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High Control, Fast Growth OEG-Based Dendron Synthesis via a Sequential Two-Step Process of Copper-Free Diazo Transfer and Click Chemistry

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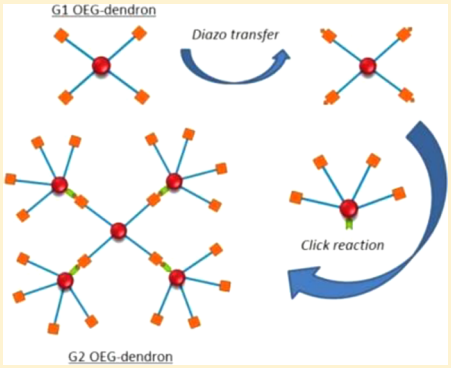
Supporting Information

ABSTRACT: Dendrons up to generation 3 were quickly synthesized using two sequential steps of diazo transfer and click chemistry reactions. The building blocks for dendron synthesis were made by incorporating monodisperse oligoethylene glycol (OEG) chains of exact length onto diethyelenetriaminepentaacetic acid (DTPA)-derived moieties to obtain first-generation dendrons. Afterward, the amine groups of the first-generation core dendron were converted to azides by means of the diazo transfer reaction. The second- and third-generation dendrons were constructed using the copper-catalyzed azide−alkyne cycloaddition (CuAAC). Hemocompatibility studies including hemolysis, red blood cell morphology, cell count and size distribution, hemostasis, and complement system activation were performed to evaluate their safety in blood.



INTRODUCTION

Dendrimers show precise architecture, a branched globular structure, and the capacity to host an abundance of functional groups. These features have led to an increased interest in their use for biomedical applications.[1] Dendrimers consist of a central core from which branching units proliferate in a treelike fashion yielding molecules of higher generations after each synthetic step.

Since its introduction in 2001 by Sharpless et al., the Huisgen 1,3-dipolar cycloaddition between azides and alkynes (CuAAC) has been widely applied in the fields of chemical biology, combinatorial chemistry, and material science.[2] The capacity of this specific type of click chemistry to produce high (almost quantitive) yields without producing difficult-to-remove byproducts makes the CuAAC an efficient tool for the construction of complex macromolecules such as dendrimers.[3] The click reaction has a high thermodynamic driving force, and therefore, when applied to dendrimers, it favors their growth in spite of the steric hindrance caused by the sheer size of the molecules. Also, because of the lack of byproducts produced, the formed dendrimers are easier to purify. In order to decorate the surfaces of a dendritic structure via click chemistry, azide functional groups need to be introduced to serve as handles onto which to click the decorative structures.

The mild and facile diazo transfer reaction applied to amines is an effective method to convert amines selectively to azides even in complex systems such as dendrimers[4] and entire proteins.[5] Our laboratory has previous experience in the synthesis and application of OEG (oligoethylene glycol)-based dendrons functionalized with amines.[6] These dendritic structures are formed by means of amide bond formation; however, this approach has the drawback of incomplete reactions and tedious purification and is therefore not the most effective method. Given that click chemistry has been proven to be an effective tool for constructing dendrimers in other systems and that the diazo transfer reaction has been used effectively to decorate the surfaces of higher generation dendrimers via amine−azide transformation and subsequent biofunctionalization by click chemistry, we hypothesized that the combination of these two synthetic methods would allow us to construct higher generation OEG-based dendrons with greater efficacy (see Scheme 1).

EXPERIMENTAL SECTION

Synthesis of Building Block Dendrons (1 and 2). The synthesis of compounds **1** and **2** has been described in previous work performed by our research group.[6,7] Nevertheless, the experimental procedure of the synthesis can be found in the Supporting Information.

