Published in final edited form as:

Biomacromolecules. 2011 February 14; 12(2): 441–449. doi:10.1021/bm1012212.

Integrin-targeting Block Copolymer Probes for Two-photon Fluorescence Bioimaging

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Abstract

Targeted molecular imaging with two-photon fluorescence microscopy (2PFM) is a powerful technique for chemical biology and, potentially, for non-invasive diagnosis and treatment of a number of diseases. The synthesis, photophysical studies, and bioimaging are reported for a versatile norbornene-based block copolymer multifunctional scaffold containing biocompatible (PEG), two-photon fluorescent dyes (fluorenyl), and targeting (cyclic-RGD peptide) moieties. The two bioconjugates, containing two different fluorenyl dyes and cRGDfK covalently attached to the polymer probe, formed a spherical micelle and self-assembled structure in water, for which size was analyzed by TEM and DLS. Cell-viability and 2PFM imaging of human epithelial U87MG cell lines that over express $\alpha_v\beta_3$ integrin was performed via incubation with the new probes, along with negative control studies using MCF-7 breast cancer cells and blocking experiments. 2PFM microscopy confirmed the high selectivity of the biocompatible probe in the integrin rich area in the U87MF cells while blocking as well as negative control MCF-7 experiments confirmed the integrin targeting ability of the new probes.

Keywords

Water-soluble block copolymer probe; ROMP; two-photon bioimaging; integrin targeting

Introduction

Two-photon fluorescence microscopy (2PFM) is fast becoming an important tool for immunological research and three-dimensional (3D) optical imaging of biological samples, ranging from cellular membranes to millimeter-thick brain slices. Several excellent reviews have appeared that describe the theory, experimentation, and applications of 2PFM.¹⁻⁷ Briefly, a fluorophore is first excited to a singlet state by the simultaneous absorption of two low energy photons, bridging the energy gap between the excited state and the ground state, followed by fluorescence emission, induced by mode-locked, high peak-power laser pulses. Two-photon absorption (2PA) processes offer several advantages over one-photon

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Supporting Information Available. All synthetic details, mass spectra of compound **4**, ¹H NMR spectral comparison of the homopolymers, block copolymers and bioconjugates, linear photophysical properties of the two polymer bioconjugates, optical imaging of the control polymer as well as bioconjugates are shown in supporting information. This material is available free of charge via the internet at <http://pubs.acs.org>.

absorption (1PA). For 2PA, fluorescence emission is quadratically dependent on the excitation irradiance (intensity of excitation light), and the volume of fluorescence emission is largely restricted to the region of the focal point (minimal out of focus emission) of interest. The longer wavelength, typically in the near-infrared (NIR) spectral range, used in 2PA is favorable for biological applications for its minimal photodamage and deeper penetration depth for imaging and therapeutic applications. For efficient two-photon assisted imaging, a fluorescent probe should be highly fluorescent (high fluorescence quantum yield) with high 2PA cross section at wavelengths suitable for biological imaging (typically 690-1000 nm to avoid scattering effect of tissues and absorption of hemoglobin), and have high photostability. Recently, remarkable progress in the design and synthesis of organic chromophores⁸ with very high 2PA cross sections (>1000 GM) has been reported.⁹⁻¹² However, most of the efficient organic 2PA chromophores are hydrophobic, posing a serious limitation for their use in biological applications.

To overcome the problem of poor water solubility, a few 2PA hydrophilic dyes¹³⁻¹⁶ have been reported, although their syntheses and purifications are tedious. Some other approaches, such as dye-doped silica nanoparticles¹⁷ and the use of amphiphilic copolymers^{18,19} has been demonstrated to make organic dyes soluble for several biological applications. The self-assembled natures of the polymer are well-known for drug/fluorescent probe delivery because of their accumulation in the tissue due to the enhanced permeability and retention (EPR) effects and longer lifetimes in bloodstream.²⁰ However, the stability (general physical-chemical stability, photo-stability, or resistance to pharmacokinetic clearance) of the micelles or nanoparticles incorporated with non-covalently attached dyes for long times in the bloodstream is matter of concern. Thus, covalent attachment of 2PA organic probes and targeting moieties, without affecting their photophysical properties, in a water-soluble block copolymer is a promising strategy for developing functional bioimaging probes (Figure 1).

