Supporting Information

Biorthogonal Click Chemistry on Poly(lactic-co-glycolic acid)- Polymeric Particles

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1. General methods and instrumentation

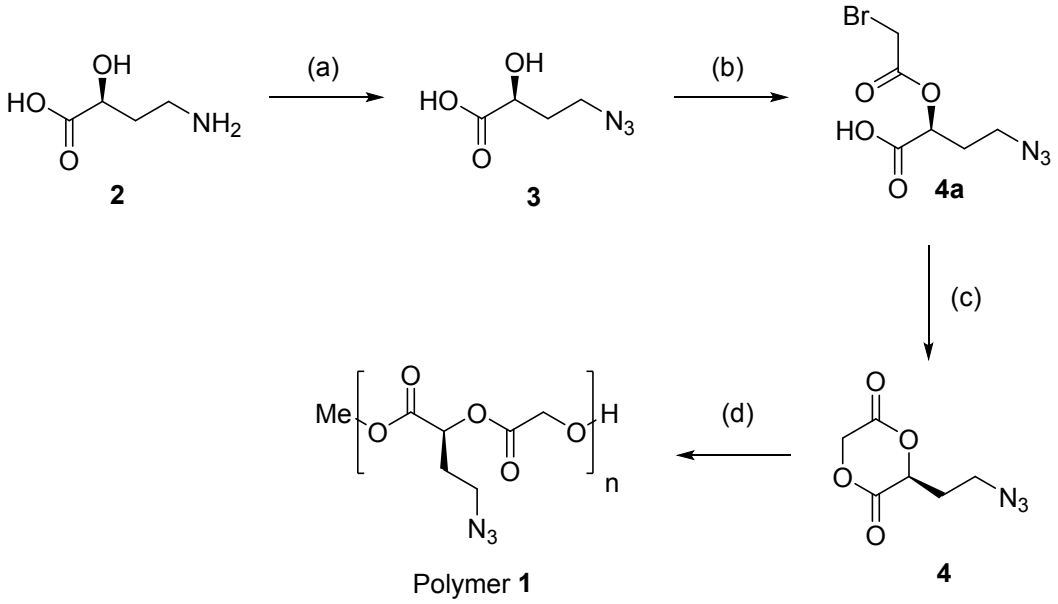
All chemicals and solvents were purchased from Sigma-Aldrich and used as received unless specified. Compound **19**,f 1-[3,5-Bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(-)-2-(dimethylamino)cyclohexyl]thiourea (R,R-TUC), was purchased from Strem Chemicals and used as received. Anhydrous solvents were acquired from a solvent purification system (LC Technology Solutions Inc., SP-1). Silica gel flash column chromatography was performed using an automated CombiFlash® Rf 200 system. Polymer molecular weights and degradation were determined by gel permeation chromatography, using a Waters e2695 instrument with a series of Styragel HR4 and Styragel HR2 columns in DMF with 0.01% LiBr at 37 ^{o}C. Monodisperse poly(methylmethacrylate) (PMMA) standards were used to determine the molecular weight and PDI of polymers. ^{1}H NMR and ^{13}C NMR spectra were acquired using a Varian spectrometer working at 600 MHz and 150 MHz respectively. Chemical shifts (δ) are reported in ppm relative to TMS, and coupling constants (*J*) are reported in hertz. High-resolution mass spectra were acquired using an Agilent 6230 ESI-TOFMS in positive ion mode. Particles were formulated using a

Qsonica Sonicator 4000 and purified by tangential flow filtration Millipore Pellicon XL, 500 kDa. Particles were characterized by DLS, Malvern Instruments Nanosizer, and transmission electron microscopy (TEM, Tecnai FEI Spirit).

