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Dendrimer Internalization and Intracellular Trafficking in Living Cells

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Abstract: The ability of dendrimers to cross cell membranes is of much interest for their application in drug and gene delivery. Recent studies demonstrate that dendrimers are capable to enter cells by endocytosis, but the intracellular pathway following their internalization remains controversial. In this study we use confocal fluorescence microscopy to elucidate the intracellular trafficking properties of PAMAM dendrimers with high spatial and temporal resolution in living HeLa cells. Macromolecules of different chemical functionality (neutral, cationic and lipidated), size (from G2 up to G6) and surface charge are investigated and their internalization properties correlated with the molecular structure. Toxicity and internalization data are discussed that allow the identification of dendrimers maximizing intracellular uptake with the minimum effect on cell viability. Time-lapse imaging and colocalization assays with fluorescent biomarkers for endocytic vesicles demonstrate that dendrimers are internalized by both clathrin-dependent endocytosis and macropinocytosis and are eventually delivered to the lysosomal compartment. Moreover we analyzed the uptake of dendrimers in additional cell lines of practical interest for therapeutic purposes. These measurements together with a direct comparison with TAT peptides demonstrate that PAMAM dendrimers possess similar properties to these widely used cell-penetrating peptides and thanks to their chemical tunability may represent a valid alternative for drug and gene delivery.

Keywords: Dendrimer; endocytosis; drug delivery; fluorescence; microscopy

Introduction

Dendrimers are very promising tools for therapeutic and diagnostic purposes.[1,2] Their unique treelike structure endows them with many desirable properties such as monodispersity, biocompatibility and tunable chemical properties.[3−6] In particular amine-terminated cationic polyamidoamine (PAMAM) dendrimers were successfully used for a wide range of biomedical applications such as drug[7,8] or gene[9−11] delivery, or as contrast agents.[12,13]

Recently a few groups reported the ability of PAMAM dendrimers to bind with high affinity the surface of cell membranes and be internalized by endocytosis.[14] The identification of the intracellular route following endocytosis, however, remains controversial. Endocytosis can take place with different mechanisms: macropinocytosis, clathrin-dependent endocytosis, caveolae and clathrin- and caveolin-independent pathways.[15] In light of drug-delivery applications it is important to consider that these intracellular

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pathways can imply distinct chains of biochemical events. For instance in the case of macropinocytosis and clathrin-dependent endocytosis vesicles undergo a gradual acidification while in caveolae the pH remains neutral.

Existing reports based on flow cytometry measurements (using specific inhibitors of endocytic pathways) and immunostaining lead to different results in the elucidation of the intracellular trafficking of PAMAM dendrimers.[16−18] Moreover these techniques can provide quantitative information about dendrimer internalization but do not allow a direct visualization of endocytic processes in living cells since they usually require sample fixation.

Here we address this issue by means of fluorescence imaging and report on the intracellular trafficking properties of PAMAM dendrimers in living HeLa cells. Exploiting the high spatial and temporal resolution of confocal fluorescence microscopy and its high biocompatibility we were able to monitor the internalization process and the subsequent intracellular trafficking in living cells obtaining information about the dynamical aspect of the process and avoiding complications related to cells fixation.

Dendrimers with different surface chemical properties (neutral, cationic and lipidated) were studied in order to dissect the impact of distinct physicochemical properties on dendrimer−cell interactions and to identify the more promising structure for biomedical applications. In particular we focused our attention on cationic dendrimers: four different structures varying in size (from G2 up to G6) and surface net positive charge were investigated.

The toxicity of these nano-objects was analyzed with particular attention to membrane and cytoskeleton integrity upon dendrimer treatment.

Time-lapse imaging and colocalization assays allowed us to determine the spatial and temporal evolution of the internalization process and to identify the intracellular vesicles involved in dendrimer trafficking in living cells. The understanding of the environment surrounding dendrimers could lead to the design of tailored nanostructures which exploit biochemical changes (i.e., pH changes or enzymatic activity) occurring in the endosome.

Finally, internalization was investigated in different cell lines such as human hepatocarcinoma, central nervous system cells and human lung fibroblast cells in order to gain information on the behavior of PAMAM dendrimers in different cell lines and show the ability of PAMAM dendrimer to deliver molecules to targets of actual therapeutic interest.

Materials and Methods

Materials. G2, G4, G6 and lipidated G4 (25% C_{12}) PAMAM dendrimers, acetic anhydride, propidium iodide, nerve growth factor (NGF), Amicon ultra 10 kDa dialysis membranes were purchased from SigmaAldrich. Alexa dyes, transferrin−Alexa488, dextran−FITC, lysosensor, DAPI and lipofectamine were purchased from Invitrogen. TAT11−TMR conjugates were purchased from Sigma Genosys. Hoechst was purchased form Gibco. For immunofluorescence we used anti-[mouse LAMP2] (BD farmingen), anti-[rabbit Giantin] (Babco), and rhodamine phalloidin (Invitrogen). Secondary antibodies: anti-rabbit and anti-mouse Alexa488-labeled (Invitrogen).

