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Fabrication of "Clickable" Hydrogels via Dendron−Polymer Conjugates

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ABSTRACT: Functionalizable hydrogels are of great interest as three-dimensional (3D) scaffolds for cell growth and tissue engineering. The ability to covalently immobilize biologically relevant molecules with accurate control of their density within the hydrogel matrix is highly desirable. Dendron−polymer conjugates prepared via Huisgen type "click" reaction provides a unique precursor for reactive hydrogels. A family of dendron−polymer conjugates were prepared by coupling second- and third-generation alkyne appended polyester dendrons with linear poly(ethylene glycol) diazides, PEG2K and PEG6K. Controlled cross-linking of alkyne-functionalized dendron−polymer−dendron conjugates with a hydrophilic diazide provides hydrogels with gelation efficiencies greater than 80%. Excess leftover alkynes can be used to functionalize these hydrogels as desired. Fine tuning of degree of cross-linking and functionalization is demonstrated by immobilization of streptavidin.

Introduction

Recent years have witnessed remarkable advances in the area of synthesis of well-defined hydrogels. Interest in developing novel synthetic approaches in this area of research is fueled by the hydrogel-based emerging technologies ranging from off-the-shelf consumer products such as contact lenses and wound dressings to medical applications such as scaffolds for tissue engineering. The impact of synthetic methodologies in macromolecular engineering is rapidly shaping the field of hydrogel synthesis.[1−10] Advances in this area have seen developments in new cross-linking methodologies and synthesis of designer hydrogels that can be precisely functionalized.[11−20] Traditional approaches for incorporating entities such as small molecules, peptides, and large biomacromolecules like enzymes or growth factors into hydrogel matrix have relied upon encapsulation and physiabsorption.[21−29] Covalent immobilization techniques have been generally limited to attachment of the molecule of interest into a polymerizable macromonomer.[32−35] Postfunctionalization of hydrogels has been evaluated as an attractive alternative in recent years.[36−39] This approach relies on the presence of reactive functional groups in the hydrogel matrix that can undergo efficient functionalization under mild reaction conditions. Advent of "click" reactions[40−47] has dramatically influenced postpolymerization functional group transformations due to their near-quantitative conversions under mild reaction conditions. Indeed, recent works from Hilborn[48] and Hawker[49] groups have reported efficient synthesis of hydrogels using Huisgen type cross-linking of telechelic PEGs with multivalent azide based cross-linkers. Since then, many other groups have reported synthesis of polymers[50−60] and hydrogels using the Huisgen click cycloaddition-based strategy.[61−75]

One strategy in hydrogel preparation has been the use of triblock copolymers.[76−87] A triblock copolymer consisting of a hydrophilic block in between two hydrophobic blocks forms a network micellar structure under aqueous conditions. Polymerizable groups such as acrylate double bonds at the end of the hydrophobic blocks allow photo-cross-linking of the structure to provide chemically cross-linked hydrogels. In recent years, dendron−polymer−dendron-based triblock copolymers[88−91] have emerged as an attractive building block for synthesis of hydrogels.[92−98] Grinstaff and co-workers reported the synthesis of biodegradable photo-cross-linkable hybrid dendritic−linear triblock copolymers and applied them to seal corneal lacerations.[99−101] We envisioned that the multivalent nature of the dendrons would allow one to utilize some of the reactive end groups for cross-linking to afford the gel, whereas the residual reactive groups would allow covalent postfunctionalization of the hydrogels with molecules of interest. In our design, we utilized the Huisgen-type copper-catalyzed click reaction[102,103] between biodegradable polyester dendrons[104−109] and biocompatible hydrophilic linear PEG polymers to access dendron−polymer−dendron conjugates necessary for the hydrogel formation. Functionalization of the dendron periphery with alkyne groups affords reactive hydrogel precursors. While some of these alkyne groups are cross-linked using a bisazide to fabricate the hydrogel (second Huisgen type "click" reaction), the residual alkynes allow efficient covalent functionalization of the hydrogel matrix with molecules of interest via the third consecutive "click" reaction (Scheme 1).

Experimental Section

Materials. 2,2-Bis(hydroxymethyl)propionic acid (BMPA), Dowex X50WX2, propargyl alcohol, and 4-pentynoic acid were purchased from Alfa Aesar. All poly(ethylene glycol) were obtained from Fluka. All solvents were purchased from Merck and used as obtained without further purification unless otherwise noted. Azide-functionalized PEGs were synthesized according to literature procedures.[110] Syntheses of dendrons **1** and **2** are given in the Supporting Information.

Methods. The monomer and copolymer characterizations involved ^{1}H NMR spectroscopy (Varian 400 MHz) and Fourier transform infrared (ATR-FTIR) spectroscopy (Thermo Fisher Scientific Inc. Nicolet 380). The molecular weights were estimated by gel permeation chromatography (GPC) analysis using a Viscotek GPCmax VE-2001 analysis system. PLgel (length/i.d. 300 mm × 7.5 mm, 5 μm particle size) Mixed-C column was caliberated with polystyrene standards (1K−150K), using a refractive index detector. THF was used as eluent at a flow rate of 1 mL/min at 30 °C. Elemental analyses were obtained from Thermo Electron SpA FlashEA 1112 elemental analyzer

