Novel Monodisperse PEG−Dendrons as New Tools for Targeted Drug Delivery: Synthesis, Characterization and Cellular Uptake

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Dendrimers, dendrons, and hyperbranched polymers are gaining popularity as novel drugs, imaging agents, and drug delivery systems. They present advantages of well-defined molecular weight, multivalent surfaces, and high drug carrying capacity. Moreover, it is emerging that such architectures can display unique endocytic properties. As poly(ethylene glycol) (PEG) is widely used for protein and drug conjugation, the aim of this study was for the first time to synthesize novel, branched PEG-based architectures, to define their cytotoxicity and, via preparation of Oregon green (OG) conjugates define the effect of structure on their cellular uptake. Five PEG-based dendrons were synthesized using monodisperse Fmoc-amino PEG propionic acid ($M_w = 840$) as a monomer, and cadaverine, tris(2-aminoethyl)amine or lysine as the branching moieties. These were diamino,bisPEG ($M_w = 1300$); triamino,trisPEG (Mw = 1946); tetraamino,tetraPEG ($M_w = 3956$); monocarboxy,diamino,bisPEG ($M_w = 1346$); and monocarboxy,tetraamino,tetraPEG ($M_w = 3999$). These products had NH_2 or both NH_2 and COOH terminal groups and the identity was verified by amino group analysis and ESI-TOF mass spectroscopy. Purity was determined by HPLC. Representative structures were not toxic towards an endothelial-like cell line (ECV304) at concentrations up to 4 mg/mL (over 72 h). At 37 °C, all of the OG-labeled PEG dendrons showed progressive uptake by ECV304 cells, but tetraamino,tetraPEG showed the greatest rate of internalization over the first 20 min. Cellular uptake was inhibited at 4 °C, and PEG dendron localization to perinuclear vesicles was confirmed by fluorescence microscopy. These well-defined novel architectures have potential for further development as targetable drug delivery systems or tools for construction of structurally defined modified surfaces.

Introduction

The search for better diagnostics and more selective medicines is leading to the clinical development of polymeric drugs, polymer−protein conjugates, polymer−drug conjugates, and polyplexes for gene delivery. These constructs have been termed "polymer therapeutics",[1] Compared to the linear polymers currently in clinical use such as poly(ethyleneglycol) (PEG),[2] *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers,[3] and polyglutamic acid,[4] dendrimers and dendronised polymers bring potential advantages arising from their well-defined chemistry and molecular weight (reviewed in refs 5−7). Moreover, our earlier experiments showed that PAMAM dendrimers, particularly those bearing −COOH terminal groups, displayed much higher rates of transport across the rat intestinal tissue in vitro compared to linear polymers.[8] Other studies have subsequently shown similarly unusual cellular pharmacokinetics with defined transport across biological barriers.[9,10] Recently, we have shown that PEG−polyester dendrons of particular molecular weight and dendron generation can show exceptionally high rates of exocytosis following internalization by the endothelial cell line ECV304.[11] These observations underline the potential importance of polymer architecture in controlling pharmacokinetics and intracellular trafficking and may bring unique opportunities from drug delivery across biological barriers, intracellular compartment-targeted drug delivery and/or design of nanosized structures to monitor intracellular environments. Dendrimer architecture could therefore provide a great opportunity to promote trans-barrier (GI or BBB) transport of drugs or peptides and better delivery of anticancer agents than is currently achieved by those linear polymers presently in the clinic.[6−11] The payload to be chosen will depend on in vivo efficiency of dendrimer delivery and must be matched to a drug payload of sufficient potency.

PEG has been widely used to prepare many polymer-protein conjugates already in routine clinical use (reviewed in refs 12 and 13) and anticancer drug conjugates in clinical development (reviewed in ref 14). Therefore, the aim of this study was to synthesize novel dendrons using PEG of narrow dispersity as a branching unit. Like all of the synthetic polymers, PEG is polydisperse.[15] The M_w/M_n depends on PEG molecular weight, but it is never below 1.01, and this can lead to heterogeneity in resultant conjugates. Therefore, to obtain stochiometrically well-tailored products, here a monodisperse Fmoc-amino $PEG_{(840)}$ propionic acid (***1***) (Scheme 1) was used as the starting material. Five PEG-dendrons were prepared (summarized in Table 1 and Schemes 1−5) of different molecular weight (generation) and having terminal NH_2 groups or both NH_2 and COOH termini. The synthesized PEG−dendrons were characterized using ESI-TOF mass spectrometry, terminal group analysis, and HPLC. Before biological evaluation, cytoxicity of the products was established using an MTT assay in the ECV 304 cell line. To enable study of endocytic properties Oregon green (OG) labeled conjugates were synthesized and flow cytometry was used to determine the rate of ECV304 association at 37 (internalization)

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Scheme 1. Synthesis of Diamino,bisPEG

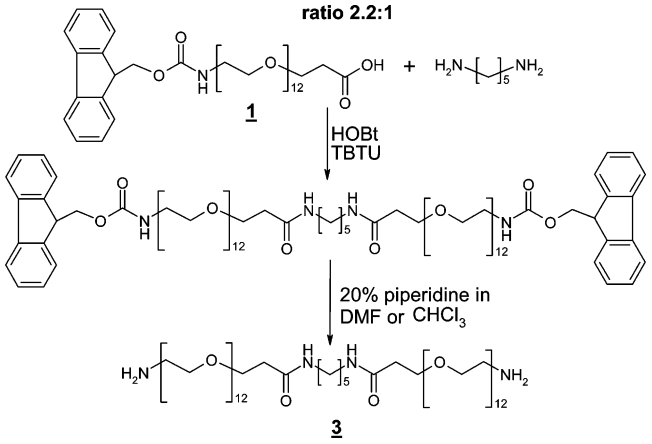
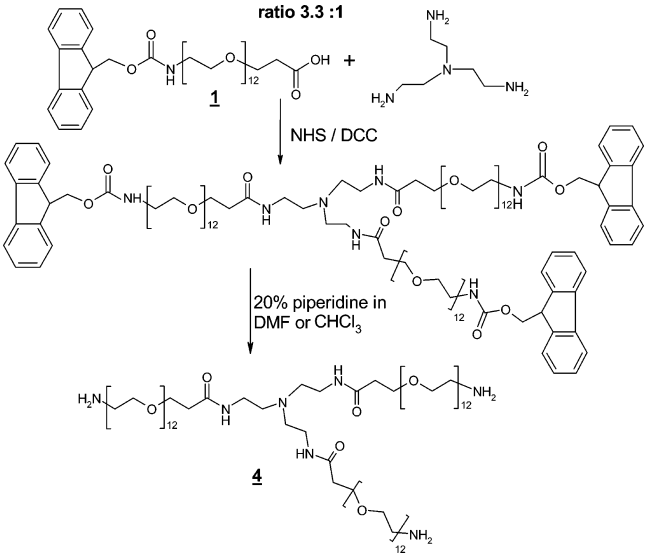