General Procedure for PAMAM Dendrimer Acetylation. Ethylenediamine core PAMAM dendrimers were dissolved in anhydrous methanol with triethylamine and acetic anhydride, and the solution was stirred overnight at room temperature. The product was diluted with deionized

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water and extensively dialyzed against water (molecular weight cutoff 10 kDa) to afford the acetylated dendrimer. In the case of G6-Ac 100 equiv of acetic anhydride was used (0.39 equiv of dendrimer primary amines), while to obtain the fully acetylated G4-Ac 640 equiv of Ac_2O was employed (10 equiv of dendrimer primary amines).

The fraction of acetylated amine groups was quantified by ^1H NMR spectra in D_2O by means of a Varian Unity 300 spectrometer (see Supporting Information). Dendrimer charge state was verified by polyacrylamide gel electrophoresis (PAGE) and by agarose gel electrophoresis (see Supporting Information). PAGE was performed following the methodology proposed by Baker Jr. and co-workers[19] for an analogous acetylated dendrimer. Briefly dendrimers were analyzed by means of a vertical electrophoresis system equipped with a power supply (Biorad). A voltage of 200 V was applied for about 40 min with reverse polarity in order to study cationic molecules. Dendrimers were moreover analyzed by electrophoresis in 1% agarose gel in TBE buffer pH = 7.5. In both electrophoretic assays dendrimers were detected exciting the Alexa488 fluorescence by means of a UV lamp.

General Procedure for Fluorophore Labeling of PAMAM Dendrimers. All PAMAM dendrimers were separately conjugated with Alexa647 and Alexa488 fluorophores to afford green- and infrared-labeled PAMAM dendrimers. Conjugation has been carried out via amide bond between the primary amine of the dendrimer and the *N*-hydroxysuccinimide activated carboxyl of the fluorophores. Dendrimers (50 nmol) were dissolved in carbonate buffer at pH = 9 and mixed with a DMSO solution of reactive dye (5 equiv for G6 and G4 or 2 equiv for G2). The solution was stirred for 4 h at room temperature and then dialyzed against water (MWCO = 10 kDa) to afford dendrimer−dye conjugates. Owing to the poor solubility of lipidated dendrimer (25% of surface groups lapidated) basic aqueous solutions the reaction was carried out in DMSO (4 h at room temperature) and then dialyzed against deionized water.

Size Exclusion Chromatography. Size exclusion chromatography was performed by means of a fast protein liquid chromatography (FPLC) system (AKTA basic 10, GE Healthcare) equipped with a Superose 6 10/300 GL column (GE Healthcare). The isocratic mobile phase was PBS buffer pH = 7.4, and the optical densities at 488 nm and 647 nm were monitored with the UV−vis detector of the FPLC system.

UV−Vis Spectrofluorimetry. Excitation and emission spectra were recorded by means of a Cary Eclipse fluorometer (Varian, Palo Alto, CA). Typically, 500 μL samples were used in quartz cuvettes (Hellma, Milan, Italy). The temperature of the cell compartment was set at 25 °C, by a built-in Peltier cooler (Varian). Excitation and emission band-pass of 5 nm was employed.

Fluorescence Correlation Spectroscopy. Fluorescence correlation spectroscopy (FCS) measurements were performed using a Microtime 200 system (Picoquant), based on an inverted confocal microscope (Olympus IX70) and equipped with two SPAD (single photon avalanche diode) detectors. Excitation was achieved by means of a 475 nm picosecond diode laser. Fluorescence emission was split with a 50/50 splitter between the two detector channels and collected through matched bandpass filters (AHF, SMDEmitter HQ 520/40).

Cell Culture and Transfection. HeLa (CCL-2), PC12 (CRL-17210), MRC5 (from normal lung tissue of a 14-week fetus, CCL-171) and HepG2 cells (HB-8065) were purchased from ATCC and cultured following manifacturer's instructions. Primary astrocytes were a kind gift of Giuseppe Bardi.[20] For live cell microscopy cells were plated onto 35 mm glass-bottom dishes (WillCo-dish GWSt-3522) and imaged at 37 °C 5% CO2. PC12 Cells were stimulated with 100 ng/mL of NGF for two days to induce differentiation. Transfection of Caveolin-E^{1}GFP[21] was carried out using lipofectamine reagent (Invitrogen) according to the manufacturer's instruction. Cells were imaged 24 h after transfection.

Confocal Imaging. Cell imaging was performed on a Leica TCS SP2 inverted confocal microscope (Leica Microsystems) equipped with a 40× 1.25 NA oil immersion objective (Leica Microsystems). Imaging was obtained illuminating the samples with the inline Ar and He−Ne lasers of the microscope and with a 403 nm pulsed diode laser (M8903-01; Hamamatsu) at 50 MHz repetition rate. Fluorescence emission was collected with the AOBS-based built-in detectors of our confocal microscope (Hamamatsu R6357).

