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Poly-(amidoamine) dendrimers with a precisely core positioned sulforhodamine B molecule for comparative biological tracing and profiling

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ABSTRACT

We report on a simple robust procedure for synthesis of generation-4 poly-(amidoamine) (PAMAM) dendrimers with a precisely core positioned single sulforhodamine B molecule. The labelled dendrimers exhibited high fluorescent quantum yields where the absorbance and fluorescence spectrum of the fluorophore was not affected by pH and temperature. Since the stoichiometry of the fluorophore to the dendrimer is 1:1, we were able to directly compare uptake kinetics, the mode of uptake, trafficking and safety of dendrimers of different end-terminal functionality (carboxylated vs. pyrrolidonated) by two phenotypically different human endothelial cell types (the human brain capillary endothelial cell line hCMEC/D3 and human umbilical vein endothelial cells), and without interference of the fluorophore in uptake processes. The results demonstrate comparable uptake kinetics and a predominantly clathrin-mediated endocytotic mechanism, irrespective of dendrimer end-terminal functionality, where the majority of dendrimers are directed to the *endo*-lysosomal compartments in both cell types. A minor fraction of dendrimers, however, localize to endoplasmic reticulum and the Golgi apparatus, presumably through the recycling endosomes. In contrast to amino-terminated PAMAM dendrimers, we confirm safety of carboxylic acid- and pyrrolidone-terminated PAMAM dendrimers through determination of cell membrane integrity and comprehensive respiratory profiling (measurements of mitochondrial oxidative phosphorylation and determination of its coupling efficiency). Our dendrimer core-labelling approach could provide a new conceptual basis for improved understanding of dendrimer performance within biological settings.

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1. Introduction

Dendrimers are receiving increasing attention as versatile macromolecular vehicles for drug solubilization, encapsulation and complexation [1]. The biological performance of dendrimers is often controlled by their physicochemical properties such as elemental composition, size, branching symmetry and surface chemistry/functionality [1–4]. Recent studies with fluorescently labelled dendrimers have demonstrated that dendrimers of different structural and surface functionalities enter mammalian cells through different modes of endocytic pathways [5–10]. Although, uptake mechanisms are cell type and cell culture-dependent, where cell confluence, passaging and media affect uptake rates, the mode of labelling may further modulate dendrimer uptake processes, intracellular trafficking and cytotoxicity [1]. For instance, surface grafting of a single fluorophore molecule may induce sufficient surface perturbation as in hydration and "structured-water" thereby influencing dendrimer–plasma protein binding constants, their stoichiometry as well as direct interaction between dendrimers and cell membranes. Uncontrolled surface (or interior) decoration with fluorophores may further promote π–π interaction between fluorophores of adjacent dendrimers triggering aggregation and forming heterogeneous entities of different sizes and shapes [1,10]. This is in contrast to biological environment where native (non-labelled) dendrimers (in macromolecular form) and dendrimer–protein complexes of different stoichiometry may interact with cells [1,11–13]. There are also limitations where fluorophore molecules are physically trapped in dendrimer voids. This may not only induce dendrimer aggregation [1], but the fluorophores may leak out prematurely in biological milieu due to partitioning [1]. Therefore, there is a need for dendrimers carrying a single well-shielded

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and core-positioned fluorophore. Two recent attempts have yielded core-labelled dendrimers of specific scaffolds where the fluorophore to dendrimer ratio was either not 1:1, or when the stoichiometry was 1:1, the fluorescent quantum yield was low [10,14]. Here, we have addressed these limitations and introduced an alternative yet robust synthetic methodology, which has allowed for precise positioning of a single fluorescent tracer (sulforhodamine B) in the core of poly-(amidoamine) (PAMAM) dendrimers. The absorbance and fluorescence spectrum of the fluorophore was unaffected by pH and temperature changes and the labelled dendrimers possessed high fluorescent quantum yields. We limited our studies to PAMAM dendrimers, and particularly to generation 4 (G4) species, since they are among the best studied of all dendrimers and suitable for biological targeting both in vitro and in vivo [1]. Amino-terminated PAMAM dendrimers can induce cytotoxicity [1,15] and are cleared rapidly from the blood on intravenous injection by reticuloendothelial system and kidneys [16,17]. Therefore, we further designed sulforhodamine B core-labelled G4-PAMAM dendrimers of different end-terminal functionality (carboxylic acid and pyrrolidone) to modulate their biological performance. Accordingly, we compared their uptake kinetics, intracellular performance and safety in two phenotypically different human endothelial cell types as examples of heterogeneous vascular targets. The carboxylic end-terminal functionality is expected to improve macromolecular compatibility with biological systems [18]. Likewise, pyrrolidonation may also improve dendrimer safety; a notion inspired from observations with polyvinyl pyrrolidone as an effective plasma substitute (Periston or Kollidon) given to over 500,000 human recipients with good safety records over years [19,20].

2. Materials and methods

2.1. General synthetic procedures for dendrimer and their characterization

Unless otherwise stated, all starting materials were used as received. All solvents were of HPLC grade and used as received. ^{1}H NMR spectra were recorded on a 500 MHz NMR (Bruker) apparatus. Chemical shifts are reported in ppm downfield of TMS (tetramethylsilane) using the resonance of the deuterated solvent as internal standard (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). High resolution MALDI (HR-MALDI-TOF(+)) was measured on a Bruker SolariX XR instrument in positive mode. For HPLC-MS analysis a Dionex Ultimate 300 PLC connected to an ESI-MS (MSQ Plus Mass Spectrometer, Dionex, Silicon Valley, CA, USA) was used. All measurements were performed on a Phenomenex Kinetex 5 μm C18 100 Å column (50 × 2.1 mm) thermostated to 42 °C with a column oven. As eluent system water and acetonitrile (containing 0.1% (*v*/*v*) formic acid) were used, starting with 5% acetonitrile for 0.5 min, then a gradient from 5% to 35% running for 0.7 min, followed by a gradient of 35% to 100% over 3 min and finally 100% acetonitrile. The flow rate was set to 0.5 mL/min (300 bar). Absorption was followed at λ = 215 nm. The injection volume was 2 μL, which contained 5 mg/mL dendrimer dissolved in acetonitrile:water (5:95).