Table 1. List of Products and Their Characteristics

name and code number	M_w	terminal groups[a]	OG content (μg of OG/ mg of polymer)[b]
Diamino,bisPEG (bisPEG) **3**	1300	2 NH_2	205.4
Trisamino,trisPEG (trisPEG) **4**	1946	3 NH_2	95.53
Tetraaminio,tetraPEG (tetraPEG) **7**	3956	4 NH_2	53.28
Monocarboxy,diamino, bisPEG (mono,bisPEG) **6**	1342	2 NH_2 1 COOH	47.23
Monocarboxy,tetraamino, tetraPEG (mono,tetraPEG) **8**	3999	4 NH_2 1 COOH	42.78

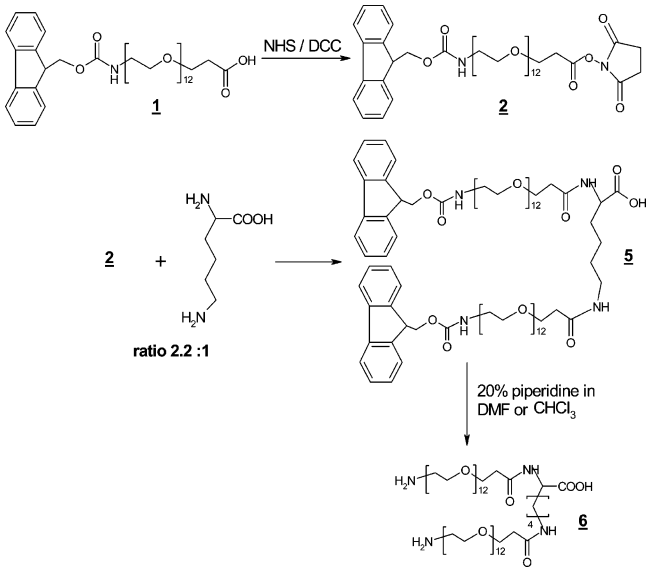
[a] The quantitative evaluation of terminal amino groups, according to the procedures of Snyder (ref 17), was consistent with the theoretical one for all the products. [b] The free OG in the sample was less than 0.5 ng/mg of PEG−dendron.

Scheme 2. Synthesis of Triamino,trisPEG

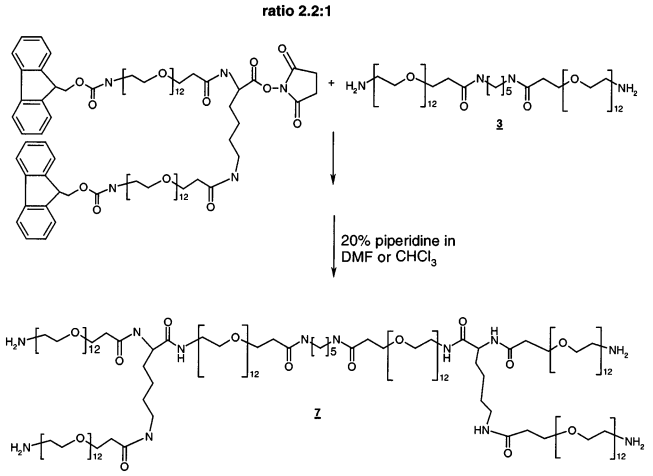


and 4 °C (binding). In parallel, fluorescence microscopy was used to visualize intracellular localization of the conjugates.

Scheme 3. Synthesis of Tetraamino,tetraPEG



Scheme 4. Synthesis of Monocarboxy,diamino,bisPEG

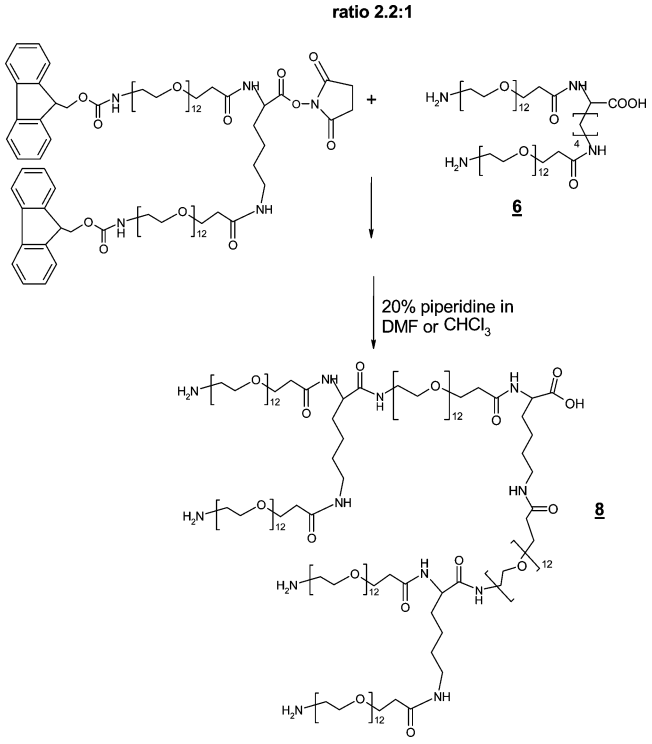


Materials and Methods

Materials. FmocPEG-propionic acid (840) was purchased from LCC Engineering & Trading GmbH, Switzerland. *N*-Hydroxybenzotriazole (HOBT) and [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophospate] (TBTU), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicycloexylcarbodiimide (DCC), cadaverine, tris(2-aminoethyl)amine, and *N*-ethyldiisopropylamine (DPEA) were obtained from Fluka Chemie (Buchs, Swiss). Tri-nitrobenzenesulfonic acid (TNBS) was purchased from Aldrich (Milwaukee, WI). OG 488-X succinimidylester was purchased by Molecular Probes Inc. (Eugene, OR). The solvents and other chemicals were of analytical grade from Merck (Rahway, NJ). 3-(4,5-Dimethylthiazol)-2-yl-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma (St. Louis, MO). Tissue culture medium M 199 (with Earle's salts and glutamax) and foetal bovine serum were from Gibco (Paisley, U.K.). The ECV 304 cells were provided by ECACC (Salisbury, U.K.). TLC silica gel on plates were from Sigma Aldrich (St. Louis, MO).

