Interaction and Transport of Poly(L-lysine) Dendrigrafts through Liposomal and Cellular Membranes: The Role of Generation and Surface Functionalization

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Two generations of poly(L-lysine) dendrigrafts (DGLs) were studied with regard to their ability to interact with and translocate through liposomal and cellular membranes. Partial guanidinylation of the surface amino groups of the starting dendrigrafts afforded the guanidinylated derivatives whose membrane translocation properties were also assessed. Mixed liposomes, consisting of dihexadecyl phosphate, phosphatidylcholine, and cholesterol, were employed as model membranes, while A549 human lung carcinoma cells were used for cellular uptake studies. At high surface group/liposomal phosphate molar ratios and depending on the structure of the DGL, the interaction led to aggregation. Dendrigraft liposomal internalization was achieved, however, at low molar ratios. Thus translocation of the second generation dendrigrafts was rather limited at 25 °C, which, however, was enhanced when the bilayer was in the liquid−crystalline phase. In contrast, third-generation counterparts exhibited minor translocational ability. Furthermore, the introduction of a guanidinium group to dendrigrafts was found to enhance their transport through liposomal membranes. On the other hand, cellular uptake by A549 cells was monitored up to 3 h incubation time via fluorescence registration employing fluorescein-labeled dendrigrafts. The efficiency of dendrigraft internalization was enhanced by the presence of the guanidinium groups, while DGLs were preferentially localized in the nucleus and nuclear membrane, as revealed by fluorescence microscopy.

Introduction

Dendrimers are highly branched, monodisperse, and symmetrical macromolecules of nanosize dimensions consisting of a central core, branching units, and terminal functional groups.[1−3] This architecture favors the formation of nanocavities, the environment of which determines the dendrimers solubilizing or encapsulating properties while the external groups primarily characterize their chemical behavior, including targeting properties. On the other hand, hyperbranched polymers[4−6] are also branched exhibiting nanocavities, but, in contrast to dendrimers, they are nonsymmetric and polydispersed. The latter polymers are, however, conveniently prepared, and therefore they are less expensive compared to dendrimers, which are prepared by tedious multistep reaction schemes. Furthermore, dendrigraft polymers,[7−9] in contrast to dendrimers, which are synthesized by conventional monomers, are prepared by employing reactive oligomers or polymers through repeated protect−deprotect grafting steps. By analogy with dendrimers, each grafting step is referred to as a generation.[9] In this manner, dendrigrafts have generally larger structures than dendrimers, grow much faster, and amplify surface groups more intensively as a function of generational development. Dendrimeric, hyperbranched, and dendrigraft polymers are collectively called dendritic polymers, a common characteristic of which is the accumulation of functional groups on their external surface. The external groups of dendritic polymers can be modified, providing diverse functional materials[10] that are employed in various applications, including drug delivery. In that sense, most of the commercially available or custom-made dendritic polymers have been appropriately functionalized to be used as drug delivery systems,[11−15] as gene delivery vectors,[16,17] or as drugs on their own.[18]

Membrane transport is crucial for drug delivery, and dendrimer translocation has recently been addressed employing either cells[19,20] or model liposomal systems.[21,22] For enhancing liposome−dendrimer or cell−dendrimer adhesion, the external amino groups of poly(propylene imine) dendrimers have been guanidinylated in order to strongly interact with the phosphate moieties of liposomal or anionic groups on the surface of cell membranes as a result of combined hydrogen-bonding and electrostatic forces.[23,24] The idea of introducing these guanidinylated poly(propylene imine) dendrimers[21,22] as transporting agents for drug delivery stems from the analogous behavior of arginine-rich peptides,[25−35] for which an appropriate number of guanidinium groups proved beneficial for enhancing their translocation ability.

On the basis of the results obtained with guanidinylated poly(propylene imine) dendrimers, an "interactive transport" mechanism was proposed,[22] which, in the present study, constitutes the basis for investigating the transport properties of poly(L-lysine) dendrigrafts (DGLs). In this context, it should be noted that the term *interactive transport* can be defined as the process in which the transport of a chemical entity through the membranes of liposomes or cells is triggered by interaction with these membranes. The above dendrigrafts that have recently been developed are promising candidates for drug delivery applications because of their biodegradability. DGLs consist of

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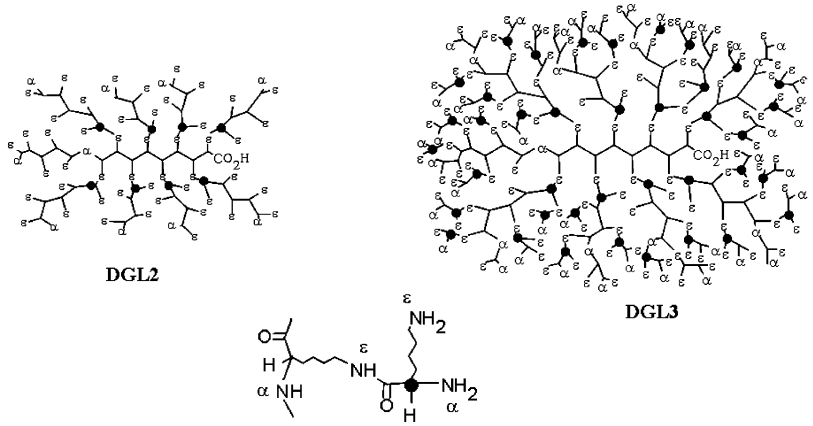