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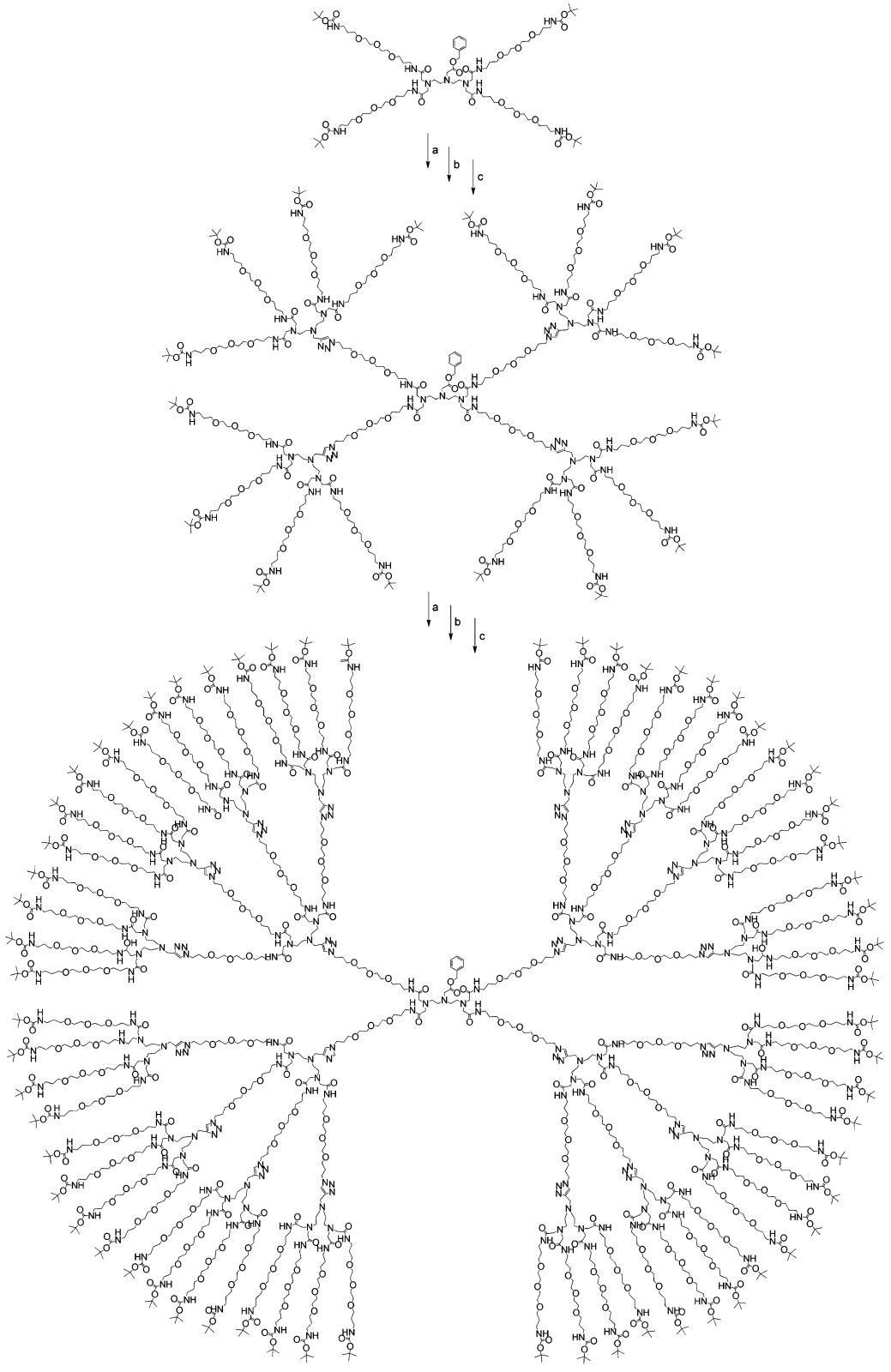
Scheme 1. Sequential Steps for Dendrimer Construction: (a) Deprotection with TFA; (b) Diazo Transfer Reaction; (c) CuAAC



Synthesis of Diazo Transfer Agent. The diazo transfer agent was synthesized as described in the literature by Goddard-Borger and Stick[8] except for the final acidic treatment which was performed with 4 M HCl in dioxane.

Synthesis of Bn-G1-N_3 (3). The Boc-protected G1 dendron **1** (500 mg, 0.2955 mmol) was dissolved in a 5 mL solution of TFA/H_2O (95:5) and stirred for 1 h. Subsequently, the TFA was evaporated using a flow of N_2, and the product was precipitated in methyl *tert*-butyl ether. After decanting the methyl *tert*-butyl ether, the pellet containing the deprotected dendron was dissolved in 10 mL of DMF and the diazo transfer agent (255 mg, 1.21 mmol) was added. The basicity was adjusted to pH 8−8.5 at regular intervals during the reaction by the addition of 1 M NaOH, and the solution was allowed to stir for 1−2 h at room temperature; meanwhile, the reaction was monitored using

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HPLC-PDA. Afterward, the reaction mixture was added to CH_2Cl_2 (50 mL) and washed with aqueous brine (3 × 50 mL) acidified to pH 3−4. The organic phase was evaporated, and the resulting crude was transferred to a 50 mL tube dissolved in CH_2Cl_2 (10 mL) and hexane (40 mL) was added. The mixture was stirred vigorously and centrifuged. The supernatant was discarded, and the remaining oily precipitate corresponded to the crude compound. The crude product was purified with flash chromatography over basis alumina oxide and was eluted with 1% MeOH in DCM as the eluent to yield the azide terminated dendron **3** (367 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.77 (q, J = 6.35 Hz, 8 H), 1.84 (q, J = 6.44 Hz, 8 H), 2.64 (m, 8 H), 3.14 (s, 8 H), 3.26−3.33 (m, 8 H), 3.33−3.41 (m, 10 H), 3.45−3.53 (m, 16 H), 3.56 (m, 16 H), 3.59 (m, 16 H), 5.11 (s, 2 H), 7.33 (m, 5 H), and 7.57 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.04, 29.42, 36.99, 48.37, 52.46, 53.38, 55.44, 59.08, 66.50, 67.80, 69.27, 70.10, 70.24, 70.39, 70.45, 128.27, 128.45, 128.62, 135.374, 170.66, and 171.26. Theoretical mass for $[C_{61}H_{109}N_{19}O_{18}]^+$: 1396.63; found by LC-MS: 699.12 (M + 2)/2 and by HRMS (ES^+): 1396.8271.