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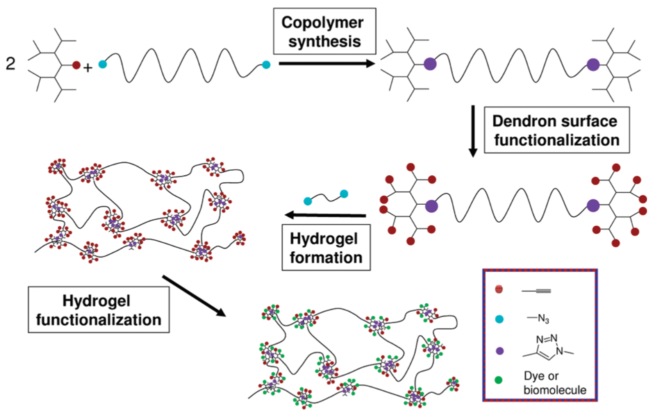
Scheme 1. General Scheme for the Synthesis of Functionalized Hydrogels



(CHNS separation column, PTFE; 2 m; 6 × 5 mm). The dry and wet surfaces of the hydrogels were observed with an ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument using an accelerating voltage of 10 kV. Functionalized hydrogels were visualized with Zeiss Observer.Z1 inverted fluorescent microscope.

Preparation of Dendron−Polymer−Dendron ABA Triblock Copolymer Systems. *Synthesis of Compound [G2]4OH[PEG2K] (5)*. PEG-2K-diazide (**3**) (512 mg, 0.25 mmol) and propargyl [G2]4[OH] (**1**) (302 mg, 0.625 mmol) were dissolved in dry THF (3 mL). In a separate flask were dissolved CuBr (3.6 mg, 0.025 mmol) and *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, 0.5 μL, 0.025 mmol) in dry THF (2 mL) and purged with N_2. The mixture was then transferred onto azide−propargyl alcohol solution and stirred at room temperature for 24 h. The solvent was then evaporated, and the crude product was dissolved in CH_2Cl_2 (100 mL) and washed with H_2O (25 mL) to remove copper salts. The solvent was concentrated in vacuo, and the desired product was precipitated with Et_2O, filtered, and dried in vacuo, yielding compound **5** (680 mg, 95%) as a yellowish-white solid. ^{1}H NMR ($CDCl_3$, δ, ppm): 7.78 (s, 1H), 5.16 (s, 2H), 4.45 (t, 2H, J = 5.0 Hz), 4.24 (d, 2H, J = 11.0 Hz), 4.17 (d, 2H, J = 11.0 Hz), 3.78 (t, 2H, J = 5.0 Hz), 3.72−3.36 (m, 90H), 2.57 (s, 4H) 1.19 (s, 3H), 0.96 (s, 6H). FTIR (cm^{-1}): 3431, 2868, 1731. $C_{126}H_{236}N_6O_{64}$ Calcd: C, 52.97; H, 8.25; N, 2.94. Found: C, 53.01; H, 8.51; N, 3.00.

Synthesis of Compound [G2]4OH[PEG6K] (6). Synthesized via the same procedure as compound **5** using PEG-6K-diazide (**4**) (1000 mg, 0.167 mmol), propargyl [G2]4[OH] (**1**) (73 mg, 0.183 mmol), CuBr (2.4 mg, 0.017 mmol), and PMDETA (3.5 μL, 0.017 mmol), yielding compound **6** (0.918 g, 80%) as a yellowish-white solid. ^{1}H NMR ($CDCl_3$, δ, ppm): 7.85 (s, 1H), 5.25 (s, 2H), 4.53 (t, 2H, J = 4.0 Hz), 4.34 (d, 2H, J = 10.8 Hz), 4.28 (d, 2H, J = 10.8 Hz), 3.86 (t, 2H, J = 4.0 Hz), 3.79−3.21 (m, 270H), 1.28 (s, 3H), 1.02 (s, 6H). FTIR (cm^{-1}): 3468, 2881, 1732. $C_{308}H_{6008}N_6O_{155}$ Calcd: C, 53.88; H, 8.78; N, 1.22. Found: C, 54.10; H, 9.01; N, 1.20.

Synthesis of Compound [G3]8OH[PEG6K] (7). Synthesized via the same procedure as compound **5** using PEG-6K-diazide (**4**) (0.100 g, 0.167 mmol), propargyl [G3]8[OH] (**2**) (0.332 g, 0.383 mmol) in dry THF (4 mL), and CuBr (2.4 mg, 0.017 mmol) and PMDETA (3.5 μL, 0.017 mmol) in dry THF (3 mL), yielding compound **7** (0.92 g, 71%) as a yellowish-white solid. ^{1}H NMR ($CDCl_3$, δ, ppm): 7.90 (s, 1H), 5.26 (s, 2H), 4.54 (t, 2H, J = 5.0 Hz), 4.19−4.28 (m, 12H), 3.85 (t, 2H, J = 5.0 Hz), 3.42−3.80 (m, 270H), 3.36 (t, 2H, J = 5.0 Hz), 1.25 (s, 3H), 1.22 (s, 6H), 1.06 (s, 12H). FTIR (cm^{-1}): 3435, 2881, 1732. $C_{348}H_{664}N_6O_{179}$ Calcd: C, 53.61; H, 8.58; N, 1.08. Found: C, 53.22; H, 8.68; N, 1.45.

