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Synthesis and Biological Evaluation of Radio and Dye Labeled Amino Functionalized Dendritic Polyglycerol Sulfates as Multivalent Anti-Inflammatory Compounds

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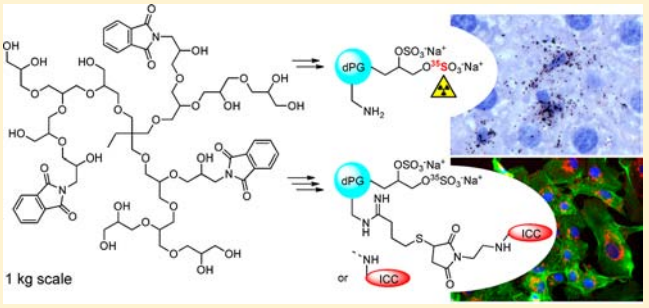
Supporting Information

ABSTRACT: Herein we describe a platform technology for the synthesis and characterization of partially aminated, ^{35}S-labeled, dendritic polyglycerol sulfate (dPG^{35}S amine) and fluorescent dPGS indocarbocyanine (ICC) dye conjugates. These polymer conjugates, based on a biocompatible dendritic polyglycerol scaffold, exhibit a high affinity to inflamed tissue in vivo and represent promising candidates for therapeutic and diagnostic applications. By utilizing a one-step sequential copolymerization approach, dendritic polyglycerol ($M_n \approx 4.5$ kDa) containing 9.4% *N*-phthalimide protected amine functionalities was prepared on a large scale. Sulfation and simultaneous radio labeling with $^{35}SO_3$ pyridine complex, followed by cleavage of the N-phthalimide protecting groups, yielded dPG^{35}S amine as a beta emitting, inflammation specific probe with free amino functionalities for conjugation. Furthermore, efficient labeling procedures with ICC via iminothiolane modification and subsequent "Michael" addition of the maleimide functionalized ICC dye, as well as by amide formation via NHS derivatized ICC on a dPGS amine scaffold, are described. The dPGS-ICC conjugates were investigated with respect to their photophysical properties, and both the radiolabeled and fluorescent compounds were comparatively visualized in histological tissue sections (radio detection and fluorescence microscopy) of animals treated with dPGS. Furthermore, cellular uptake of dPGS-ICC was found in endothelial cord blood (HUVEC) and the epithelial lung cells (A549). The presented synthetic routes allow a reproducible, controlled synthesis of dPGS amine on kilogram scale applying a one-pot batch reaction process. dPGS amine can be used for analysis via radioactivity or fluorescence, thereby creating a new platform for inflammation specific, multimodal imaging purposes using other attachable probes or contrast agents.



INTRODUCTION

The development of macromolecular diagnostics and therapeutics has gained increasing interest in the past few years.[1] Achieving high selectivity for a specific kind of tissue or disease is very challenging when designing molecules that either exhibit therapeutic activity or serve as a carrier for controlled drug delivery and release.[2] In both cases, it is crucial to understand the physiological fate of the nanoconstruct in detail, especially with respect to its administration/absorption, distribution, metabolism, and excretion/elimination (ADME) behavior, including bioavailability, pharmacokinetics, and uptake into the desired target tissue or cell. In order to investigate the distribution in cells and in vivo, qualitative and quantitative detection methods require labeling of a certain candidate with an appropriate tracer. For low molecular weight compounds, e.g., cytostatics, it is an established practice to tag the molecule with an emitting moiety, either through a radioisotope or a fluorescent label. However, the effect of the chosen label on the chemical behavior has to be carefully considered. Usually, radiolabeling with long-lived tritium (^3H) or carbon (^{14}C) is the method of choice for ADME studies, but this can require laborious synthetic work and careful handling. The covalent attachment of organic fluorophores, such as cyanine dyes, on the other hand, is a widely applied and straightforward methodology but causes substantial changes in the chemical structure and possibly function. As a result, the properties of the labeled candidate might be completely different compared to the unlabeled, native compound. In this work, we present a new, general synthetic approach toward multivalent polymeric and therapeutically active macromolecule, dendritic polyglycerol sulfates (dPGS),[3] which has been designed for labeling with either a radioisotope or fluorophore using a single polymeric

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precursor. Dendritic polyglycerol (dPG), which is based on a soft, spherical polyether polyol scaffold, is known to be highly biocompatible and suitable for biomedical applications,[2,4,5] and its sulfated derivative dPGS expresses exceptionally high affinities toward the inflammation targets, e.g., the cell adhesion glycoproteins L- and P-selectin.[6−8] This strong multivalent interaction enables molecular imaging of inflammatory diseases after intravenous (i.v.) administration of dPGS-based probes, which was recently shown with a multifunctional dendritic polyglycerol sulfate (dPGS) near-IR conjugate that was applied in an arthritis rat model and an acute dermatitis model.[9,10] Based on an easily accessible general platform polymer, dPG N-phthalimide, we present two efficient synthetic routes for the preparation of ^{35}S radiolabeled dPGS containing free amino functionalities (dPG^{35}S amine) for further conjugation and the synthesis of dPGS indocarbocyanine (ICC) dye conjugates using mild and straightforward bioconjugation protocols. In contrast to previously published routes to modify polyglycerol scaffolds,[8,9] the described procedures deliberately avoid copper-catalyzed azide−alkyne "click" chemistry (CuAAC) for better biological applications.