Figure 1. Second- and third-generation DGLs (DGL2 and DGL3, respectively). The structure of a branch is also shown.

a linear octylysine core moiety acting as a backbone for the polymerization of ϵ-protected lysine *N*-carboxyanhydride, leading to the preparation of various generations of the so-called "poly(L-lysine) dendrigraft" or DGL.[36]

In the present study, two generations of these dendrigrafts were studied with regard to their ability to interact with and, under certain conditions, transverse model liposomal and cellular membranes. In addition, partial surface guanidinylation yielded the corresponding guanidinylated derivatives, whose membrane transport properties were also assessed by the same model liposomal system, consisting of dihexadecyl phosphate (DHP), phosphatidylcholine (PC), and cholesterol (Chol). Moreover, for evaluating whether this interactive-transport mechanism applies to cellular membranes leading to uptake of the dendrigrafts, A549 lung carcinoma cells were employed. In fact, several reports,[37−44] discussed in two reviews,[45,46] refer to analogies between liposome−liposome and cell−cell interactions, while, for the transport through cell membranes, the ability of guanidinium-rich beta-peptides to cross phospholipid bilayers was assessed both in a model liposomal system as well as in Hela cells.[47]

Experimental Section

Materials. Soybean hydrogenated PC (Phospholipon 90H) was purchased from Nattermann Phospholipid GmbH. DHP was purchased from Sigma and used as received. Chol was recrystallized twice from ethanol before use. 1*H*-pyrazole-1-carboxamidine hydrochloride (99%) and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Sigma. Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes. SEPHADEX G-50 was obtained from Pharmacia, while Nucleopore filters of 100 nm pore size (Whatman) were employed for liposome extrusion.

RPMI 1640 without phenol red, fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, phosphate buffered saline (PBS), and trypsin/versene were purchased from Invitrogen, Ltd. (Paisley, UK). 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) and digitonin were purchased from Sigma-Aldrich. Triton X-100 was purchased from Panreac.

Cell Culture. Cells used in this study were the human lung carcinoma cell line A549. The cells were grown in RPMI 1640 with 10% FBS and penicillin/streptomycin at 37 °C in a 5% CO_2-humidified atmosphere. Cells were inoculated into either 96-well plates (2×10^4 cells/100 μL media per well) or 35 mm dishes with 2 cm radius round glass coverslips (5×10^4 cells/2 mL media per well per dish) 24 h before experiments.

Synthesis of DGLs. DGLs were prepared as described in a recent patent.[36] In brief, N$^\epsilon$-TFA-L-lysine-NCA (10 g) prepared according to a method reported in the literature[48] was dissolved in an aqueous H_2O/HCO_3Na solution (pH 6.5)[49] leading, after 30 min, to oligo-N$^\epsilon$-TFA-L-lysine, which precipitates and is isolated by filtration. The protecting group is removed with 150 mL of water−methanol−ammonia solution at pH 11 (15 h at 40 °C). Following partial solvent removal at reduced pressure, the remaining solution was freeze-dried, affording 5 g of first-generation oligo(L-lysine) of DPn = 8 and low polydispersity (1.2). By repeating the same polymerization procedure in the presence of 3 g of first-generation oligo(L-lysine), 6 g of second-generation DGL (DGL2) was obtained (DPn = 48 and polydispersity = 1.3) following deprotection and freeze-drying. The third-generation DGL (DGL3) was obtained (DPn = 123 and polydispersity = 1.4) under the same conditions using 3 g of DGL2 (Figure 1). The structures of DGL2 and DGL3 were established by ^{1}H and ^{13}C NMR.

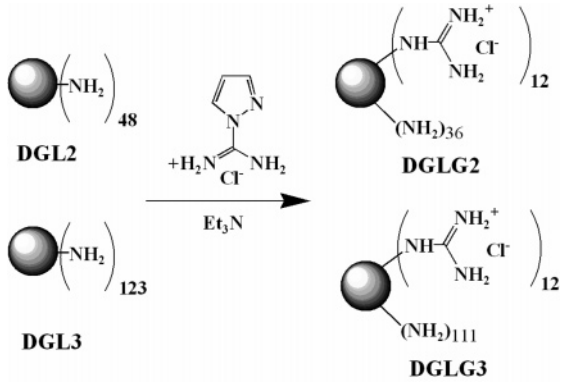

Figure 2. Partial surface guanidinylation of the second- and third-generation DGLs affording the final products DGLG2 and DGLG3, respectively.

^{1}H NMR (500 MHz, D_2O) δ = 4.30 (broad s, NHC*H*CO), 4.05 (s, NHC*H*COOH), 3.35 (s, NH_2CH_2CO), 2.90 (m, NH_2CH_2), 1.50−1.85 (m, β and δ CH_2 of lysine), 1.10−1.50 (m, γ-CH_2 of lysine). ^{13}C NMR (62.9 MHz, D_2O) δ = 172 (*C*O), 56−57 (α-*C*H$_2$ of lysine), 41.5 (NH_2CH_2), 33.5 (δ-CH_2 of lysine), 29 (β-*C*H$_2$ of lysine), 25 (γ-*C*H$_2$ of lysine).