Instrumentation and Methods. Reversed phase chromatography was performed with a Gilson analytical and semipreparative HPLC system, using an Agilent C18 column (250 × 4.6 mm, particle size 5 μm) and a Phenomenex C18 column (250 × 21.2 mm, particle size 10 μm) respectively, with UV detector. Mass spectra were obtained using an API-TOF Mariner (Applied Biosystems) instrument. PEG−OG conjugates were purified using a preparative Sephadex-LH 20 column (Amersham Biosciences, U.K.) eluted with methanol. Sephadex-G25

Scheme 5. Synthesis of MonoCarboxy,tetraamino,tetraPEG



disposable PD 10 analytical columns (Amersham Biosciences, U.K.), with phosphate buffer as eluent, were used to assess the purity and the stability of the PEG−OG conjugates during tissue culture. Thin layer chromatogaphy (TLC), using methanol as mobile phase, was used to analyze the amount of free OG after the labeling of PEG−dendrons.

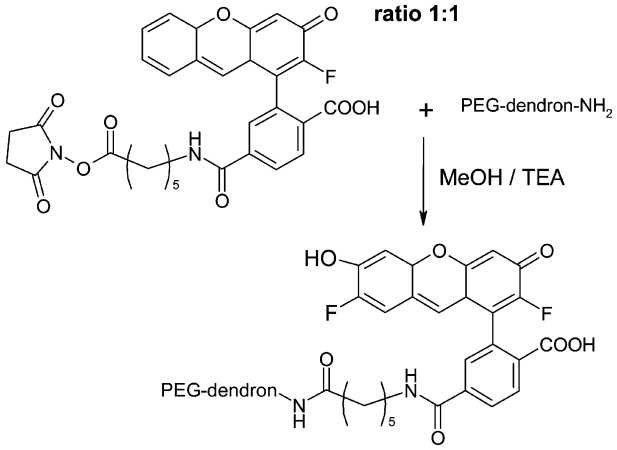
Synthesis of PEG Dendrons. *Preparation of Diamino,bisPEG (3).* The method used is shown in Scheme 1. To 0.3 g (0.36 mmol) of **1** were added 52.46 mg of HOBT (0.39 mmol) and 12.46 mg of TBTU (0.39 mmol) in anhydrous chloroform. After 30 min, this was followed by 15.8 mg (0.156 mmol) of cadaverine in chloroform brought to pH 8 with *N*-ethyldiisopropylamine (DPEA). The mixture was maintained at room-temperature overnight and purified by extraction into a $KHSO_4$ buffer. The water was removed by Na_2SO_4 and this product precipitated by addition of diethyl ether. The product appeared as a light yellow oil at room temperature but turned to wax at low temperature. The yield of reaction was 60%. Product ***2*** was characterized by ESI-TOF mass spectrometry by infusion of a solution of ~5 μM into the spray of the instrument and recording the spectra every 10 s.

The Fmoc groups were then removed by 30 min incubation in chloroform-20% piperidine. Then the dibenzofulvene-piperidine produced was removed by diethyl ether precipitation. The final product **3** was analyzed by ESI-TOF mass spectrometry. A procedure using TNBS, as described by Habeeb,[16,17] was used to determine the amino groups content of the product.

Preparation of Triamino,trisPEG (4). The method used is shown in Scheme 2. To 1 g (1.19 mmol) of **1** dissolved in chloroform were added 0.15 g of NHS (1.3 mmol) and 0.27 mg of DCC (1.31 mmol). The reaction was stirred for 15 min, tris(2-aminoethyl)amine dissolved in chloroform and *N*-ethyldiisopropylamine were added, and the reaction was stirred overnight. The dicycloexylurea was filtered off and the product precipitated twice from chloroform solution by diethyl ether addition. The purity of the Fmoc-protected intermediate was evaluated as previously described by ESI-TOF spectrometry. To generate the final product, the Fmoc group was removed, as described above using piperidine, and the product was again characterized by amino groups analysis and mass spectrometry. The yield of the reaction was 60%.

Preparation of Monocarboxy,diamino,bisPEG (6). The method used is shown in Scheme 3. To 1 g (1.19 mmmol) of **1** dissolved in 5 mL of chloroform were added 0.15 g of *N*-hydroxysuccinimide (1.3 mmol)

Scheme 6. Synthesis of PEG−OG Conjugates



and 0.27 mg of *N*,*N*′-dicycloexylcarbodiimide (1.31 mmol), and the reaction mixture was stirred overnight at room temperature. The dicycloexylurea was filtered off and the product **2** precipitated from chloroform solution by diethyl ether addition (yield 60%). The obtained **2**, 2.2 equiv, was added to 1 equiv of lysine dissolved in acetonitrile: H_2O (1.3:1) and *N*-ethyldiisopropylamine. The reaction mixture was stirred at room temperature and the dicycloexylurea removed as above. The reaction mixture was then purified by RP-HPLC chromatography using H_2O-0.05% TFA as eluent (A) and acetonitrile-0.05% TFA as eluent (B). A gradient system was used as follows: 5 min at 5% (B), then from 5% (B) to 50% (B) over 2 min, then raising to 70% (B) over 15 min, then from 70% (B) to 95% (B) over 3 min, and finally over 3 min raising to 95% (B). The eluted fractions were analyzed spectrophotometrically at 320 nm to detect Fmoc. The polymer-containing fractions were then pooled and lyophilised to give a light yellow oil (product **5**) that was characterized by ESI-TOF mass spectrometry. Following deprotection, as described above to give product **6**, and amino-end group analysis was also performed as previously described. The yield of the final step was 50% whereas the overall yield was 25%.