2. Abbreviations

DMF = dimethylformamide, EtOAc = ethyl acetate, Et_3N = triethylamine, MeCN = acetonitrile, MeOH = methanol, DCM = dichloromethane, THF = tetrahydrofuranPDI = polydispersity index, UV = ultraviolet, DLS = dynamic light scattering, GPC = gel permeation chromatography

3. Synthesis of Azide Polymer



Scheme S1. Synthesis of the desired polymer. a) MeOH, H_2O, $CuSO_4{\cdot}5H_2O$, K_2CO_3, Imidazole-1-sulfonyl azide, 78 %; b) bromoacetyl bromide, MeCN, 0 ^oC, c) $NaHCO_3$, DMF, 32% (over two steps), d) 1-[3,5-Bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(-)-2-(dimethylamino)cyclohexyl]thiourea (R,R-TUC), methanol, triazabicyclo[4.4.0]dec-5-ene, CH_2Cl_2, 71%.

Compound 3. Compound **2** (591 mg, 4.96 mmol), K_2CO_3 (1.05 g, 7.63 mmol) and $CuSO_4 \cdot 5H_2O$ (95 mg, 0.38 mmol) were dissolved in a 1:1 mixture of MeOH and H_2O (12 mL). A solution of Imidazole-1-sulfonyl azide in a 1:1 mixture of MeOH and H_2O (4 mL) was added dropwise. The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated to remove MeOH, but not to dryness due to the potential explosive hazard of Imidazole-1-sulfonyl azide, by rotary evaporator. Acidified concentrated solution with 1 M HCl until pH 3 then extracted 9X with DCM (10 mL). Dried organic over $MgSO_4$ then concentrated to yield Compound **3** as a colorless oil (0.432 g, 78%).

HRMS: composition: C4 H6 N3 O3; measured mass 144.0414; theoretical mass: 144.0415.

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.03 (bs, 1H), 4.40 (dd, *J*= 7.8, 3.6 Hz, 1H), 3.92 (s, 1H), 3.54 (t, *J*= 6 Hz, 2H), 2.18-2.12 (m, 1H), 1.99-1.94 (m, 1H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 178.7, 67.7, 47.4, 33.0.

Compound 4a. Compound **3** (432 mg, 2.98 mmol) was dissolved in MeCN (42 mL). Et_3N (1.1 mL, 7.75 mmol) was dripped into the reaction mixture and it was chilled to 0 ^{o}C. A solution of bromoacetyl bromide in MeCN (14 mL) was added to the reaction dropwise. The reaction was stirred at 0 ^{o}C for 1 h then quenched with 1 M HCl (60 mL). Extracted 3X with EtOAc (60 mL), dried over $MgSO_4$ and concentrated. Used directly without further purification in the preparation of compound **4**.

Compound 4. Compound **4a** (7.93 mg, 2.98 mmol) used without purification was dissolved in DMF (45 mL). This solution was added via syringe pump to a suspension of $NaHCO_3$ (375 mg, 4.47 mmol) in DMF (90 mL) over 28 h. The reaction mixture was stirred a further 8 h at room temperature. The reaction mixture was then filtered and concentrated. The resulting oil was purified by silica column (4:1 Hex/EtOAc) to yield compound **4** as a colorless oil (178 mg, 32%).

HRMS: composition: $C_6 H_6 N_3 O_4$; measured mass 184.0362; theoretical mass: 184.0364.

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.07 (dd, *J*= 8.4, 4.2 Hz, 1H), 4.98 (d, *J*= 16.2 Hz, 1H), 4.93 (d, *J*= 16.2, 1H), 3.66-3.57 (m, 2H), 2.41-2.36 (m, 1H), 2.24-2.18 (m, 1H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 165.4, 163.9, 72.3, 65.6, 46.3, 30.3.