Synthesis of Bn-G2-Boc (4). The azide-terminated dendron Bn-G1-N_3 **3** (0.200 g, 0.14 mmol) and the Boc-terminated dendron Alkyne-G1-Boc **2** (0.905 g, 0.57 mmol) were dissolved in 40 mL of anhydrous CH_2Cl_2 in a two-necked round-bottom flask purged with N_2. DIEA (0.1 mL, 0.57 mmol) was added followed by the addition of CuCl (42 mg, 0.42 mmol). The reaction mixture slowly turned blue. The solution was allowed to stir for 1 h at room temperature; meanwhile, the reaction was monitored using HPLC-PDA and LC-MS. When the reaction was finished, 40 mL of an aqueous 0.05 M EDTA 5% $NaHCO_3$ solution was added. The mixture was vigorously stirred for a couple of minutes, and the two layers were separated. The organic phase was collected, and this washing process was repeated twice in order to remove the free copper as well the copper entrapped in the core of the dendron. Afterward, the organic phase was evaporated to yield the Boc-terminated second generation dendron **4** (1.010 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.43 (s, 144 H), 1.71−1.82 (m, 72 H), 2.16 (m, 8 H), 2.57 (t, J = 5.4 Hz, 20 H), 2.68 (t, J = 5.4 Hz, 20 H), 3.15−3.24 (m, 72 H), 3.28−3.35 (m, 40 H), 3.41 (s, 2 H), 3.42−3.46 (m, 8 H), 3.48−3.53 (m, 72 H), 3.56−3.61 (m, 80 H), 3.6−3.65 (80 H), 3.72 (s, 8 H), 4.46 (t, J = 13.7 Hz, 8 H), 5.12 (s, 2 H), 7.34 (m, 5 H), 7.61 (s, 4 H), and 7.86 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.56, 29.62, 29.79, 30.43, 37.06, 38.54, 47.44, 48.02, 51.53, 52.84, 59.01, 66.06, 67.44, 69.33, 69.39, 59.56, 70.26, 70.41, 70.51, 70.57, 70.60, 78.93, 123.38, 128.38, 128.59, 128.76, 123.52, 143.58, 156.16, 170.84, and 170.93. Theoretical mass for $[C_{361}H_{681}N_{63}O_{114}]^+$: 7723.9422; found by LC-MS: 1106.19 (M + 7)/7, 1290.41 (M + 6)/6, 1548.31 (M + 5)/5 and by HRMS (ES^+): 7723.9012.

Synthesis of Bn-G2-N_3 (5). The Boc-protected G2 dendron **4** (200 mg, 0.026 mmol) was dissolved in a solution of TFA/H_2O (95:5) and stirred for 1 h. Subsequently, the TFA was evaporated using a flow of N_2, and the product was precipitated in methyl *tert*-butyl. After decanting the methyl *tert*-butyl ether, the pellet containing the deprotected dendron was dissolved in 10 mL of DMF, and the diazo transfer agent (93 mg, 0.44 mmol) was added. The basicity was adjusted to pH 8−8.5 at regular intervals during the reaction by the addition of 1 M NaOH, and the solution was allowed to stir for 3 h at room temperature; meanwhile, the reaction was monitored using HPLC-PDA. Afterward, the reaction mixture was added to CH_2Cl_2 (50 mL) and washed three times with an aqueous brine (50 mL) acidified to pH 3−4. The organic phase was evaporated, and the resulting crude was transferred to a 50 mL tube dissolved in CH_2Cl_2 (10 mL) and hexane (40 mL) was added. The mixture was stirred vigorously and centrifuged. The supernatant was discarded, and the remaining oily precipitate corresponded to the crude compound. The pure compound was isolated by means of semiprep purification using a Sunfire C_{18} column with a gradient of 20−80% CH_3CN in H_2O, rendering compound **5** (43 mg, 25%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.74 (q, J = 6.55 Hz, 40 H), 1.81 (q, J = 6.26, 32 H), 2.14 (m, 8 H), 3.11 (bs, 20 H), 3.19−3.31 (m, 60 H), 3.35 (t, J = 6.70 Hz, 32 H), 3.43 (bs, 40 H), 3.45−3.52 (m, 82 H), 3.52−3.62 (m, 160 H), 3.85 (s, 8 H), 4.47 (m, 8 H), 5.12 (s, 2 H), 7.31 (m, 5 H), 7.88 (bs, NH), and 8.20 (s, 4 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 29.01, 29.17, 29.97, 37.13, 45.99, 47.58, 48.38, 49.82, 50.89, 53.41, 57.76, 67.09, 67.81, 68.76, 69.10, 70.01, 70.20, 70.33, 70.40, 115.04, 117.95, 127.28, 128.36, 128.62, 135.46, 160.89, and 170.56. Theoretical mass for $[C_{281}H_{521}N_{95}O_{82}]^+$: 6538.9519; found by LC-MS: 936.48 (M + 7)/7, 1092.41 (M + 6)/6, 1310.76 (M + 5)/5, 1638.32 (M + 4)/4, and by HRMS (ES^+): 6538.9725.