Propidium Iodide (PI) Assay. HeLa cells were incubated for 1 h at 37 °C with DMEM containing 8 μg/mL propidium iodide and different concentrations of dendrimer (100 nM, 500 nM and 1.5 μM). The medium was then discarded and the cells were washed with PBS buffer containing the same concentration of propidium iodide before confocal imaging.

Flow Cytometry Measurements. HeLa cells were grown in a six well plate and after treatment with Alexa488-labeled dendrimer were detached using trypsin-EDTA, washed with PBS buffer and fixed with 4% paraformaldheyde (PFA). Cells were washed with PBS until complete removal of PFA and finally resuspended in 250 μL of PBS. Flow cytometry was performed on a FACScalibur system (BD biosciences) by counting 10,000 events. Histogram plots were analyzed using WinMDI 2.9 (http://facs.scripps.edu/software.html).

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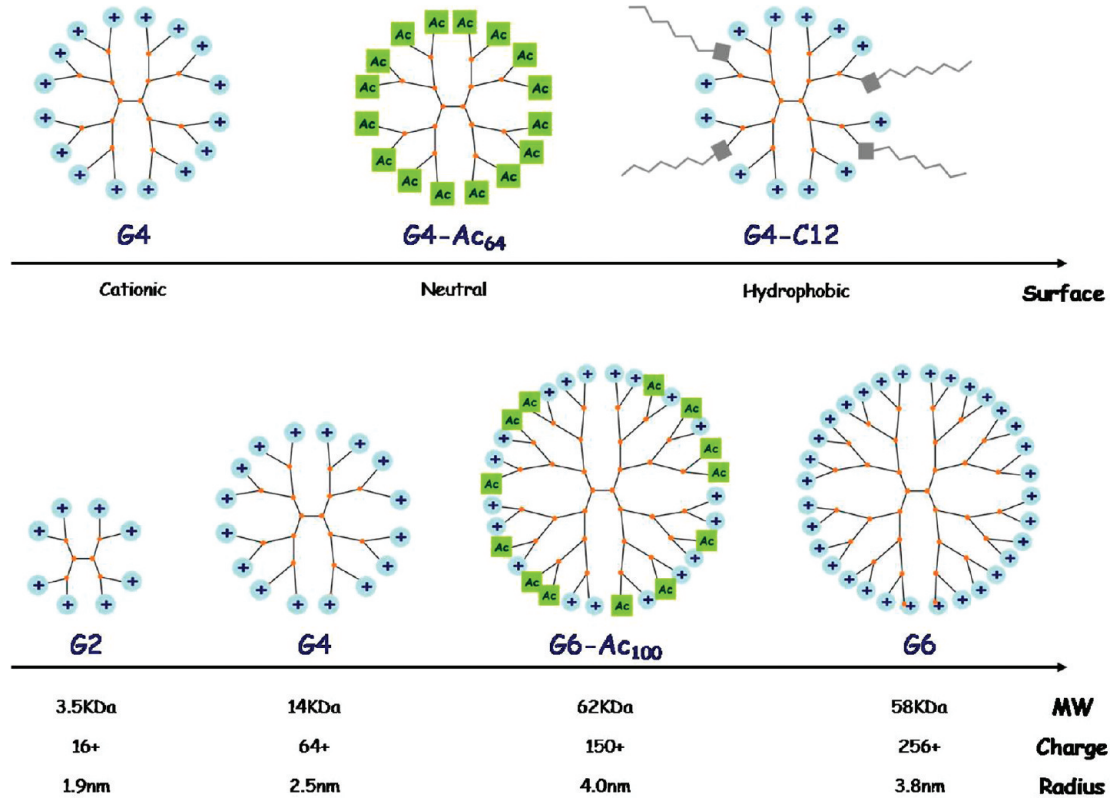

Figure 1. Schematic diagram of dendrimers analyzed in this work. Dendrimers with different physicochemical surface properties, cationic, neutral and hydrophobic (top), and cationic dendrimers varying in size and charge (bottom) were studied. Amine terminated PAMAM dendrimers are positively charged at physiological pH owing to primary amine group protonation (pale blue). Acetylation of primary amines (in green) reduces the surface charge density. Dendrimer hydrophobicity was modulated by adding C12 fatty acid chains (gray).