EXPERIMENTAL PROCEDURES

General. Chemicals and reagents were obtained from Acros Organics, Sigma-Aldrich, Merck, and ABCR, and were reagent grade and used as received unless otherwise stated. Dry reactions were performed in flame-dried glassware under argon atmosphere. Radioactive $^{35}S-H_2SO_4$ was purchased from Hartmann Analytics (Braunschweig, Germany). Purification by ultrafiltration was performed in solvent resistant stirred cells with PLAC regenerated cellulose membranes with a molecular weight cutoff (MWCO) of 1000 g mol^{-1} (Millipore, USA). ICC NHS ester and ICC maleimide are commercially available from mivenion GmbH (Berlin, Germany) or IRIS Biotech (Marktredwitz, Germany) under the product line MiDye550. Dialysis was performed with membranes of benzoylated cellulose or regenerated cellulose (MWCO 2000; Sigma-Aldrich). Size exclusion chromatography was performed with Sephadex G-25 superfine (Sigma-Aldrich) using a syringe as column with a diameter of 1.3 and 6.0 cm length under ambient pressure and temperature. Reversed-phase chromatography was performed in glass columns with MPLC pumps using LiChroprep RP-18 (40−63 μm; Merck KG, Darmstadt, Germany). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Jeol ECX 400 or on a Bruker BioSpin AV 700 spectrometer. IR measurements were performed on a Nicolet AVATAR 320 FT-IR 5 SXC with a DTGS detector. MALDI-TOF measurements were performed on a Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer in linear or reflection mode using a α-cyano-4-hydroxycinnamic acid (CHCA) matrix. The laser repetition rate was 40−45 Hz and the acceleration voltage was 25 kV. Elemental analysis was performed on a Vario EL III elemental analyzer using sulfanilic acid as standard. Molecular weight distributions were determined by size exclusion chromatography (SEC) equipped with a refractive index detector (operated at 50 °C) providing the parameters M_n, M_p, M_w, and PDI. Measurements were performed under diluted conditions (10 mg mL^{-1}, injected volume 20 μL) using an Agilent 1100 solvent delivery system with ISOpump, manual injector, and an Agilent 1100 differential refractometer. Three 30 cm columns in row (PSS SUPREMA, 5 μm particle size) were used at room temperature with a flow rate of 1.0 mL min^{-1} using an aqueous 0.1 M $NaNO_3$ solution as mobile phase. DIN certified Pullulan provided by PSS was used as polymeric standard. Samples with M_n = 342, 1080, 5500, 9200, 20k, 100k, 188.5k, 358k, and 636k and PDI = 1.00−1.30 were used for regression. WinGPC Unity from PSS was used for data acquirement and interpretation. Liquid scintillation counting (LSC) was performed in triplicate on a Hidex 300 SL (Hidex, Finland) using a Tritium Standard and a duration of 180 s for each measurement in 10 mL Rotiszint eco plus (Roth, Germany). Absorption spectra were recorded on a LAMBDA 950 UV/vis/NIR spectrometer (PerkinElmer, USA), fluorescence spectra were measured with a JASCO FP-6500 spectrofluorometer (150 W xenon lamp, R928 Hamamatsu photomultiplier).

Synthesis and Characterization. *dPG Phthalimide (**3**).* (a) 2-Ethyl-2-(hydroxymethyl)-1,3-propanediol (**1**, (TMP) 2.68 g, 20 mmol, 1 equiv) was placed in an extensively dried 1 L-batch reactor and melted at 60 °C in vacuo until a colorless liquid was obtained. KO*t*Bu (1 M in THF, 6.00 mL, 2 mmol, 0.3 equiv) was added and the resulting salt immediately dissolved by adding anhydrous NMP (10 mL). Under rapid stirring (anker stirrer, 250 U min^{-1}) the temperature was elevated to 120 °C. Within 18 h, freshly distilled glycidol (89.7 mL, 100.0 g, 67.5 equiv) in anhydrous THF (225 mL) was added using a precision dosing pump (17.48 mL h^{-1}), followed by 30 min of stirring. For structural evaluation of the dPG a small aliquot was removed. ^{1}H NMR (700 MHz, CD_3OD, δ): 4.37−3.40 (dPG backbone), 0.91 (s, C$\underline{H}_3$ TMP, 3 H) ppm. Reference CD_3OD quintuplet at 3.3 ppm. ^{13}C NMR (176 MHz, CD_3OD, δ): 81.5, 80.0, 73.9, 73.3−71.9 (m), 70.8, 64.4, 62.8 ppm. Reference CD_3OD septuplet at 49.15 ppm. GPC (0.1 M $NaNO_3$, 10 mg mL^{-1}): M_n = 3256 g mol^{-1}, PDI = 1.72. Subsequently, *N*-(2,3-epoxypropyl)phthalimid (**2**, 27.43 g, 135 mmol, 6.75 equiv) in anhydrous NMP (100 mL) was added over two hours at 120 °C and the mixture was stirred overnight. The reaction was cooled down to ambient temperature and quenched by the addition of 400 mL MeOH and cationic ion-exchange resin (DOWEX Monosphere 650C) and stirred at rt overnight. Precipitation in acetone and diethyl ether and subsequent drying in vacuo yielded the final polymer **3** in quantitative yield. ^{1}H NMR (700 MHz, CD_3OD, δ): 8.25−7.61 (m, Ar, 3 H), 4.37−3.40 (m, dPG backbone, 5 H), 0.91 (s, 3 H) ppm. Reference CD_3OD quintuplet at 3.31 ppm. ^{13}C NMR (176 MHz, CD_3OD, δ): C resonance peaks from phthalimide group: 169.9, 135.3, 133.4, 124.1 ppm. C resonance peaks from polymer backbone: 81.5, 80.0, 73.9, 73.3−71.93 (m), 70.8, 64.37, 62.8 ppm. Reference CD_3OD septuplet at 49.15 ppm. MALDI-ToF-MS: m/z = 3082.04, 3506.69, 3708.39, 4281.97 ± x·74.08 [dPG-(phthalimide)$_n$ + Na^+] with n = 2, 4, 6, 8.