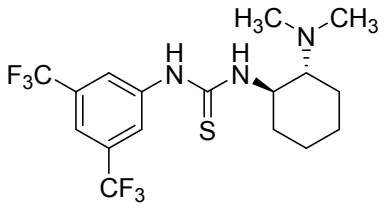


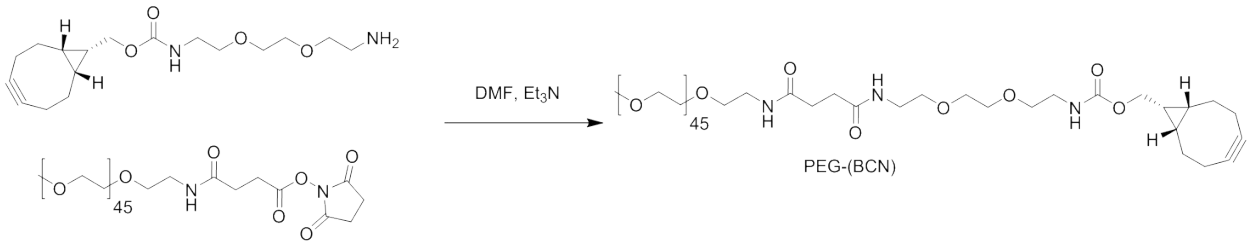
Figure S1. 1-[3,5-Bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(-)-2-(dimethylamino)cyclohexyl]thiourea (R,R-TUC).

Polymer 1. Compound **4** (154 mg,1.00 mmol) and 1-[3,5-Bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(-)-2-(dimethylamino)cyclohexyl]thiourea (R,R-TUC) (43 mg, 0.125 mmol) were dissolved in CH_2Cl_2 (0.7 mL). 208 μL of 0.1 M methanol in CH_2Cl_2 were added dropwise to the reaction mixture. 208 μL of 0.1 M triazabicyclo[4.4.0]dec-5-ene in CH_2Cl_2 were added dropwise to the reaction mixture. The reaction was allowed to proceed for 1 h. The polymer was then purified by repeated precipitation into cold ethyl ether from a CH_2Cl_2 solution to yield polymer **1** (109 mg, 71%) as a colorless oil.

Mw = 12,800 Da PDI= 1.5 (determined by gel-permeation chromatography (GPC) relative to Poly(methyl methacrylate)standards).

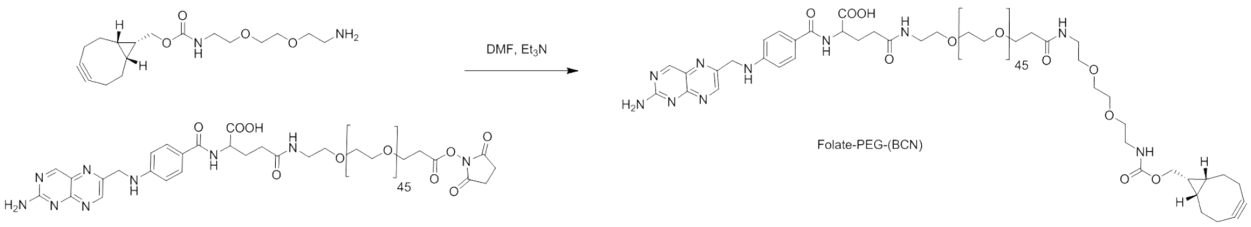
^{1}H NMR (600 MHz, $CDCl_3$) δ 5.36-5.34 (m, 1H), 4.96-4.67 (m, 2H), 3.58-3.40 (m, 2H), 2.32-2.13 (m, 2H).

4. Preparation of PEG-(BCN) and Folate-PEG-(BCN)



Scheme S2. Preparation of PEG-(BCN).

PEG-(BCN). *O*-[(*N*-Succinimidyl)succinyl-aminoethyl]-*O′*-methylpolyethylene glycol 2′000 (Sigma-Aldrich) (17.42 mg, 0.00767 mmol) and *N*-[(1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl]-1,8-diamino-3,6-dioxaoctane (Sigma-Aldrich) (2.5 mg, 0.00767 mmol) were dissolved in DMF (0.5 mL) and trimethylamine (10.7 μL, 0.0767 mmol) was dripped in. After 4 days the reaction was concentrated and used in particle functionalization without purification.



Scheme S3. Preparation of Folate-PEG-cyclooctyne (BCN).