Internalization Assay and Colocalization Studies. To monitor dendrimers internalization cells were incubated with 100 nM Alexa647-labeled dendrimers in DMEM for 1 h at 37 °C. To remove unbound molecules in the medium, cells were rinsed two times with PBS. After the initial preloading and subsequent washing, cells were incubated again in DMEM and imaged at the indicated time point. In order to identify the endocytic vesicles involved in dendrimer internalization, we performed colocalization assays in living cells. HeLa cells were coincubated with dendrimers (as described above) and different dyes: with 1 mg/mL 70 kDa dextran−FITC conjugate at 37 °C for 30 min to label macropinosomes, with 50 mM Lysosensor for 10 min to label lysosomes, with 2 μg/mL transferring Alexa488 conjugate to label recycling and sorting endosomes and with 2 μM TAT−FITC conjugates. Images were analyzed using ImageJ software version 1.37 (NIH Image; http://rsbweb.nih.gov/ij/). Immunostaining was used to investigate dendrimer intracellular fate. After 12 h from dendrimer incubation cells were fixed with PFA 4% for 10 min at room temperature, permeabilized with 0.5% Triton-X100, incubated with 1% BSA and labeled with specific antibodies for Golgi network (Anti-Giantin). Phalloidin−rhodamine (Invitrogen) conjugate was used to label actin filaments.

Results

Dendrimer Surface Modification and Labeling. In this study we analyze the internalization properties of different PAMAM dendrimers in HeLa cells with particular attention to cationic dendrimers since these show high affinity for the negatively charged cell surface.[14] Figure 1 schematically shows the dendrimers investigated in this work. Macromolecules with three different chemical surfaces were studied: (i) cationic amine-terminated (primary amines are protonated at physiological pH), (ii) neutral acetylated amines and (iii) a cationic lipidated dendrimer (25% of surface groups lipidated by C12 chains and 75% of primary cationic amines). Notably although fully acetylated PAMAM dendrimers display neutral acetamide groups on the surface, they retain a dynamic pH-dependent cationic behavior.[19]

Additionally a series of cationic dendrimers varying in size and surface charge were investigated in order to dissect the influence of each of these properties on cell uptake. G2, G4 and G6 dendrimers were chosen in order to analyze internalization properties on a broad range of sizes and surface charges. Moreover the highly charged G6 dendrimer was partially acetylated (100 amine groups out of 256 were acetylated) to reduce the molecule surface charge.[22] For our endocytosis assays dendrimers were separately labeled with the Alexa488 and Alexa647 dyes to afford green- and red-

labeled dendrimers for internalization assays. Labeled dendrimer properties were studied by NMR, UV−vis spectrofluorimetry, size exclusion chromatography and FCS (see Supporting Information) revealing the formation of bright and stable dendrimer−dye conjugates.

Cytotoxicity and Flow Cytometry. The effect on cell viability of PAMAM dendrimers and dependence on concentration was assessed by a propidium iodide (PI) assay. This dye cannot permeate intact cell membranes while it is internalized when the membrane is defective. With this assay we could monitor two distinct phenomena: (a) the reduction on cell viability as propidium permeates apoptotic and dead cells; (b) the reversible dendrimer-induced permeation of cell membranes as reported by Hong et al.[23] Figure 2a shows our PI assay results after treatment of HeLa cells with dendrimers at 100 nM, 500 nM and 1.5 μM. The acetylation of primary amine in G6-Ac and the consequent lower surface charge strongly reduced toxicity, in line with the results by Stasko et al.[24] Moreover the lipidation of the G4 dendrimer induces a small reduction of cell viability (about 10%) in comparison with the amine-terminated dendrimer of the same size. All experiments in the following were performed with 100 nM dendrimer concentration. At this value none of the dendrimers display a relevant effect on cell viability and membrane permeability. Cytotoxicity should also be analyzed considering the large differences in molecular weight of the dendrimers studied in this work. To this end Supplementary Table 1 in the Supporting Information reports the molecular weight and the concentration of dendrimers administered for PI assay expressed in μg/mL.

Since previous reports demonstrated that some cell penetrating peptides (e.g., HIV-1 TAT derived peptide) can induce actin reorganization causing loss of stress fibers and peripheral retraction,[25] we monitored the effect of dendrimers on cytoskeleton integrity. Supplementary Figure 1 in the Supporting Information shows phalloidin staining of actin filaments indicating that no cytoskeleton perturbation occurred following dendrimer administration.

A flow cytometry assay was performed, and the results are reported in Figure 2b. The experiment was carried out incubating for 1 h HeLa cells; as discussed in the following section in these conditions dendrimers enrich only cell membranes while internalization is negligible. As a consequence we can present a quantitative assay of membrane affinity of different PAMAM dendrimers, a crucial aspect for cellular uptake. Data presented in Figure 2b were normalized taking into account the single-dendrimer brightness values (determined by FCS, see Supporting Information).