*dPGS Amine (**5**).* (b) dPG phthalimide (**3**, M_n = 4560 g mol^{-1}, 208 mg, 34.7 μmol, 2.8 mmol OH groups) was dried under vacuum at 60 °C for two days and dissolved in anhydrous DMF (2 mL). At 60 °C, dry SO_3 pyridine complex (492.3 mg, 3.1 mmol, 1.1 equiv/OH group) as a mixture with the pyridinium chloride (ratio 2:3) in anhydrous DMF (5 mL) was added at 60 °C over a period of 30 min and the reaction was stirred for further 15 h. Water (5 mL) was added and the pH of the resulting solution was immediately set to 8 by addition of aqueous NaOH. The solvent was evaporated in vacuo and the crude intermediate dPGS N-phthalimide was dissolved in H_2O (10 mL). Details for the preparation of SO_3 pyridine complex are provided in the Supporting Information.

(c) Sodium borohydride (63.3 mg, 1.7 mmol, 48 equiv) was slowly added to the aqueous solution of dPGS N-phthalimide

at rt over a period of 30 min and the reaction was stirred for 15 h at 50 °C. Acetic acid (300 μL) was added dropwise, the mixture was stirred for another 7 h at 85 °C, and the solvent was evaporated. Toluene (15 mL) was added, the mixture was stirred for 10 min, and the solvent was evaporated in order to coevaporate residual acetic acid. This process was repeated twice. To the crude product was added methanol (100 mL), the mixture was stirred for one hour, and the solvent was decanted (three times). The solvent was evaporated in vacuo and the crude product subjected to ultrafiltration (MWCO 1000 g mol^{-1}) using a half concentrated sodium chloride solution followed by water until no residual salt was observed in the filtrate (monitored by precipitation using silver nitrate). The solution was filtered through a 0.2 μm syringe filter (Roth, Germany), concentrated and purified by size exclusion chromatography (Sephadex G-25). The polymer fraction was freeze-dried to yield the title compound as a colorless salt with a calculated molecular weight of M_n = 10 050 g mol^{-1} in 71% yield (325 mg). ^{1}H NMR (700 MHz, CD_3OD, δ): 4.89−4.65 ($C_{sec}\underline{H}$-OSO_3Na), 4.48−4.17 ($C_{prim}\underline{H}_2$-OSO_3Na), 4.15−3.30 (m, dPG backbone), 1.53 (s, $C\underline{H}_2CH_3$, TMP, 2 H), 1.00 (s, $CH_2C\underline{H}_3$, TMP, 3 H) ppm. Reference: D_2O singulett at 4.79 ppm. ^{13}C NMR (176 MHz, CD_3OD, δ): 78.5, 77.3, 76.1, 75.9, 73.8, 71.1, 70.3, 69.7, 69.5, 68.9, 68.7, 68.3, 67.6, 66.9, 41.2 ($\underline{C}(CH_2)_4$, TMP), 22.0 ($\underline{C}H_2CH_3$, TMP), 7.0 ($CH_2\underline{C}H_3$, TMP) ppm. IR (bulk) $\tilde{\nu}_{max}$: 3460, 2952, 2884, 1640, 1460, 1220, 1071, 1027, 1006, 931, 770 cm^{-1}. Sulfur content from elemental analysis: 16.6% corresponding to a degree of sulfation of 85%.

*dPG^{35}S Amine (**6**).* Compound **6** was synthesized under identical reaction parameters (b) and (c) as for **5**, but using $^{35}SO_3$ pyridine complex (**4**) as a mixture with the pyridinium chloride salt (2:3) for radio sulfation. Details for the synthesis of the sulfation agent **4** are provided in the Supporting Information. Identical workup and freeze-drying gave dPG^{35}S amine (**6**) as a colorless salt with a specific activity of 1.09 MBq mg^{-1}, determined by liquid scintillation counting (LSC), in 70% yield (319 mg). Details are provided in the Supporting Information. Analytical data are similar to those of **5**. The radioactivity elemental analysis was not measured, but the procedure has been optimized with "cold" substrates and polymers with a degree of sulfation of 85% and 4−5 amino groups have reproducibly been obtained (by C, H, N, S analysis).

*dPGS ICC Conjugate (**7**).* (d) To a solution of dPGS amine (**5**, 5 mg, 0.43 μmol) in DMF/PBS (9:1, 12 μL) iminothiolane (1.72 μmol, 4 equiv), DIPEA (1.72 μmol, 4 equiv) and ICC maleimide (1.72 μmol, 4 equiv) were added. The solution was stirred for 48 h at room temperature. All solvents were evaporated to dryness followed by purification with RP C-18 chromatography (solvent: dist. water) and dialysis (reg. cellulose; solvents: sat. NaCl solution, dist. water). Lyophilization yielded 5.4 mg of a red solid. Spectroscopic data (water): $\lambda_{max,abs}$ 552 nm, $\lambda_{max,em}$ 567 nm. The average dye-to-polymer ratio was determined with a 5 μM solution using a molar absorption coefficient λ (552 nm) for ICC of 120 000 M^{-1} cm^{-1} giving a value of 0.45 dye molecules/polymer.

*dPGS ICC Conjugate (**8**).* (e) To a solution of dPGS amine (**5**, 30 mg, 2.59 μmol) in a DMF:water mixture (9:1, 500 μL) was added DIPEA (10.34 μmol, 4 equiv) and ICC NHS ester (10.34 μmol, 4 equiv), respectively. The reaction was stirred 72 h at 50 °C. All solvents were evaporated to dryness followed by purification with RP C-18 chromatography (solvent: dist. water) and dialysis (reg. cellulose; solvents: sat. NaCl solution, dist. water). Lyophilization yielded 31.3 mg of a red solid. Spectroscopic data (water): $\lambda_{max,abs}$ 551 nm, $\lambda_{max,em}$ 570 nm. The average dye-to-polymer ratio was as described above giving a value of 1.1 dye molecules/polymer.

