Shell Cross-Linked Nanoparticles Designed To Target Angiogenic Blood Vessels via $\alpha_v\beta_3$ Receptor−Ligand Interactions

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ABSTRACT: The design, synthesis, and characterization of a novel polymeric nanostructured material bearing surface-attached integrin antagonists are demonstrated. The covalent coupling of a complex and biologically active small molecule to a well-defined nanostructured material combines the elegance of synthetic organic chemistry with the state-of-the-art polymer chemistry. This unique material offers potential in targeting to tumor neovasculature and delivery of diagnostic and therapeutic agents.

Introduction

For the general detection, diagnosis, and treatment of tumors, targeting of the vasculature has received considerable attention. Identification of cell surface receptors that are overexpressed and ligands that can interact selectively with those receptors[1] is a key area in which rapid developments are being assisted by advances in proteomics and genomics and by the development of analytical tools. From the many possibilities, the integrin $\alpha_v\beta_3$, which is found in large numbers upon the endothelial cells of many angiogenic tumor-associated blood vessels and is known to play a key role in cell matrix recognition and facilitate gene transfer, has been the focus of several studies aimed at the detection[2] and treatment[3] of cancers. To accomplish therapeutic efficacy, Hood et al.[4] demonstrated that the attachment of a ligand that targets $\alpha_v\beta_3$ to cationic nanoparticles allowed for packaging, transport, and delivery of antiangiogenic genes to $\alpha_v\beta_3$-overexpressing M21 melanoma cells in mice, resulting in tumor regression. Given the promising in vivo data that were reported for this $\alpha_v\beta_3$ targeting ligand, our interest has been its implementation as a targeting moiety for well-defined nanostructured materials, comprised of an amphiphilic core−shell morphology, which can be utilized for the sequestration of multiple and variable guest molecules.[5]

To develop a novel, endothelial cell specific delivery vehicle, the preparation of a shell cross-linked (SCK) nanoparticle[6,7] bearing an integrin $\alpha_v\beta_3$-targeting ligand (**1**) was undertaken. The SCK was also labeled fluorescently to allow for characterization and tracking of the nanostructure. Intracellular pH plays an important role in many cellular events including receptor-mediated signal transduction, ion transport, endocytosis, cell adhesion, and various other cellular processes.[8] In recent years, pH-sensitive fluorescent dyes are often applied to monitor changes in intracellular pH.

Experimental Section

Materials. Unless otherwise listed, all solvents and reagents were purchased from Aldrich Chemical Co. (St. Louis, MO) and used as received. Tetrahydrofuran (HPLC grade, inhibitor free, Aldrich) was dried over calcium hydride and distilled from sodium/benzophenone. Isoprene was purified by passing through a neutral alumina column to remove the inhibitor. *N*,*N*-Dimethylformamide (DMF, 99.8%) and *tert*-butyl acrylate (98%) were purchased from Aldrich Chemical Co. and distilled over calcium hydride prior to use. Nitrogen (UHP, 99.99%) was used for polymerization and storage of materials. Flash column chromatography was performed using 32−63 D 60 Å silica gel from ICN SiliTech (ICN Biomedicals GmbH, Eschwege, Germany). Spectra/Por membrane (MWCO: 12 000−14 000 Da) used for dialysis was obtained from Spectrum Medical Industries, Inc. (Laguna Hills, CA).

Measurements. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were recorded as solutions on a Varian Mercury 300 MHz spectrometer with the solvent signal as standard. Hydrodynamic diameter distribution and distribution averages for the SCKs in PBS solutions were determined by dynamic light scattering. A Brookhaven Instruments Co. (Holtsville, NY) DLS system equipped with a model BI-9000AT digital correlator, a model EMI-9865 photomultiplier, and a model 95-2 Ar ion laser (Lexel Corp., Fremont, CA), operated at 514.5 nm, was used. Measurements were made at 20 ± 0.1 °C. Nanoparticles were dialyzed into 50 mM PBS, pH 7.2, prior to analysis. Buffered nanoparticle solutions were either centrifuged in a model 5414 microfuge (Brinkman Instrument Co., Westbury, NY) for 4 min or filtered through 0.22 μm poly(vinylidene fluoride) and 0.1 μm poly(ether sulfone) membrane filters (Pall Gelman, East Hills, NY) to remove dust particles. Scattered light was collected at a fixed angle of 90°. The digital correlator was operated with 522 ratio spaced channels, an initial delay of 1.4 μs, a final delay of 10 ms, and a duration time of 15 min. A photomultiplier aperture of 400 μm was used, and the incident laser power was adjusted to obtain a photon counting rate between 200 and 300 kcps. Only measurements for which the measured and calculated baselines of the intensity autocorrelation function agreed to within ±0.1% was used to calculate nanoparticle hydrodynamic diameter values. All determinations were made in triplicate. The calculations of the nanoparticle diameter distributions and distribution averages were performed with the ISDA software package (Brookhaven Instruments Co.), which employed single-exponential fitting, cumulants analysis, and nonnegatively constrained least-squares particle size distribution analysis routines. Solution state hydrodynamic diameter determination of the fluoresceinated SCK samples were performed by using Brookhaven Instruments Co. 90 PLUS particle size analyzer equipped with a solid-state laser operated at 658 nm. All the UV−vis spectroscopy data were acquired on a Varian Cary 1E UV−vis spectrophotometer. SCK solutions (~0.3 mg/mL) and a solution of $\alpha_v\beta_3$-targeting ligand (0.2 mg/mL) were made in deionized water prior to the absorbance measurements. All