Synthesis of Bn-G3-Boc (6). The azide-terminated dendrimer Bn-G2-N_3 **5** (20 mg, 0.031 mmol) and the alkyne dendron **4** (82 mg, 0.53 mmol) were dissolved in 10 mL of anhydrous CH_2Cl_2 in a two-necked round-bottom flask purged with N_2. DIEA (94 μL, 0.53 mmol) was added followed by the addition of CuCl (52 mg, 0.53 mmol). The reaction mixture slowly turned blue. The solution was stirred magnetically for several hours, and the disappearance of the alkyne-functionalized building block dendron was monitored by HPLC-MS. When the reaction was finished, 40 mL of an aqueous 0.05 M EDTA 5% $NaHCO_3$ solution was added. The mixture was vigorously stirred for a couple of minutes, and the two layers were separated. The organic phase was collected, and this process was repeated twice in order to remove the free copper as well the copper entrapped in the core of the dendron. Afterward, the organic phase was evaporated, and the crude was dialyzed in EtOH/H_2O (1:1) using a 3 kDa MWCO membrane to remove the excess building block and the Boc-terminated third-generation dendrimer **6** was obtained (85 mg, 84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.42 (s, 576 H), 1.71−1.82 (m, 296 H), 2.10−2.19 (m, 40 H), 2.50−2.62 (m, 84 H), 2.62−2.76 (m, 84 H), 3.15−3.24 (m, 336 H), 3.31 (m, 168 H), 3.38−3.53 (m, 336 H), 3.59 (m, 336 H), 3.63 (m, 336 H), 3.76 (s, 20 H), 4.46 (t, J = 6.81 Hz, 40 H), 7.34 (m, 5 H), 7.61 (s, 20 H), and 7.85 (bs). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.57, 29.62, 29.80, 37.04, 38.54, 47.45, 48.01, 51.53, 52.84, 59.03, 67.64, 69.38, 69.56, 70.27, 70.58, 70.60, 78.95, 123.24, 128.39, 128.77, 143.56, 156.17, 170.96.

Synthesis of Dendron Library. The synthesis and characterization of the small dendron library used in the hemocompatibility studies are described in the Supporting Information.

Dynamic Light Scattering (DLS). For dendron size determination, DLS measurements were performed on a Malvern Zetasizer Nano-S Zen1600 using an He−Ne 125 mW 633 nm laser. The refractive index of the material and the dispersant were set to 1.33. The dendrons were dissolved in a 0.2 M PBS 0.05 M NaCl buffer at a concentration of 5 mg/mL and filtered with a 0.2 μm filter to remove dust particles. Twenty runs per measurement were performed, and the measurements were carried out in triplicate.

ζ-Potential Experiments. Measurements for ζ-potentials were made with a Malvern ZetaSizer Nano ZS instrument. Dendrons dissolved in distilled water at a 10 mg/mL concentration and passed through a 0.2 μm filter were placed in a specific cuvette, and certain parameters of the refractive index and the absorption coefficient of the material and the viscosity of the solvent were introduced into the software.

Size Exclusion Chromatography (SEC). SEC was carried out on a Waters instrument comprising of a Ultrahydrogel 250 column, 6 μm, 7.8 × 300 mm, a separation module (Waters 2695), an automatic injector, and a photodiode array detector (Waters 2298). Data were managed with Empower 2 software. UV detection was performed at 210 nm, and a 0.2 M PBS, 0.05 M NaCl buffer (pH 7.2) was used as the mobile phase to perform SEC at a flow rate of 1.0 mL/min.

General Considerations Hemocompatibility Studies. In-vitro hemocompatibility tests of the liposomal formulations were performed according to ISO standards (10993-4). Normal human blood from healthy volunteer donors was collected in Terumo Venosafe citrated tubes (Terumo Europe N. V., Belgium). Experiments were done within 2 h after blood collection. All tests were performed with the agreement of the local ethical committee of the Medicine Faculty of the University of Liège.

Blood Smears for the Control of the RBC (Red Blood Cell) Morphology. Dendrons were dissolved in PBS (or DMSO/PBS (1:9)) at a 1000 μM concentration and further diluted in whole blood in order to obtain final dendron concentrations of 100, 10, 1, and 0.1 μM. Samples were incubated for 15 min at 37 °C under lateral agitation (250 rpm). After blood incubation, 5 μL of the blood was withdrawn and spread on a microscopy glass slide. Blood cells were observed with an Olympus Provis microscope at 20× and 50× magnification in transmission mode. At least two representative pictures were acquired per sample with a digital camera (VisiCam (5 megapixels), VWR International).