Another important dimension is for the probe is to specifically target particular moieties of a cell for imaging purposes. Identification of the $\alpha_v\beta_3$ integrin that regulates angiogenesis, to understand the process of angiogenic cascade role in tumor growth, and anti-integrin treatment efficiency are major concerns facing researchers in the cancer field. Certain integrin proteins are significantly up-regulated in growing tumor cells and their expression levels correlate well with the aggressiveness of the disease.²¹⁻²⁴ Endothelial tumor cells that over express $\alpha_v\beta_3$ integrin selectively bind the short peptide sequence arginine-glycine-aspartic (RGD).²⁵⁻²⁷ Preclinical studies as well as phase I/II clinical trials showed that RGD-containing peptides inhibit metastasis and tumor growth, while a number of studies demonstrated the effectiveness of RGD as a target to specifically bind $\alpha_v\beta_3$ integrin in PET, SPECT, and NIR imaging.²⁸⁻³³ It has been demonstrated that cyclic (RGD) is more stable and selective with respect to the linear analog.^{34,35} Cyclic RGD acts as an antagonist of $\alpha_v\beta_3$ integrin which suppress the angiogenesis process, thus preventing tumor growth.³⁶ Grubbs *et al.* reported that ring-opening metathesis polymerization (ROMP) based copolymers, substituted with (glycine-arginine-glycine-aspartate-serine) (GRGDS) peptides, enhanced the inhibitory effect for cell-adhesion to the extracellular matrix (ECM) fibronectin protein significantly (3300%) compared to the GRGDS peptide itself.³⁷ Also, recent reports claim that synthetic polymer bioconjugates based on N-(2-hydroxypropyl) methacrylamide (HPMA-RGDfK) selectively delivers the diagnostic agent/therapeutic agent efficiently due to its multivalency, combining active targeting and passive tumor localization, and extravascularization.³⁸ Multimeric RGD has even superior activity in targeting the integrins with respect to monomeric analogs.³⁹⁻⁴¹ But, to the best of our knowledge, this is one of the first reports of two-photon fluorescence bioimaging with multi-scaffold polymeric bioimaging probes.

Herein, we report novel norbornene-based block copolymers, comprised of polyethylene glycol (PEG) groups in one block, to impart hydrophilicity and biocompatibility, and succinimidyl ester groups in the other block, to facilitate covalent conjugation with amine-terminated 2PA fluorenyl probes and an amine-terminated cyclic-RGD (c-RGDfk) peptide. PEG groups are known to impart not only aqueous solubility but also to prevent non-specific adsorption to general substances in a living body, reducing toxicity and immunogenicity.^{20,42-44} Post polymerization modification was performed on the succinimidyl-containing block copolymers by reacting with specific quantities of the two different amine-containing 2PA fluorenyl dyes and cRGDfk, yielding polymeric multi-scaffold bioimaging probes. To compare and investigate the photophysical behavior of the polymeric probes, two model monomer adducts were synthesized. Synthesis and characterization with NMR, High resolution mass spectrometry (HRMS), and Gel permeation chromatography (GPC) of monomers, dyes, model compounds and polymers are reported. Both one-photon (linear) and two-photon (nonlinear) photophysical studies of the model compounds and polymers are reported. Transmission electron microscopy (TEM) analysis on the polymer micelles provided the size of the micelles. Cell viability (MTS) assay of the polymers demonstrated the high biocompatibility (low cytotoxicity) of the probes. 2PFM imaging with human glioblastoma U87MG cell lines that over express $\alpha_v\beta_3$ integrin, along with control studies using human breast cancer MCF-7 cells ($\alpha_v\beta_3$ integrin negative) and blocking experiments, showed high targeting efficiency of the polymeric probe at the integrin region of the U87MG cell, useful for further investigation of the angiogenesis and early detection of cancer metastasis by 2PFM.

Experimental Section

Materials

Bicyclo[2.2.1]hept-5-ene 2-carboxylic acid (98% mixture of *endo* and *exo*), norbornene (99%), thionyl chloride (99.5%), trimethylchlorosilane, triethylamine (99.5%), polyethyleneglycol monomethyl ether ($M_n = 550$), 6-aminocaproic acid, N-hydroxysuccinimide, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-methyl morpholine (NMM), and Grubbs second generation catalyst were purchased from Aldrich or ACROS and used as received. CH_2Cl_2 and CHCl_3 were dried over CaCl_2 and distilled. THF was distilled over sodium and benzophenone ketyl under N_2 before use. All catalyst solutions were prepared in a glovebox. N,N'-Dicyclohexylcarbodiimide was distilled before use. 7-(Benzo[thiazol-2-yl]-9,9-di(2-(2-methoxyethoxy)-ethyl)fluoren-2-amine was prepared as described previously.⁴⁵ Promega CellTiter 96® Aqueous One Solution Reagent was purchased from Fisher.