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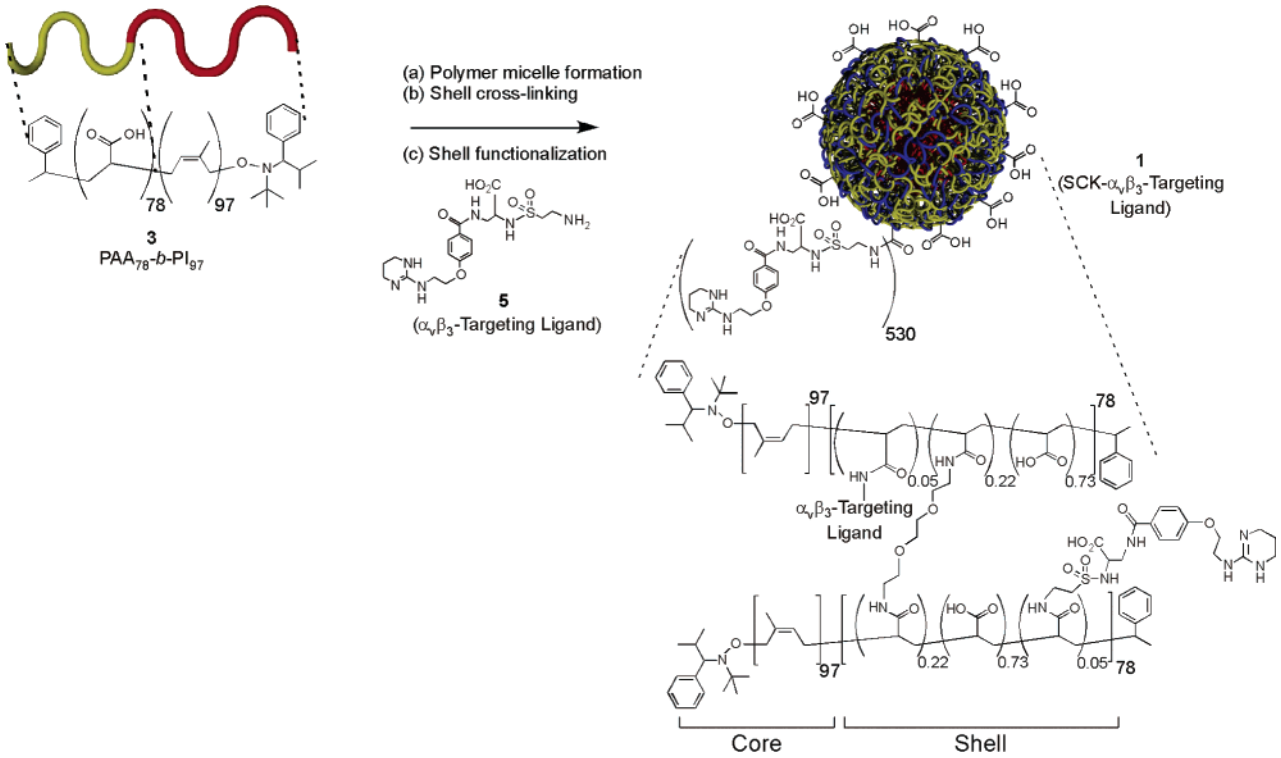
the SCK concentrations have been determined by lypholization of the aqueous sample solutions. All the fluorescence spectroscopy data were acquired on a Varian Cary Eclipse fluorescence spectrophotometer. Each sample was prepared independently from a stock solution of **7** at 0.25 mg/mL. Sample solutions at pH values 5.9, 6.3, 6.9, 7.3, and 7.8 were excited at $\lambda_{ex} = 488$ nm. Fluorescence emission spectra ($\lambda_{em} = 517$ nm) from **7** at different pH values in 50 mM sodium phosphate buffered saline were recorded. Zeta potential (ζ) values for the SCKs were determined with a Brookhaven Instrument Co. (Holtsville, NY) model Zeta Plus zeta potential analyzer. Measurements were made following dialysis (MWCO 12−14 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of SCK solutions into 1 mM KH_2PO_4, 1 mM KCl, pH 7.1 buffer. Data were acquired in the phase analysis light scattering (PALS) mode following solution equilibration at 25 °C. Calculation of ζ from the measured nanoparticle electrophoretic mobility (μ) employed the Smoluchowski equation: $\mu = \epsilon\zeta/\eta$, where ϵ and η are the dielectric constant and the absolute viscosity of the medium, respectively. Measurements of ζ were reproducible to within ±2 mV of the mean value given by 16 determinations of 10 data accumulations. Transmission electron microscopy carbon grids were prepared by oxygen plasma treatment to make the surface hydrophilic. Particle samples were diluted 9:1 in water and further diluted 1:1 with a 1% phosphotungstic acid (PTA) stain. Micrographs were collected at 100 000× magnification. The number-average particle diameter values and standard deviations were generated from the analyses of a minimum of 100 particles from three micrographs. Tapping-mode atomic force microscopy measurements were conducted in air with a Nanoscope III BioScope system (Digital Instruments, Santa Barbara, CA) operated under ambient conditions with standard silicon tips (type, OTESPA-70; *L*, 160 μm; normal spring constant, 50 N/m; resonance frequency, 246−282 kHz). The samples were prepared for AFM analysis by depositing a 2 μL drop of the solution onto freshly cleaved mica and allowing it to dry freely in air. The number-average particle height values and standard deviations were generated from the section analysis of a minimum of 100 particles from at least five different analysis regions. SEC was conducted on a Waters Chromatography Inc. model 150-CV, equipped with a model 410 differential refractometer, a Precision Detectors Inc. model PD2040 dual-angle (15° and 90°) light scattering detector, and a three-column series of Polymer Laboratories Inc. PL_{gel} 10 μm mixed B 300 × 7.5 mm columns. The system was equilibrated at 35 °C in anhydrous THF, which served as the polymer solvent and eluent (flow rate set to 1.00 mL/min, then determined gravimetrically). Data collection was performed with Precision Detectors Inc. Precision Acquire software. Data analysis was performed with Precision Detectors Inc. Discovery 32 software. Interdetector delay volume and the light scattering detector calibration constant were determined from a nearly monodisperse polystyrene calibrant (Pressure Chemical Co., M_p = 90 000 g/mol, $M_w/M_n < 1.04$). The differential refractometer was calibrated with standard polystyrene reference material (SRM 706 NIST), of known specific refractive index increment d*n*/d*c* (0.184 mL/g). The d*n*/d*c* values of the analyzed polymers were then determined from the differential refractometer response. Glass transition temperatures (T_g) were measured by differential scanning calorimetry on either a Perkin-Elmer DSC-4 (polymer samples) or Mettler Toledo DSC822^e (SCK). The Perkin-Elmer instrument was upgraded with an Instrument Specialties (Antioch, IL) temperature program interface PE, and data were acquired and analyzed with TA-PC software (version 2.11, Instrument Specialists). Measurements obtained using the Mettler Toledo DSC822^e were analyzed using Mettler Toledo Star SW 7.01 software. Heating rates for both instruments were 10 °C/min, and the T_g values were taken at the midpoint of the inflection tangent, upon the third heating scan.