Functionalization of the Dendronized Polymers. *Synthesis of Compound [G2]4OR[PEG2K] (8)*. [G2]4OH[PEG2K] (**5**) (0.100 g, 0.035 mmol), pyridine (0.30 mL), and DMAP (0.003 g, 0.028 mmol) were dissolved in dry CH_2Cl_2 (5 mL) in a 10 mL round-bottom flask. To the stirring reaction mixture was added 4-pentynoic acid anhydride (0.075 g, 0.42 mmol) and continued stirring for 24 h at room temperature under N_2. Pyridine:water solution (1 mL, 1:1) was added to the reaction mixture and stirred at room temperature for 5 h. The reaction mixture was diluted with CH_2Cl_2 (30 mL) and then extracted with 1 M $NaHSO_4$ (3 × 30 mL), 10% Na_2CO_3 (3 × 30 mL), and then with brine (1 × 30 mL). Combined organic layers were dried over anhydrous Na_2SO_4, and the residue was concentrated in vacuo. The crude product was purified by precipitation in diethyl ether to give 90 mg of **8** as a yellowish-brown viscous liquid (74% yield). ^{1}H NMR ($CDCl_3$, δ, ppm): 7.79 (s, 1H), 5.21 (s, 2H), 4.52 (t, 2H, J = 5.0 Hz), 4.13−4.23 (m, 12H), 3.84 (t, 2H, J = 5.0 Hz), 3.40−3.78 (m, 90H), 2.51 (t, 8H, J = 6.6 Hz), 2.43 (t, 8H, J = 6.6 Hz), 1.95 (s, 4H), 1.20 (s, 3H), 1.17 (s, 6H). FTIR (cm^{-1}): 3262, 2868, 1736. $C_{166}H_{278}N_6O_{72}$ Calcd: C, 56.84; H, 7.93; N, 2.40. Found: C, 56.82; H, 7.60; N, 2.45.

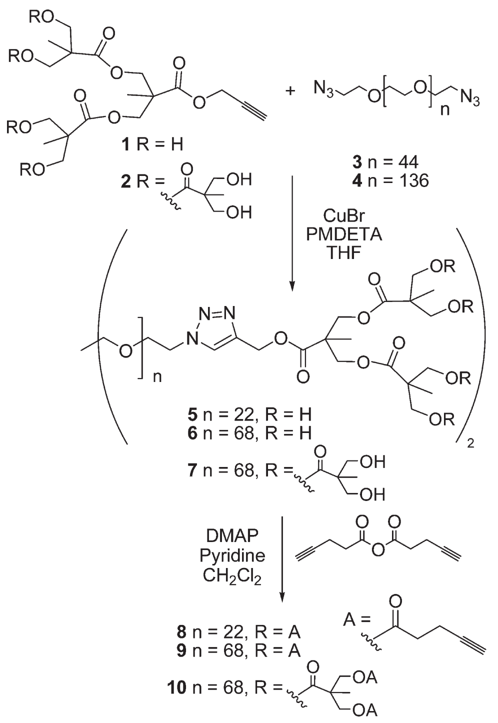
Synthesis of Compound [G2]4OR[PEG6K] (9). Synthesized via the same procedure as compound **8** using [G2]4OH[PEG6K (**6**) (0.250 g, 0.036 mmol), pyridine (0.30 mL), DMAP (0.004 g, 0.029 mmol), and 4-pentynoic acid anhydride (0.078 g, 0.440 mmol) to give 0.227 g of **9** as a yellowish-white solid (83% yield). ^{1}H NMR ($CDCl_3$, δ, ppm): 7.78 (s, 1H), 5.20 (s, 2H), 4.51 (t, 2H, J = 5.0 Hz), 4.12−4.22 (m, 12H), 3.84 (t, 2H, J = 5.0 Hz), 3.32−3.77 (m, 270H), 2.51 (t, 8H, J = 6.8 Hz), 2.43 (t, 8H, J = 6.8 Hz), 1.94 (s, 4H), 1.19 (s, 3H), 1.16 (s, 6H). FTIR (cm^{-1}): 3265, 2882, 1740. $C_{348}H_{632}N_6O_{163}$ Calcd: C, 55.68; H, 8.46; N, 1.12. Found: C, 55.05; H, 8.67; N, 0.84.

Synthesis of Compound [G3]8OR[PEG6K] (10). Synthesized via the same procedure as compound **8** using [G3]8OH[PEG6K] (**7**) (0.043 g, 0.0055 mmol), pyridine (0.30 mL), DMAP (0.005 g, 0.0044 mmol), and 4-pentynoic acid anhydride (0.024 g, 0.135 mmol) to give 65 mg of **10** as a yellowish-white solid (79% yield). ^{1}H NMR ($CDCl_3$, δ, ppm): 7.80 (s, 1H), 5.23 (s, 2H), 4.53 (t, 2H, J = 5.0 Hz), 4.18−4.25 (m, 28H), 3.86 (t, 2H, J = 5.0 Hz), 3.42−3.80 (m, 270H), 2.54 (t, 16H, J = 6.8 Hz), 2.45 (t, 16H, J = 6.8 Hz), 1.97 (s, 8H), 1.24 (s, 3H), 1.22 (s, 12H), 1.19 (s, 3H). FTIR (cm^{-1}): 3279, 2882, 1739. $C_{428}H_{728}N_6O_{195}$ Calcd: C, 56.63; H, 8.08; N, 0.93. Found: C, 56.51; H, 8.27; N, 1.27.