Surface Modification of DGLs. DGLs of the second and third generation were dissolved in freshly distilled methanol, and an excess of DIEA was added followed by the addition of a methanolic solution of 1*H*-pyrazole-1-carboxamidine hydrochloride, as described for an analogous guanidinylation of poly(propylene imine) dendrimers.[22] The mixture was allowed to react for 24 h under argon atmosphere (Figure 2). Following removal of the solvent under vacuum, the crude product was dissolved in water and was subjected to dialysis (mol weight cutoff: 1200) to remove byproducts. Lyophilization afforded the final products (DGLG2 and DGLG3, respectively) and the degree of substitution was determined by inverse-gated ^{13}C NMR. Twelve guanidinium groups were introduced into both generations of DGLs,

yielding the guanidinylated DGLG2 and DGLG3 derivatives. The structures of DGLG2 and DGLG3 were established by ^{1}H and ^{13}C NMR.

^{1}H NMR (500 MHz, D_2O and DMSO-d_6) δ = 7.80−8.00 (broad s, N*H* of guanidinium group), 6.90−7.6 (broad d, NH_2^+), 4.30 (broad s, NHC*H*CO), 4.05 (s, NHC*H*COOH), 3.35 (s, NH_2CH_2CO), 3.00−3.20 (m, $CH_2NHC(NH_2^+)_2$), 2.90 (m, NH_2CH_2), 1.50−1.85 (m, β and δ CH_2 of lysine), 1.10−1.50 (m, γ-CH_2 of lysine). ^{13}C NMR (62.9 MHz, D_2O) δ = 172 (*C*O), 159 ($NHC(NH_2^+)_2$), 56−57 (α-*C*H_2 of lysine), 43.5 ($CH_2NHC(NH_2^+)_2$), 41.5 (NH_2CH_2), 33.5 (δ-CH_2 of lysine), 30.5 (CH_2-$CH_2NHC(NH_2^+)_2$), 29 (β-*C*H_2 of lysine), 25 (γ-*C*H_2 of lysine).

FITC-Labeled DGLs. The interaction of the DGLs (DGL2, DGLG2, DGL3, and DGLG3) with the liposomal and cellular membranes was monitored by fluorescence experiments employing FITC-labeled dendrigrafts. The latter were prepared by the interaction of the dendrimeric compounds with a 10% molar excess of FITC in distilled methanol for 24 h at room temperature and in the dark to avoid photobleaching of the probe. Following the removal of the solvents under vacuum, the resulting products were dissolved in water, and the unreacted FITC was removed by dialysis against water (mol. weight cutoff: 1200). Subsequent lyophilization afforded the desired products FDGL2, FDGLG2, FDLG3, and FDGLG3, respectively. ^{1}H NMR established that approximately one fluorescein moiety was attached per molecule of the dendrigrafts.

Preparation of Liposomes. Large unilamellar liposomes of about 100 nm diameter were prepared using the extrusion method employing the protocol previously described.[22,44] In a typical experiment, for the preparation of a 4 mL liposome dispersion, 2.19 mg (0.004 mmol) of DHP, 60.80 mg (0.076 mmol) of PC, and 14.71 mg (0.038 mmol) of Chol were used.

Characterization. Liposomes were characterized by dynamic light scattering (DLS). The interaction of liposomes with DGLs and their guanidinylated counterparts was indirectly monitored by DLS, optical microscopy, and fluorescence experiments.

For the size determination of liposomes, an AXIOS-150/EX (Triton Hellas) DLS apparatus equipped with a 30 mW He−Ne laser emitting at 658 nm and an Avalance detector at 90° was employed. In a typical experiment, 50 μL of a liposomal dispersion, after the addition of increasing quantities of dendrimeric compounds, was diluted to 0.8 mL of phosphate buffer. Ten measurements were collected per experiment, and the results were averaged.

Zeta-potential measurements for both the original and guanidinylated dendrigrafts were performed employing a ZetaPlus (Brookhaven Instruments Corporation), yielding 36.0 ± 1.9 mV for DGL2, 51.92 ± 0.83 mV for DGL3, 54.72 ± 1.82 mV for DGLG2, and 69.77 ± 1.56 for DGLG3. These results represent the mean values of 10 zeta-potential measurements collected for each solution.

Fluorescence measurements employing a Cary Eclipse spectrophotometer (Varian) were performed using calcein-loaded liposomes to investigate whether the interaction of dendrigrafts with the liposomal membrane can lead to leakage of calcein from the aqueous interior of liposomes to the bulk aqueous phase. The preparation of calcein-loaded liposomes was achieved by hydration of the lipid film with a 40 mM solution of calcein in phosphate buffer, as previously described.[50,51] Experiments were performed by incubating the samples for 20 min at 25 °C or 65 °C after the addition of dendrigrafts at various surface group/phosphate (of liposomes) molar ratios. The calcein leakage was determined as described in earlier reports.[44]

In order to investigate the ability of the dendrigrafts to penetrate the liposomal membrane, fluorescence quenching experiments were also performed employing I^- as a quencher of fluorescein.[52] The quenching constants of FITC-labeled polymeric derivatives added in liposomal dispersions were determined upon the addition of a NaI solution. Thus, 100 μL of the liposomal dispersion was diluted to 2.5 mL with phosphate buffer. FITC-labeled dendrigrafts at a certain surface group/phosphate molar ratio were added, and the dispersions were titrated with a 20% (w/v) NaI solution. Samples were incubated for 20 min at 25 °C and 65 °C, and the fluorescence intensity was registered (excitation, 490 nm emission, 520 nm). The quenching constants K_{SV} were determined by a linear regression employing the Stern−Volmer equation, as described in our previous work.[22]