Preparation of Tetraamino,tetraPEG (7). The method used is shown in Scheme 4. The Fmoc-protected intermediate **5** (105.6 mg; 0.059 mmol) was dissolved in 6 mL of chloroform, and 7.5 mg (0.065 mmol) of NHS and 13.4 mg (0.065 mmol) of DCC were added and the reaction mixture was stirred overnight at room temperature. Dicycloexylurea (DCC) was then filtered off, and the intermediate with the activated carboxylic group was precipitated from chloroform by addition of diethyl ether. The yield of this reaction was 50% and the amount of activation was evaluated by Snyder assay (refs 16 and 17). Then 25 mg (0.013 mmol) of the product **5** obtained was added to an anhydrous solution of chloroform containing 7.8 mg (0.006 mmol) of **3** and DPEA to reach a final pH of 7.5. The reaction was stirred at room temperature under N_2 atmosphere for 3 h, and the mixture was purified by RP-HPLC as described above. The polymer-containing fractions were pooled, and the solvent was evaporated to give light yellow oil that was analyzed by ESI-TOF mass spectroscopy.

The Fmoc groups were again removed by piperidine treatment as described above, and the product **7** was characterized by mass spectrometry confirming a molecular weight of 3956. The terminal amino group content was again detected colorimetrically. The yield of the reaction was 40%.

Preparation of Monocarboxy,tetraamino,tetraPEG (8). The method used is shown in Scheme 5. The carboxylic group of the Fmoc-protected intermediate was activated using DCC and NHS as described for product ***7***. To 23 mg (0.012 mmol) of this activated intermediate was added 7.4 mg (0.005 mmol) of **6** dissolved in anhydrous chloroform and DPEA. The reaction mixture was stirred at room temperature under N_2 atmosphere for 3 h, and the mixture was purified by RP-HPLC as described above. The polymer-containing fractions were pooled, and the solvent was evaporated to give a light yellow oil. This was analyzed

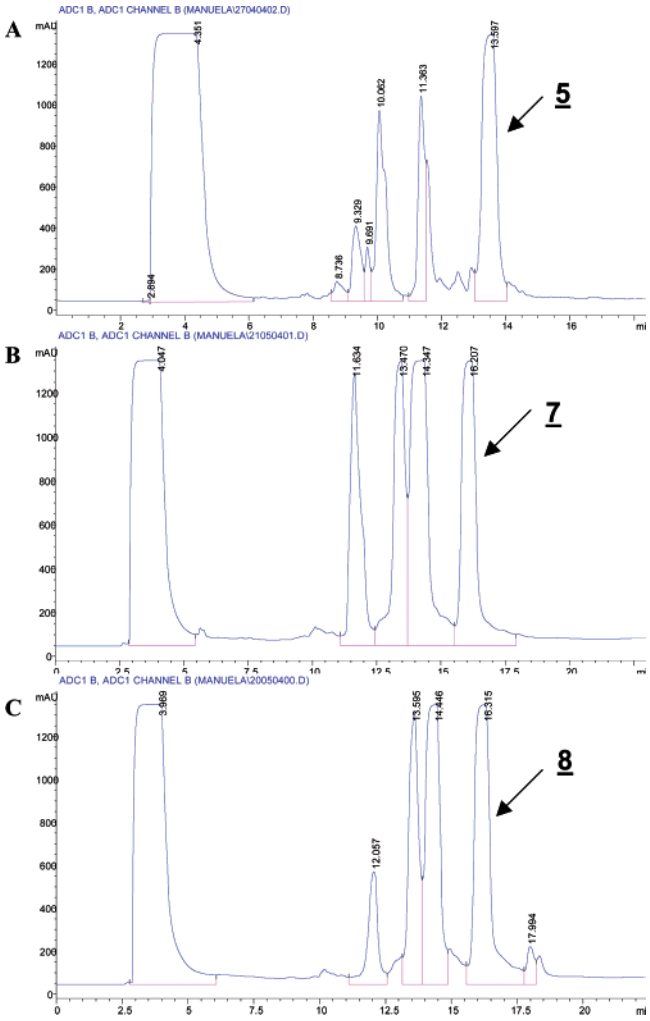


Figure 1. HPLC analysis of the reaction mixtures analyzed by ESI TOF mass spectrometry. Panel (a) shows the elution pattern of the reaction mixture of product **5**. The peaks represent: at 11.3 min product **1**, and at 13.59 min the desired product **5**. Panel (b) shows the elution pattern of reaction mixture product **7**. The peaks represent: at 11.63 min the starting product **1**, at 13.47 min the product **5**, at 14.3 min the product **5** −NHS activated, and at 16.2 min the desired Fmoc-product **7**. Panel (c) shows the elution pattern of reaction mixture of Fmoc-product **8**. The peaks represent: at 12.05 min the starting product **1**, at 13.5 min the product **5**, at 14.44 min the product **5** −NHS activated and at 16.3 min the desired Fmoc-product **8**.