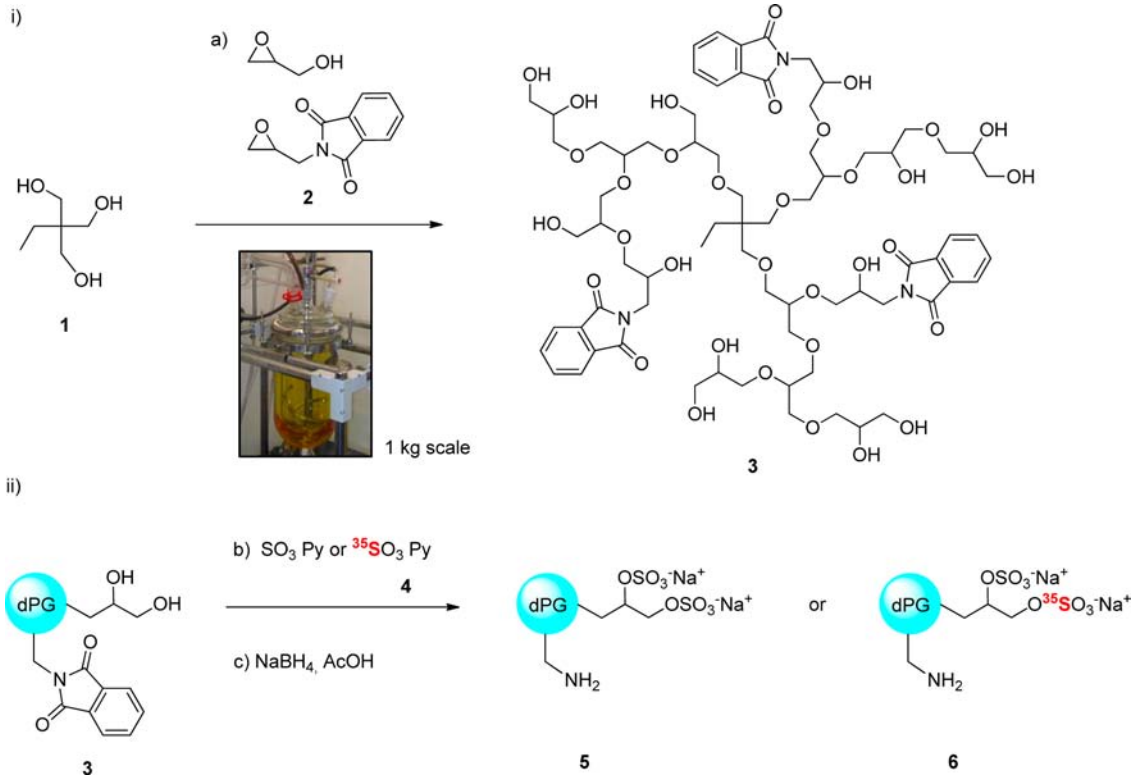
Fluorescence Histology, Radio Detection, and Cell Microscopy. Animals in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. All experiments were approved by the local animal welfare committee.

Fluorescence Histochemistry. Healthy female BALB/c mice with a bodyweight of 25 ± 3 g on arrival and fed a normal diet were used (Pia). The animals (n = 5) were treated once with 2 mg kg^{-1} b.w.$^{-1}$ dPGS-ICC **8**, dPG-OH-ICC, or 0.5 mL of 0.9% NaCl, pH 7.4 (control). Three hours after i.v. administration into the tail vein the animals were killed in deep anesthesia by intravenous injection of T61 ad us. vet. (Intervet) at a dose of 2 mL $animal^{-1}$. Tissues were removed and histochemical staining was performed on 4 μm sections of formalin-fixed, paraffin-embedded probes. Paraffin tissue sections were deparaffinated and fixed with acetone. 4,6-Diamidino-2-phenylindole (DAPI, Abcam) was used for nuclear counter stain. Image acquisition was performed using a Leica DMRB microscope (Leica). Images were taken with 250× magnification with a digital camera (Spot 32, Diagnostic Instruments). The tissue sections were analyzed under identical instrument settings to allow semiquantitative evaluation of the fluorescence intensities.

Cellular Fluorescence Microscopy. The epithelial human lung cancer cell line A549 was routinely propagated as follows: DEMEM medium, with 10% fetal calf serum (FCS), 2% glutamine, and penicillin/streptomycin (all from PAN Biotech) were added. Cells were seeded into medium at 1 × 10^5 cells mL^{-1}, cultured at 37 °C with 5% CO_2, and split 1:5 two times a week. Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial cell growth medium with a supplement mix (Promocell). Cells were seeded into medium at 2 × 10^4 cells mL^{-1}, cultured at 37 °C with 5% CO_2 up to 80% of confluence, and then split 1:4. For cytochemistry the cells were seeded at 5 × 10^4 cells mL^{-1} in a 24-well culture plate on glass coverslips (Sigma), and cultured for 24 h at 37 °C. Thereafter, cells were incubated with medium containing 10^{-6} M of dye-labeled test substances for 3 h at 37 °C. The cells were fixed with cold acetone, rinsed, and covered with Alexa Fluor 488 Phalloidin (1:300, Molecular Probes). 4,6-Diamidino-2-phenylindole (DAPI, Abcam) was used for nuclear counterstain. Image acquisition was performed using a Leica DMRB microscope (Leica) equipped with a digital camera (Spot 32, Diagnostic Instruments) maintaining exposure time and setting constant during all picture acquisitions.

Light Microscopic Autoradiography Studies. Healthy male MNRI mice with a bodyweight of 26 ± 3 g on arrival were treated once with 30 mg kg^{-1} b.w. dPG^{35}S amine **6** (i.v. administration into the tail vein) with a specific activity of 0.3 MBq mg^{-1} polymer. This activity was obtained by mixing **6** with inactive dPGS amine **5** which was synthesized under the identical reaction parameters. Control experiments were performed with pure dPGS amine **5** and 0.9% aqueous NaCl. The animals were fed and held normally; they maintained their bodyweight constant and did not show any abnormalities. After 21 days the mice were sacrificed by cervical dislocation and the organs were dissected, sectioned, and analyzed via light microscopic autoradiography.