Folate-PEG-(BCN). Folate-PEG-NHS (NANOCS, PG2-FANS-2k) (20 mg, 0.00767 mmol) and *N*-[(1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl]-1,8-diamino-3,6-dioxaoctane (Sigma-Aldrich) (2.5 mg, 0.00767 mmol) were dissolved in DMF (0.5 mL) and trimethylamine (10.7 μL, 0.0767 mmol) was dripped in. After 4 days the reaction was concentrated and used in particle functionalization without purification.

5. Particle Formulation and Characterization

5.1 Particle Formulation (microparticles) for Fluorescence Microscopy and GPC. 11 mg polymer **1** and 11 mg of poly(lactic-co-glycolic acid) (MW: 7-17 k) were dissolved in 1 mL dichloromethane, which was then added to 20 mL of 10% Pluronic F-127 in water. The mixture was sonicated and then injected through a 10 μm pore size membrane into a flask. The particle solution was stirred at r.t. for 4 h to completely remove the organic solvent. The particle solution was then washed with molecular biology grade water by centrifugation at 4500 rpm with five washes of 40 mL each. 118 mg of trehalose was added to the particle solution, which was then frozen with liquid nitrogen and lyophilized.

5.2 Particle Surface Functionalization (microparticles) for Fluorescence Microscopy and GPC.

Particle trehalose mixture was used such that 5.3 mg of particles would be used in each batch. The particle trehalose mixture was suspended in 1 mL molecular biology grade water with either 0.5 mg of (DIBAC)-PEG-TAMRA or 0.5 mg of (DIBAC)-PEG-TAMRA and 0.5 mg of PEG-(BCN) depending on the desired surface functionalization. The solution was stirred for 96 h then the particle solution was washed with molecular biology grade water by centrifugation at 4500 rpm with three washes of 40 mL each. For fluorescence microscopy the (DIBAC)-PEG-TAMRA and PEG-(BCN) modified particles were resuspended in molecular biology grade water, drop casted onto a glass slide, and air dried then imaged. Microparticle size was measured to be 1.4 ± 0.6 μm from microscope images using ImageJ. For GPC the particles were lyophilized then dissolved in DMF with 0.01% LiBr, and analyzed by gel permeation chromatography monitoring the absorbance at 280 nm.

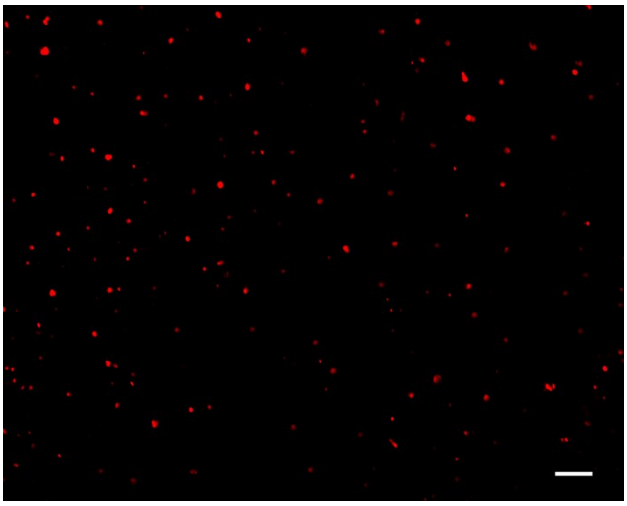


Figure S2. Additional fluorecence microscopy image of polymer **1** and PLGA blended microparticles functionalized with (DIBAC)-PEG-TAMRA and PEG-(BCN). (scale bar = 10 μm).