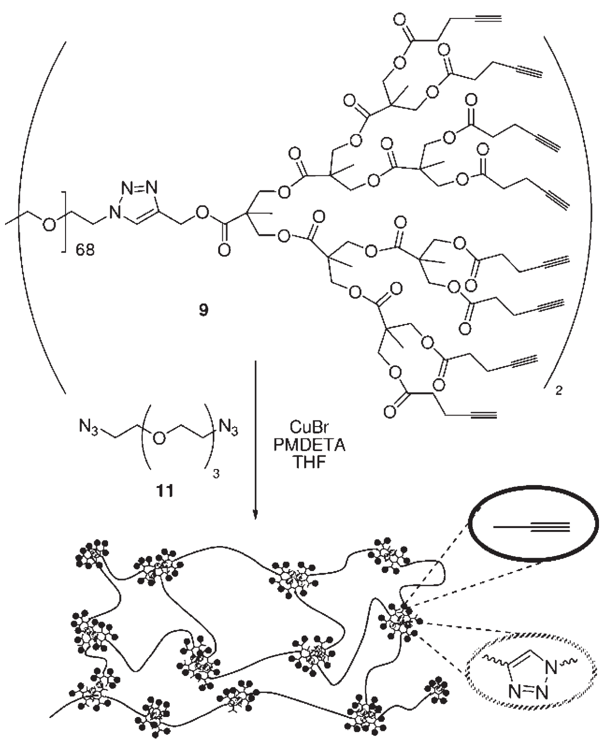
General Synthesis of Hydrogels via [3 + 2] Huisgen “Click” Chemistry. Formation of hydrogel via click chemistry was achieved according to literature protocol with minor changes.[49] To a small vial was added poly(ethylene glycol)−bis(tetraacetylene) **9** (20 mg, 2.67 μmol) and tetra(ethylene glycol) diazide **11** (0.652 mg, 2.67 μmol, 1.0 eqv) in H_2O (18 μL) and ethanol (50 μL). To the vial was added deionized H_2O (110 μL) containing sodium ascorbate (1.0 mg, 5.04 μmol), and the mixture was mixed under ultrasound to give a clear solution. Copper sulfate (1.0 mg, 6.28 μmol) in water (25 μL) was added, and after stirring for 10 s, the reaction mixture was poured into a Teflon O-ring with 1.5 mm height and 1.0 cm diameter. The bottom of the ring was capped with a Teflon rod, and upon addition of the reaction mixture, the ring was covered with a glass slide. The solution was allowed to react for 10 min at room temperature, and then the glass slide was removed. The formed gel was taken out of the ring with the help of the Teflon rod and then was submerged into an aqueous EDTA solution (5%, pH ∼7−8) to extract the trapped $CuSO_4$ and ethanol and finally was washed with deionized water. The hydrogel sample was quickly frozen and further freeze-dried in vacuo until the solvent was sublimed, yielding 6KG2$_{(1:1)}$ (17 mg, 82% gel content).

Functionalization of Hydrogels. *Functionalization with 4-Azido-N-ethyl-1,8-naphthalimide (12)*. Two identical cylindrically shaped hydrogels (**6KG2$_{(1:1)}$**) were synthesized (each about 20 mg) and placed into two different vials. 4-Azido-*N*-ethyl-1,8-naphthalimide (**12**, 0.25 mg, 0.94 μmol), deionized water (2.5 mL), and ethanol (2.5 mL) were added to each of the vials. Sodium ascorbate (1.00 mg, 0.005 mmol) and $CuSO_4$ (1.00 mg, 0.004 mmol) was added to vial 1 for copper-catalyzed cycloaddition. Vial 2 was left without catalyst as a control for nonspecific adsorptions.

Scheme 2. Synthesis and Functionalization of G2 and G3 Triblock Copolymers



Scheme 3. Hydrogel Formation via Cross-Linking of Dendron−Polymer Conjugates



The reaction mixtures were stirred at room temperature for 12 h. After the reaction was completed, hydrogels were first transferred to an aqueous EDTA solution (5%, pH ~7−8) to extract the trapped $CuSO_4$ and ethanol and finally washed with deionized water. Near-quantitative amounts of gels were recovered upon lypholization.

*Functionalization with BODIPY Azide **13***. Two identical cylindrically shaped hydrogels ($6KG3_{(1:1)}$) were synthesized (each about 20 mg) and placed into two different vials. Prior to the reaction, the hydrogels were washed with THF to remove water. Bodipy azide **13** (0.25 mg, 0.58 μmol) in THF (2.5 mL) was added to each of the vials. PMDETA (1.20 μL, 0.0058 mmol) and CuBr (1.00 mg, 0.007 mmol) were added to vial 1 for copper-catalyzed cycloaddition. Vial 2 was left without catalyst addition as control for nonspecific adsorptions. The reactions were stirred at room temperature for 12 h. After the reaction was completed, hydrogels were washed with THF to remove any trapped dye molecules and then washed with an aqueous EDTA solution (5%, pH ~7−8) to extract the trapped Cu(I)Br and finally washed with deionized water. Near-quantitative amounts of gels were recovered upon lypholization.

*Functionalization with Biotin Azide (**14**)*. Biotin azide was synthesized according to the literature procedure.[111] $6KG3_{(1:1)}$ hydrogel (containing 0.016 mmol alkyne) was reacted with biotin azide in the presence of $CuSO_4$ (1.0 mg, 0.0063 mmol) and sodium ascorbate (1.0 mg, 0.005 mmol) for 12 h at room temperature. As a control, the same hydrogel was treated with biotin azide in the absence of $CuSO_4$ and sodium ascorbate. After the reaction was completed, the gel was first transferred to a pH ~7−8 EDTA water solution (5%) and then washed with deionized water. Near-quantitative amounts of gels were recovered upon lypholization. The hydrogels were incubated with FITC-labeled streptavidin (0.1 mg/mL of PBS buffer, pH 7.4) for 30 min. After the incubation, hydrogels were rinsed with PBS, kept in water for 15 min, and washed with water several times.