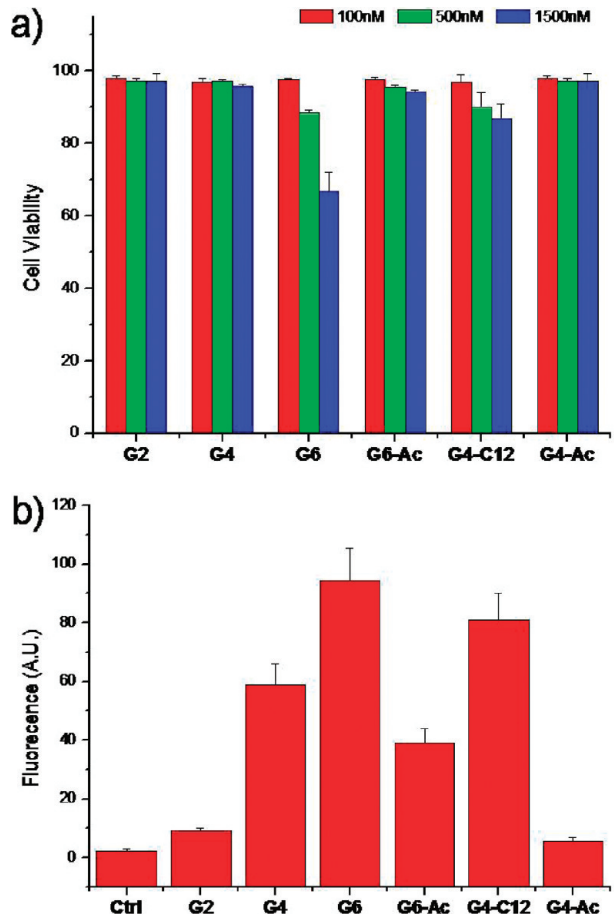

Figure 2. (a) Propidium iodide assay on HeLa cells. Cells were treated with different amounts of dendrimer (100 nM, 500 nM and 1.5 μM) for 1 h, and cell viability is reported. Dendrimers display a low cytotoxicity in this concentration range except for the highly charged G6. (b) FACS analysis performed after 1 h treatment with different dendrimers (100 nM concentration).

Time Lapse Imaging and Colocalization Assay. In order to investigate the mechanism of dendrimer internalization in living cells we performed time-lapse imaging of HeLa cells treated with dendrimer−Alexa647 conjugates at different temperatures of incubation. Supplementary Figure 2 in the Supporting Information shows dendrimer localization after 2 h of cells incubated either at 4 or 37 °C. In both cases dendrimers bind the cell membrane, but at 4 °C no internalization occurs. This confirms that cationic dendrimers have a strong electrostatic affinity for negatively charged membrane proteoglycans and phospholipids and indicates that they are internalized by an active energy-dependent process.

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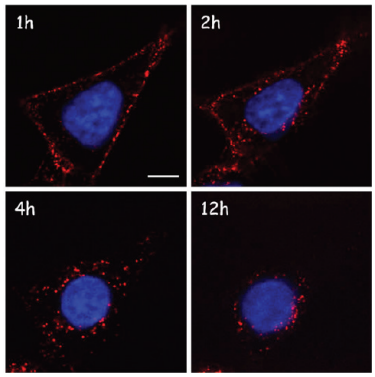

Figure 3. Time lapse imaging of HeLa cell treated with G4 dendrimer (red). After membrane binding (1 h) dendrimer is internalized and gradually delivered to the perinuclear region. Nuclei are stained with Hoechst (blue). Scale bar = 10 μm.

Figure 3 shows time-lapse imaging of a HeLa cell treated with the G4 dendrimer. Three main steps in dendrimer internalization can be identified. First the cell was rapidly coated by fluorescent dendrimers bound to the membrane surface. In the first hour no significant internalization was observed. At a later stage vesicles moving toward the nucleus were observed. After 4 h no membrane fluorescence was detectable. Dendrimers were delivered to their intracellular fate in the perinuclear region in 12 h. Time lapse imaging (up to 48 h) did not reveal any further dendrimer-localization changes (data not shown).

In order to identify the biological compartments involved in dendrimer internalization we performed colocalization assays with biomarkers of different endocytic vesicles. Transferrin−Alexa488 and 70 kDa dextran−FITC were used to trace clathrin-dependent pathway and macropinocytosis,[26,27] respectively, and a transfection of caveolin-E[1]GFP was used to label caveolae structure.[21] Figure 4 shows colocalization data between the dendrimer (red channel) and the endocytic vesicles (green channel). Table 1 provides Pearson's coefficients for each colocalization. Data show that all dendrimers examined strongly colocalize with transferrin and dextran (yellow vesicles) while there is no significant correlation with the caveolin-E[1]GFP fluorescence signal. Regardless of size, surface functionality and total charge, all dendrimers studied appear to follow the same route. Time lapse imaging demonstrates that dendrimers are eventually delivered to a vesicular structure in the perinuclear region. In order to identify the latter we performed colocalization assays with lysosensor and Giantin known markers for lysosome and Golgi network respectively. Figure 5 shows these colocalization results: we observe very high colocalization with lysosensor while no dendrimer signal is observed in the Golgi. We moreover report the movie of a time series of lysosome-dendrimer colocalization (supporting movie 1 in the Supporting Information) where the colocalization and the dynamics of the vesicles can be appreciated.