Fluorescence quenching experiments were also performed using NaI-loaded liposomes. Loading was performed by hydration of the lipid film with phosphate buffer containing 0.142 M NaI. Removal of the nonencapsulated NaI was performed by column chromatography using SEPHADEX G-50. To avoid leakage of NaI due to osmotic effects, phosphate buffer containing 0.142 M NaCl was used as the eluent. FITC-labeled dendrigrafts up to a 5% surface group/phosphate molar ratio were added to the resulting dispersions. Fluorescence intensity was measured immediately following mixing and after incubating the samples for 20 min at 25 °C or 65 °C. The F_0/F ratio was used to evaluate the percentage of dendrigraft translocating the bilayer, where F_0 is the fluorescence intensity of the sample immediately after mixing, and F is the intensity after each incubation period. The translocation ability was determined as in our previous reports.[22]

Fluorescence Microscopy in Cells. An Olympus BX-50 microscope coupled with an Olympus DP71 digital color camera was used to obtain both bright-field as well as fluorescence microscopy images of cells incubated with FITC-labeled dendrimers. Fluorescence excitation was facilitated by a Mercury USH 102D lamp (Ushio Electric), while FITC emission was imaged through an Olympus UPLFLN40×/0.75 objective, using a U-M41001 FITC/EGFP/Biodipy/Fluo3/Dio filter (Chroma Technology Corp). Cells inoculated on coverslips in 35 mm Petri dishes, as elaborated in the cell culture section, were incubated for 1.5 and 3 h with 20 μM FDLG2 and FDGLG2. These concentrations were found to be relatively nontoxic by standard XTT assays, performed immediately following incubation.

Cytotoxicity Assessment. Cells inoculated in 96-well plates, as explained earlier, were incubated with various concentrations of the dendrigrafts (1, 10, 20, 50, 100 μM) with/without FBS for 3 h, while control cells were allowed in media not containing any polymeric derivatives. Following incubation, the cells were washed twice in PBS and media replaced with fresh RPMI 1640 containing 10% FBS without phenol red. XTT assays were performed immediately following incubation to determine the dendrigraft toxicity.

Mitochondrial redox function was assessed by the XTT assay. This relies on the reduction of the tetrazolium salt to a formazan formation by mitochondrial matrix reductive enzymes. In non-redox competent mitochondria (e.g., uncoupled mitochondria or dead cells), no formazan formation occurs. The assay was performed by adding 50 μL of XTT salts (1 mg/mL) and 1 μL of phenazine methosulfate (0.383 mg/mL) to 100 μL of cell media and incubating at 37 °C in a 5% CO_2 atmosphere for 2 h. The endpoint absorbance measurement was performed at 492 nm in a Fluostar Galaxy plate reader (BMG Labtechnologies). Blank values measured in wells with no cells were subtracted.

Fluorescence Time-Course Experiment. Dendrigraft loading of A549 cells was studied through FITC fluorescence. Second-generation dendrigrafts coupled to FITC (FDGL2 and FDGLG2) were added to cell media with/without 10% FBS at a 20 μM final concentration. The cells were washed twice with PBS, and fluorescence was registered versus incubation time from 0 to 3 h in a Fluostar Galaxy plate reader (BMG Labtechnologies) with the excitation wavelength set to 492 nm and emission set to 520 nm.

The fluorescence time-course uptake study was performed in intact cells, in their supernatant, as well as following selective plasma membrane permeabilization with 4 μM digitonin (5 min) and complete disruption of intracellular membranes with Triton X-100 (0.25%). Blank values measured in control cells (not incubated with dendrigrafts) were subtracted in each case.

Results and Discussion

The interaction and translocation of biodegradable DGLs and of their partially guanidinylated derivatives were initially