Measurements. *Scanning Electron Microscopy (SEM) Analysis of the Hydrogel Samples*. The hydrogel samples were first equilibrated in H_2O at room temperature. The equilibrated hydrogel samples were quickly frozen and further freeze-dried in vacuo until the solvent was sublimed. The freeze-dried samples were then studied by using a scanning electron microscope. Wet sample was studied without the lyophilization step.

Physical Property Analysis (Water Uptake). Physical property analysis (water uptake) was done according to the previously reported methods.[112] The swelling behavior of the hydrogels was characterized as a function of time. The experiments were carried out by measuring the weight gain as a function of immersion time in 10 mL of deionized water. At given times the disks were removed from the water, blotted with absorbent tissue paper to eliminate excess water, and weighed. Measurements were taken until equilibrium hydration degree was reached, considered when three consecutive determinations gave the same weight (±0.001 g). These experiments were always done for a minimum of three samples of a particular hydrogel. The ability for swelling was expressed as the swelling ratio percent, W, via eq 1 in which M_w and M_d are the weights of wet and dry samples, respectively:

$$W = (M_w - M_d)/M_d \times 100 \tag{1}$$

Results and Discussion

Synthesis of three different G2 and G3 dendron−polymer−dendron ABA triblock copolymers via Huisgen "click" reaction and their functionalization with alkyne groups are shown in Scheme 1. Aliphatic polyester dendrons were chosen as the A block of the copolymer. For the synthesis of the alkyne core bearing protected polyester dendrons literature procedure was followed (see Supporting Information).[113] Deprotection of the acetonide groups of these G2 and G3 dendrons was achieved via treatment with DOWEX, H^+, yielding second-generation dendron **1** and third-generation dendron **2**, respectively. Azide-containing telechelic PEG polymers **3** and **4** were synthesized according to literature protocols.[110] These triblock copolymers are named according to the dendron generations and length of PEG used: i.e., [G2]4OH[PEG2K] represents two G2 dendrons

Table 1. Properties of Hydrogels with Variations in Dendron−Polymer Structure and Linker Quantity

item	hydrogel[a]	PEG	dendron	copolymer:cross-linker mole ratio	water uptake %	available functional groups
1	$2KG2_{(1:3)}$	2000	G2	1:3	904	2
2	$6KG2_{(1:3)}$	6000	G2	1:3	1282	2
3	$6KG2_{(1:2)}$	6000	G2	1:2	1107	4
4	$6KG2_{(1:1.5)}$	6000	G2	1:1.5	1054	5
5	$6KG2_{(1:1)}$	6000	G2	1:1	998	6
6	$6KG3_{(1:4)}$	6000	G3	1:4	2015	8
7	$6KG3_{(1:3)}$	6000	G3	1:3	1286	10
8	$6KG3_{(1:2)}$	6000	G3	1:2	1135	12
9	$6KG3_{(1:1)}$	6000	G3	1:1	1038	14

[a] The nomenclature of the hydrogels were made according to the molecular weight of the PEG and the generation of the dendron, i.e., second-generation dendron−PEG 2K = 2KG2.

with alcohol as the surface functionality bearing four alcohol groups as the A block and PEG polymer with MW = 2000 as the middle (B) block. Two different PEG lengths, 2K and 6K, were employed in this study. The desired copolymers [G2]4OH-[PEG2K] (**5**) and [G2]4OH[PEG6K] (**6**) were obtained via the reaction of alkyne core functionalized G2 polyester dendron **1** and bisazido PEG **3** or **4** in the presence of CuBr and PMDETA in THF. Functionalization of the surface alcohol groups on the obtained triblock copolymers were realized via the acylation reaction with pentynoic anhydride in the presence of pyridine yielding copolymers **8** and **9**. Similar procedures were applied for the G3 dendron **2** (Scheme 2) yielding [G3]8OH[PEG6K] (**7**) and alkyne-functionalized ABA block copolymer **10**. The presence of alkyne groups on the surface of the dendrons enables utilization of a second "click" reaction to afford the hydrogels.

Hydrogels were prepared by the Huisgen type "click" reaction between alkyne-functionalized dendron−PEG−dendron triblock copolymers **8**, **9**, and **10** and tetraethylene glycol bisazide (**11**) (Scheme 3). Although dendron−polymer conjugates **5**, **6**, and **7** have good water solubility, functionalization of the surface hydroxyl groups with alkyne moieties changed this property. Conjugate **8** required utilization of water-miscible organic solvents such as THF as cosolvent during gelation. Utilization of neat ethanol during the gelation procedure was also successful and led to clear gels. In order to obtain a copolymer with higher water solubility, longer PEG chain (MW = 6000) was employed with G2 dendron, and the resulting conjugate **9** was gelled in water without any solubility problems. On the other hand, hydrogel formation with the third-generation dendron-based conjugate **10** in water as the solvent was not successful and required cosolvent addition. One can envision that as the number of end groups increase with increasing generation, the hydrophobicity of the conjugate will increase rendering the overall copolymer less soluble in water. Thus, conjugate **10** was gelled in H_2O with the addition of THF and/or EtOH. The synthesized gels were subsequently placed in distilled water for 2 days with periodic replacement of the water to remove unreacted monomer and for leaching the organic solvents used for gelation.