Cell lines—U87MG cells and MCF7 cells were purchased from ATCC (America Type Culture Collection, Manassas, VA, USA). All cells were incubated in Gibco RPMI-1640 medium without phenol red (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin-streptomycin (Atlanta Biologicals, Lawrenceville, GA, USA), and incubated at 37 °C in a 95% humidified atmosphere containing 5% CO_2 .

Measurements

The ^1H and ^{13}C NMR spectroscopic measurements were performed using a Varian 500 NMR spectrometer at 500 MHz with tetramethylsilane (TMS) as internal reference; ^1H (referenced to TMS at $\delta = 0.0$ ppm) and ^{13}C (125 MHz, referenced to CDCl_3 at $\delta = 77.0$ ppm). Chemical shifts of ^1H and ^{13}C spectra were interpreted with the support of CS ChemDraw Ultra version 11.0 and NMR spectra were analyzed with Mestrec software. HR-MS analysis was performed in the Department of Chemistry, University of Florida,

Gainesville, FL. GPC was conducted with a Waters 2414 refractive index detector, Waters 2996 photodiode array, and Waters 1525 binary HPLC pump (THF as the mobile phase, flow rate of 1.5 mL/min) using Waters styragel HR3, and HR5E columns, with reference to polystyrene standards. TEM was done using JEOL 1011, operated at 100 kV and the column vacuum was less than 1×10^{-4} Pa. The samples were prepared on carbon coated copper grid by drop cast the polymer solution in water, followed by air dry at room temperature overnight and TEM size distribution was evaluated by Image-J software. Hydrodynamic diameter of the polymer was determined by dynamic light scattering (DLS) method on a Nanosizer (Nano-ZS90, Malvern, UK) instrument and the data was processed by number particle size distribution method.

Synthesis

All synthetic details and characterization data of the monomers, block copolymers, 2PA dyes, model compounds and post modification on the block copolymers are described in the Supporting Information.

Linear Photophysical Measurements

Linear photophysical properties of the block copolymer fluorescent probes were investigated in spectroscopic-grade solvents (DMSO and ultrapure water) at room temperature. The steady-state absorption spectra were obtained with Agilent 8453 UV-visible spectrophotometer using 1 cm path length quartz cuvettes with dye concentrations of 1×10^{-5} M. The steady state fluorescence spectra and excitation anisotropy spectra were obtained with a Photon Technologies, Inc. (PTI) QuantaMaster spectrofluorimeter, using 10 mm spectrofluorometric quartz cuvettes and low concentration solutions $C \leq 10^{-6}$ M. All fluorescence spectra were corrected for the spectral sensitivity of the PTI emission monochromator and photomultiplier tube (PMT) detector. Excitation anisotropy spectra were measured with viscous poly-THF (polytetrahydrofuran) at room temperature and using an L-format configuration geometry,⁴⁶ with extraction of the scattered light and solvent emission. Fluorescence quantum yields of the compounds were determined by a relative method with 9,10 diphenylanthracene in cyclohexane as a standard.⁴⁶ Fluorescence lifetimes were measured with a time-correlated single photon counting system PicoQuant PicoHarp 300) under linear polarized 76 MHz femtosecond excitation (MIRA 900, Coherent) oriented by the magic angle 55° , with time resolution ~ 80 ps.

Two-photon Absorption Measurement

The 2PA spectra of the compounds were obtained over a broad spectral region via a typical two-photon induced fluorescence (2PF) method relative to Rhodamine B in methanol as a standard.⁴⁷ A PTI QuantaMaster spectrofluorimeter and femtosecond Coherent Mira Ti:sapphire laser with pulse duration, ~ 200 fs, (FWHM), tuning range 690-1000 nm, and 76 MHz repetition rate. Two-photon fluorescence measurements were performed in 10 mm fluorometric quartz cuvettes with dye concentrations of 3×10^{-5} M in DMSO. The quadratic dependence of two-photon induced fluorescence intensity on the excitation power was confirmed for each excitation wavelength.