Preparation of P*t*BA Homopolymer. A 100 mL Schlenk tube connected to a double manifold under N_2, fitted with a stir bar (oven-dried at 110 °C overnight and flame-dried under vacuum immediately prior to use), was covered with a rubber septum and charged with 43 mL (38 g, 300 mmol) of *t*BA. To this solution was added alkoxyamine (2,2,5-trimethyl-3-(1-phenylethoxy)-4-phenyl-3-azahexane) (401 mg, 1.23 mmol) and nitroxide (2,2,5-trimethyl-4-phenyl-3-azahexane 3-nitroxide) (13 mg, 0.062 mmol). The mixture was allowed to stir at room temperature for 10 min to ensure homogeneity, prior to performing three freeze−pump−thaw cycles. After the final degassing, the tube was sealed, allowed to warm to room temperature, and placed in an oil bath at 130 °C. After 47 h, the tube was immersed in an ice bath to quench the polymerization. Excess *t*BA was removed via distillation, and 15 mL of tetrahydrofuran was added to the tube and allowed to stir until complete dissolution had occurred. The resultant polymer was precipitated three times into a 50% methanol/water (1 L) solution at 4 °C. The white polymer product was collected via filtration and dried under vacuum overnight to give poly(*tert*-butyl acrylate) (P*t*BA). Yield: 10.3 g (76%). $M_n^{NMR} = 10\,300$ Da, $M_n^{GPC} = 11\,100$ Da, $M_w/M_n = 1.14$. $T_g = 33$ °C. IR: 3437, 2972, 1723, 1443, 1357, 1252, 1163, 753 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 1.4 (−COOC(C*H*$_3$)$_3$), 1.6−2.0 (−C*H*$_2$−CH(COOC(CH$_3$)$_3$)−), 2.0−2.4 (−CH$_2$−C*H*(COOC(CH$_3$)$_3$)−), 7.0−7.2 (aromatic H's from initiator). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): δ 28.1, 36.0−39.2, 41.4−43.8, 81.4, 173.8.

Preparation of P*t*BA-*b*-PI Diblock Copolymer. A 100 mL Schlenk tube connected to a double manifold under N_2, fitted with a stir bar (oven-dried at 110 °C overnight), was charged with P*t*BA macroinitiator (2.11 g, 0.205 mmol) and placed under vacuum (<0.1 mm Hg) for 24 h. The tube was backfilled with N_2 and fitted with a rubber septum. Isoprene (20 mL, 200 mmol, freshly distilled from CaH_2) was transferred to the Schlenk tube via cannula followed by the addition of nitroxide via a gastight syringe. The mixture was allowed to stir at room temperature for 10 min to ensure homogeneity, prior to performing three freeze−pump−thaw cycles. After the final degassing, the tube was sealed, allowed to warm to room temperature, and placed in an oil bath at 143 °C. After 72 h, the tube was immersed in an ice bath to quench the polymerization. Excess isoprene monomer was removed via distillation, and 25 mL of tetrahydrofuran was added to the tube and allowed to stir until complete dissolution had occurred. The resultant polymer was precipitated three times into a 70% methanol/water (1 L) solution at 4 °C. A white solid was collected via filtration and dried under vacuum overnight to give P*t*BA-*b*-PI diblock copolymer. Yield: 2.63 g (77%). $M_n^{NMR} = 16\,700$ Da, $M_n^{GPC} = 15\,100$ Da, $M_w/M_n = 1.11$. $(T_g)_{PI} = -74$ °C, $(T_g)_{PtBA} = 32$ °C. IR: 3437, 3079, 2972, 1723, 2640, 2443, 1357, 1234, 1159, 860, 783, 750, 783 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 1.4 (−COOC(C*H*$_3$)$_3$), 0.9−2.1 (br, backbone H's and side-chain CH$_3$'s), 2.1−2.3 (−CH$_2$−C*H*(COOC(CH$_3$)$_3$)−), 4.4−5.0 (br, 1,2 −CH=C***H***$_2$ and 3,4 −C(CH3)=C***H***$_2$), 5.0−5.2 (1,4-, −CH$_2$−C(CH$_3$)=C*H*−CH$_2$−), 5.7−5.9 (br, 1,2 −C***H***=CH$_2$), 7.0−7.2 (aromatic H's from end groups). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): δ 15.8, 23.2, 26.5−27.8, 30.7, 39.5, 41.7−42.1, 80.1−80.8, 124.1−124.8, 127.2−128.2, 130.3, 134.8, 147.9, 173.7−174.

Preparation of PAA-*b*-PI Diblock Copolymer 3. To a 250 mL two-necked round-bottom flask equipped with a stir bar and West condenser was added P*t*BA-*b*-PI (2.48 g, 0.148 mmol (polymer), 11.5 mmol (ester)). The polymer was dissolved into an 80:20 toluene:acetic acid (v:v) solution (250 mL) and allowed to stir for 20 min to ensure homogeneity. To this solution was added methanesulfonic acid (0.0519 mL, 0.800 mmol) neat via a syringe. The solution was allowed to stir at reflux and monitored via ^{1}H NMR. The reaction was stopped after 1.5 h at reflux, and excess solvent was removed under vacuum. The resultant solid was taken up into tetrahydrofuran (100 mL), and 100 mL of water was added dropwise over a 30 min period. Purification was afforded through dialysis (MWCO 3500 Da) against water for 4 days. Lyophilization of the resultant solution afforded pure PAA-*b*-PI as a colorless solid. Yield: 1.89 g (100%). $(T_g)_{PI} = -73$ °C, $(T_g)_{PAA} = 127$ °C. IR: 3100−3600, 3079, 2972, 1680, 1640, 1601, 1443, 1347, 1243, 1212, 860, 783 cm^{-1}. ^{1}H NMR (300 MHz, THF-d_8, ppm): δ 1.3−2.1 (br, backbone H's and side-chain CH$_3$'s), 2.3−2.5 (−CH$_2$−C*H*(COOH)−), 4.5−5.0 (br, 1,2 −CH=C***H***$_2$ and 3,4-, −C(CH3)=C***H***$_2$), 5.2 (1,4 −CH$_2$−C(CH$_3$)=C***H***−CH$_2$−), 5.0−5.2 (1,4-,