Gelation of PEG−dendron conjugates **8**, **9**, and **10** with different ratios of tetraethylene glycol bisazide (**11**) cross-linker allowed to obtain hydrogels with different number of available residual alkyne functional groups (Scheme 3). 2KG2 and 6KG2 dendron−PEG copolymers have a total of eight alkynes on the periphery. Since tetraethylene glycol bisazide (**11**) has two azide groups, 3 equiv of the azide would react with six of the peripheral alkynes, leaving two alkynes for further functionalization (Table 1, items 1 and 2). 2 equiv of the diazide **11** would result in four available alkynes (Table 1, item 3), and decreasing amounts (1.5 and 1 equiv) of the diazide **11** would leave five and six available alkynes, respectively (Table 1, items 4 and 5). 6KG3 dendron−PEG copolymers on the other hand have a total of 16 peripheral alkynes. 4 equiv of the diazide **11** would leave eight alkynes for functionalization (Table 1, item 6), and

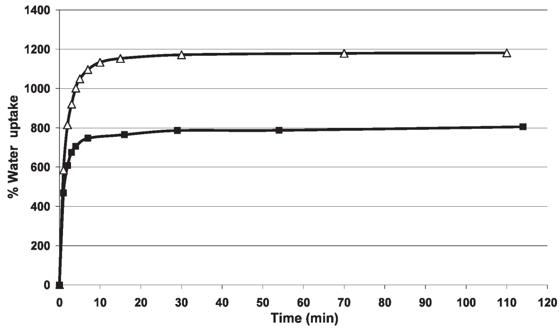


Figure 1. Water uptake comparison of $2KG2_{(1:3)}$ (■) and $6KG2_{(1:3)}$ (△) hydrogels.

decreasing equivalents of the diazide **11** would result in increasing available alkynes in the hydrogel (Table 1, items 7−9). Reaction of bisazide with alkyne groups on the same dendron cannot be ruled out, but this event would not affect the number of residual alkyne groups after gelation.

The swelling properties of the hydrogels were examined. Water uptake of two different constructs in which the length of the hydrophilic section was varied is shown in Figure 1. The hydrogel formation of the constructs $2KG2_{(1:3)}$ and $6KG2_{(1:3)}$ having PEG molecular weights of 2000 and 6000, respectively, was achieved with the same mole ratio of the diazide compound (1:3 alkyne: azide), and water uptake measurements were undertaken as explained above. As expected, increase in the length of the hydrophilic PEG segment resulted in an increase in the water uptake of the hydrogel (Figure 1).

Comparative swelling behavior of $6KG2_{(1:3)\to(1:1)}$ and $6KG3_{(1:4)\to(1:1)}$ hydrogels was investigated as a function of time, and the equilibrium hydration levels are reported in Table 1. The hydrogels prepared with more equivalents of the diazide **11**, namely $6KG2_{(1:3)}$ and $6KG3_{(1:4)}$, showed slightly higher degree of swelling than the less cross-linked counterparts $6KG2_{(1:1)}$ and $6KG3_{(1:1)}$ (Figures 2 and 3). The increasing amount of the diazide, though increasing the cross-linking density, would also decrease the amount of hydrophobicity, thus making the hydrogel slightly more hydrophilic and ready to absorb water.

Morphological Studies. To investigate the microstructure of the resultant hydrogel, the gels were allowed to reach fully swollen state in H_2O, quickly frozen, and then dried under vacuum. The investigation of the sample surface and cross section under SEM did not reveal any difference. SEM micrographs of dry and swollen hydrogels are presented in Figure 4. The morphological differences between dry and wet state of hydrogel can be clearly observed. No visible pores are seen in the "wet" pictures (Figure 4a) due to swelling. Dry hydrogel presents an interconnected porous structure with various pore sizes from submicrometers to few micrometers. SEM images of 6KG3(1:2) revealed larger macropores along with the similar micropores observed for other hydrogels (Figure 4d).

For full characterization of the hydrogel precursors and for establishing the presence of residual alkyne functionality on the hydrogels, infrared spectra were taken. Figure 5 shows the IR spectra of starting components, namely the G3 dendron (a), the bisazido PEG 6K (b), the copolymer (c), the alkyne-functionalized copolymer (d), and finally the $6KG3_{(1:4)}$ hydrogel (e). The IR spectra of the G3 dendron and the PEG diazide are given for comparison. The azide strech can be seen clearly at 2099 cm^{-1} for bisazido PEG (b) and is not present for the copolymers as expected. The alcohol surface groups in copolymer [G3]8OH[PEG6K] (**7**) (3435 cm^{-1}) are converted to alkyne groups in [G3]8OR-[PEG6K] (**10**). The C−H stretch of the alkyne functionality is observed at 3279 cm^{-1} for the copolymer [G3]8OR-[PEG6K] (**10**), and this peak is noticeable at 3249 cm^{-1} for the hydrogel $6KG3_{(1:4)}$ with eight remaining alkynes, theoretically demonstrating the presence of unreacted triple bonds.