Cytotoxicity (MTS) Assay

To assess the cytotoxicity of conjugates 16 and 17, 5×10^3 cells/well of U87MG cells in 96-well plates were incubated in 90 μ L of RPMI-1640 without phenol red, supplemented with 10% FBS and 1% penicillin-streptomycin for 24 h. Then the cells were incubated with various amounts of polymer probes **16** or **17** (5 μ M, 1 μ M, 0.5 μ M, 0.1 μ M), respectively, for an additional 20 h. Subsequently, 20 μ L of CellTiter 96® AQueous One Solution reagent was added into each well, followed by further incubation for 4 h at 37 °C. The relative

viability of the cells incubated with the polymer probe to untreated cells was determined by measuring the MTS-formazan absorbance on a microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA) at 490 nm with subtraction of the absorbance of cell-free blank volume at 490 nm. The results from three individual experiments were averaged.

Cell culture and Incubation

U87MG cells or MCF-7 cells were placed onto poly-D-lysine coated glass coverslips (12mm, #1) in 24-well plates (40,000 cells per well), and the cells were incubated for 48 h before incubating with the fluorescent polymer conjugate. Stock solution of fluorescent conjugate **16** or **17** dissolved in water was prepared as 10^{-4} M solution. The solution was diluted to 1 μ M with complete growth medium, RPMI-1640, and then incubated for a 2 h period. After incubation, the cells were washed with PBS (3–5 $\times$) and fixed using 3.7% formaldehyde solution for 15 min at 37 °C. Then freshly prepared NaBH₄ (1 mg/mL, prepared by adding few drops of 6N NaOH solution into PBS (pH=7.2)) solution in PBS (pH=8.0) was added to each well (0.5 mL/well) for 15 min. Then the plates were washed PBS (2 $\times$) and water (1 $\times$). Finally, the glass coverslips were mounted using Prolong Gold mounting media (Invitrogen) for microscopy.

Blocking Experiment

The blocking experiment was performed to verify the integrin targeting ability of the RGD containing polymeric probe **16** and **17**. U87MG cells were placed onto poly-D-lysine coated glasses in 24-well plates (40,000 cells per well), and the cells were incubated for 48 h. Then, the cells were incubated with unlabeled cRGDfK (2 mg/mL of RPMI-1640) for 1 h. After that, 1 μ M solution of **16** or **17** in RPMI-1640 was added over the cells and incubated for a 2 h period. After incubation, cells were washed, treated and mounted similar way as stated above.

One-photon and Two-photon Fluorescence Imaging

Conventional single-photon fluorescence images were obtained using an inverted microscope (Olympus IX70) equipped with a QImaging cooled CCD (Model Retiga EXi) and mercury lamp 100 W. In order to improve the fluorescence background-to-image ratios, a customized filter cube (Ex 377/50, DM 409, Em 460/50) was used for the one-photon fluorescence images. The specifications of the filter cube were tailored to match the excitation wavelength of the probe, and to capture most of the probe's emission profile.

Two-photon fluorescence microscopic (2PFM) images were obtained with a modified Olympus Fluoview FV300 microscope system combined with a tunable Coherent Mira 900F Ti:sapphire laser, pumped by a 10W Coherent Verdi frequency doubled Nd:YAG laser. The femtosecond NIR laser beam (with 220 fs pulse width and 76 MHz repetition rate) was tuned to 740/820 nm as required and used as the two-photon excitation source. The two-photon induced fluorescence was collected by a 60 $\times$ microscopic objective (UPlanSApo 60 $\times$, NA = 1.35, Olympus). A high-transmittance (>95%) short-pass filter (cutoff 685 nm, Semrock) was placed in front of the PMT detector of the FV300 scan head in order to filter off background radiation from the laser source (740/820 nm).

Results and Discussion

Synthesis of Monomers and Block Copolymers

The two monomers, one containing a succinimidyl ester (**3**)⁴⁸ and another containing PEG (**4**), were synthesized according to Scheme 1. The succinimidyl ester is a well-known amine reactive group, forming an amide under very mild conditions, and is widely used in

biological chemistry. Hence, a norbornene derivative with the succinimidyl ester group was prepared⁴⁹ for further derivatization to react with amine-containing two-photon fluorescent dyes as well as lysine terminated cyclic-RGD. Compound **2** was prepared according to literature with little modification. Briefly, first the carboxyl group was protected with trimethylsilylchloride and then amidation with **1**⁵⁰, followed by the deprotection under basic condition was conducted. Then, succinimidyl ester group was introduced to **2** via esterification to obtain the norbornenyl NHS monomer **3**. PEG-functionalized monomer **4** was also prepared from intermediate **1** to prepare the hydrophilic block (See Supporting Information).