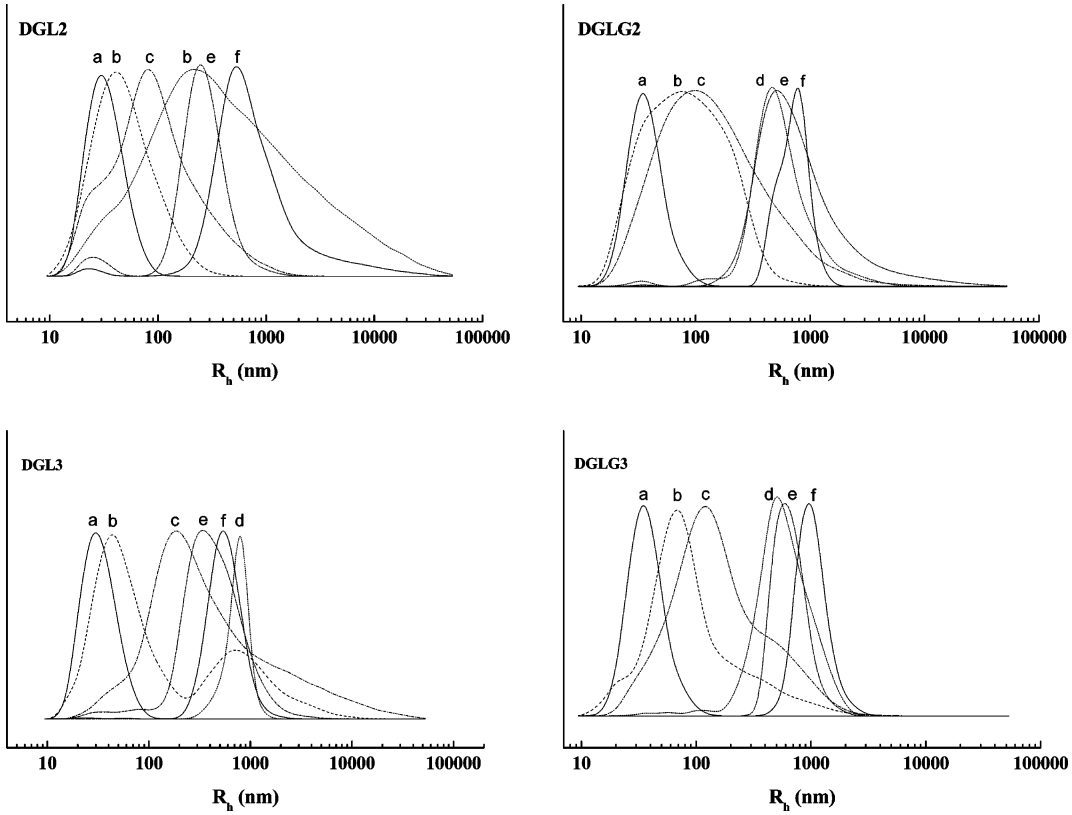

Figure 3. Size distribution of liposomal dispersions following the addition of DGLs and their guanidinylated derivatives, and subsequent incubation at 25 °C for 20 min at increasing surface group/phosphate molar ratios: 0% (a), 5% (b), 20% (c), 50% (d), 70% (e) and 100% (f).

investigated performing experiments with liposomal model membranes. Experiments with cells followed at a second stage. Interaction of dendrigrafts with liposomes was indirectly assessed by DLS measurements, as will be discussed immediately below, while translocation was monitored through fluorescence. In addition, selected dendrigrafts from those employed with liposomes were initially tested for their cell toxicity with A549 lung carcinoma cells prior to incubation with the same cells, for investigating whether translocation of these dendrigrafts leads to efficient in vitro uptake.

DLS Studies. Liposomes interacted effectively with all dendrigrafts, leading to aggregate formation. Their size distribution is shown in Figure 3. For DGLG2, 25% of the surface amino groups of DGL2 were guanidinylated, and aggregates with diameter sizes up to 200 nm were formed even at low surface group/phosphate molar ratios (0.2), while, for the DGL2, interaction was weaker at low molar ratios because of a lack of strong guanidinium−phosphate binding.[23,24] For higher molar ratios (>50%) for both dendrigrafts, aggregates with diameter size of 2 μm predominate. In the case of DGLG3, only 10% of the surface amino groups of DGL3 were modified, and the observed aggregation profiles between the unmodified and the guanidinylated dendrigrafts were almost similar. From the DLS data it is evident that, at low surface group/phosphate molar ratios (5%), the size increase is minor compared to that of the initially prepared liposomes, indicating that the dendrigrafts do not cause extensive adhesion of liposomes.

Toxicity of Dendrigrafts. The ultimate objective of dendrigrafts and of their guanidinylated derivatives is their application as drug delivery systems, and therefore toxicity experiments need to precede cellular transport experiments in order to determine dendrimer sublethal doses. For this purpose, the toxicity of dendrigrafts was investigated by employing A549 human lung carcinoma cells by use of a standard XTT assay immediately following 3 h of incubation. The results appear in Figure 4. From that figure it can be seen that DGL2 was nontoxic to the cells for incubation with 10, 20, and 50 μM dendrigraft concentration, while 30% cytotoxicity was observed for incubation with 100 μM of the compound. The third-generation counterpart, DGL3, proved quite toxic to the cells. The cell death values for incubation with 10, 20, 50, and 100 μM DGL3 concentration were 60%, 85%, 85%, and 85% in the presence of FBS and 70%, 85%, 90%, and 90% in the absence of FBS, respectively. With regard to the corresponding guanidinylated compounds, DGLG2 exhibited toxicity following 3 h of incubation with 50 and 100 μM (<10% survival in both cases) in the presence of FBS, while incubation without FBS also incurred a 30% cell toxicity at 10 μM incubation and a 40% toxicity at 20 μM. The third-generation guanidinylated dendrigraft exhibited high toxicity following 3 h of incubation at 10 and 20 μM (~5% survival in both cases ±10% FBS) but was nontoxic following incubation at 1 μM.

Liposomal Stability. The effect of dendrigrafts, both original and guanidinylated, on the liposomal bilayer stability was assessed by employing calcein-loaded liposomes as described in detail in a previous report.[50,51] Any disruption of the bilayer would result in calcein leakage from the aqueous core of liposomes to the bulk aqueous phase, leading to a strong fluorescence signal. The phase transition temperature, T_m, from ordered to disordered liquid crystalline phases of the liposomal bilayer, was found at 53 °C.[22] For this reason, experiments were carried out at 25 and 65 °C, that is, well below and well above the phase transition of the liposomal membranes. At room temperature, the concomitant leakage was negligible (~1.5%) at a 50% surface group/phosphate molar ratio. The fluid phase of the bilayer had a minor effect on the bilayer stability, resulting in a slightly increased leakage (~2.5%) at a 50% surface group/phosphate molar ratio. This indicates that the interaction of the