To further demonstrate the presence and the reactivity of remaining alkyne groups, the hydrogels were reacted with azide bearing molecules in a [3 + 2] Huisgen-type cycloaddition. The choices of azides were as follows: The first dye, 4-azido-*N*-ethyl-1,8-naphthalimide (**12**),[114] shows no fluorescence but has click-activated fluorogenic properties showing

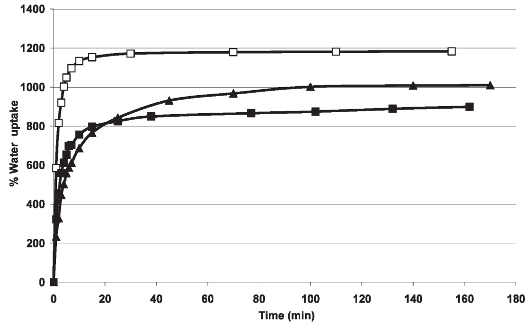


Figure 2. Water uptake comparison of $6KG2_{(1:3)}$ (□), $6KG2_{(1:2)}$ (▲), and $6KG2_{(1:1)}$ (■) hydrogels.

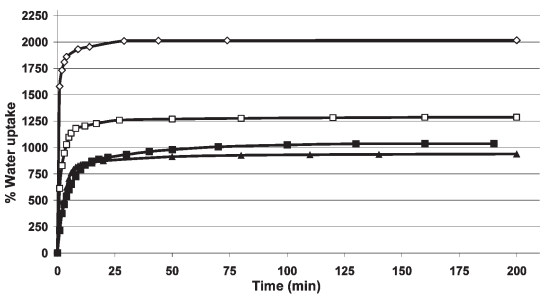


Figure 3. Comparison of $6KG3_{(1:4)}$ (◇), $6KG3_{(1:3)}$ (□), $6KG3_{(1:2)}$ (■), and $6KG3_{(1:1)}$ (▲) water uptake.

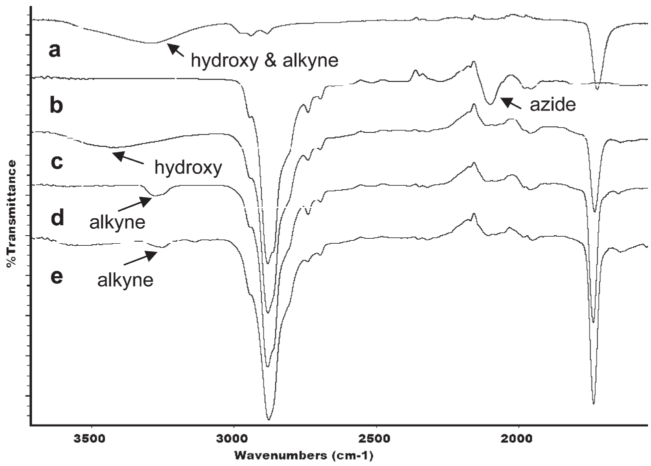


Figure 5. FTIR spectra of (a) G3 dendron **2**, (b) PEG-6K bis-azide **4**, (c) PEG dendron copolymer **7**, (d) PEG−dendron alkynyl-functionalized copolymer **10**, and (e) functional $6KG3_{(1:4)}$ hydrogel.

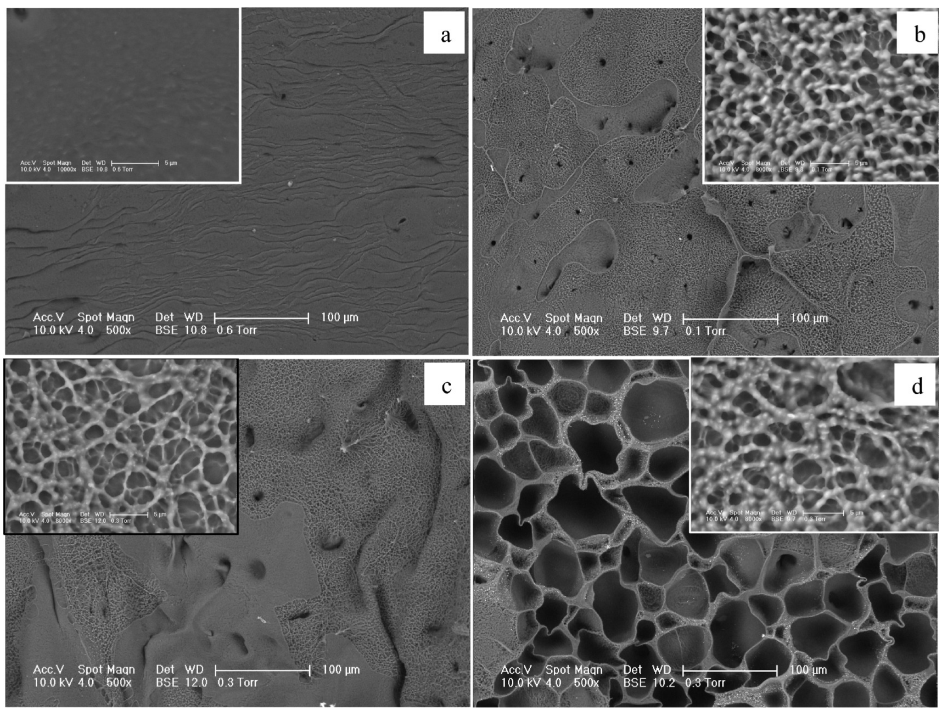


Figure 4. Representative SEM images of hydrogels (a) wet $6KG2_{(1:1.5)}$, (b) dry $6KG2_{(1:1.5)}$, (c) dry $6KG3_{(1:4)}$, and (d) dry $6KG3_{(1:2)}$. Large images scale bar = 100 μm; inset scale bar = 5 μm.