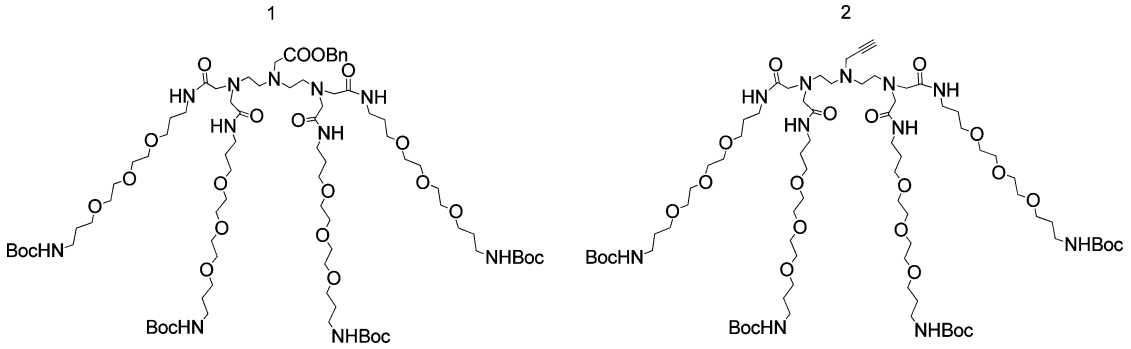
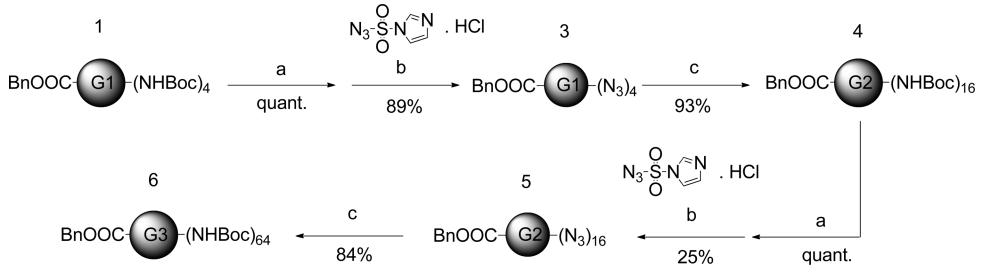


Figure 1. Molecular structure of first-generation dendrons with either a benzylester carboxylic acid (**1**) or an alkyne (**2**) in the focal point.

Scheme 2. Dendron Synthesis: (a) TFA/H_2O (95:5); (b) NaOH (aq, 1 M), DMF; (c) Dendron 2, CuCl, DIEA, DCM



Hemolysis. Dendron solutions and blood were prepared and incubated as described above. The hemolytic test was performed following Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM designation F 756-00). Briefly, after incubation, the samples were centrifuged at 600*g* for 5 min at rt, and supernatants were collected and mixed with the cyanmethemoglobin reagent. The hemoglobin released was measured by reading the absorbance of 100-fold dilution of whole blood in Drabkin's reagent at 540 nm in a microplate reader (Anthos HT III, type 12600, Anthos, Salzburg, AU). A calibration curve was established using bovine hemoglobin as the standard. Saponine (0.8 mg mL^{-1}), and PBS were used as positive and negative controls, respectively. Hemolysis was expressed as the percentage of hemoglobin released to total hemoglobin content, taking the positive control as 100% of hemolysis. The tests were done in triplicate. In view to assess the influence of plasma protein on the hemolytic action of the dendrons, this test was also performed using washed RBCs (4% RBC suspension) instead of whole blood.

Count and Size Distribution of RBCs, Platelets, and White Blood Cells. Dendron solutions and blood were prepared and incubated as described before. After 15 min of incubation, blood cells were counted and their size distribution was determined with CELL-DYN 18 Emerald (Abbott Diagnostics). Three analyses were conducted per sample.

Complement Activation. Complement activation was assesed using the Human C3a ELISA kit for quantification of Human C3a-des-Arg (Becton Dickinson). After a 15 min incubation of blood and dendron mixtures, EDTA (1 mM final) was added to block any future complement activation. Samples were centrifuged at 2000*g* for 5 min at rt, and supernatants were used for the analysis of complement activation following the kit protocol (BD OptEIA, Human C3a ELISA, Instruction Manual. Cat. No.550499). Absorbance was measured at 450 nm with a microplate reader (Anthos HT III, type 12600). Plasma containing 2 mg mL^{-1} of Zymosan was taken as a positive control and plasma without additives as a negative control. The concentration of C3a was expressed as a percentage of activation by reference to the negative control incubated set at a value of 100% of complement activation. Measurements were done in duplicate.

Coagulation Experiments. Whole blood and dendrimer solutions were mixed and incubated as described before. Samples were centrifuged at 2000*g* for 5 min at rt, and the supernatants were collected, recalcified to reverse the effect of citrate anticoagulant, and supplied with the specific activators of coagulation (thromboplastin). Prothrombin time (PT), to evaluate the extrinsic pathway, and activated partial thromboplastin time (aPTT), to evaluate the intrinsic pathway, were measured directly with a Dade Behring coagulation timer analyzer (BCT) (Siemens Healthcare Diagnostics NV/SA, Belgium) using commercial reagents (Thromborel S, Dade Behring/Siemens, for PT determination and C.K. PREST kit, Roche Diagnostics, France, for aPTT). Kaolin reagent was used as a positive control and PBS as a negative control. Clotting time was measured for each sample, and coagulation capacity was expressed as a percentage, taking the value of standard human plasma (Dade Behring/Siemens) as 100%. Measurements were done in duplicate.