TAT−Dendrimer Comparison. Dendrimer endocytic properties were compared to those of a much-studied cell-penetrating peptide (CPP): the arginine-rich motif derived from the HIV protein TAT. This arginine-rich peptide binds aspecifically cell membranes and is mainly internalized by macropinocytosis and clathrin-dependent endocytosis.[28,29] Figure 6a shows HeLa cells imaged 30 min after coincubation with G4−Alexa647 and TAT−Rhodamine conjugates. As previously showed G4-dendrimers at early time points are localized on the membrane while TAT displays a faster endocytosis and at about 1 h is completely localized in the perinuclear region. By incubating HeLa cells with dendrimers 2 h before TAT treatment marked colocalization could be observed (Figure 6b). Notably in the cotreatment a 10-fold higher concentration of TAT was necessary owing to the larger affinity of dendrimers for cell membranes.

Cell Line Analysis. All experiments reported so far were carried out using HeLa cells as a model for endocytosis. We investigated dendrimer internalization also in other cell lines. In particular we considered PC-12 (neuronal-like), HepG2 (human hepatocarcinoma), MRC5 (human lung fibroblast) and primary astrocytes. Figure 7 shows that cationic PAM-AM dendrimers can cross cell membranes in all these cell types but with some notable differences. Efficient membrane binding was observed with PC-12 cells, but after 3 h only a small amount of the fluorescent dendrimer was internalized. On the contrary HepG2 cells displayed faster delivery of dendrimers to their intracellular fate. G4 displayed very efficient internalization in both primary lines evaluated. The observed behavior is very similar to what reported for HeLa cells.

Discussion

Dendrimers can be of much relevance for a number of biomedical applications such as drug delivery, molecular imaging and gene therapy.[30] The successful delivery of therapeutics to the designated cells and their availability at the intracellular target location are crucial requirements for all these applications. Recent findings demonstrate that dendrimers can cross cell membranes by endocytosis.[14] Endocytosis is a constitutive cell mechanism for molecule

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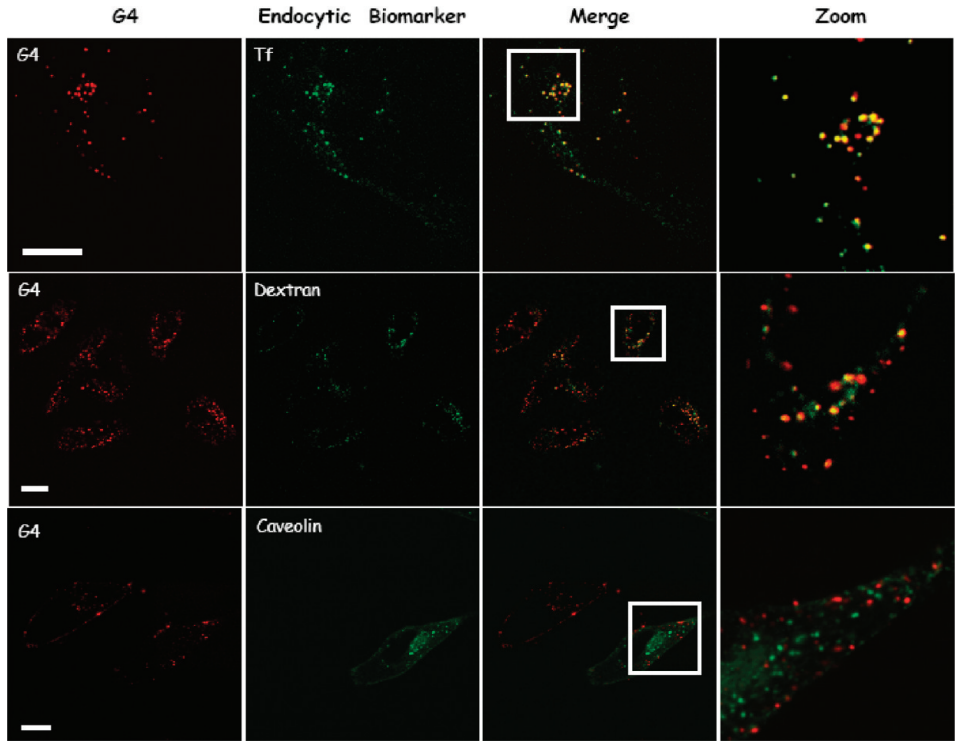

Figure 4. G4 dendrimer (red) colocalization assay with in vivo endocytic markers (green). High colocalization (yellow vesicles) was observed with transferrin (clathrin pathway) and dextran (macropinocytosis) while no correlation with caveolin signal was observed. Scale bar = 15 μm.

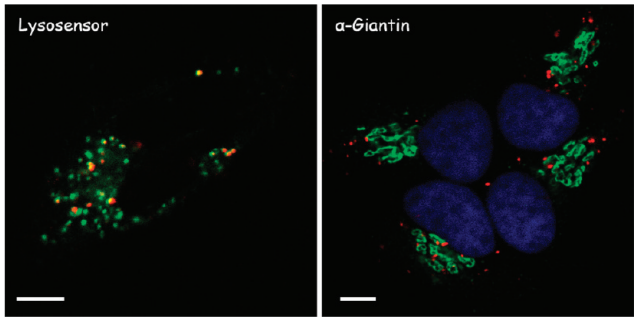

Figure 5. Colocalization of G4 dendrimers (red) with lysosensor (lysosome marker, left), anti-Giantin (Golgi marker, red) after 12 h from dendrimer treatment reveals that dendrimers are completely delivered to the lysosomal compartment. Scale bar = 5 μm.