Scheme 1. Reaction Pathway for the Synthesis of dPG N-Phthalimide (3), dPGS Amine 5, Radiolabeled dPGS Amine (dPG^{35}S amine 6)[a]

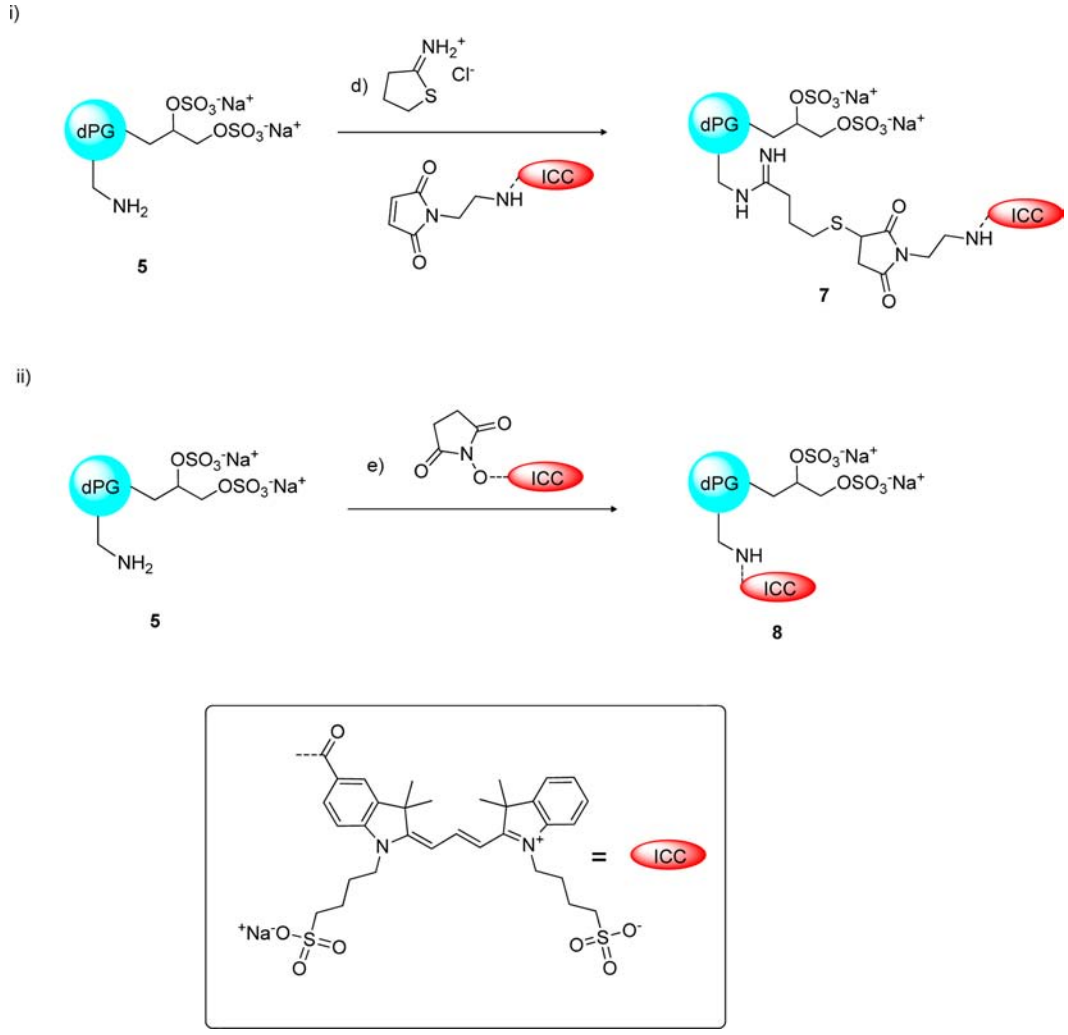


[a](i) (a) KO*t*Bu, glycidol, NMP, THF, 120 °C, o.n.; N-(2,3-epoxypropyl)phthalimide (**2**), NMP, THF, 120 °C, 2 h; rt, 30 min, H^+ resin, MeOH, rt, o.n. (ii) (b) SO_3 pyridine complex or $^{35}SO_3$ pyridine complex/pyridinium chloride (ratio 2:3) (**4**), DMF, 60 °C, 24 h, H_2O, NaOH (c) $NaBH_4$, H_2O, 50 °C, 15 h, AcOH, 85 °C, 7 h. The chemical structure of the dPG scaffold is representative. Detailed reaction conditions, purification procedures, the synthesis of the noncommercial, radioactive sulfation reagent $^{35}SO_3$ pyridine (**4**), and the synthesis of **5** on large scale are provided in the Supporting Information (SI).