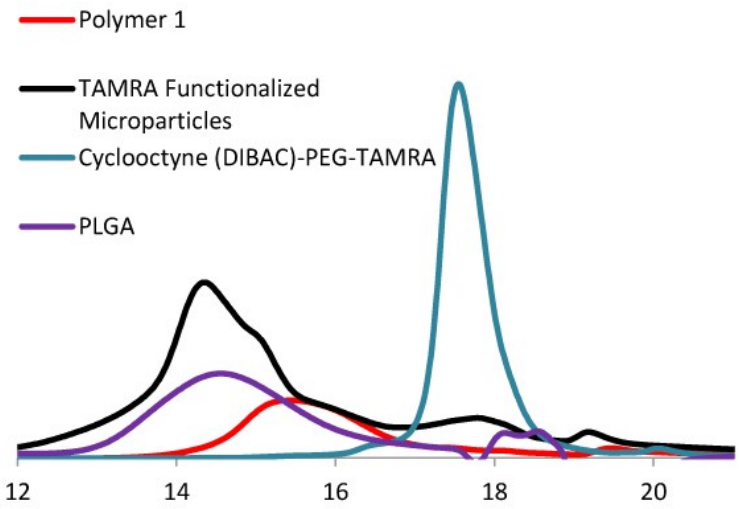


Figure S3. GPC traces (monitored by absorbance at 280 nm) of Polymer **1** alone, (DIBAC)-PEG-TAMRA, microparticles functionalized with (DIBAC)-PEG-TAMRA, PLGA (monitored by refractive index).

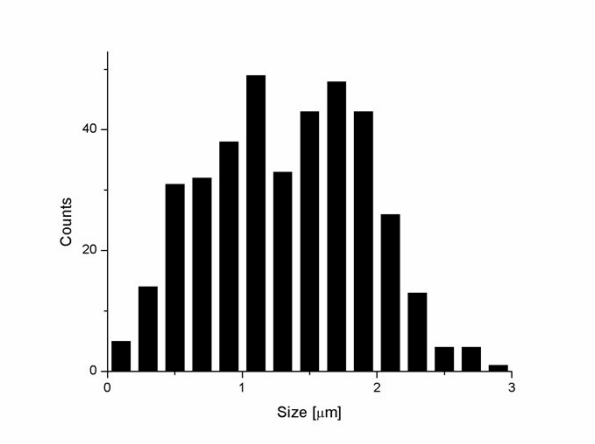


Figure S4. Histogram showing the size distribution of functionalized microparticles.

5.3 Particle Formulation (nanoparticles) for GPC and Targeting. 12 mg polymer **1** and 12 mg of poly(lactic-co-glycolic acid) (MW: 7-17 k) were dissolved in 400 μL chloroform, which was then added to 8 mL of 3% polyvinyl alcohol in water. The mixture was sonicated using a 1/8 inch tip sonicator (Misonix S-4000) at about 9.5 W for 5 min. Particle solution was stirred at r.t. for 2 h then concentrated by rotovap at 40 ^{o}C to completely remove the organic solvent. The particle solution was then washed with molecular biology grade water by tangential flow filtration through 500 kDa Pellicon XL cassettes (Millipore). 202 mg of trehalose was added to the particle solution, which was then frozen by liquid nitrogen and lyophilized. The size and distribution of particles were determined by dynamic light scattering (DLS, Malvern Instruments Nanosizer).

5.4 Particle Surface Functionalization (nanoparticles) for GPC and Targeting.

The particle trehalose mixture was used such that 4 mg of particles would be used in each batch. The particle trehalose mixture was suspended in 2 mL molecular biology grade water with either 1 mg of (DIBAC)-PEG-TAMRA for GPC, 1 mg of (DIBAC)-PEG-TAMRA and 3 mg of PEG-(BCN) for a targeting control, or 1 mg of (DIBAC)-PEG-TAMRA and 3 mg Folate-PEG-(BCN) for folate targeted particles. The solution was stirred for 96 h then the particle solution was washed with molecular biology grade water using 100 kDa Amicon Ultra 0.5 mL centrifugal filters five times. For GPC particles

functionalized with only (DIBAC)-PEG-TAMRA were lyophilized then dissolved in DMF with 0.01% LiBr, and analyzed by gel permeation chromatography monitoring the absorbance at 280 nm. The size and distribution of particles were determined to be 157 ± 37 nm by dynamic light scattering (DLS, Malvern Instruments Nanosizer).