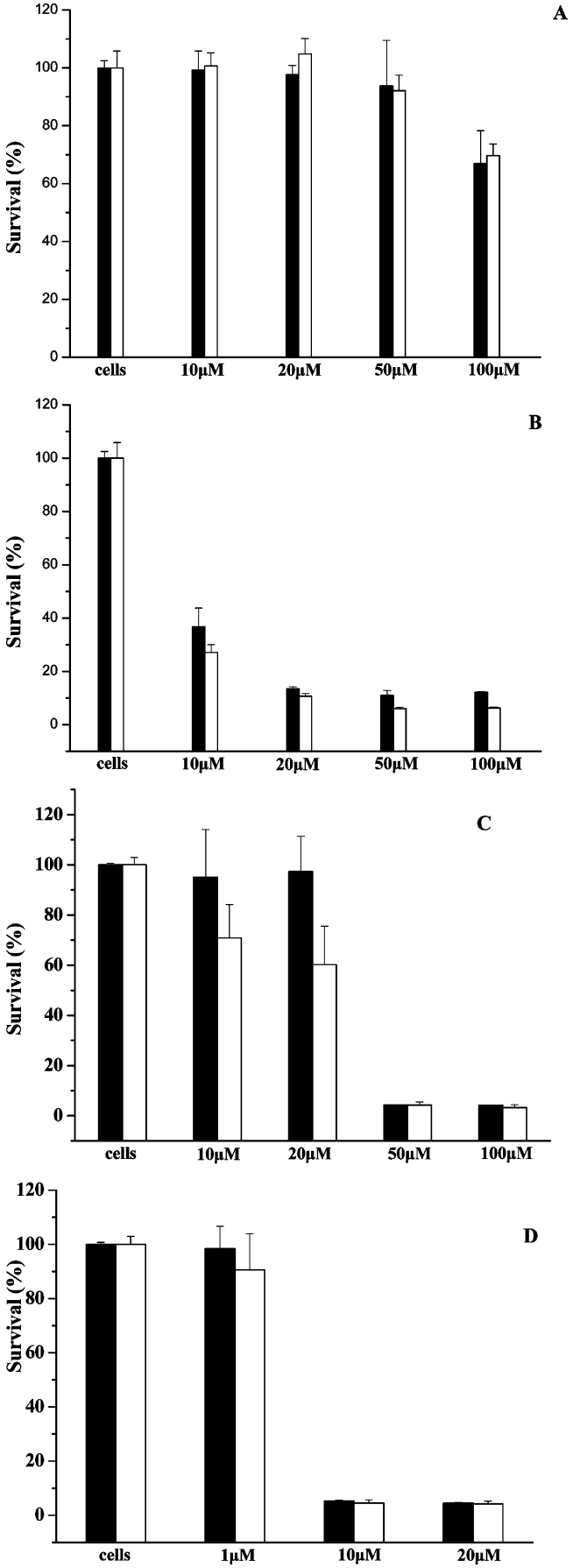

Figure 4. Cell viability following 3 h of incubation at various concentrations of DGL2 (A), DGL3 (B), DGLG2 (C), and DGLG3 (D) in the presence of 10% FBS (black columns) and without FBS (white columns).

Table 1. Calculated K_{SV} of FITC-Labeled Dendrigrafts in the Absence of Liposomes in Isotropic Media or Following Their Interaction with Liposomes at 5% Surface Group/Phosphate Molar Ratio after Incubation for 20 min at 25 and 65 °C

	in isotropic media	in the presence of liposomes	
dendrigraft	K_{SV} at 25 °C	K_{SV} at 25 °C	K_{SV} at 65 °C
FDGL2	40.5	17.5	5.5
FDGLG2	28.0	6.5	5.0
FDGL3	5.5	1.0	1.0
FDGLG3	28.5	5.5	4.5

Table 2. Calculated Translocation of FITC-Labeled DGLs Following Interaction with NaI-Loaded Liposomes at 5% Surface Group/Phosphate Molar Ratio after Incubation for 20 min at 25 and 65 °C

dendrigraft	translocation (%) at 25 °C	translocation (%) at 65 °C
DGL2	7.5	57.0
DGL3	3.0	4.0
DGLG2	10.5	78.5
DGLG3	2.0	9.0

original and guanidinylated dendrigrafts with the liposomal membrane does not disrupt its lipid bilayer.

Liposomal Membrane Transport. Florescence quenching experiments employing FITC-labeled derivatives and I^- as a quencher showed a reduction in the quenching constants K_{SV} calculated in the absence and presence of liposomes. Experiments were performed at a 5% surface group/phosphate molar ratio, since, at this molar ratio, minor aggregation of the liposomes is observed. The results are given in Table 1. It is obvious that the presence of liposomes caused a significant reduction in K_{SV}, which was further reduced for samples incubated at 65 °C. At this high temperature, the bilayer membrane is in its liquid crystalline phase, in which the lipids possess higher mobility. These results indicate less effective quenching and a reduction of the dendrigraft concentration in the bulk aqueous phase. Therefore dendrigrafts have either accumulated in the liposomal bilayer or penetrated it, residing in the aqueous liposomal core.