Detailed synthetic steps for intermediate compounds **1–14** and products **15** (Rhodamine Dendrimer–G4–Carboxy, Rh-G4-COOH) and **16** (Rhodamine Dendrimer–G4–Pyrrolidone, Rh-G4-Pyr) together with their corresponding ^{1}H NMR, ^{13}C NMR, HR-MALDI-TOF(+), HPLC chromatogram and size exclusion chromatography-multi angular light scattering (SEC-MALS), where applicable, are presented in the Supporting Information File.

2.2. Steady-state measurements

Absorption and emission spectra of dendrimer products **15** and **16** were collected in a Jasco V-650 spectrophotometer (JASCO, Japan) and a Jasco Model FP-6200 spectrofluorometer (JASCO, Japan), respectively. MilliQ water was used as solvent, unless otherwise stated. The values of fluorescence quantum yield were obtained using a diluted method and Rhodamine 101 in ethanol as fluorescence standard ($\varphi_{st} = 0.915$) [21].

To investigate temperature and pH stability of sulforhodamine B labelled dendrimers, Rh-G4-Pyr and Rh-G4-COOH were added to different pH buffered solutions (0.1 M citrate buffer pH 2.1; 0.1 M citrate buffer pH 4.8; 0.1 M PBS pH 7.5; and 0.1 M carbonate buffer pH 10.1) or the cell culture media (Dulbecco's Modified Eagle's medium, DMEM, supplemented with 20% *v*/*v* FBS) to yield absorbance values below 0.1. Absorption and emission spectra were collected at 20 °C and after incubation at 37 °C for 16 h.

2.3. Fluorescence anisotropy and hydrodynamic radius determination

Time-resolved fluorescence anisotropy decays were acquired on Fluotime 300 instrument (Picoquant, Berlin, Germany) using picosecond laser diodes with a wavelength of 507 ± 3 nm as excitation source. The detection wavelength was set at the emission maximum. The long rotational correlation time component obtained from the fit was used in the Perrin equation [22] to calculate the hydrodynamic diameters of Rh-G4-Pyr and Rh-G4-COOH.

2.4. Size exclusion chromatography–multi angular light scattering (SEC-MALS)

The number and weight average molecular weights were obtained using a size exclusion system (HPLC: Dionex Ultimate ® 3000, Column: TSKgel® GMPWXL HPLC Column) coupled with a multi-angle light scattering (miniDAWN TREOS-AQUEOUS) and refractive index (RI Detector Refractomax 521) detectors. The data were analysed and processed with ASTRA software. The SEC-MALS-RI apparatus was from Wyatt Technology Europe (Dernbach, Germany) and the column was obtained from Sigma Aldrich (Denmark). PBS tablets (Sigma Aldrich) were dissolved in MilliQ to yield 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH 7.4). The PBS buffer was used as solvent. A 50 μL volume of sample solution pre-filtered with 0.2 μm Acrodisc ® Syringe Filter (Supor ® Membrane) was injected into the system with a flow rate of 0.5 mL/min.

2.5. Cell uptake and trafficking studies

The human brain capillary endothelial cell line hCMEC/D3, transduced by lentiviral vectors incorporating human telomerase or SV40 T antigen, were grown in EBM-2 medium supplemented with 5.0% (*v*/*v*) FBS, hydrocortisone (1.4×10^{-6} M), basic fibroblast growth factor (1 ng/mL), 1.0% (*w*/*v*) penstrep and 10×10^{-9} M HEPES [23]. Human umbilical vein endothelial cells (HUVECs) were grown in DMEM with 20.0% (*v*/*v*) FBS on gelatin-coated flasks. All cells were maintained at 37 °C in a humidified atmosphere (air supplemented with 5% CO_2). For the uptake study, hCMEC/D3 cells and HUVECs (2×10^4/cm^2) were seeded on 24-well plates (Corning, USA) and grown for 1 day at 37 °C, and 5% CO_2 to 60%–70% confluence. Afterwards, cells were washed three times with pre-warmed PBS and the uptake studies (in the presence of serum) were initiated by adding different concentration of Rh-G4-Pyr and Rh-G4-COOH dendrimers. After dendrimer exposure for specified times, the cells were harvested by trypsinization and a total of 10,000 cells were analysed by flow cytometry (FACS Array Cell Analysis, BD, USA). For live-cell microscopy and organelle tracking, cells were seeded on eight-well Lab—-Tek chamber slides (Nunc, Naperville, IL, USA) for 1 day and then labelled with CellLight Reagents BacMam 2.0 actin–GFP, tubulin–GFP, early endosomes–GFP, lysosomes–GFP, Golgi–GFP, and endoplasmic reticulum (ER)–GFP in accordance with the manufacturer's protocol (Molecular Probes, Life Technologies, CA, USA) [23]. Live cell imaging was performed on a Leica AF6000LX microscope equipped with a 63× (numerical aperture 1.47) oil objective using 1.6× magnification and analysed [23]. The co-

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