The homopolymers of each monomer (**3** and **4**) were synthesized using ROMP in order to establish the reactivity and time for polymerization. This also determined the order of monomer addition during synthesis of the block copolymers with narrow PDI; in general, slower reacting monomers are added first. To accomplish this, each monomer was dissolved in deuterated THF and ¹H NMR spectra were collected at different time intervals (Supporting Information Figure S2) until it was observed that all the monomer was transformed to the corresponding polymer (with the progress of polymerization, the signal for the monomer alkene CH at ~6 ppm diminished and a new polymer olefinic CH signal at ~5.3 ppm was observed). ¹H NMR studies showed that PEG containing monomer **4** reacted slower to yield the corresponding homopolymer compare to **3** (see Supporting Information). Two different series of block copolymers (**5** and **6**) were synthesized by ROMP, using Grubbs second generation catalyst in THF, by varying the ratio of the monomers **3** and **4**, according to Scheme 1. For bioimaging purposes, the solubility of the polymer in water was essential. Both of the block copolymers that were prepared were water soluble. Comparison ¹H NMR spectra of different polymers are shown in Supporting Information (Figure S3-S5).

Synthesis of 2PA Dyes (**10**, **11**) and Model Compounds (**12**, **13**)

The details of amine-terminated 2PA probe (**10**, **11**) and intermediates **7**, **8**, and **9** are described in the Supporting Information, as are the structure of **7**, **8**, and **9**. Key intermediate **7** was prepared following our previously published procedure.⁴⁵ Considering the efficiency of the reaction of a primary alkyl amine with the succinimidyl ester group, a linker on the 2PA probe was introduced to provide a primary amine for conjugation. Hence, intermediate **8** was prepared by protecting the amine group, followed by coupling with **7** to give **9**. The protecting group was then removed under acidic conditions, affording primary amine-containing 2PA probe **10**. Another fluorenyl based dye **11**, containing different electron donor (diphenylamine) and acceptor group and amine linker was synthesized (detailed synthesis will be published elsewhere). Two monomeric model compounds, bearing the same chromophore, were synthesized to optimize the conjugation chemistry as well as investigate photophysical properties. The model compounds were prepared by simple reaction with the succinimidyl derivative of norbornene **3** with **10**, or **11**, as shown in Scheme 2. The reaction was carried out in DMSO at room temperature using NMM. Structures of all new compounds were confirmed by ¹H and ¹³C NMR spectra along with HRMS, with exception of the oxidatively labile amine **10**, which was used immediately after isolation.

Post-modification on Block Copolymer

Post-modification on polymers provides an efficient tool to prepare functionalized polymers, depending on the desired applications. Complex, bulky substituents with multiple heteroatoms often interfere with the catalyst reactivity, initiation, and propagation reactions in ROMP, often resulting in poorly controlled polymerization reactions with broad polydispersity index (PDI). Also, the solubility and purification of polymers with complex

functional groups are challenging issues.^{51,52} Thus, post-modification is an attractive strategy for the synthesis of highly functionalized polymers. Post-modifications on block copolymers were performed by reaction of the succinimidyl ester-containing block and amine reactive 2PA probe **10** as well as the integrin-targeting small cyclic peptide cRGDfK at room temperature under mild conditions. In order to monitor the post-modification process, both block copolymers were reacted with excess dye, followed by purification. After conjugation, the ¹H NMR spectrum showed the disappearance of succinimidyl peaks (2.74-2.78 ppm) and the appearance of new aromatic peaks (7-8.2 ppm) from the fluorescent dyes. ¹H NMR spectral analysis also demonstrated the desired block ratio formation from the integration of the proton adjacent to the benzothiazole group (8.15 ppm-1H) to the alkene proton at (5.35-5.20 ppm). The solubility of the block copolymer containing a 2:1 ratio of PEG and dye-containing blocks (**15**) was more favorable in water compared to its 1:1 analog (**14**), as expected due to the greater amount of PEG (Supporting Information). This result led to selection of block copolymer **6** for further modification as a better candidate for polymeric 2PA bioimaging probes.