Translocation of the dendrigrafts across the liposomal bilayer was confirmed with fluorescence experiments employing NaI-loaded liposomes. Quenching of FITC-labeled dendrigrafts can only occur if they reach the liposomal aqueous core. The results are presented in Table 2. Translocation of FDGL2 and FDGLG2 was rather limited at 25 °C; however, it was enhanced when the bilayer was in the liquid−crystalline phase. On the other hand, FDGL3 and FDGLG3 had minor translocational ability. The enhanced internalization of the second-generation dendrigrafts with respect to their third-generation counterparts could be attributed to their smaller size (hence a smaller number of surface amino groups) and the flexibility of their branches.

In line with guanidinylated poly(propylene imine) dendrimers,[22] the introduction of a guanidinium group in dendrigrafts was also found to enhance their transport through membranes. It is evident that the steps of the previously proposed mechanism[22] also apply to dendrigrafts. Thus, guanidinium groups (and ammonium groups to a lesser extent) adhere to liposomal membranes following their interaction with the negatively charged phosphate group, a process enhanced by multivalent effects.[53−55] The adhered dendrigrafts or their guanidinylated derivatives become less polar due to charge neutralization, and are therefore more prone to enter the hydrophobic bilayer. Synergistically to charge neutralization, the adaptive solubility behavior ("chameleon behavior" established for dendrimers) of

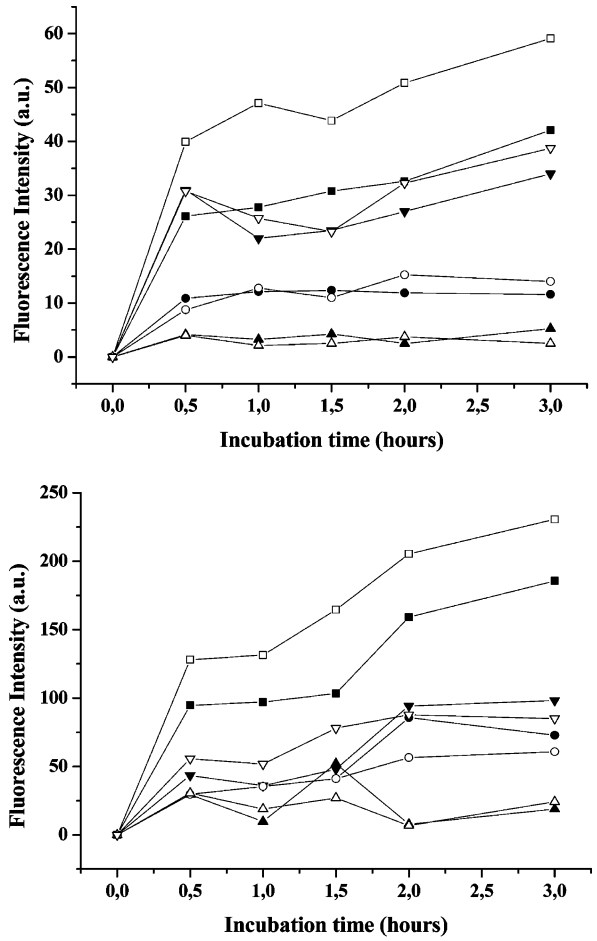

Figure 5. Fluorescence intensity, following incubation of cells with FDGL2 (top) and FDGLG2 (bottom), of intact cells after incubation with 20 μM dendrigrafts (squares), the cell supernatant (circles), the supernatant following treatment with 4 μM digitonin for 5 min (up triangles), and cell lysates following the addition of TRITON X-100 (down triangles). Solid symbols represent cells incubated in the presence of 10% FBS, while open symbols represent cells incubated without FBS.

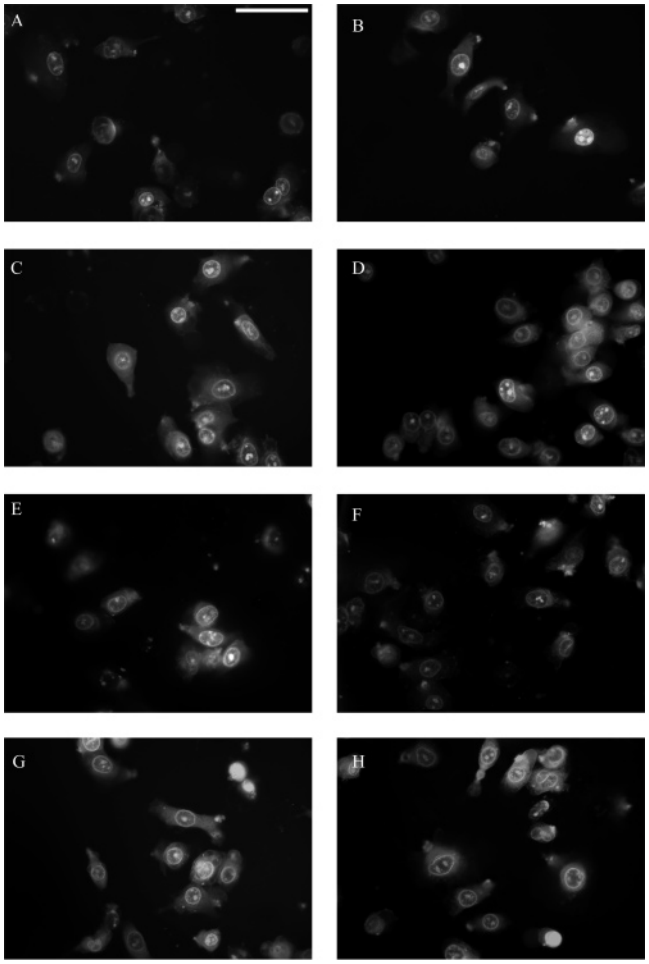

Figure 6. Fluorescence imaging of A549 cells following incubation with FDGL2 (A,B) and FDGLG2 (C,D) for 1.5 h and FDGL2 (E,F) and FDGLG2 (G,H) for 3 h. Incubation was performed either in the presence of 10% FBS (left) or in the absence of FBS (right). The bar represents 200 μm.

the dendrigrafts begins to function, rendering them even more hydrophobic and, consequently, appropriate for transport through the hydrophobic liposomal membrane.