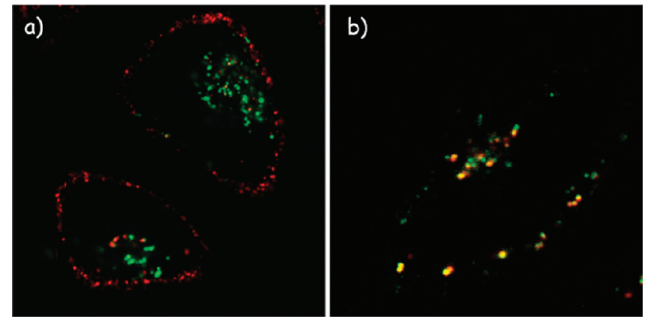

Figure 6. G4−AF647 (red) TAT−Rhodamine (green) colocalization. (a) Cotreatment reveals faster internalization of TAT in HeLa cells. (b) TAT treatment after 2 h of incubation with dendrimer displays strong colocalization indicating that both vectors follow the same internalization pathway but with different kinetics. Scale bar = 5 μm.

Table 1. Pearson's Coefficients for Dendrimer Colocalization with Endocytic Markers

	dextran	transferrin	caveolin
G2	0.09 ± 0.07	0.10 ± 0.08	0.02 ± 0.05
G4	0.19 ± 0.11	0.17 ± 0.07	−0.04 ± 0.08
G6	0.24 ± 0.03	0.17 ± 0.06	−0.05 ± 0.08
G6-Ac	0.30 ± 0.09	0.18 ± 0.12	−0.06 ± 0.10
G4-C12	0.26 ± 010	0.18 ± 0.09	−0.03 ± 0.08

internalization and targeting to a specific intracellular compartment.[15] Endocytosis can take place with different mechanism: macropinocytosis,[31] clathrin-dependent pathway,[32] caveolae[33] and clathrin- and caveolin-independent pathway.[34] Macropinocytosis is an actin-driven endocytic process resulting in an elevation of actin-mediated ruffling and ultimately macropinosome formation in the cytoplasm. On the contrary other mechanisms involve the formation of vesicles on the inner leaflet of the cell surface coated of specific proteins. Eventually these transport vesicles are gathered and fused to an intracellular acceptor compartment. Knowledge of the internalization mechanism in the desired cell line is a prerequirement for the rational application of these potential

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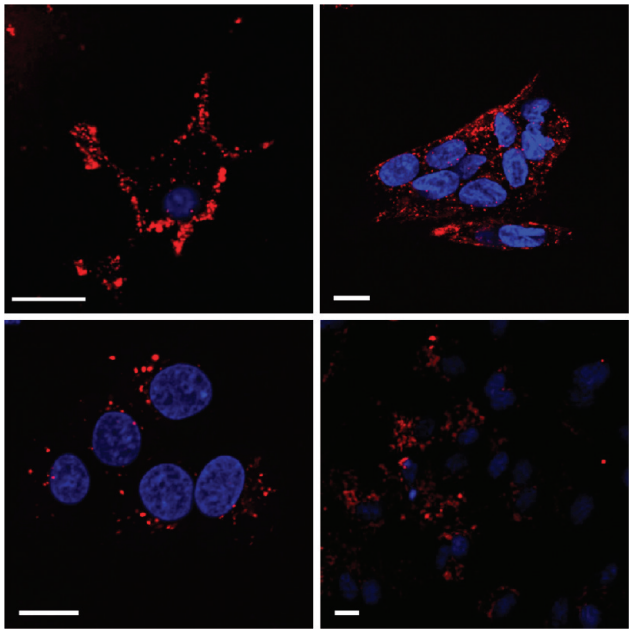

Figure 7. G4 (in red) endocytosis in different cell lines: PC-12 (upper left), MRC5 (upper right), HepG2 (bottom left) and primary astrocyte cultures (bottom right). Nuclei are stained with Hoechst (blue). Scale bar = 15 μm.

carriers since the chemical environment of the different pathways can significantly vary and affect the delivery effectiveness.[35,36]

Existing reports on internalization and intracellular trafficking following endocytosis are controversial. Flow-cytometry measurements and immunostaining on PAMAM dendrimers lead to different results in the identification of the endocytic pathway in different cell lines.[16−18] Interestingly the conjugation of a cargo molecule to the endocytic vector was reported to be able to alter the internalization pathway in a size-dependent manner.[37,38]

In the present work we utilized biophysical techniques to investigate cell−dendrimer interactions in living cells with high spatial and temporal resolution obtaining information about the dynamics and the pathways involved in dendrimer trafficking in living HeLa cells.