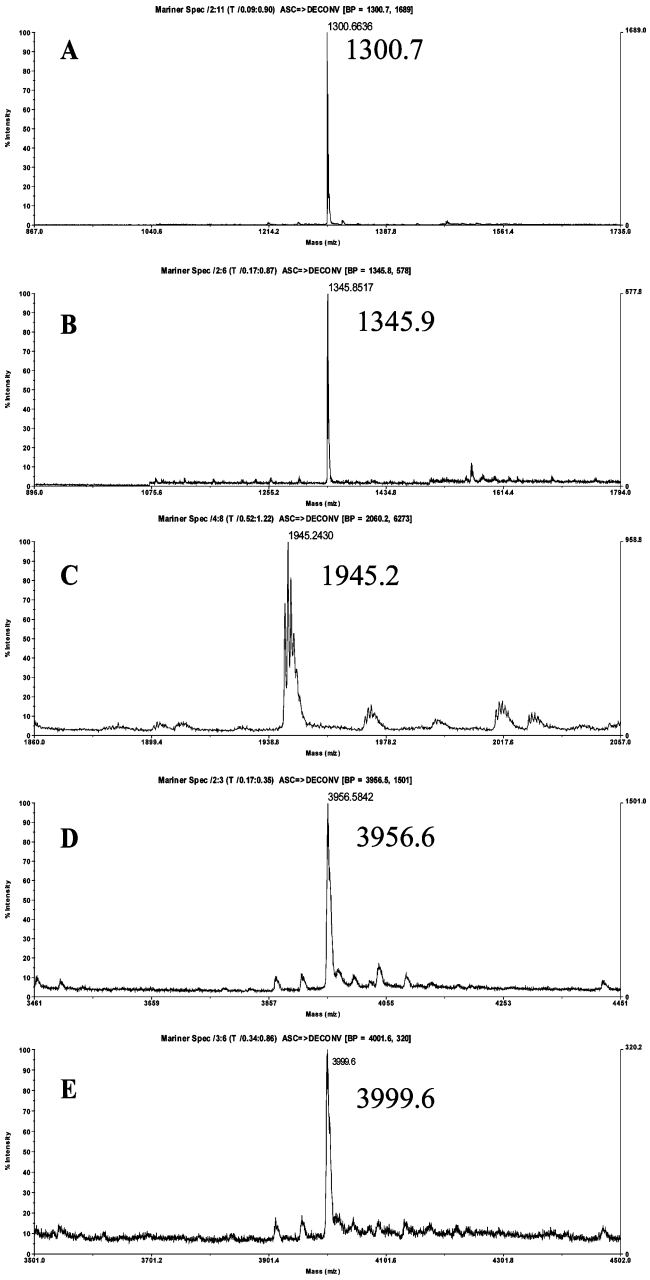


Figure 2. ESI-TOFF mass spectra of the final products. Panel (a) shows product **3**, panel (b) product **6**, panel (c) product **4**, panel (d) product **7**, and panel (e) product **8**. The dispersity of the peaks correspond to their isotopic distribution.

by ESI-TOF mass spectroscopy. Again the Fmoc groups were removed by piperidine treatment, as described above, and the final product **8** was characterized by mass and amino group analysis. The yield of this reaction was 30%.

Synthesis and Characterization of PEG Dendron-OG Conjugates. The method used is outlined in Scheme 6 (the method was used for each of the five samples). OG-succinimidyl ester was added to each PEG dendrons in a 1:1 molar ratio between fluorescence dye and polymer (references [18,19]). In the case of tetraamino,tetraPEG, 3.18 mg (0.8 nmol) was dissolved in 700 μL of methanol and 0.5 mg (0.8 nmol) of OG were added. The reaction mixture was stirred for 1 h at room temperature under N_2 atmosphere, in the dark, at pH 8 for TEA and the solvent then evaporated under reduced pressure. The yellow-orange solid obtained was dissolved in 1 mL of methanol and purified by application to a Sephadex LH 20 column eluted with methanol to remove any remaining unreacted OG. The PEG−OG containing fractions were pooled and the solvent removed. TLC examination as well as PD 10 column elution profile before and after purification were used to assess the purity of the conjugates (see Methods). Since the yield of coupling of OG to dendrons was not complete (in the range of 30−60% only), a purification step was needed to remove the unbound form. The amount of OG present in each conjugate was evaluated spectrophotometrically, by comparison with the extinction coefficient of OG (ϵ = 70 000 cm^{-1} M^{-1}, at λ_{MAX} of 496 nm, measured at pH 8), whereas the amount of polymer was evaluated by weight.

It is well-known that fluorescent probes used routinely for cell-based assays can display concentration- and pH-dependent fluorescent quenching. This can bias interpretation of the results obtained. Therefore the fluorescence of all PEG−OG conjugates was determined at pH 7.4, 6.5, 5.5 (corresponding to the endocytic pathway) and over a range of concentrations.

Evaluation of PEG Dendron Cytotoxicity in Vitro. ECV 304 cells were maintained in medium 199 supplemented 10% foetal bovine serum at 37 °C, in humidified atmosphere with 5% CO_2 atmosphere. Cells were sub-cultured every 2−3 days using trypsin-EDTA for their removal from the flask, and the number of viable cells was assessed by measuring trypan blue exclusion. To assess polymer cytotoxicity, ECV304 cells were seeded (4×10^4 cells) in a 96 well plate. After 24

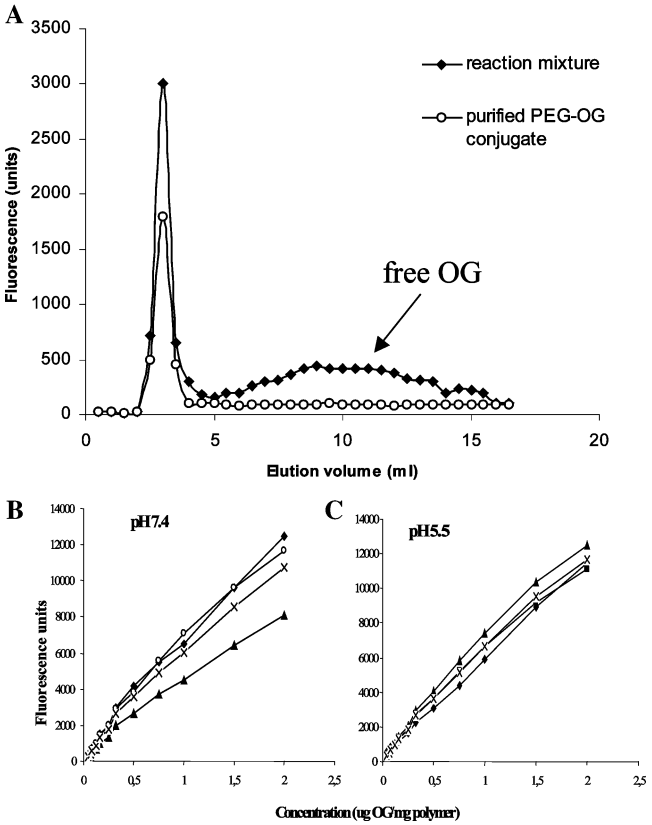


Figure 3. Purification and characterization of OG-labeled PEG dendrons. Panel (a) shows the elution of the OG−tetra-amino,tetraPEG though a Sephadex LH 20 column eluted with methanol. The first peak corresponds to the conjugate, the second fraction to free OG. Panels (b) pH 7.4 and (c) pH 5.5 show the concentration-dependence of fluorescence output. Key to the symbol: ▲, bisPEG-OG; O, trisPEG-OG; ×, monoBisPEG-OG; ◆, tetraPEG-OG.

h, the culture medium was replaced with 100 μL of fresh medium containing serial dilutions of the PEG dendrons (diamino,bisPEG, **2**, triamino,trisPEG, **3**, tetraamino,tetraPEG, **5**, monocarboxy,di-amino,bisPEG, **4**, monocarboxy,tetraamino,tetraPEG, **6**) to a final concentration of 0−4 mg/mL. All stock solutions were filtered sterilized using a 0.22 μm membrane filter. The cells were incubated for 67 h before addition of 20 μL of MTT to each well (5 mg/mL in PBS filtered using 0.22 μm filters). After further 5 h, the medium was removed and 100 μL of optical grade DMSO added to dissolve the insoluble formazan dye crystals present. Absorbance at 550 nm was measured using a microtiter plate reader and cell viability was expressed as percentage of the viability versus untreated control cells.