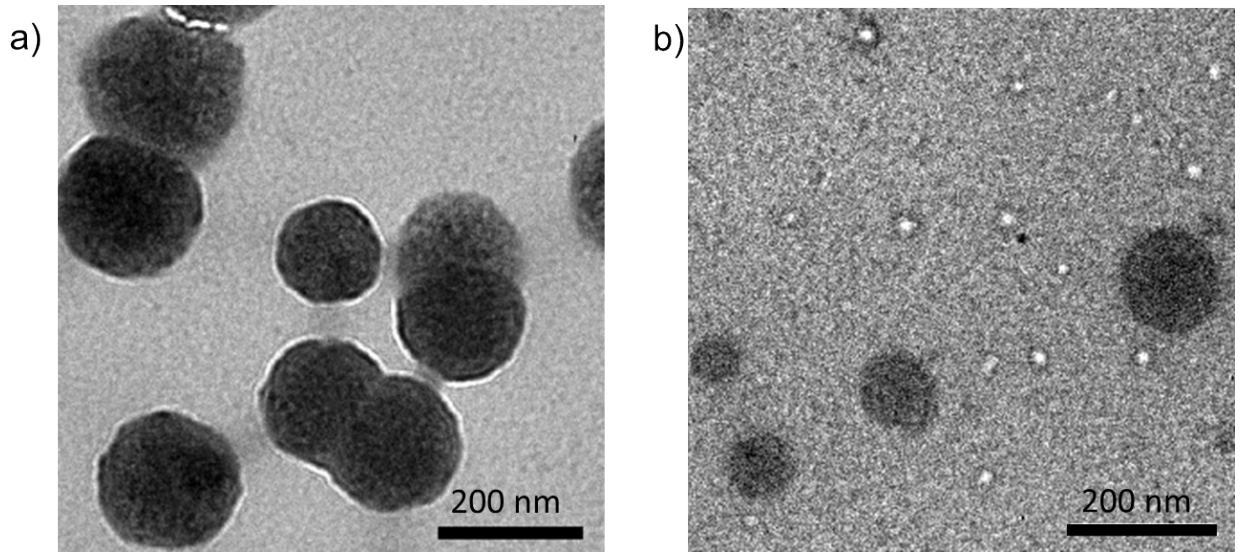


Figure S5. TEM images of a) (DIBAC)-PEG-TAMRA and Folate-PEG-(BCN) functionalized folate targeted nanoparticles and b) (DIBAC)-PEG-TAMRA and PEG-(BCN) functionalized non-targeted control nanoparticles.

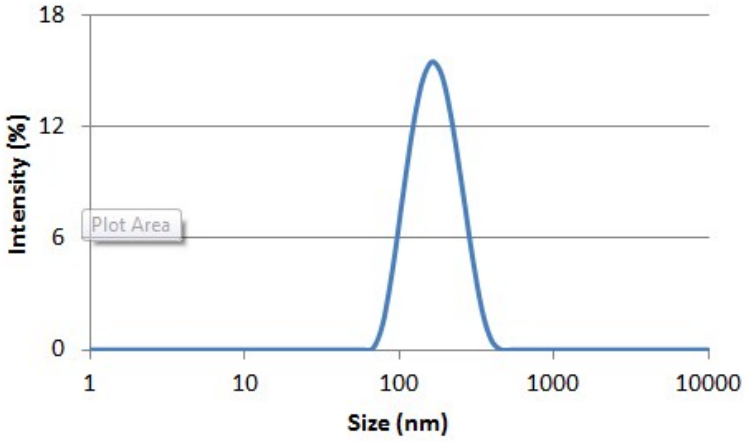


Figure S6. DLS histogram plot showing size distribution of non-functionalized nanoparticles.

