cell internalisation (and hence lower transfection efficiency) because of the smaller number of surface guanidinium groups. In short, functionalisation leads to dendrimeric derivatives that combine satisfactory transfection efficiency with minor concomitant cytotoxicity.

Outlook

In summary, the role of the guanidinium functional group is crucial for dendritic polymers in inducing and facilitating their transport through liposomal and cell membranes. However,

other structural features fine-tune these transport properties and even determine their subcellular destinations. Specifically, an ideal dendritic molecular transporter needs to possess the optimised size of dendritic scaffold and degree of guanidinylation, flexibility of the guanidinium moiety, and an appropriate balance between hydrophilic and hydrophobic moieties on the dendritic surface. Design of novel guanidinylated dendritic systems that can be directed, together with their load, to specific subcellular organelles is the main future challenge. Towards this end, efforts should be made in establishing the structural characteristics that will allow the guanidinylated dendritic polymers to localise in specific subcellular compartments.

Acknowledgements

The work was partially supported by the GSRT, Greek Ministry of Development (Greece) under the ENTEP, 04EP 61 program, as well as by the EU under the 'NMP' Integrated Project, NMP4-CT-2006-026723

Keywords: cell transfection · drug delivery · guanidinylated dendrimers · liposomes · molecular transporters

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Received: June 25, 2008
Revised: August 27, 2008
Published online on November 4, 2008