Scheme 1[a]



[a] Reagents and conditions: (a) tetrahydrofuran, followed by addition of water and dialysis against water; (b) 2,2′-(ethylenedioxy)bis(ethylamine), 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide, rt, overnight, followed by dialysis against water; (c) **5**, 1-(3′-dimethylaminopropyl)-3-ethylcarbodiimidemethiodide, rt, overnight, followed by dialysis against sodium phosphate buffered saline at pH 7.2.

$-CH_2-C(CH_3)=CH-CH_2-$), 5.7−5.9 (1,2-, $-CH=CH_2$), 7.0−7.2 (aromatic H's from end groups). ^{13}C NMR (75 MHz, THF-d_8, ppm): δ 15.9, 23.1, 26.7−27.1, 31.2, 35.5−36.2, 40.7−42.0, 80.2−80.8, 124.1−124.9, 127.3−128.8, 130.4, 134.8, 148.9, 175.2.

Preparation of PAA-*b*-PI Micelle 4. Micellization of the PAA-*b*-PI diblock copolymer was induced by dissolving the polymer (950 mg) into tetrahydrofuran (475 mL), followed by the addition of Nanopure water at a rate of ∼10 mL/h over 2 days. Afterward, a second 500 mL of water was added neat, and the resulting solution was allowed to stir 2 h before being transferred to dialysis bags (MWCO 3500 Da), and dialysis was performed against water for 5 days. Final concentration = 0.435 mg/mL. D_h (DLS) = 52 ± 9 nm.

Preparation of Shell Cross-Linked (SCK) Nanoparticles 2. To a stirred solution of micelles (500 mL, 218 mg, 0.0177 mmol (polymer), 1.4 mmol (acid residues)) was added a solution of 2,2′-(ethylenedioxy)bis(ethylamine) (0.0190 g, 0.152 mmol) in water (20 mL) dropwise over a 10 min period. The resultant solution was allowed to stir for 1 h at ambient temperature. To this solution was added a solution of 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (0.0903 g, 0.304 mmol) in water (20 mL) dropwise over 10 min. The resultant solution was allowed to stir overnight under ambient conditions. Removal of small molecule contaminants was achieved through dialysis through a 10−12 kDa MWCO membrane against pure water for 1 week. DSC (T_g) = −70 °C (an endotherm for the transition of the PI core only was observed). IR (KBr): 3560−3200, 2930, 2440, 1651, 1556, 1450, 1387, 1339, 1155, 1075, 1036, 874 cm^{-1}. ^{1}H NMR [D_2O(1):THF-d_8(1), overnight]: δ 0.98−3.3 (aliphatic protons of polymer backbone), 7.2 ppm (aromatic end groups). DLS volume-average D_h (λ = 514.5 nm): 31 ± 1 nm; DLS volume-average D_h (λ = 658 nm): 33 ± 1 nm; D_{ah} (TEM): 17 ± 3 nm; H_{av} (AFM): 2.7 ± 1 nm; zeta (ζ): −26 ± 5 mV.

Preparation of the $\alpha_v\beta_3$-Targeting Ligand Functionalized Shell Cross-Linked Nanoparticles 1. To a stirred solution of SCK **2** (0.20 mg/mL, 0.0092 mmol of acrylic acid residues available) in a round-bottom flask was added a solution of 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (0.4 mg, 0.0013 mmol) in deionized water (1 mL). The solution was allowed to stir for 20 min at room temperature. To this reaction mixture was added a solution of **5** (0.3 mg, 6.5 × 10^{-4} mmol), dissolved in deionized water (1 mL). The reaction mixture was allowed to react overnight at room temperature. The solution was then transferred to a presoaked dialysis membrane tube (MWCO 12−14 kDa) and allowed to dialyze for 3 days against 50 mM sodium phosphate, 50 mM sodium chloride, pH 7.2. IR (KBr): 3430, 3070, 2980−2860, 2440, 2390, 2350, 2344, 2300, 1734, 1728, 1710, 1690, 1680, 1650, 1630, 1555, 1536, 1500, 1459, 1438, 1250, 1130, 1050, 990, 950, 860, 810−630 cm^{-1}. ^{1}H NMR [D_2O(2):THF-d_8 (1), overnight]: δ 0.98−3.4 (aliphatic protons along the backbones of the core polymer chains). DLS volume-average D_h: (λ = 514.5 nm): 42 ± 5 nm; DLS volume-average D_h (λ = 658 nm): 48 ± 7 nm; D_{ah} (TEM): 21 ± 8 nm; H_{av} (AFM): 2.8 ± 1 nm; zeta (ζ): −20 ± 3 mV.

Fluorescence Labeling of the $\alpha_v\beta_3$-Targeting Ligand Functionalized Shell Cross-Linked Nanoparticles 7. To a stirred solution of SCK-$\alpha_v\beta_3$ **1** (0.20 mg/mL, 0.0043 mmol of acrylic acid residues available) in a round-bottom flask was added a solution of 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (0.3 mg, 0.001 mmol) in deionized water (1 mL). The solution was allowed to stir for 20 min at room temperature. To this reaction mixture was added a solution of **6** (0.2 mg, 3.9 × 10^{-4} mmol), dissolved in deionized water (1 mL). The reaction mixture was allowed to react overnight at room temperature. The solution was then transferred to a presoaked dialysis membrane tube (MWCO 12−14 kDa) and allowed to dialyze for 7 days against 50 mM sodium phosphate, 50 mM sodium chloride, pH 7.3. DLS volume-average D_h (λ = 658 nm): 46 ± 6 nm; D_{ah} (TEM): 24 ± 8 nm; H_{av} (AFM): 2.6 ± 2 nm; zeta (ζ): −27 ± 2 mV.