RESULTS AND DISCUSSION

The building of dendrons of generation 2 and 3 consisted of two main steps: (1) conversion of amines to azides via the diazo transfer reaction and (2) incorporation of the alkyne building block dendron by CuAAC. To achieve this goal, the synthesis of two types of building block dendrons was required: the core dendron (**1**) and the alkyne-functionalized building block dendron (**2**) (see Figure 1). The two OEG-based dendrons consisted of a branching unit derived from diethylene triamine pentaacetic acid (DTPA), to which monodisperse OEG-chains, Boc-TOTA (1-(*tert*-butoxycarbonylamino)-4,7,10-trioxa-13-tridecanamine) were incorporated. The DTPA derivatives, and the corresponding first-generation dendrons, contain either an alkyne or a benzyl ester carboxylic acid in their focal point and were synthesized as described in previous work.[6,7]

The conversion of amines to azides was carried out with imidazole-1-sulfonyl azide hydrochloride as the diazo transfer agent (see Scheme 2). This reagent was developed by Goddard-Borger and Stick as a nonexplosive, shelf-stable, and efficient alternative to TfN_3.[8] With the aforementioned reagent, the diazo transfer reaction is usually performed in a protic solvent such as methanol, ethanol, or 2-propanol, using K_2CO_3 or NEt_3 as base and $CuSO_4$ as the source of Cu^{II} catalyst. At first, in our case, the solvent of choice was either ethanol or 2-propanol because methanol leads to transesterification of the benzylic ester of the

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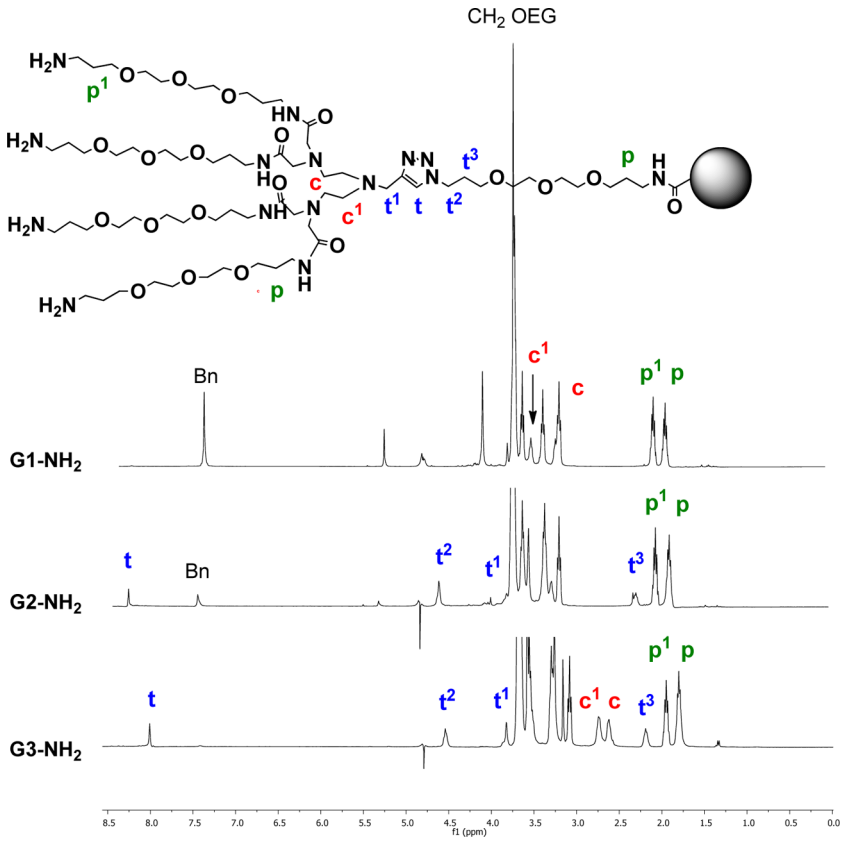

Figure 2. ^{1}H NMR spectra of first-, second-, and third-generation amine-terminated dendrons (in D_2O).

core dendron under basic conditions. Given that the dendron contains a chelating DTPA derivative, a portion of the $CuSO_4$ added to the reaction mixture was trapped by coordination in the DTPA derivative, which was detectable by HPLC-MS, thereby impeding its catalytic performance. Therefore, higher amounts of $CuSO_4$ were required to drive the reaction to completion. Other metals, such as Zn^{II}, Co^{II}, and Ni^{II}, were tested for the catalysis of the diazo transfer reaction, but none gave any improvement. It came to our attention that this new diazo transfer agent is so effective that no Cu^{II} catalyst is needed to convert the amines to azides, as reported in the literature for the introduction of azides into proteins.[9] In fact, the choice of solvent and the pH are the factors that influence the rate of the diazo transfer reaction the most. Polar solvents such as DMF and H_2O turn out to be more suitable solvents than the conventionally used protic solvents, such as MeOH. Maintaining the pH of the reaction mixture between 8 and 8.5 was crucial for clean conversion of the amines to azides. The azide dendrons **3** and **5** were obtained with 89% and 25% yields in the diazotransfer reaction, respectively. Yield differences can be attributed to the high ratio of amine groups that are transformed to azides to obtain dendron **5** (4 times more than dendron **3**) and required longer reaction time, causing the appearance of more undesired impurities. Therefore, dendron **5** could only be isolated by reverse phase chromatography, in contrast to dendron **3**, which was easily purified by flash chromatography.