RESULTS AND DISCUSSION

Synthesis of Partially Aminated Dendritic Polyglycerol Sulfate (dPGS amine, 5). In order to provide the option of attaching active agents such as dyes or drugs covalently to the highly inflammation specific dPGS, the synthesis of a multifunctional dendritic polyglycerol core employing an adjustable number of protected amines on the surface was established. This platform was prepared according to Scheme 1(i): dendritic polyglycerol containing 9.4% N-phthalimide functionalities (dPG N-phthalimide **3**) was synthesized by utilizing a sequential anionic ring-opening, multibranching copolymerization (ROMBP) approach by applying slow monomer addition (SMA) of glycidol,[11] which was followed by N-(2,3-epoxypropyl)phthalimide (**2**) on a partly deprotonated trimethylol propane (TMP) starter (**1**). Quenching with methanol and treatment with cationic exchange resin, followed by repeated precipitation in acetone and diethyl ether, yielded dPG phthalimide **3** with a molecular weight of M_n = 4560 g mol^{-1}, a PDI = 1.52, and degree of branching of 62%, which was determined by ^{1}H, inverse gated ^{13}C NMR spectroscopy, GPC, and MALDI-TOF MS. N-Phthalimide functionalized dendritic polyglycerol sulfate (dPGS N-phthalimide) was obtained by sulfation of dPG N-phthalimide (**3**) with SO_3 pyridine complex in dry DMF at 60 °C followed by conversion to the sodium salt. Free amino functionalities were generated in a Gabriel-type hydride mediated hydrolysis of the N-phthalimide groups using sodium borohydride and acetic acid. Purification by ultrafiltration (MWCO = 2000 g mol^{-1}) and size exclusion chromatography (Sephadex G-25) gave partially aminated, dendritic polyglycerol sulfate (dPGS amine **5**) with a degree of sulfation of 85%, as determined by elemental analysis, with a calculated average molecular weight of M_n = 10 050 g mol^{-1}. The described polymer synthesis and purification process was scaled up to a semi-industrial level by using a three-liter batch reactor yielding about 700 g of **5** from approximately 1 kg of reactants (Supporting Information). In this process, the toxic SO_3 pyridine complex was replaced by a less toxic SO_3 trimethyl amine complex. Detailed information about the upscaling process is provided in the Supporting Information. The amine content of polymer **5** was deduced from ^{1}H NMR of polymer **3** by integrating the aromatic protons of the phthalimide protecting group versus the polymer backbone. Deprotection was confirmed by a strong reduction of the aromatic signals of polymer **5**. The final polymer bears 9.4% amino groups, corresponding to approximately 5 amino functionalities on average per polymer, as determined by NMR. These amino groups are required for conjugation and dPGS amine is only an intermediate polymer. Once derivatized, ideally no free amino groups are present anymore. Hence, biological effects originated from the amino groups can be excluded. Dynamic light scattering (DLS) in phosphate buffered saline at pH 7.4 revealed a hydrodynamic diameter of 5.5 ± 2.0 nm (distribution by volume), a size previously found for dPGS with a comparable molecular weight and degree of sulfation, however, without amino functionalities.[7,8]

Scheme 2. Synthesis of dPGS-ICC Conjugates (7, 8)[a]



[a](i) Applying iminothiolane and subsequent addition of ICC maleimide (d) 4 equiv iminothiolane in DMF/PBS (9:1), 4 equiv DIPEA, 4 equiv ICC maleimide, rt, 48 h, and (ii) conjugation through amide formation (e) 4 equiv DIPEA in DMF/water (9:1), 4 equiv ICC NHS ester, 50 °C, 72 h. Dye loadings of (i) 0.45 and (ii) 1.1 dye molecule per polymer were obtained, determined by UV-VIS spectroscopy (data not shown).

Under the applied physiological conditions, partial aggregation was observed in DLS, probably due to intermolecular electrostatic interactions of sulfate and amino functionalities. A zeta potential of −31 ± 6 mV was determined in 10 mM phosphate buffer, pH 7.4, which is in the range between small (5 nm) and larger (12 nm) dPGS macromolecules.[7] The developed method avoids post amine modification of the polymer, usually comprising a multistep conversion of hydroxyl into azides and subsequent reduction to amines. More importantly, this strategy enables the generation of free amines in the last step without interfering with the sulfation process. The synthesis of radiolabeled dPGS amine (**6**) and dPGS amine (**5**) for bioconjugation purposes is illustrated in Scheme 1.

Synthesis of ^{35}S Labeled Dendritic Polyglycerol Sulfate Amine (dPG^{35}S amine, 6). The above-described pathway was also followed for the radiosulfation yielding dPG^{35}S amine (**6**). The most conveniently accessible sulfation agent $^{35}SO_3$ pyridine complex (**4**) was obtained from pyridine and ^{35}S-chlorosulfonic acid which was previously synthesized by reacting ^{35}S-sulfuric acid with phosphoryl chloride (details are provided in the SI).[12,13] Complex **4** was obtained as a mixture of the $^{35}SO_3$ pyridine complex and the pyridine hydrochloric acid salt in the ratio 2:3, as determined by ^{1}H NMR spectroscopy. The absence of phosphorus species in the sulfation reagent was proven by ^{31}P NMR spectroscopy. Inactive SO_3 pyridine complex was prepared in analogy to **4** under the same reaction conditions. The radiolabeled intermediate, N-phthalimide functionalized dendritic polyglycerol sulfate, was obtained by sulfation of dPG N-phthalimide (**3**) with $^{35}SO_3$ pyridine complex (**4**) in dry DMF at 60 °C. Hydrolysis of the N-phthalimide groups generated free amino functions. Purification by ultrafiltration (MWCO = 1000 g mol^{-1}) and size exclusion chromatography (Sephadex G-25) yielded the aminated, ^{35}S-labeled dendritic polyglycerol sulfate (dPG^{35}S amine **6**) with a specific activity of 1.09 MBq mg^{-1}. The degree of sulfation was assumed to be similar to the "cold" synthesis of **5**, since the parameters were kept identical but combustion analysis of the radioactive material was not possible. Labeling with ^{35}S (energy: 0.167 MeV) is a convenient approach since the half-life of 87.4 days offers an ideal time frame for qualitative and quantitative in vitro or in vivo studies.[14] Furthermore, polymer **6** is chemically identical to its