Synthesis of 5- and (6)-Carboxyfluorescein Succinimidyl Active Ester (CF-Osu) 9. To a solution of 5- and (6)-carboxyfluorescein (**8**) (0.25 g, 0.66 mmol) in anhydrous DMF

Table 1. Summary of Characterization Data for SCK 2, SCK 1, Labeled with the $\alpha_v\beta_3$ Targeting Ligand, and SCK 7, Labeled with the $\alpha_v\beta_3$ Targeting Ligand and 5- and (6-)Carboxyfluorescein

property	SCK (**2**)	SCK-$\alpha_v\beta_3$-targeting ligand (**1**)	SCK-$\alpha_v\beta_3$-targeting ligand-carboxy fluorescein (**7**)
D_h/DLS[a]	33 ± 6 nm	42 ± 5 nm	
D_h/DLS[b]	33 ± 1 nm	48 ± 7 nm	46 ± 6 nm
D_{ah}/TEM	17 ± 3 nm	21 ± 8 nm	24 ± 8 nm
H_{av}/AFM	2.7 ± 1 nm	2.8 ± 1 nm	2.6 ± 2 nm
ζ/ZETA	−26 ± 5 mV	−20 ± 3 mV	−27 ± 2 nm

[a] Using an Ar ion laser at $\lambda = 514.5$ nm. [b] Using a solid-state laser at $\lambda = 658$ nm.

(5 mL) was added EDAC (0.16 g, 0.8 mmol) followed by *N*-hydroxysuccinimide (HOSu, 0.095 g, 0.8 mmol). The reaction was covered with foil, stirred under N_2. After 4.5 h, additional EDAC (0.025 g) was added, and the reaction mixture was allowed to stir for an additional 18.5 h. Reaction mixture was rinsed into a separatory funnel with DMF (2−3 mL) and diluted with acetone (10 mL). Buffer (0.05 M, pH 6.0 phosphate buffer Na_3PO_4, 13 mL) was added, and the mixture was extracted with ether/ethyl acetate (2:1) (50 mL). The combined organic layer was washed with water three times, dried over $MgSO_4$, and solvent removed to furnish 0.1 g of crude 5- and (6)-carboxyfluorescein succinimidyl esters. This compound was immediately used for the next step without further purification. Yield: 32%. ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 2.88 (s) and 2.93 (s) (total 4H), 6.50−6.60 (m) (total 2H), 6.60−6.75 (m) (total 4H), 7.56 (d, $J = 8.2$ Hz) and 8.20 (d, $J = 8.0$ Hz) (total 1H), 7.9 (s) and 8.54 (s) (total 1H), 8.30−8.45 (m) (total 2H), 10.20 (br s) (total 1H).

Preparation of 2-Amino-2′-[(*tert*-butoxycarbonyl)amino]ethylene Glycol Diethyl Ether (11).[9] To a solution of 2,2′-(ethylenedioxy)bis(ethylamine) (**10**) (4.15 mL, 0.0275 mol) in dioxane (20 mL) was added dropwise at ambient temperature over 20 min a solution of di-*tert*-butyl dicarbonate (1.02 g, 0.0046 mol) in dioxane (5 mL). The mixture was allowed to stir for 24 h and concentrated. 50 mL of water was added to it, and it was rinsed with 50 mL of dichloromethane. The combined organic layer was dried over $MgSO_4$ and concentrated to give 0.44 g of the desired product. Yield: 37%. ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 1.4 (s, 9H, *t*Bu), 1.5 (brs, 2H, NH_2), 2.8 (t, 2H), 3.3 (m, 2H), 3.5−3.6 (m, 4H), 3.7 (s, 4H), 5.2 (brs, 1H, N*H*Boc).

Reaction of 5- and (6)-Carboxyfluorescein Succinimidyl Ester (9) with 2-Amino-2′-[(*tert*-butoxycarbonyl)amino]ethylene Glycol Diethyl Ether (11) 12. To a stirred solution of CF-Osu (**9**) (0.05 g, 0.11 mmol) in 4 mL of dry DMF, a solution of mono-boc-protected diamine (**11**) was added (0.029 g, 0.12 mmol) in 2 mL of dry DMF. The reaction mixture was allowed to stir for 3 h and 40 min. Reaction was monitored by TLC. It was then poured in 10 mL of deionized water, mixed well, and extracted with ethyl acetate. The organic layer was washed with water (two times), dried ($MgSO_4$), and evaporation of solvent afforded the crude product. This was purified by column chromatography (silica gel, chloroform and methanol; 2:1). Yield: 0.04 g (66%). ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 1.3 (s, 9H), 2.94−3.60 (m, 12H), 6.50−6.75 (m, 4H), 7.35 (d, $J = 8.2$ Hz) and 8.24 (d, $J = 8.0$ Hz) (total 1H), 7.62 (s) and 8.42 (s) (total 1H), 8.05 (d, $J = 8.0$ Hz) and 8.21 (d, $J = 8.0$ Hz) (total 2H), 8.67 (m) and 8.80 (m) (total 1H), 10.20 (brs) (total 1H).