Cell-Association of PEG Dendron-OG Assessed by FACS. ECV304 cells were seeded (5×10^5 cells/well) in 12-well plates. After 24 h, the medium was removed, and the cells were washed with PBS. A total of 1 μg of OG-dendron dissolved in 250 μL of medium was added to each well. The cells were incubated for period between 0 and 2 h at either 4 or 37 °C. At each time point, the adherent cells were washed three times with cold PBS and then harvested by scraping. The cell-associated fluorescence was determined by flow cytometry (FACScalibur, 488 nm argon-ion laser, Becton Dickinson, Oxford, U.K.). For each time point 10 000 cells were acquired. Cell-association of PEG-dendrons was evaluated by median fluorescence intensity.

The stability of the OG conjugates during incubation with cells was assessed by liquid chromatograpy (PD 10 column) of the tissue culture medium i.e., after 2 h incubation, to estimate the presence of free OG present at that time.

Fluorescence Microscopy. A total of 1 mL of ECV304 cells (1×10^6 c/w) was seeded on glass bottomed 35 mm culture dishes (MatTek Corporation, Ashland U.S.A.) and allowed to adhere for 24 h. The cells

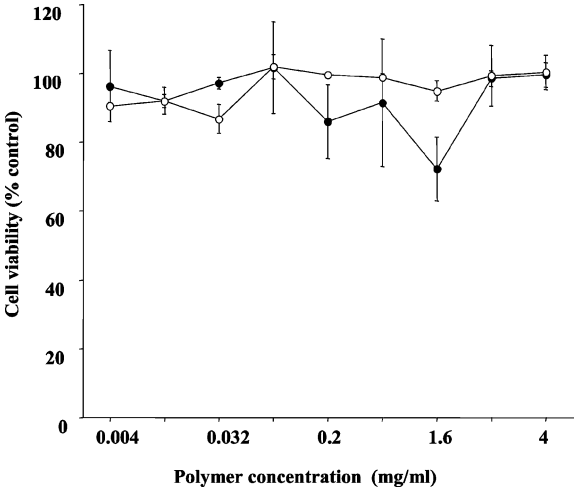


Figure 4. Cytotoxicity of PEG-dendrons incubated with ECV304 cells for 72 h. The cell viability in the presence of triamino,trisPEG (●) and, tetraamino,monocarboxy tetraPEG (○) is shown, mean ± S. E. (n = 6).

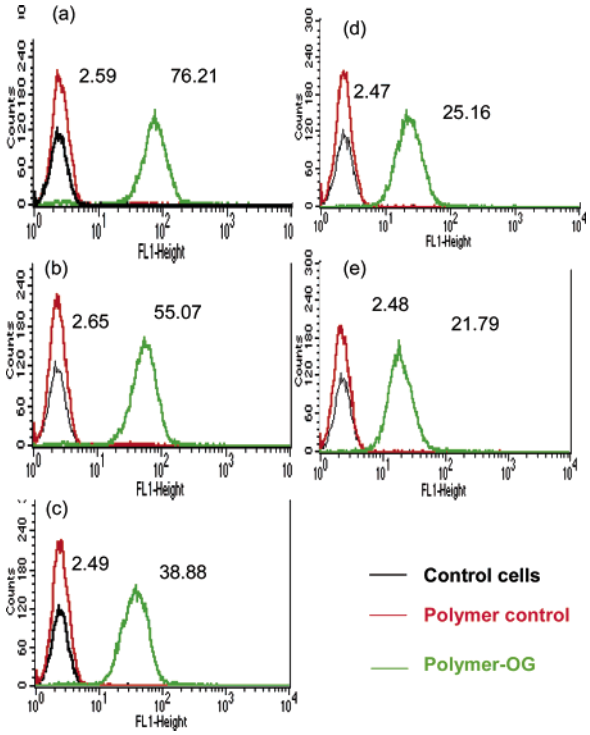


Figure 5. Flow cytometry showing the association of OG-PEG dendrons (4 μg/mL) with ECV304 cells at 37 °C after 2 h. Cells and cells incubated with non labeled polymers were used as controls to eliminate the autofluorescence of cells and the possible influence of the polymers in the cell fluorescence.

were then incubated under tissue culture conditions for 7 h in 600 μL of complete medium containing 4 μg of PEG dendrons-OG conjugates (expressed as μg of OG per mg of compound). Figure 8 shows ECV304 cells after the incubation with triamino,trisPEG-OG conjugate, as typical example. At the end of the incubation, the cells were washed 4× in PBS containing 0.2% w/v BSA and then washed once with imaging medium (MEM lacking Phenol Red containing 25mM HEPES pH 7.4). The live cells, in imaging medium, were then viewed by epifluorescence microscopy using a Leica DMIRB inverted fluorescent microscope equipped with a 40× objective and using an excitation filter 450−490 nm and suppression filter 515 nm. Images were captured with a Digital CCD Retiga 1300 camera and processed using Improvision software.