5.5 UV-visible absorption spectra of disassembled particles

Particles, labeled with the (DIBAC)-PEG-TAMRA or unlabeled, were dispersed at 250 mg/mL in a solution of water/DMSO (50:50 vol/vol) to be disassembled. 200 µL of the resulting solution containing dissolved components was used to measure the absorbance spectrum from 300 to 700 nm. The cyclooctyne (DIBAC)-PEG-TAMRA spectrum was measured at 20 µM in a solution of water/DMSO (50:50 vol/vol). All spectra were collected using a Shimadzu UV-3600 UV−visible spectrophotometer.

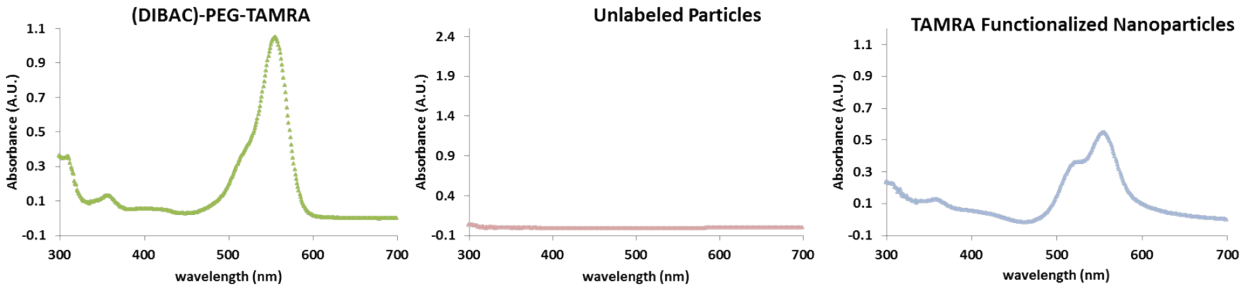


Figure S7. The UV-visible absorption spectra of (DIBAC)-PEG-TAMRA, unlabeled particles, and TAMRA functionalized nanoparticles.

5. Evaluation of targeting properties and cytotoxicity

5.1 Cell culture. Human adenocarcinoma HeLa cells were cultured in RPMI 1640 with L-glutamine (Gibco Invitrogen) supplemented with 10 % (vol:vol) fetal bovine serum (FBS, heat inactivated, Omega Scientific), 10 µg/ml ciprofloxacine HCl (bioWorld). Cells were routinely cultured at 37 °C in a humidified incubator in a 95 % air/5 % CO_2 atmosphere and passaged by detaching them with 0.25 % trypsin-0.05 % EDTA (w/v) solution (Gibco Invitrogen).

5.2 Cell targeting. The targeting efficient of FA-functionalized nanoparticles was determined by in vitro incubation with HeLa cells. A fibronectin coating of the cell culture plastic surface of the 96 well plate was performed by overnight incubation of a 10 µg/mL solution in PBS at 37 °C, 5% CO_2, high humidity

environment. Cells were seeded at 5 x 10^3 cells/well and cultured in folate-free RPMI 1640 for 72 hours. After removing the cell culture medium, cells were washed once with PBS. FA-PEG-NPs and the PEG-NPs (control) were dispersed at 50 µg NPs/mL in folate-free RPMI 1640 and incubated with cells, 100 µL/well, for 1 hour at 37 °C, 5% CO_2, high humidity environment. The specific binding of FA–PEG–NPs by HeLa cells was also examined in a competitive assay, where nanoparticles were co-incubated 1 hour with cells in the presence of free FA 100 µM. This method is designed to saturate folate receptors to prevent receptor-specific binding by FA-PEG-NPs, thereby distinguishing between receptor-mediated and non-specific NPs binding. At the end of the incubation time, cells were washed two times with PBS, and fixed during 30 minutes at 37 °C by a 4% PFA solution, (paraformaldehyde, w/v in PBS). After the fixation step, cells were washed two times with PBS and then labelled with a 10 µg/mL DAPI solution (w/v in PBS). Labelled cells were then observed by fluorescence microscopy.