Deprotection of the *tert*-Boc: Preparation of Carboxyfluoresceinamine (6). *tert*-Boc-protected fluoresceinamine (**12**) (20 mg) was taken in a round-bottom flask, and to it 3−4 mL of a 1:2 mixture of dichloromethane and trifluoroacetic acid (cold) was added and allowed to stir for 1 h at 0−5 °C and an additional 2.5 h at ambient temperature. Excess

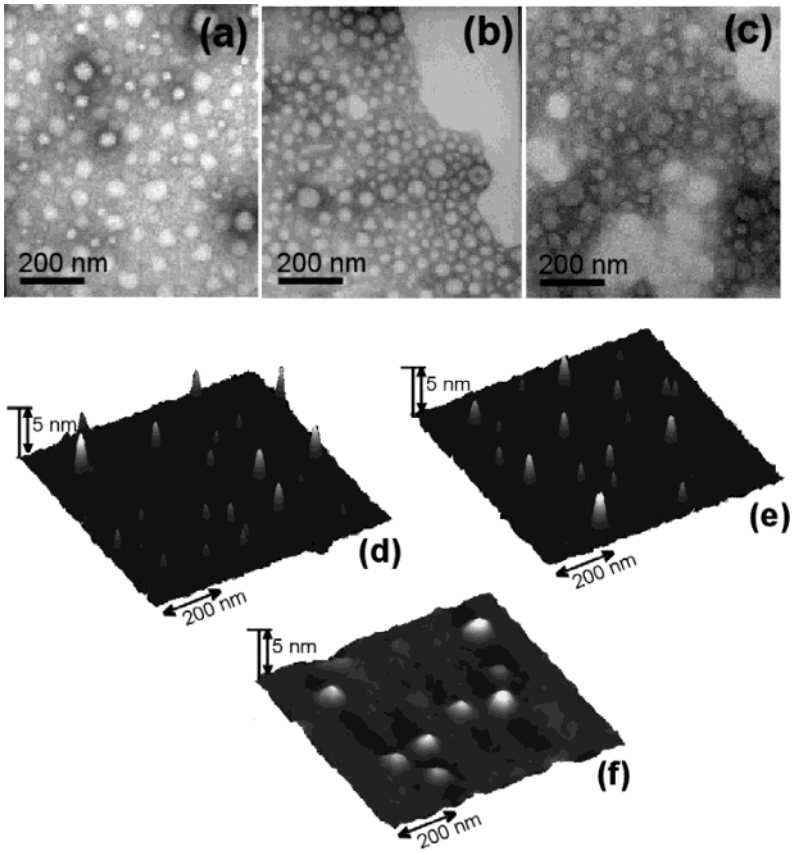


Figure 1. (a, b, c) Transmission electron microscopy images of **2**, **1**, and **7**, respectively. The samples were prepared by addition of 1% phosphotungstic acid, followed by drop deposition from the aqueous solution upon a carbon-coated copper grid and allowing to dry freely in air. (d, e, f) Tapping-mode AFM images of **2**, **1**, and **7**, respectively. The samples were prepared by drop deposition from aqueous solution onto freshly cleaved mica and allowing to dry freely in air.

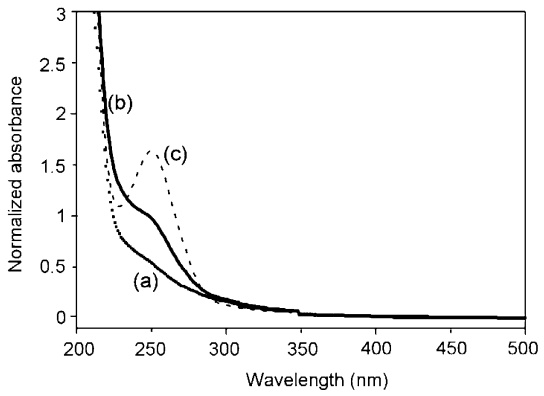


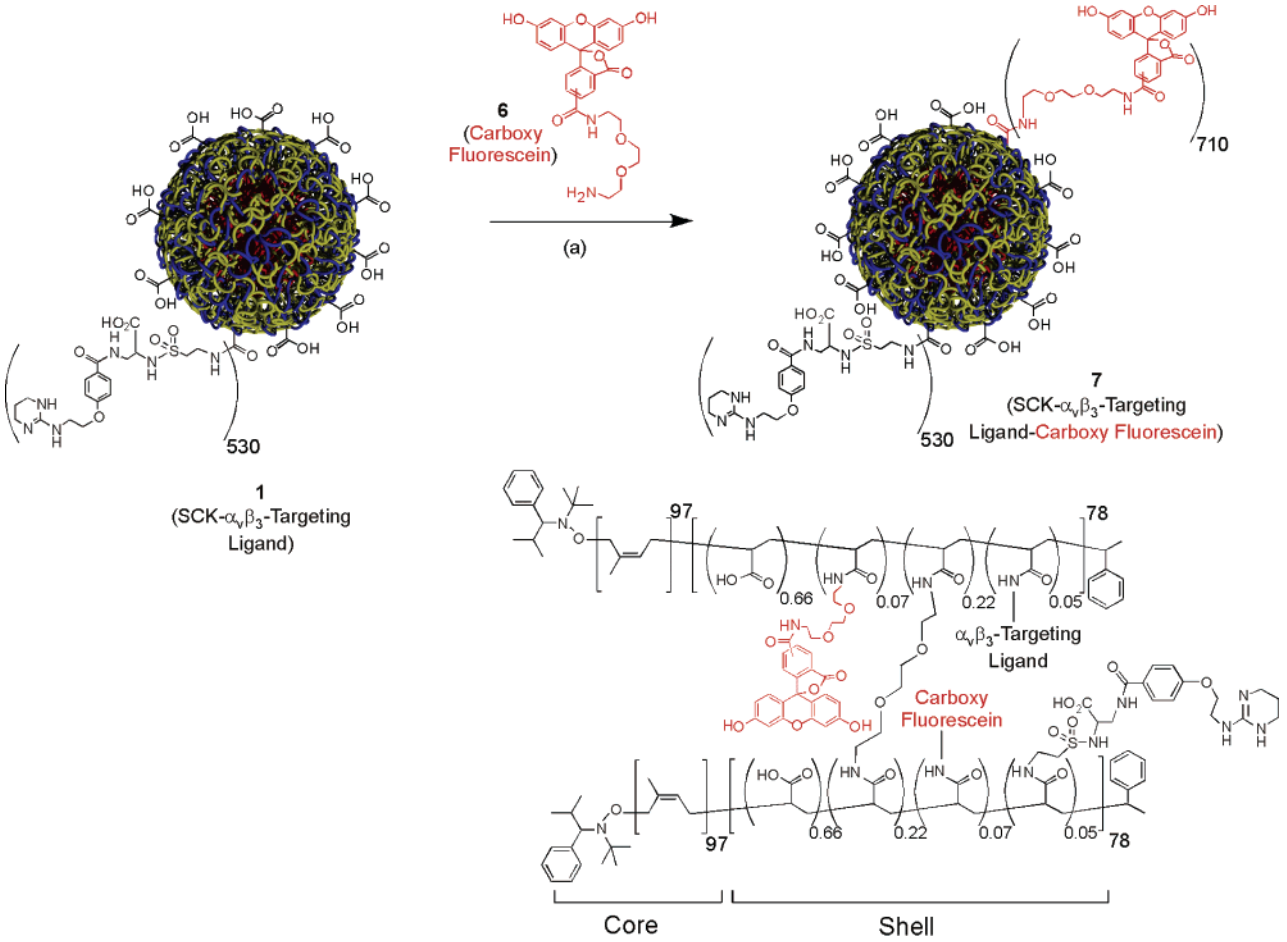
Figure 2. Overlaid UV−vis absorption spectra of (a) SCK **2** (0.3 mg/mL), (b) SCK **1**, labeled with $\alpha_v\beta_3$ targeting ligand (0.3 mg/mL), and (c) $\alpha_v\beta_3$-targeting ligand (**5**) (0.2 mg/mL).