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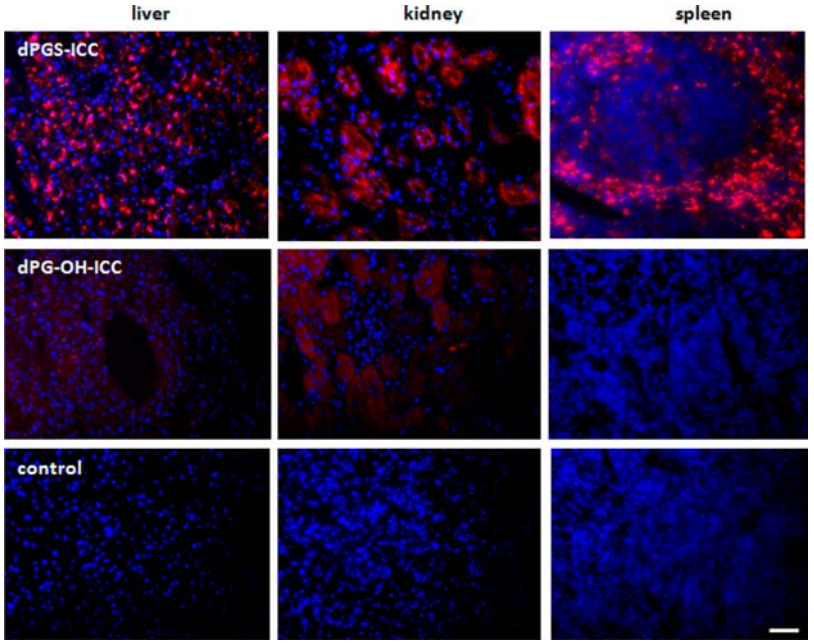

Figure 1. Cryosections of tissues from mice treated with a single dose of 2 mg kg^{-1} b.w. of dPGS-ICC **8** (red; 1st row), with nonsulfated control conjugate dPG-OH-ICC (2nd row) or aqueous NaCl (control, 3rd row). The tissue sections were analyzed three hours after i.v. administration (tail vein). Nuclei were stained with DAPI (blue). Magnification 250×; one representative example of $n = 5$. Scalebar: 50 μm.

inactive analog, which is subsequently applied for fluorophore conjugation[9] using dyes suited for histological analysis.

Synthesis and Characterization of Dendritic Polyglycerol Sulfate Indocarbocyanine Conjugates (dPGS-ICC). For the synthesis of fluorescent conjugates of dPGS, we employed the reactive fluorophore indocarbocyanine (ICC), a well-known label used as free carboxylic acid, NHS ester, or maleimido derivative.[15] Here the identical nonradioactive polymer precursor, dPGS amine (**5**, Scheme 2) was used in order to be able to perform "cold" experiments and accurately characterize the probes. Scheme 2 illustrates the synthesis of dPGS-ICC conjugates (**7** and **8**) following two different routes with the goal of achieving an average dye-to-polymer ratio of approximately 1. The first approach (Scheme 2, i) uses ICC with maleimido functionality obtained by derivatization of ICC carboxylic acid with 2-(aminoethyl)maleimide.[15] Conjugation was conducted by in situ modification of **5** with 2-iminothiolane followed by subsequent coupling to the maleimido function of the ICC dye. The second approach involved direct conjugation with dye NHS esters in a DMF:water mixture. In both cases, purification was achieved by RP C-18 chromatography (eluent: dist. water) and dialysis (MWCO = 2000 g mol^{-1}) against NaCl solution followed by dist. water. Freeze-drying afforded the dPGS-ICC conjugates as red solids in >95% yield. In order to evaluate the efficiency of the labeling reactions using ICC NHS ester or ICC maleimide, respectively, the dye-to-polymer ratio was determined by UV–vis spectroscopy on the basis of a 5 μM aqueous solution obtained from a weighted material. With an estimated molar extinction coefficient of 120 000 M^{-1} cm^{-1}, which takes into account a certain loss in the absorption maximum of the dye due to quenching,[9] dye-to-polymer values of 0.45 for compound **7** (iminothiolane/maleimido labeling) and 1.1 for conjugate **8** (NHS ester labeling) were obtained. Thus, the latter method more effectively resulted in a desired average number of one dye per polymer molecule, at least under the reaction conditions chosen. The fluorescence emission with a maximum at approximately 570 nm is optimal for applications with conventional microscopy equipment as performed in subsequent studies (absorption and fluorescence spectra are provided in the SI).

Histological and Cellular Characterization with Fluorescence and Radioimaging. The qualitative tissue uptake of fluorescence labeled dPGS-ICC conjugate **8** and dPG-OH-ICC was studied in histological sections from liver, kidney, and spleen tissue of mice three hours after a single i.v. injection of 2 mg kg^{-1} b.w.$^{-1}$ (Figure 1). dPG–OH-ICC was prepared according to a recently published procedure[15] with a molecular weight of $M_n \approx 4500$ g mol^{-1} a PDI < 1.6 and a dye loading of one dye per polymer. This conjugate is comparable to the nonsulfated precursor of dPGS-ICC **7** and **8**.[15] In the liver, dPGS-ICC **8** accumulated in specialized liver macrophages (Kupffer cells) and sinusoidal endothelial cells, both endocytotic competent cells. As a result, dPGS-ICC was not only bound at the endothelial selectins but also accumulated inside the cells.[16] In the spleen, which is a lymphatic tissue, dPGS-ICC fluorescence signals were observed in the endothelial cells of the blood sinusoids and in macrophages of the marginal zone around the lymphoid follicles. This is in accordance with results recently described for this kind of polysulfates.[9] In order to study the role of the sulfate groups in inflammatory enrichment of dendritic polyglycerols, the distribution of a nonsulfated dendritic polyglycerol-ICC (dPG-OH-ICC) conjugate was studied in mouse tissue. As can be seen from Figure 1, dPG-ICC must have been eliminated by the urinary tract much faster than dPGS-ICC. No accumulation was detected in the spleen, and only marginal signals in the liver and kidney (Figure 1) were found under identical instrumentation settings. To demonstrate the uptake of dPGS-ICC conjugates inside the cell, two different cell types were incubated in vitro. In human endothelial cord blood cells (HUVEC) as well as epithelial lung cells (A549) dPGS-ICC was accumulated in the cytoplasm of the cells (Figure 2). In accordance with previous results,[4] no significant uptake of dPG-