Despite avoiding Cu to catalyze the diazo transfer reaction, the chelating capacity of the DTPA derivative dendrons still complicates the CuAAC as the Cu^{I} required for the click reaction is sequestered. In order to make the reaction proceed, 0.5 equiv of Cu^{I} catalyst per DTPA derivative dendron was needed. This amount was determined by performing a series of trial experiments in which the amount of Cu^{I} was increased in a stepwise manner until the click reaction occurred. The most conventional procedure for click chemistry involves the use of $CuSO_4$ as the source of copper and sodium ascorbate as the agent to reduce *in situ* the Cu^{II} to Cu^{I} species which actually serves as the catalyst. Two or more equivalents of ascorbic acid with respect to $CuSO_4$ are generally used to reduce Cu^{II} sufficiently. In our case, the minimal required amounts of $CuSO_4$ and sodium ascorbate were 2.5 and 5 equiv (with respect to the core dendron with azides), respectively, in order to build the second-generation dendrimer. Using these conditions, the dendron G2 was formed but was accompanied by other impurities (60% purity as determined by HPLC). The best results were achieved using 3 equiv of CuCl, a direct source of Cu^{I}, together with DIEA, which makes the alkyne more accessible,[10] in dichloromethane under a N_2 atmosphere. The entrapped copper was removed afterward by washes with an EDTA solution. Using these conditions, the dendron G2 (**4**) was obtained at a scale of 1 g at high purity (>95%) and with a CuAAC reaction yield of 93%. To build the third-generation dendron (**6**), the same CuAAC conditions were used with similar results (84% CuAAC reaction yield). The global yield of G2 (**4**) and G3 dendron (**6**) from Boc protected G1 (**1**) was 87% and 35%, respectively. The formation of the second-generation G2 was confirmed by NMR and ES-MS. The use of mass spectrometry to confirm the formation of the third-generation dendron G3 was not possible; therefore, this molecule was characterized by NMR (see Figure 2). Integration of the peaks indicated a total of 20 protons corresponding to the triazole signal. Integration of the signals corresponding to the protons adjacent to the triazole ring also gave the correct values.

This corresponding increase in molecular weight of the dendrons was also confirmed by aqueous SEC of the free-amine surface dendrons G1, G2, and G3 which eluted at retention times

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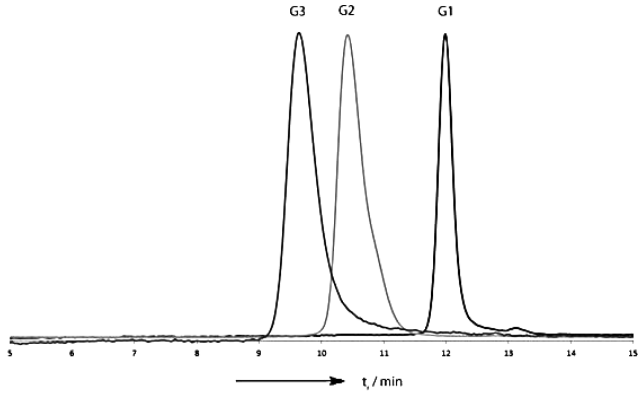

Figure 3. SEC traces of dendrons G1-NH_2, G2-NH_2, and G3-NH_2.

of 12.0, 10.4, and 9.6 min, respectively (see Figure 3). The presence of a small shoulder in SEC traces of the second- and third-generation dendron indicates that to a certain degree there are defects in the formed structures.

First-, second-, and third-generation dendrons with free or acetylated surface amines (G1-NH_2 (**7**), G1-Ac (**8**), G2-NH_2 (**9**), G2-Ac (**10**), G3-NH_2 (**11**), and G3-Ac (**12**)) were chosen to determine their size and the electrical potential at the dendron surface. Dynamic light scattering (DLS) measurements (see Table 1) showed the relative difference in size between amine-functionalized or acetylated dendrons G1, G2, and G3 with no apparent variation between the surface functionalities. The size of the OEG-dendrons G1 and G2 was consistent with PAMAM dendrimers of generations G0 (1.5 nm) and G3 (3.6 nm), respectively. The third-generation OEG-dendron has a molecular weight (25−28 kDa) comparable to a G5 PAMAM dendrimer (28 kDa) but in terms of hydrodynamic diameter is similar to a G7 PAMAM dendrimer (8.1 nm). This means that with an increase in generation the OEG-dendrons are becoming less dense than the PAMAM dendrimers.

The electrical potential at the dendron surface was determined by ζ-potential measurements. As expected, the influence of the surface functionalization was clear as the dendrons with the free amines in the exterior had a positive ζ-potential ranging from 12 to 43 mV, and the number of positive charges is directly related to an increase in the ζ-potential. The dendrons with the acetyl groups, on the other hand, were practically neutral.

To evaluate whether OEG-based dendrons synthesized in this study can be used for further biomedical applications, their biocompatibilty was assessed by means of hemocompatibility assays. For this purpose, we prepared a small library of eight dendrons of generation 1−3 with different functional groups in the periphery: G1-NH_2 (**7**), G1-Ac (**8**), G1-N_3 (**3**); G2-NH_2 (**9**), G2-Ac (**10**), G2-N_3 (**5**); G3-NH_2 (**11**), G3-Ac (**12**). Despite its successful synthesis, the dendron G3-NH_2 was omitted from these studies because of its insolubility in the isotonic buffer at the assessed doses. Previous results in the literature show that large cationic dendrimers with a high number of free amines can negatively interfere with human blood.[11] Therefore, we also included acetylated dendrons in order to verify whether the anticipated cytotoxicity of large dendrons is the result of their size or of their surface charge. Furthermore, the acetyl analogues could be suitable mimics of the potential structures to be synthesized and used for further applications. Dendrons functionalized with azides were evaluated for their biocompatibility because they might serve as cross-linkers for biomaterials. Hemocompatibility was assessed following ISO-10.993, and these assays should be considered as prescreening tests. They consisted of microscopic evaluation of red blood cell morphology, hemolysis testing, blood cell counting and size distribution, assaying the activation of the complement immune system, and finally evaluating hemostasis (intrinsic and extrinsic pathways). The results of the blood cell counting and size distribution testing showed that no cell type (erythrocytes, leukocytes, and platelets) was affected by the presence of the dendrimers whatever the concentration. The total count number and size distribution did not show any deviation from the control values (results in Supporting Information). In the case of the erythrocytes, this result was also confirmed by the hemolysis assay and microscopic observation of cell morphology (see Figure 4).