The bioconjugate polymeric probe was prepared from block copolymer **6** by the post-modification of the succinimidyl block with a calculated amount of 2PA dye **10** or **11** and cRGDfK, determined by ¹H NMR analysis (Scheme 3). The polymer was purified by passing through a SEC column, using water as eluent, to remove the traces of excess of unreacted dye and cRGD. The formation of the target polymer was confirmed by the ¹H NMR as well as GPC studies. The appearance of a new peak at about 8.59 ppm, in addition to the dye's proton resonances in the aromatic region, due to the -COOH presence in cRGD was observed.

Photophysical Properties of the Model Compounds and Polymers

Linear photophysical properties of model compounds **12** and **13** were thoroughly characterized by UV-vis absorption, steady-state fluorescence, fluorescence quantum yield, lifetime, and anisotropy in poly-THF and DMSO (Figure 2). The linear absorption and emission maxima of **12** and **13**, with considerably high fluorescence quantum yield of 0.95 using DPA as standard, are shown in the Table 1. Compounds **12** and **13** exhibited a single exponential fluorescence decay process with lifetimes of 1.36 and 2.79 ns, respectively.

2PA cross sections were determined by the upconversion fluorescence method, using a femtosecond Ti:sapphire laser as the excitation source. The 2PA cross section of **12** was 30 GM at 740 nm, while **13** exhibited a higher 2PA cross-section of 100 GM at 820 nm, shown in Figure 2. The result of the dye conjugated polymer **15** showed good agreement with the model compound, although in the more polar solvent water, a slight blue shift of the absorption peak (355 nm) and red shift of the emission peak (447 nm) were observed along with a reduction in the fluorescence quantum yield (0.47). Also, it was noticed, **16** had slightly lower fluorescence compared to **15**. Similarly, for polymeric bioconjugate **17**, with higher 2PA cross section, the fluorescence quantum yield was 0.82 in DMSO and 0.25 in water.

The photophysical characterization of the model compounds is representative since the bioconjugate polymers bear the same chromophore responsible for the photophysical properties, while the model is a more well-defined system for careful study. The linear photophysical properties of the two polymer conjugates **16** and **17** were evaluated (because of their aqueous solubility) in both DMSO and in water and compared with the model adduct, shown in Table 2

Size Analysis of the Polymer Bioconjugate

The morphology and size distribution of the water soluble polymer bioconjugates in water were evaluated by TEM and dynamic light scattering (DLS) analysis, which is shown in the Figure 3. The TEM image shows the appearance of the polymeric nanoparticles as the spherical grey spots (without any staining). The diameters of the dried polymeric nanoparticles, **17**, on a copper grid coated carbon film were about 90 nm, while the larger nanoparticles of size ~ 120-130 nm suggests the self-assembled nature of the polymer.⁵³ The size distribution analysis of the polymeric micelles can be correlated to the difference in the PEG chain at the pendant group in the hydrophilic block. The hydrodynamic diameter of **17** was about 100 nm (measured by DLS by number distribution method) and some of the larger polymeric particles result with the average of 230 nm diameter in aqueous solution, which is comparable to the TEM result. The larger particles results from possible self-assembly nature of the amphiphilic polymeric structures.

Cell-viability Study

Cytotoxicity of the polymers (**16** and **17**) was investigated using an MTS assay prior to biological applications, shown below in Figure 5(I). Both of the conjugated copolymers showed excellent cell viability for the U87MG cell lines after incubation for 24 h. Fluorenyl dye conjugated polymer **15** was incubated with 1-30 μ M dye concentration, resulting in more than 85% viable cells, demonstrating the minimal cytotoxicity of the functionalized block copolymer (results not shown here). Greater than 90% of the U87MG cells were viable after incubation with the cRGDfK/dye-containing polymer **16** and **17** at 0.1-5 μ M concentration of the dye. These results confirmed the non-toxicity of the both cRGDfK and dye-conjugated polymers.