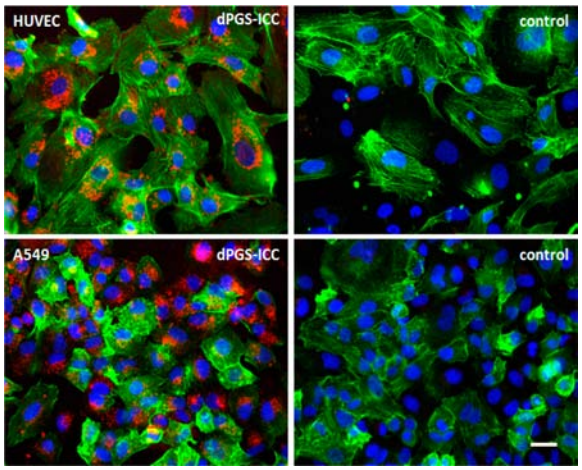

Figure 2. In vitro cell cultures incubated with dPGS-ICC **8** (red) for 3 h. dPG-OH-ICC was used as control. The cytoskeleton was stained with phalloidin-Alexa488 (green). Nuclei were stained with DAPI (blue). Scalebar: 10 μm.

OH-ICC was observed, confirming that cellular uptake is strongly charge and size dependent.[15] An administration of radioactive dPG^{35}S amine **6** into mice now allows one to determine the dPGS amine distribution in vivo, e.g., via autoradiography and liquid scintillation counting (LSC). Figure 3 exemplarily shows the liver of healthy mice 21 days after a single i.v. administration of dPG^{35}S amine **6** into the tail vein (dose: 30 mg kg^{-1} b.w.$^{-1}$). Black dots indicate accumulation in and adjacent to hepatic Kupffer cells and clearly show that qualitative and quantitative distribution data can be deduced. A detailed in vivo biodistribution analysis of dPG^{35}S amine **6** in mice is currently under investigation. Preliminary studies with

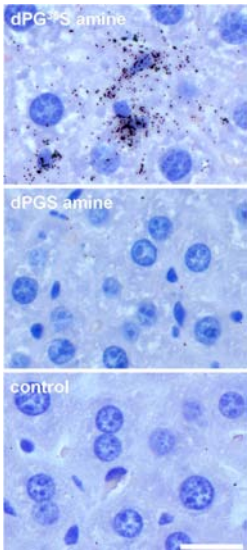

Figure 3. Light microscopic autoradiography of mouse liver sections covered with a photoemulsion layer 21 days after a single i.v. injection of dPG^{35}S amine **6** into the tail vein (dose: 30 mg kg^{-1} b.w., specific activity: 0.3 MBq mg^{-1} polymer). (top): Radioactivity (black dots) was almost exclusively located over or adjacent to hepatic Kupffer cells. dPGS amine (middle) and 0.9% aqueous NaCl, (bottom): Randomly distributed radioactivity as a result of background activity without any association to a specific cell type or tissue structure. Staining: Hematoxylin and Eosin. Scalebar: 20 μm.

radiolabeled dPGS without amino functionalities show that the presence of amino groups does not significantly alter the organ distribution profile. It must be noted that the amino groups are only intermediate and derivatized once tracers are conjugated. Ideally, if a multimodal imaging probe is prepared, no residual amino group is left. An influence of the coupled moiety, e.g., flourophore, on biological studies is by far more likely than an effect of a free amino group. The herein presented qualitative detection of dPGS conjugates clearly demonstrates the suitability for diagnostic applications in vitro and in vivo and underlines the potential of the general precursor N-phthalimide **3**, which can be synthesized in kilogram quantities in a one-pot approach.

CONCLUSION

In this article we have presented a readily accessible polymer precursor dPG N-phthalimide (**3**) for the synthesis of multifunctional dendritic polyglycerol sulfate derivatives, which has been recently identified as a highly anti-inflammatory drug.[6] The dPG N-phthalimide (**3**) represents an excellent platform for further conjugation of active agents and can be reproducibly prepared on a kilogram scale in a one-pot batch reactor process. An efficient route for the synthesis of partially aminated, ^{35}S-labeled dendritic polyglycerol (dPG^{35}S amine, **6**) has also been established that simultaneously provides free amino functionalities to label the polymeric backbone with a second modality, in this case the fluorescent dye. Conjugates with the red emitting dye indocarbocyanine (ICC), which are perfectly suited for cellular analysis, spectroscopy, and microscopy, have been prepared and analyzed. Tissue distribution and uptake could be monitored qualitatively and visualized ex vivo in histological specimens of liver, kidney, and spleen, respectively. Via radioactive detection and in comparison to fluorescence-based histological studies with the chosen target-specific polymer, a cross comparison between the two different methods was shown to be possible and yielded qualitatively corresponding results. The development of dendritic polymers as drug candidates represents a rather new and emerging field of research. Hence, methods for their biological characterization, including labeling techniques for visualization and quantification, are crucial. By comparing the results of histological examinations of radiolabeled and fluorescence labeled versions, we have shown how multivalent macromolecular targeting molecules can be readily prepared with this approach for biological studies. Furthermore, this now allows assessment of whether or not a fluorescent label would alter the properties of this macromolecular carriers in comparison with the nonconjugated radiolabeled (here the pure ^{35}S-labeled polymer) drug entity. Accordingly, other combinations such as labeling with ^{14}C or ^{3}H may now be envisaged and multimodality pathways for drug characterization via in vivo imaging modalities[17,18] can play a major role. The simplicity of the described synthesis and scalability is a great advantage when developing fully synthetic, dPGS-based polymers as preclinical diagnostic or therapeutic candidates.

ASSOCIATED CONTENT

Supporting Information

Detailed reaction conditions, purification procedures, the synthesis of noncommercial, radioactive sulfation reagent $^{35}SO_3$ pyridine **4**, and the semi-industrial scale batch reactor synthesis of dPG N-phthalimide **3** and dPGS amine **5**.

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Spectroscopical data of **3**, **5**, and photophysical data of **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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