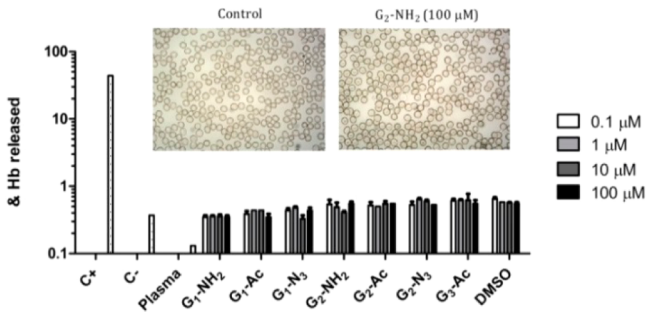

Figure 4. Hemolysis induced by dendrons in whole blood, expressed as percent to whole blood hemoglobin content. Inset: microscopic images of erythrocytes after dendron incubation.

No significant increase in the hemolysis rate was detected. In addition, the erythrocytes remained round after being in contact with the dendrimers. These results indicate that the dendrimers did not damage the integrity of the cellular components of blood—a result that is particularly interesting regarding platelets, which are known to be particularly sensitive to polycations. The coagulation cascade leading to the formation of blood clots involves two separate, but linked, pathways: the intrinsic and the extrinsic pathway. The former, as assayed by the PT assay, was not inhibited by any type of dendrimer. The intrinsic pathway was assayed by the aPTT assay. In this case the polycationic dendrimers G2-NH_2 caused up to 50% inhibition at the highest dose of 100 μM. This inhibition, also found with other synthetic polycations,[12] was most probably caused by electrostatic interference with the signaling cascade as a result of electrostatic interactions between the polycationic dendrimers and several negatively charged coagulation factors involved in this pathway. Finally, activation of the complement immune system was tested by measuring the concentration of the anaphylatoxin C3a by

Table 1. Zeta-Potential and Measured Diameters of the Dendrons

	G1		G2		G3	
	G1-NH_2	G1-Ac	G2-NH_2	G2-Ac	G3-NH_2	G3-Ac
ξ-potential (mV)	12.3 ± 1.5	−1.6 ± 0.6	33.9 ± 3.0	−5.65 ± 1.3	43.5 ± 0.6	−4.2 ± 0.2
diameter (nm)	1.2 ± 0.1	1.2 ± 0.12	4.2 ± 0.2	4.2 ± 0.1	8.7 ± 1.2	8.4 ± 1.9

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ELISA. In similar fashion to coagulation, the complement system was activated by the polycationic dendrimers G2-NH_2, showing activation at 100 μM. The other types of dendrimers did not cause any significant activation In comparison to the typical hemoreactivity of polycations, these dendrimers showed better hemocompatibility in *in vitro* tests at a concentration range typically used for pharmacological purposes (0.1−100 μM).

In conclusion, the platform described here allows the synthesis of dendrons of low (G1), medium (G2), and high (G3) molecular weight with potential use in biomedical applications, including intravenous administration. The dendrons can be adapted to specific requirements by incorporation of OEG-chains of variable length and distinct functional moieties due to the orthogonality of the click reaction. To the best of our knowledge, this is the first study to report the use of the diazo transfer reaction to build dendritic molecules. Also, this reaction allows switching between azides and amines, a feature enabling the functionalization of the dendrons through click chemistry (for G1 and G2) and amide bond formation (G1, G2 and G3). Finally, the DTPA-derived units that form part of the dendritic scaffold offer metal chelation sites, a feature that deserves further attention for potential use in medical imaging.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and characterization data for all the new compounds, i.e., branching units and dendrons, and hemocompatibility assays results for all dendrons. 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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■ ABBREVIATIONS

aPTT, activated partial thromboplastin assay; BOC-TOTA, 1-(*tert*-butoxycarbonylamino)-4,7,10-trioxa-13-tridecanamine; CuAAC, copper-catalyzed azide−alkyne cycloaddition; DIEA, *N*,*N*-diisopropylethylamine; DLS, dynamic light scattering; DMF, dimethylformamide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraactic acid; ELISA, enzyme-linked immunosorbent assay; ES-MS, electrospray mass spectrometry; EtOH, ethanol; HPLC-PDA, high performance liquid chromatography−photodiode array; HRMS, high resolution mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; OEG, oligoethylene glycol; PBS, phosphate buffered saline; PT, prothrombin time; SEC, size exclusion chromatography; TFA, trifluoroacetic acid.

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