One- and Two-photon Mediated Fluorescence Bioimaging

Receptor affinity of the polymeric bioconjugate containing a 2PA dye and cRGD (**16**) for integrin $\alpha_v\beta_3$ positive human glioblastoma U87MG tumor cells was investigated. To demonstrate and compare the integrin specificity and localization, polymer **16** was incubated for 2 h with a U87MG cell line and a $\alpha_v\beta_3$ negative control human breast cancer cell line MCF7 under the same experimental conditions. Images were taken by both one-photon and two-photon fluorescence microscopy. The imaging results demonstrated the specificity of the cRGDs at the peri-nuclear area of the cell, after presumed receptor-mediated endocytosis, by strong fluorescence in the U87MG cells, even at very low concentration of the dye (1 μ M) by both techniques. Meanwhile, in the negative control MCF7 cells there was only dim/no fluorescence without any specificity (largely autofluorescence). Integrin receptor specificity was further validated by a blocking experiment. The U87MG cells were blocked (incubated) with the unlabeled cRGDfK peptide (2 mg/mL) prior to the incubation with the polymeric conjugate **16** (1 μ M), followed by fluorescence microscopy, resulting in reduced tumor contrast. Another interesting finding of the polymer mediated integrin targeted bioimaging with the 2PA dye-containing copolymer **16** was that it exhibited better localization, higher affinity, and brighter fluorescence in the cytoplasmic region of the U87MG cells at much lower probe concentration and low power with respect to free dye possibly due to the multivalency of the polymeric probe.⁵⁴ Figure 4 IIA shows the 3D reconstruction of the 2PFM images, comparing the effectiveness of the polymeric probe in U87MG cells with respect to a blocking experiment (Figure 4 IIB) and integrin negative MCF7 cells (Figure 4 IIC).

Since bioconjugate **16** had a low 2PA cross section a polymeric probe bearing a fluorene derivative with a higher 2PA cross section, along with cRGDfK, **17**, was prepared and studied with U87MG cells, along with blocking experiments (Figure 5). The differential interference contrast (DIC), one-photon fluorescence image, two-photon fluorescence

image, and fluorescence lifetime imaging with probe **17** strengthened our previous finding with a higher degree of localization, accompanied by brighter, higher contrast images (Figure 5 IIa). Integrin plays a significant role in regulating cellular adhesion, binding with extracellular ligands near focal adhesions (FAs), an actin and actin-like protein rich area.⁵⁵⁻⁵⁷ Magnification of the two-photon image with probe **17** (Figure 5 IIb) clearly shows the details of the integrin localized area of the FA's spots near culling, persistence and motile zone of a polarized cell as reported earlier.¹³ This multi-scaffold polymeric probe demonstrated its high selectivity and effectiveness as carrier of both fluorescent probe for 2PFM and cell-specific targeting. In addition, the PEG group attached to the polymer imparted biocompatibility and should provide longer circulatory retention time for *in vivo* use, an aspect under current investigation.

Conclusion

In this study, we report two multi-scaffold copolymer probes conjugated with a 2PA fluorenyl dye and cRGDfK peptide to target human $\alpha_v\beta_3$ integrin in U87MG glioblastoma cancer cells and 2PFM bioimaging. The biocompatible PEG-containing block copolymers were synthesized by ROMP polymerization using Grubbs second generation catalyst and further post-modified with an amine-reactive fluorene dye and cRGDfK, and characterized.

The formation of polymeric nanoparticles in water with an average value of 100 nm was examined by TEM microscopy. The biocompatibilities of the system were evaluated by an MTS assay, revealing minimal cytotoxicity of the system in U87MG cells. The efficiency of the targeting ability of the polymeric probe was studied by both conventional (one-photon) and two-photon fluorescence microscopy of the $\alpha_v\beta_3$ positive U87MG cells with respect to a negative control (MCF7 cells) and a blocking experiment with U87MG cells incubated first with unlabeled cRGDfK, demonstrating very good integrin selectivity and bioimaging. This research opens a new dimension to the design and use of multi-scaffold copolymers to prepare target-specific probes for 2PFM with potential applications in angiogenesis imaging and cancer detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to acknowledge the National Institutes of Health (1 R15 EB008858-01), the U.S. Civilian Research and Development Foundation (UKB2-2923-KV-07), and the National Science Foundation (CHE-0832622 and CHE-0840431) for support of this work.

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Figure 1.
Design of multi-scaffold copolymer.

Figure 2.

Normalized absorption (black), fluorescence (blue), excitation (red) in DMSO and excitation anisotropy (green) spectra in poly-THF for **12** (A) and **13** (C) and corresponding two-photon cross-section spectra in DMSO for **12** (B) and **13** (D).