Dendrimers studied in this work vary in size and surface charge (Figure 1) but do not display a significant reduction of cell viability up to 1.5 μM except for the highly charged G6 dendrimer (this negative impact is much reduced in less charged G6-Ac). Notably owing to the differences in molecular weight the dendrimer dose calculated on a mass basis is much higher for G6 molecules (see Supplementary Table 1 in the Supporting Information). Therefore the mass of G6 dendrimer in the lowest concentration tested with PI corresponds to the highest one for G2. This should be taken into account considering cytotoxicity data. However from the comparison between G6 and G6-Ac data it appears that the main factor affecting cytotoxicity is charge regardless of size and mass.

Moreover propidium iodide assay and phalloidin staining revealed the integrity of cell membranes and cytoskeleton, respectively, upon dendrimer treatment.

FACS analysis on HeLa cells demonstrates that membrane affinity depends on the amount of positive charge on the dendrimer surface (G6 > G4 > G2) and can be modulated by acetylation (G6 > G6-Ac > G4-Ac). Moreover lipidated dendrimers show more affinity for cell surface than the corresponding cationic molecules.

We can conclude that the same groups (cationic amines and lipid chains) that promote membrane binding (and thus cell uptake) are responsible for cytotoxicity. The realization of an ideal vector relies on the balance between these two aspects: according to our study G4, G4-C12 and acetylated G6 offer the best compromise between effective internalization and toxicity.

Time-lapse imaging was performed on HeLa cells and revealed the kinetics of dendrimer internalization. Dendrimers displayed rather long residence times on the cell membrane and correspondingly slow internalization kinetics probably owing to their strong interactions with negatively charged membrane proteoglycans. The long permanence on the membrane is moreover highlighted by the comparison with the TAT peptide that displays a faster internalization (Figure 6). In this framework the functionalization of PAMAM dendrimers with TAT, or TAT-derived peptides with improved properties,[39] could lead to an enhancement of the internalization properties of the dendrimer.

Dendrimer internalization in HeLa cells seems also slower than in other cell lines;[16] this may be linked to membrane composition and constitutive cellular endocytosis regulation.

Colocalization assays with endocytic structure markers revealed that both clathrin-mediated endocytosis and macropinocytosis are involved in dendrimer internalization in HeLa cells, while caveolin does not play an important role. These assumptions are also supported by the colocalization

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with TAT peptide (Figure 5b) that is known to follow these two pathways[28,29] and by the effective internalization observed in caveolin-lacking HepG2 cells (Figure 7). These observations agree with Kitchens et al.[17,40] that reported clathrin-mediated internalization for PAMAM dendrimers in Caco-2 monolayer (macropinocytosis was not investigated), but differs from what was observed by Perumal et al.[16] This can be explained with the different behavior of the A549 cell line used in that study for which even internalization of neutral dendrimers was reported. Colocalization with lysosensor and specific antibodies revealed that dendrimers are eventually delivered to the lysosomal compartment and no interactions with trans Golgi network were observed. Thus cationic dendrimers bind the negatively charged cell exterior and are internalized in HeLa cells via the clathrin-dependent pathway and macropinocytosis and populate early endosomes and macropinosomes that are progressively acidificated until lysosome delivery. These observations are of much interest for drug-delivery purposes for two main reasons: (a) in this acidic environment dendrimer tertiary amine protonation contributes to vesicle swelling and destabilization thus facilitating vesicle escape;[41] (b) pH-triggered release based on pH cleavable linkers can be a viable strategy for the release of small molecules that can permeate destabilized acidic vesicles and diffuse in the cytoplasm.[7,42]

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In this work we studied extensively dendrimer internalization in HeLa cells. We extended these observations by evaluating the interactions of G4 dendrimers with four different cell lines: human hepatocarcinoma liver carcinoma cell line HepG2 cells, neuronal-like PC12 cells and two primary cultures (MRC5 human lung fibroblast and astrocytes). Dendrimers were internalized in all these cell lines but differences in the dynamics and in the localization were observed. Dendrimers in HepG2 cells display a strong and faster endocytosis, and after 4 h they reach their intracellular fate in the perinuclear region. On the contrary in the case of PC-12 cells, dendrimers display high membrane affinity but very slow internalization as shown in Figure 7. After 4 h the fluorescence signal is mainly localized on the membrane and few endocytic vesicles are observed inside the cell, probably owing to slow membrane recyling. Finally internalization in primary cell cultures was investigated. Both astrocytes and lung fibroblasts display G4 internalization with a similar evolution of what observed in HeLa and analogous lysosomal delivery. This cell-line analysis reveals that cationic dendrimers are capable of crossing cell membranes of all tested cellular types but with different effectiveness and dynamics that we argue are linked to the differences in membrane chemical composition and detailed cell-type recycling kinetics.

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Supporting Information Available: Supporting figures and dendrimer characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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