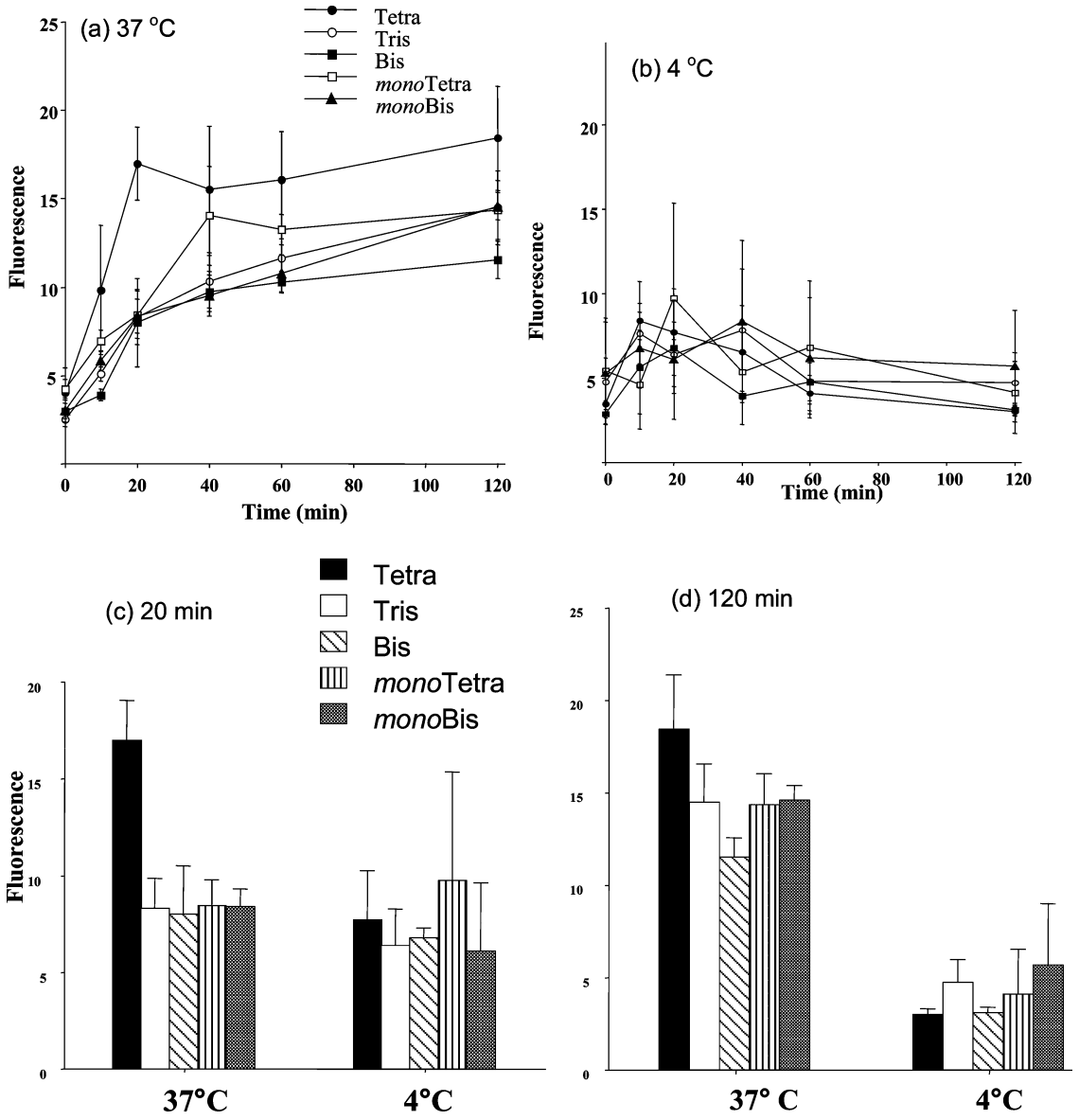


Figure 6. Cell-association of OG-labeled PEG conjugates with ECV304 cells over time at 37 and 4 °C, by flow cytometry. Panel (a) shows the time-dependent association at and panel (b) at 4 °C. Panel (d) compares the cellular fluorescence seen at 37 and 4 °C for all compounds at 20 min and panel (d) makes this comparison at 2 h.

Results and Discussion

Since the synthesis of the first dendrimers by convergent and divergent routes,[20−22] interest in their use for biomedical applications has been growing exponentially. Over recent years several families of linear (mono-functional and diol) and branched (tetraol) PEGs have been developed by Frechet and colleagues as a platform for the attachment polyester dendrons (generation 1 to 4) to which drugs (such as the anticancer agent doxorubicin) have been bound.[23−26] Observation that PEG-polyester dendrons show interesting intracellular trafficking properties in ECV304 cells, stimulated our interest in these architectures. When the cells are grown as an adherent monolayer linear low molecular weight constructs predominantly trafficked to lysosomes after endocytic uptake (only ∼34% exocytosed), whereas the higher molecular weight, more branched PEG-polyester dendrons were predominantly exocytosed rapidly after internalization (∼75%).[11] Examining the transport of such PEG−polyester dendrons across ECV304 cells, grown as a monolayer, it was shown that linear lower molecular weight constructs rapidly traversed the monolayer at rates ∼3-fold faster than FITC-dextran. These observations were the motivation for design the new PEG-based dendritic building blocks described here.

The PEG-based dendrons were synthesized to contain either NH_2, or both NH_2 and COOH terminal groups (Table 1). The products **3** and **4** were easily obtained in homogeneous form by precipitation. In contrast, for products **6**−**8**, the reaction mixture revealed both starting products and intermediates as impurities (Figure 1). Therefore an additional HPLC-based purification step was introduced before removal of Fmoc. ESI-TOF mass spectroscopy and amino terminal end group analysis on the final products were used to establish products identity. Mass spectroscopy confirmed purity since the obtained values were consistent with the theoretical ones, and the expected number of amino terminal groups was also found (Table 1 and Figure 2).

To create probes able to monitor endocytosis, the PEG dendrons synthesized were labeled using OG activated as succinimidyl ester.[10,11] The reaction conditions were aimed at obtaining one OG label per polymer molecule, and therefore a