5.3 Microscopy observations. Fluorescent pictures were obtained with a KEYENCE BZ-X700 all-in-one fluorescence microscope (Keyence, Osaka, Japan) equipped with charge-coupled device (CCD) camera. An 80 W metal halide lamp was used as excitation source. DAPI fluorescence was detected with a filter cube composed of a 360 ± 40 nm excitation filter, 400 nm beam splitter, and 460 ± 50 nm emission filter. TexasRed fluorescence was detected with a filter cube composed of a 560 ± 20 nm excitation filter, 660 nm beam splitter, and 630 ± 37.5 nm long-pass emission filter. All acquisitions were performed with a 20X objective PlanApoλ N.A 0.75. Twelve fields of view were acquired per condition and quantified with ImageJ software extracting from each single picture the mean fluorescence intensity. All values were expressed as means ± SD. Statistical analysis was conducted by Student *t* test. Group differences resulting in p = 0.05 by student *t* test were considered statistically significant and indicated with *, whereas p = 0.01 with **, and p 0.001 with ***.

5.4 Cell viability. The toxicity of PLGA-N3-based nanoparticles and the resulting degradation products was evaluated in vitro by Alamar Blue™ assay. HeLa cells were seeded at 5 x 10^3 cells/well in a 96-well plate and incubated at 37 °C in a humidified atmosphere with 5% CO_2 during 48 h into 200 µL/well of

complete RPMI (supplemented with 10 % FBS and 10 µg/ml ciprofloxacine). Then, the culture supernatants were removed and replaced by the nanoparticles and the degradation product diluted at various concentration (0.2-50 µg/mL) in complete RPMI. After 24 h and 48 h incubation, cells were washed once with 100 µL of complete RPMI and incubated for 3 h in RPMI with 10% Alamar Blue™ reagent (100 µL/well final) before measuring the fluorescence of each well with a plate reader (FlexStation 3, Molecular devices, λ_{ex} = 560 nm, λ_{em} = 585 nm, cutoff 570 nm). Data were normalized on untreated cells (empty complete RPMI) and considered as 100 % viability. All values were expressed as means ± SD.

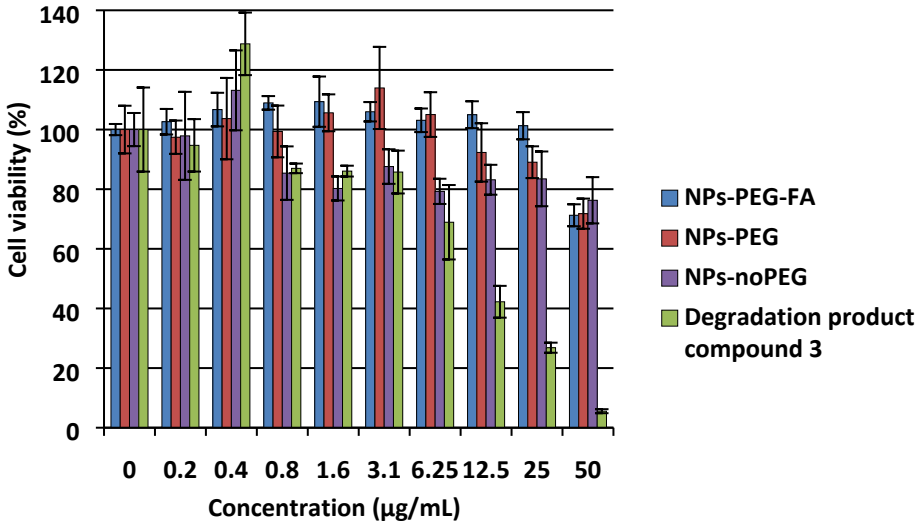


Figure S8. Cell viability after 48 h incubation of HeLa cells with either the NPs-PEG-FA particles (blue), or the nontargeted NPs-PEG (red), or the unfunctionalized NPs-noPEG (purple). The presumed degradation product compound **3** is presented in green. Means ± SD.