Cellular Membrane Transport. Fluorescence time-course experiments with A549 cells revealed that the second-generation dendrigraft was efficiently internalized. From Figure 5 it is evident that the fluorescence signal from cells incubated with FDGLG2 is approximately 4 times that of cells incubated with the same concentration of FDGL2, both in the presence and absence of FBS. It should be noted, however, that the fluorescence intensity values were significantly higher in cells incubated with dendrigraft in the absence of FBS, despite the ~40% cytotoxicity associated with the guanidinylated compounds. Given the fact that cell populations were similar in all cases, that experimental conditions were otherwise kept the same, and that the fluorescence of the two compounds (FDGL2 and FDGLG2) is comparable in equimolar aqueous solutions ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm, $I_{FDGL2} \sim 1.1 \times I_{FDGLG2}$), there was at least a 4-fold internalization ability in the case of FDGLG2 incubation. This can be attributed to the introduction of the guanidinium groups to the dendrigraft surface which interact effectively with the available anionic polar groups located on the outer cell membrane (e.g., phosphate, carboxylate, etc.), in a fashion analogous to that of liposomal membranes, despite the complexity of cellular membranes. It is also clear from Figure 5 that, in both cases, fluorescence rapidly increased up to 0.5 h of incubation, indicating a continuous upload of the dendrimers during that period. The rate of dendrigraft uptake diminished after that initial period and up to 3 h of incubation. This trend was partly followed in the case of fluorescence registration from the cell supernatant, although the rate of dendrimer efflux from the cells seemed to remain constant after 0.5 h of incubation, suggesting steady-state conditions reached at that time point. Selective plasma membrane permeabilization by digitonin revealed a negligible amount of cytoplasmic dendrigraft in the case of DGL2, while a slightly higher cytosolic localization was observed in the case of DGLG2. Fluorescence registration following complete cell rapture by the addition of Triton X-100 (0.25%) followed the pattern of intact cells, demonstrating the efficient localization of the dendrigrafts in internal cell organelles and, more specifically, in the nuclear organelles and the nuclear membrane, as shown by fluorescence microscopy in Figure 6. The third-generation derivatives were not tested for their cell loading ability because of their high toxicity, as discussed earlier (Figure 4).

Subcellular Localization of Dendrigrafts. Cells were seeded on round glass coverslips in 35 mm Petri dishes. The cells were incubated with 20 μM FDGL2 and FDGLG2 for 1.5 and 3 h in the absence and presence of FBS and subsequently imaged for FITC fluorescence as detailed earlier. The four images on the left in Figure 6 depict cells incubated with 10% FBS, while the four on the right depict cells incubated in the absence of FBS.

The four top images show cells incubated with dendrigrafts for 1.5 h, while the four bottom ones represent cell incubation with dendrigrafts for 3 h. These images further confirm our findings from the fluorescence uptake time-course study. All dendrigrafts very efficiently localize in the cell nucleoli and nuclear membrane, while, in the case of the guanidinylated compounds (C,D,G,H), there is a more pronounced cytosolic localization. This most probably indicates a difference in the transport mechanism in the two cases. In principle, images acquired from cells incubated in the absence of FBS (B,D,F,H) exhibited more intense fluorescence. The same can be deduced for images obtained from the guanidinylated compounds (C,D,G,H) in comparison to those from their unmodified counterparts (A,B,E,F). This difference in fluorescence intensity between the unmodified and guanidinylated compounds is actually more pronounced than what is shown in Figure 6 since image acquisition was performed at different exposure times for the two groups (18 ms for the guanidinylated dendrigrafts and 28 ms for the unmodified ones). Once again, this difference can be attributed to the functionality of the guanidinium moiety and its affinity to the various anionic groups resident in the outer cell membrane.

Conclusions

The interaction of DGLs and their guanidinylated counterparts with liposomes induced their interactive transport, depending on the generation and phase organization of the lipid bilayer. Thus, the translocational ability of the second-generation dendrigrafts was more efficient compared to that of the third generation, apparently because of less constraints imposed by the size, the charge, and the flexibility of the branches. Cellular uptake by A549 cells revealed efficient dendrimer internalization. Specifically, the DGLs were preferentially localized in the nucleus and nuclear membrane, as revealed by fluorescence microscopy. Overall, from the present studies with cells it can be deduced that both original and guanidinylated DGLs of the third generation are quite toxic and probably not optimal for use as drug or gene delivery systems. Conversely, their second-generation counterparts are relatively nontoxic, and, taking into account their biodegradable character, these derivatives, especially the guanidinylated derivatives, are promising candidates for drug and gene delivery. It can be further deduced that liposomal membranes, consisting of PC and Chol (which are both lipids also present in cell membranes) and DHP, behave in an analogous manner to cell membranes as far as the transport of dendrigrafts is concerned. This is in agreement with the assumption that liposomes are characterized as the closest models of cells.

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