molar ratio of 1:1 of OG per polymer was used. However, since the reaction did not reach completion the unbound OG was removed by column chromatography and the final content in each polymer is reported in Table 1. Fluorescent probes are notoriously difficult to use since they often display both pH- and concentration-dependent quenching. However, since the OG probes used here displayed an approximately linear relationship between concentration and fluorescence up to 2 μg of bound OG to avoid any quenching the maximum amount used in the study was 1 μg OG. Fluorescence output was not affected by pH values in the range of pH 7.4−5.5, the physiological values that would be encountered during endocytic uptake (Figure 3b,c). Before studying the cellular uptake properties, it was considered important to verify the lack of toxicity of the amino group-containing PEG dendrons prepared here. It is in fact known that the amino containing polymers are usually cytotoxic. Many studies have already examined dendrimer cytotoxicity in vitro using different cell lines, a variety of incubation times (hours to days), and various assay methods (reviewed in ref 27). General trends are clear: dendrimers bearing $-NH_2$ termini display concentration- and usually generation-dependent cytotoxicity. For example, we had already showed that generation 4 PAMAM, DAB, and DAE dendrimers had IC_{50} values = 50−300 μg/mL when incubated for 72 h with B16F10 cells.[28−30] In addition, cell morphology visualized by scanning electron microscopy (SEM) showed that cell morphology changed dramatically on exposure to PAMAM and DAB (1 mg/mL) dendrimers.[29] Dendrimer cytotoxicity is dependent on the chemistry of the core but is more strongly influenced by the nature of the dendrimer surface. For example, the cytotoxicity (clone 9 cells; 3h, MTT assay) of cationic dendrimers selected from a melamine-based dendrimer library including amine, guanidine, carboxylate, sulfonate, phosphonate, or PEGylated surfaces, showed that cationic dendrimers were much more cytotoxic than anionic or PEGylated dendrimers.[30] In fact, we found that PEG-poly(ester) dendritic hybrids were non toxic toward B16F10 cells (IC_{50} > 1 mg/mL; 72 h; MTT),[11] and Frechet and colleagues reported that a three-arm PEG star (M_w = 22 550 g/mol) bearing generation 2 poly(ester) dendrons (final M_w = 23 500 g/mol) had an IC_{50} of 40 mg/mL toward B16F10 cells during a 48 h incubation.[24] When compounds **4** and **8** (considered representative of amino or amino-carboxylic species) were incubated with ECV304 cells for 72 h, they were not toxic at concentration up to 4 mg/mL (Figure 4).

All of the OG-labeled PEG dendrons showed time-dependent cell-association at 37 °C (Figures 5 and 6a,c), and the increase in cell fluorescence was biphasic. An initial more rapid association (up to ∼20 min) was followed by a slower but sustained increase over 2 h. At 4 °C, the cell association was significantly reduced after 2 h for all PEG dendrons; however, over the first 20 min, only the OG−tetraamino,tetraPEG dendron showed greater uptake at 37 °C then seen at 4 °C (Figure 6b,c). These observations suggest that most PEG dendrons show little or no internalization in the early time frame, but they are simply membrane bound. The OG−PEG dendron linkage should be stable under the neutral and mild acidic conditions of the assay and thus we wanted confirm by gel permeation chromatography of the tissue culture medium at the end of the 2 h incubation the absence of free OG released from the probe (Figure 7).

Fluorescence microscopy analysis showing the presence of TrisPEG−OG fluorescence in the perinuclear region suggested that the polymer was delivered during the incubation period to late endosomes and lysosomes that are often enriched in this region[33] (Figure 8). In agreement with endosomal traffic, more

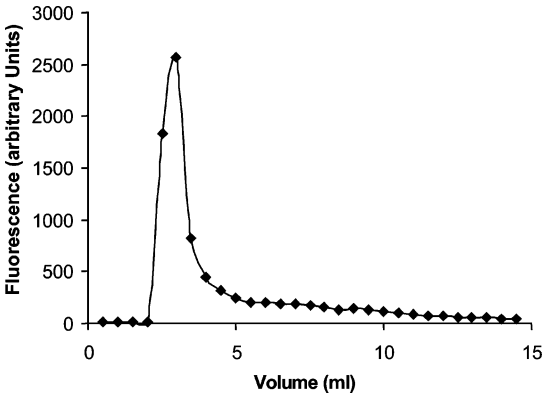


Figure 7. PD10 column chromatography of tissue culture medium after a 2 h incubation of diamino,monocarboxy,bisPEG with ECV 304 cells. The peak at 4 mL represents OG conjugate and absence of free OG at 10 mL of elution confirms the stability during the incubation period.

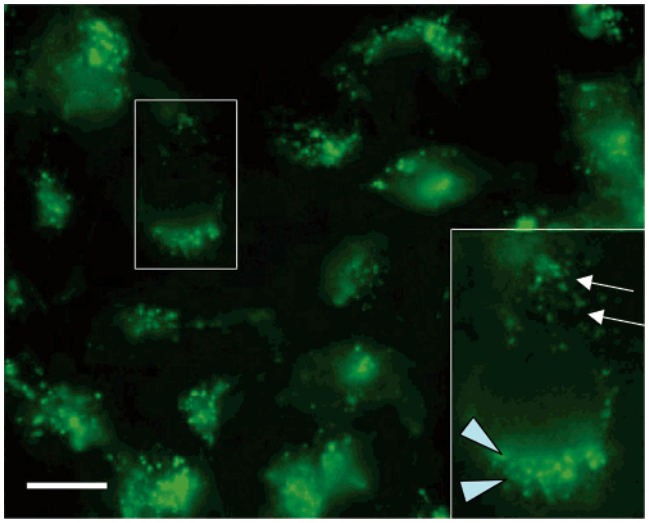


Figure 8. Fluorescence microscopy of trisaminotrisPEG−OG in ECV304 cells. Cells were incubated with trisaminotrisPEG-OG polymer for 7 h. The perinuclear region was shown to be enriched with fluorescence. The enlarged image shows fluorescence positive vesicular structures in the perinuclear (arrowhead) and peripheral regions (arrows). Scale bar 10 μM.

peripheral labeled vesicles were observed (arrows in enlarged image) and these probably correspond to early endosomes. Based on the observation that OG−conjugates are stable over the course of the cell incubation and that fluorescence would not be significantly influenced by differences in intracellular vesicular pH, we can conclude that the fluorescence observed by microscopy and FACS analysis is due to PEG-dendron uptake.

4. Conclusions

Polymer architecture is emerging as an important parameter when designing synthetic macromolecular carriers for therapeutic use.[11,23,31,32] Here, novel monodisperse PEG−dendrons have been synthesized with amino or carboxylic terminal groups. These novel constructs, non toxic, over the concentration range tested, have broad potential for further development in view of the fact that using convergent assembly it would be also possible to generate higher molecular weight dendrimers. They are tools for synthesis of new "bow-tie" architecture dendrimers, and they could also be used for surface modification or synthesis of dendronised polymers. Not least these PEG dendrons have

potential for further development as drug or gene delivery systems, as they are internalized by endocytosis. The polymer charge appears to play a greater role on cell-association/internalization than the polymer molecular weight. The tetraamino,tetraPEG was taken up most rapidly over the initial 20 min. Further experiments are ongoing to define the precise intracellular trafficking pathways utilised by these constructs and moreover define their rates, endocytosis and transcytosis. This profile will allow optimized design for specific biomedical application.

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