Figure 3. (a) TEM analysis of the bioconjugated polymer **17**, (b) its corresponding size distribution (by Image-J analysis of TEM) and (c) size distribution (hydrodynamic diameter, d) of **17** in water at 25 °C.

Figure. 4.

(I) Nonradioactive MTS-based cell proliferation assay was applied with U87MG cell lines. The cell was incubated with various amounts of fluorene dye conjugated polymer derivatives without RGD, **16** (A) and with RGD, **17** (B) for 24 hours at 37 °C. The relative viability of the cells incubated with the derivatives to untreated cells was determined by measuring the MTS-formazan absorbance on a Kinetic microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA) at 490 nm. (II) (A) Images of U87MG cells incubated with fluorescence conjugate **16** (1 μM , 2 h); (B) Images of U87MG cells incubated first with 2 mg/mL RGD for 2 h and then with fluorescence conjugate **16** (1 μM , 2 h); (C) Images of MCF-7 cells incubated with fluorescent conjugate **16** (1 μM , 2 h) all taken with 60x, oil immersion objective. a. DIC image, 40 ms b. One-photon fluorescence image, 100 ms (filter cube Ex: 377/50 DM: 409 Em: 525/40); c. 3D reconstruction from overlaid 2PFM of different cells incubated with fluorescence conjugate **16** (1 μM , 2h) (Ex: 740 nm; power: 90mW (12 mW on the focal plane); Em. short-pass filter 690 nm, 10 μm grid)

Figure 5.

Images of U87MG cells incubated with polymer probe **17** (1 μ M, 2 h) all taken with 60 $\times$, oil immersion objective (NA = 1.35, Olympus). I. (A) DIC (B) One-photon image. (C) 3D reconstruction from overlaid two-photon fluorescence images [76 MHz, 115 fs pulse, Ex: 820 nm; Power: 90mW (12.6 mW on the focal plane); Em. short-pass filter 690 nm], Scale: 10 μ m grid. (D) Two-photon Fluorescence lifetime image [76 MHz, 115 fs pulse, Ex: 820 nm; Power: 90 mW (12.6 mW on the focal plane); Em. short-pass filter 690 nm]. II. Detail images of U87MG cells incubated with polymer probe **17** (A) 3D reconstruction from overlaid two-photon fluorescence images [76 MHz, 115 fs pulse, Ex: 820 nm; Power: 90 mW (12 mW on the focal plane); Em. short-pass filter 690 nm], Scale: 10 μ m grid. (B) and (C) Magnification of volume indicated by cubes in A, Scale: 5 μ m grid.

Scheme 1.
Synthesis of Monomers and Block Copolymers

Scheme 2.

Structure of Amine Terminated 2PA Dyes and Model Compounds

Scheme 3.
Synthesis of Multifunctional Polymer-2PA dye-cRGDfK Probes

Table 1

Photophysical Properties of Model Compound

Entry	$\lambda_{\text{Abs}}^{\text{max}}$ (nm)	$\lambda_{\text{Em}}^{\text{max}}$ (nm)	Stokes Shift (nm)	ϵ_{max} ($10^{-3}\text{M}^{-1}\text{cm}^{-1}$)	Φ_{Fl}	R	τ (ns)	R^2	δ (GM)
12	360	439	79	51	0.95	0.38	1.36	0.99	30 at 740nm
13	393	552	159	47	0.95	0.19	2.79	0.99	100 at 820 nm

All photophysical studies were done in DMSO.

Maxima of absorption, $\lambda_{\text{Abs}}^{\text{max}}$; fluorescence, $\lambda_{\text{Em}}^{\text{max}}$; extinction coefficients, ϵ_{max} ; fluorescence quantum yield, Φ_{Fl} ; lifetimes, τ ; correlation coefficient, R^2 and anisotropy (R) measured in Poly-THF.

Table 2

Linear Photophysical Properties of Bioconjugated Polymer

	Solvent	$\lambda_{\text{Abs}}^{\text{max}}$ (nm)	$\lambda_{\text{Em}}^{\text{max}}$ (nm)	Stokes Shift (nm)	Φ_{FL}
15	H ₂ O	355	447	91	0.47
16	H ₂ O	355	447	91	0.40
16	DMSO	360	439	79	0.78
17	H ₂ O	394	523	129	0.25
17	DMSO	394	552	158	0.82