trifluoroacetic acid and dichloromethane were removed by rotary evaporator. It was then dissolved in excess amount of water and lyophilized. The crude product was then purified by flash column chromatography (silica, chloroform: methanol 1:1). Yield: (0.01 g) 63%. ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 2.82−3.60 (m, 12H), 6.46−6.75 (m, 4H), 7.35 (d, J = 8.2 Hz) and 8.22 (d, J = 8.0 Hz) (total 1H), 7.62 (s) and 8.42 (s) (total 1H), 8.70 (brs) (total 1H), 8.10 (d, J = 8.0 Hz) and 8.20 (d, J = 8.0 Hz) (total 2H), 8.67 (m) and 8.80 (m) (total 1H). MALDI−TOF (matrix: α-cyanocinnamic acid): $(M + H)^+$ 507.9 (observed) (calculated 507.3).

Results and Discussion

As is illustrated in Scheme 1, the SCK nanoparticles (**2**) were prepared in two steps from the diblock copolymer poly(acrylic acid-*b*-isoprene) (**3**, PAA_{78}-*b*-PI_{97}).[10] Briefly, a diblock copolymer composed of polyisoprene (1,4-isomer predominant) and poly(*tert*-butyl acrylate) blocks was synthesized by sequential nitroxide-mediated radical polymerization (NMRP). The nitroxide and alkoxyamine initiator were prepared and utilized in the polymerization of *tert*-butyl acrylate according to the method of Hawker and co-workers.[11] The resultant alkoxyamine end-functionalized P*t*BA homopolymer was then used as a macroinitiator for the polymerization of isoprene to obtain the diblock copolymer (poly(*tert*-butyl acrylate)-*b*-polyisoprene). The *tert*-butyl ester groups were hydrolyzed by treatment of the polymer with methanesulfonic acid in a mixture of acetic acid and toluene at reflux to produce the amphiphilic diblock copolymer **3**.[10b,d] The hydrolysis reaction was continued for 1.5 h and was monitored by the disappearance of *tert*-butyl groups resonances in the ^{1}H NMR spectroscopy. An aqueous solution of micelles **4** composed of PAA_{78}-*b*-PI_{97} were formed by controlled addition of deionized water to a solution of **3** in THF, followed by dialysis against deionized water. The 20% cross-linking of the total acrylic acid residues constituting the micellar periphery was accomplished by condensation reactions with 2,2′-(ethylenedioxy)bis(ethylamine) as a diamino cross-linker (0.22 mol equiv, amine-to-acid ratio),

Scheme 2[a]



[a] Reagents and conditions: (a) **6,** 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide, rt, overnight, followed by dialysis against sodium phosphate buffered saline at pH 7.3, 7 days.

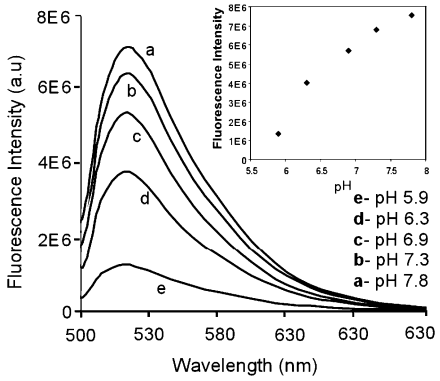


Figure 3. Fluorescence emission spectra (excitation wavelength $\lambda_{ex} = 488$ nm) from **7** at different pH values in 50 mM sodium phosphate buffered saline. Inset: plot of fluorescence emission intensities from **7** (at $\lambda_{em} = 517$ nm) against pH. Each sample was prepared independently from a stock solution at 0.25 mg/mL.

facilitated by 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (0.22 mol equiv relative to the carboxylic acids). The SCK nanoparticles were purified by dialysis, using a 10 kDa molecular weight cutoff (MWCO) cellulose membrane, against deionized water.