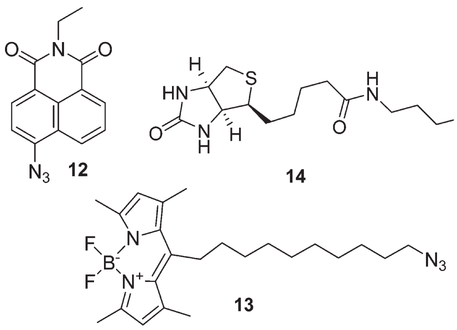


Figure 6. Azides utilized for hydrogel functionalization.

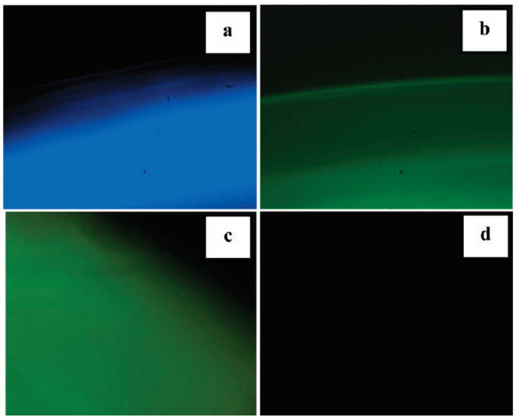


Figure 7. Fluorescence microscope images of functionalized hydrogels (a) with 4-azido-*N*-ethyl-1,8-naphthalimide (**12**), (b) with bodipy azide **13**, (c) with biotin azide (**14**)−streptavidin, and (d) control without catalyst.

an emission maximum between 400 and 500 nm (Figure 6). The second dye, bodipy azide **13**,[115] has an emission at green-shifted wavelengths. The third and last azide, biotin azide (**14**),[116,117] is attached for immobilization of streptavidin as an example of protein attachment on the hydrogels.

The functionalized hydrogels were visualized under the fluorescent microscope (Figure 7). The cylindrical hydrogel was divided into two parts, and as one part was treated with the desired azide and the catalyst system, the other part was treated with only the azide as a control. $CuSO_4$−sodium ascorbate was used as a catalyst for cycloadditions with the naphthalimide **12** and the biotin azide (**14**), whereas CuBr−PMDETA was used for the bodipy azide (**13**) due to the hydrophobic nature of the dye. The fluorescence microscope images of the catalyst bearing reactions with compounds **12** and **13** produced colored images (Figure 7, a and b, respectively). The images were taken at the edge of the hydrogels for easier visualization of the contrast. For the demonstration of facile and selective enzyme immobilization, the hydrogels were appended with biotin, a streptavidin binding ligand. Hydrogels were treated with biotin azide (**14**) in the presence of the Cu(I) to covalently attach the biotin ligands. After thorough rinsing with water, the sample was incubated with FITC-labeled streptavidin. As a control, a hydrogel sample was incubated with biotin azide in the absence of Cu(I). Incubation with streptavidin followed by thorough rinsing with water provided the control sample. The resulting fluorescence images of streptavidin immobilization are shown in Figure 7c,d.

To establish the relationship between number of remaining alkyne groups in the hydrogel and the ability to functionalize, four hydrogel samples ($6KG3_{(1:1)}$−$6KG3_{(1:4)}$) were

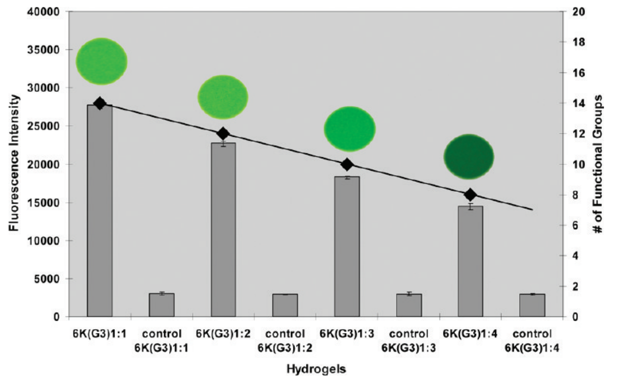


Figure 8. Relative fluorescence intensities of functionalized hydrogels with streptavidin and their controls that contain descending amount of functional groups.

first reacted with excess amounts of biotin azide (**14**) and then incubated with FITC-labeled streptavidin. Each hydrogel was accompanied by the control gel, going through the same treatments, albeit without the Cu(I) catalyst system. Relative fluorescence intensities were calculated using Jimage 1.41 software and plotted in Figure 8. As can be clearly seen from the graph, there was a direct correlation between the number of alkynes in the hydrogel and the fluorescence intensity after functionalization. Fluorescence microscope images of the samples are shown above the bars.

Conclusions

Synthesis of dendron−polymer conjugates was accomplished via [3 + 2] Huisgen "click" chemistry of biodegradable polyester dendrons and PEG bisazides with high yields. The copolymers have been successfully functionalized with alkyne groups, preparing the system for the second [3 + 2] Huisgen "click" reaction. This reaction was achieved with utilizing a variety of cross-linker ratios yielding hydrogels with different amounts of functionalizable groups, namely leftover alkynes. It has been shown that these alkynes effectively participate in the third "click" reaction to afford functionalized hydrogels. Conjugation of various dye molecules and attachment of streptavidin on the hydrogels have been illustrated. The FITC-labeled streptavidin attachment on the hydrogels established the direct correlation between the remaining alkyne groups and the fluorescence observed after the conjugation.

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Supporting Information Available: Experimental details about synthesis of materials and NMR and IR spectroscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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