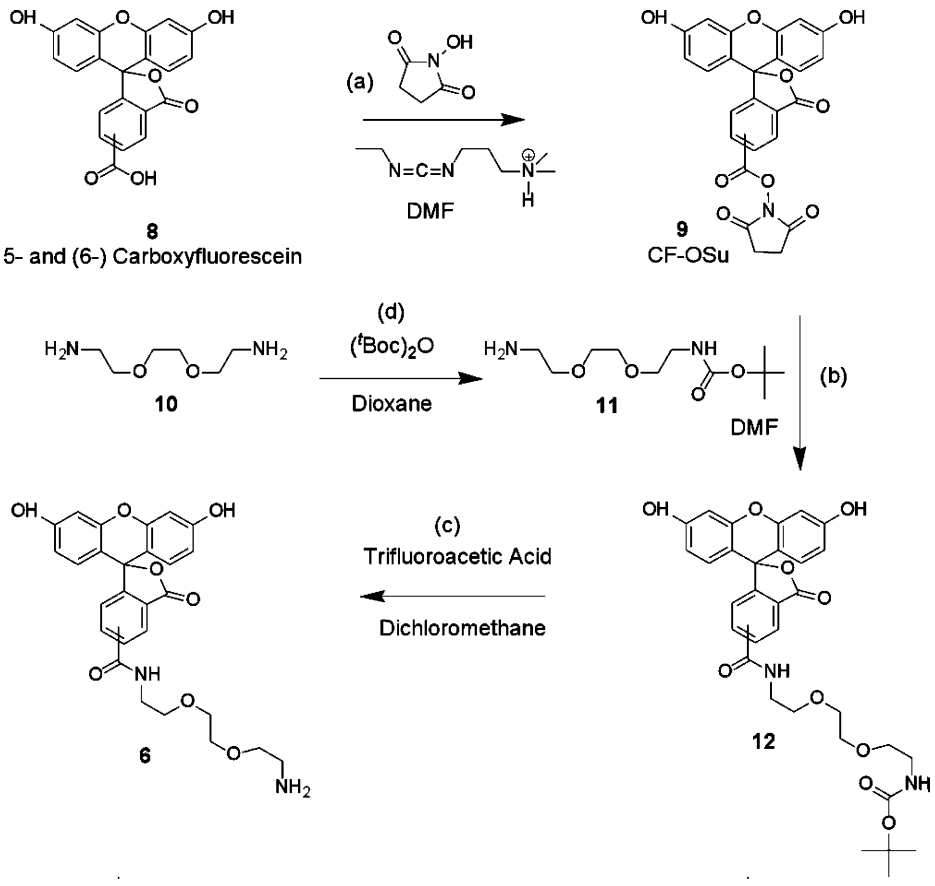
The peptidomimetic integrin antagonist, $\alpha_v\beta_3$-targeting ligand, **5**, was prepared starting from commercially available taurine following reported procedures[4,12] and was then attached within the shell of the SCK (**2**). Activation of a fraction of the available surface carboxylic acid groups of the SCKs with 1-[3′-(dimethylamino)-propyl]-3-ethylcarbodiimide methiodide (0.14 equiv based upon the remaining acrylic acid residues) was followed by the addition of **5** (0.07 equiv amine-to-acid ratio), and the reaction was allowed to proceed overnight at ambient temperature in aqueous solution. This resulted in the attachment of nominally 530 ligands per SCK nanoparticle. The SCKs (**1**), labeled with **5**, were purified by exhaustive dialysis using a 12−14 kDa MWCO membrane against sodium phosphate buffered saline at pH 7.2.

Detailed characterization of these nanoparticles was performed by multiple analytical techniques. Dynamic light scattering (DLS) measurements were made to obtain the hydrodynamic diameters (D_h) of the micelles and SCKs in aqueous solution. The SCKs exhibited a slight increase in D_h upon functionalization with the $\alpha_v\beta_3$ targeting ligand (Table 1). Transmission electron microscopy (TEM) and atomic force microscopy (AFM) gave smaller diameter and height values, respectively, in comparison to the DLS D_h values, obtained in a hydrated state.

These findings are consistent with the expected reduction in SCK dimensions due to the dehydration of the SCK shell and also to the effects of particle shape distortion that occur as the fluidlike polyisoprene-filled SCKs flatten upon adsorption onto the substrates required for TEM and AFM imaging.[10b,c]

Zeta potential values (ζ) of these nanoparticles were also determined by electrophoretic light scattering measurements (Table 1). In each case, the ζ value was

Scheme 3[a]



[a] Reagents and conditions: (a) *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide hydrochloride, *N*-hydroxysuccinimide (HOSu), anhy DMF, rt, 23 h; (b) 2-amino-2′-[(*tert*-butoxycarbonyl)amino]ethylene glycol diethyl ether (**11**), DMSO, rt, 3 h 40 min; (c) trifluoroacetic acid (TFA):dichloromethane (2:1), rt, 3.5 h; (d) di-*tert*-butyl dicarbonate (^{t}Boc$_2$O), dioxane, rt, 24 h.

negative, owing to the presence of remaining carboxylate groups on the SCK surface. UV−vis spectroscopy of the functionalized nanoparticles demonstrated the successful covalent attachment of ligand **5** (Figure 2).

Even though the SCK **2** lacks significant concentrations of UV chromophores, there is a shoulder in the UV−vis spectrum that results from the presence of the aromatic rings as polymer chain termini and/or scattering of light from the nanoparticles. Upon reaction with **5**, the intensity of this shoulder ($\lambda_{max} = 250$) increases, indicating the conjugation to produce **1**.

Fluorescence labeling of the nanoparticles was achieved by activating a portion of the available carboxylic acid groups of **1** upon reaction with 1-[3′-(dimethylamino)-propyl]-3-ethylcarbodiimide methiodide (0.23 mol equiv based upon the remaining acrylic acid residues) and then allowing reaction to occur with the 5- and (6-)-carboxyfluorescein derivative (**6**) (0.1 mol equiv, amine-to-acid ratio) overnight at ambient temperature. Fluoresceinated SCKs (**7**) were purified by exhaustive dialysis through 12−14 kDa MWCO membrane against sodium phosphate buffered saline at pH 7.3 for a prolonged period of time (Scheme 2). This resulted in the attachment of nominally 710 fluoresceins per SCK nanoparticle.

DLS studies showed that there is no significant change in D_h after the fluorescence tagging (Table 1). Fluorescence spectroscopy, performed as a function of pH, illustrated the pH dependence of the emission intensity from **7** (Figure 3). The carboxyfluorescein derivative, **6**, was also synthesized in a straightforward way from the commercially available mixture of 5- and (6-)carboxyfluorescein (**8**) (Scheme 3).

Conclusions

In summary, a peptidomimetic $\alpha_v\beta_3$-targeting ligand was synthesized, well-defined SCKs were prepared by the cross-linking of supramolecular assemblies of amphiphilic block copolymers, and these two components were then conjugated to produce a nanoscale vessel that is decorated with biologically active ligands. With the additional introduction of a pH-sensitive fluorophore, which can act as an optical marker, these materials are now undergoing advanced characterization studies to probe their behavior when challenged under in vitro and in vivo conditions. Studies are in progress to test the efficacy of this integrin mediated polymeric drug delivery vehicle in targeting tumor vasculature.

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