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Double Click: Dual Functionalized Polymeric Micelles with Antibodies and Peptides

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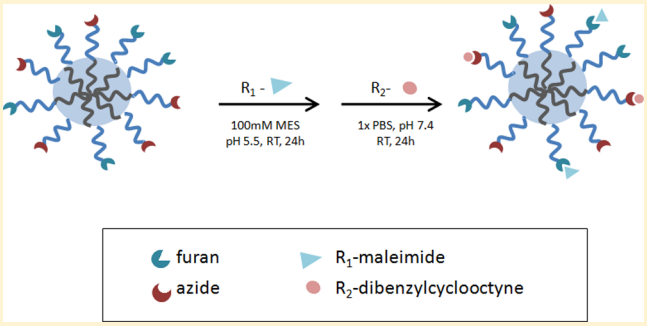
Supporting Information

ABSTRACT: Polymeric nanoparticle micelles provide a possible platform for theranostic delivery, combining the role of therapeutics and diagnostics in one vehicle. To explore dual-functional micelles, the amphiphilic copolymer of poly(D,L-lactide-*co*-2-methyl-2-carboxytrimethylene carbonate)-*graft*-poly(ethylene glycol)-X (P(LA-*co*-TMCC)-*g*-PEG-X) was self-assembled to form micelles, with X representing either azide or furan. Micelles of P(LA-*co*-TMCC)-*g*-PEG-azide and P(LA-*co*-TMCC)-*g*-PEG-furan terminal functional groups were used to conjugate dibenzylcyclooctyne and maleimide-modified probes, respectively, taking advantage of orthogonal coupling chemistry. To verify the utility of the dual-functional micelles, trastuzumab-maleimide antibodies and FLAG-dibenzylcyclooctyne peptides were covalently bound by sequential click chemistry reactions. SKOV-3luc cells that were treated with the dual-functionalized micelles showed colocalization of the antibodies and peptides by confocal imaging, demonstrating the promise of this approach.



INTRODUCTION

Biodegradable copolymers that self-assemble into nanoparticle micelles are promising drug delivery vehicles due to their tunable physical properties.[1] Polymeric micelles can be tailored to improve the solubility of hydrophobic drugs, control the release rate of drugs, reduce the elimination rate associated with the host immune system, increase the circulation time, and enhance the preferential delivery of drugs to solid tumors.[2] Polymeric micelles are unique drug delivery vehicles because they remain intact at concentrations above the critical micelle concentration (CMC) and dissociate slowly when below the CMC to release encapsulated drugs.[2]

Polymeric micelles can capitalize on their unique amphiphilic properties by loading hydrophobic drugs into the core to improve drug solubility while incorporating targeting and/or imaging ligands on the surface as well. For example, micelles can be conjugated to antibodies, peptides, lectins, saccharides, or hormones[3] for targeted drug delivery. Targeting cell surface receptors using antibodies is a promising method to improve the specific binding of micelles to desired cells;[4] however, intracellular trafficking may be required if the target is within the nucleus or organelles. Cell penetrating peptides and fusogenic proteins facilitate the targeting to intracellular locations and can be added to the drug carrier system.[5,6] The codelivery of additional therapeutics can also improve the overall efficacy.[7] Likewise, micelles that are labeled with a molecular probe or contrast agent can aid in diagnosing disease states or track the kinetics of the drug delivery system.[8] A recent shift toward multifunctional "theranostic" treatments aims to use the polymeric micelle as a drug carrier with diagnostic capacity, thereby serving a dual purpose.

To covalently attach multiple, unique diagnostic agents, targeting ligands, or therapeutic agents, the micelle must have multiple, accessible functional groups.[2] A mixed micelle system comprising a blend of block copolymers could produce multifunctional micelles. Mixed micellar systems are formed by weak hydrophobic interactions, hydrogen bonding, stereocomplexation, ionic interaction, or chemical cross-linking.[1] In one system of self-assembled micelles, a graft copolymer was combined with a diblock copolymer to conjugate two moieties, Cy5.5 and folate, with the size and polydispersity dependent on the proportion of each polymer.[9]

Micelles have been surface-functionalized with a single type of functional group, using either Diels−Alder or Huisgen 1,3-cycloaddition click chemistries,[4,10,11] but never both. While the most common click reaction is the copper-catalyzed alkyne−azide (CuAAC) reaction,[12] the copper-free reaction using a strain-promoted alkyne−azide cycloaddition (SPAAC) reaction

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is gaining popularity as it overcomes the potential cytotoxicity associated with copper ion contaminatio.[13] The Diels–Alder reaction similarly avoids potential metal ion contamination and is a clean and simple click reaction that is effective in aqueous conditions.

Poly(D,L-lactide-*co*-2-methyl-2-carboxytrimethylene carbonate)-*graft*-poly(ethylene glycol) (P(LA-*co*-TMCC)-*g*-PEG) is an amphiphilic polymer that self-assembles to form stable micelles with the capacity to encapsulate hydrophobic drugs and to be covalently modified with targeting ligands.[14−16] Here, we show that a P(LA-*co*-TMCC)-*g*-PEG-X copolymer, where X represents one of two click-able functional groups (azide or furan), can self-assemble into micelles and be functionalized with two distinct biomolecules (dibenzylcyclooctyne-FLAG peptide and maleimide-trastuzumab), thereby achieving dual functionalized micelles through click chemistry (Figure 1). The

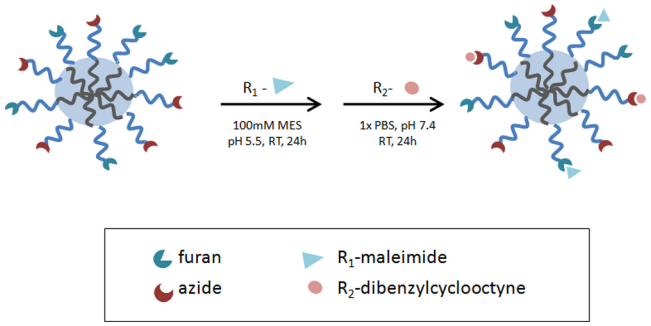

Figure 1. Dual functionalized polymeric micelles facilitated by click coupling chemistry.

FLAG octapeptide is a protein tag commonly used for recombinant protein purification or immunofluorescence and was selected as a mimic for other small (<20 residue), functional peptides.[17] Trastuzumab is a humanized antibody, used clinically, that binds human epidermal growth factor receptor type 2 (HER2) and was selected as to target cell-surface receptors of HER2-overexpressing cells to enhance receptor mediated endocytosis.[18,19]

In contrast to mixed micelles formed from two dissimilar block copolymers, the use of a single copolymer with two functional groups produces a stable micelle that retains the characteristics of a single population. We take advantage of the copper-free reaction between azide and dibenzylcyclooctyne and the Diels–Alder reaction between furan and maleimide on preformed micelles in aqueous conditions. The strained alkyne bond of dibenzylcyclooctyne has increased electrophilicity over simple alkynes, allowing the reaction to proceed without a catalyst.[20] The versatility of binding different moieties with click chemistry reactions provides a platform for the incorporation of unique combinations of ligands with potential use in therapeutic and diagnostic applications or in primary/secondary targeting approaches (e.g., primary—cell surface, secondary—intracellular or organelle). The ability to conjugate biomolecules on the surface of preformed micelles in aqueous conditions is particularly important for ligands that are sensitive to the organic solvents used during micellization, for targeting ligands that require direct binding to cells, or for MRI contrast agents that have enhanced contrast properties when exposed to aqueous environments.[21]

EXPERIMENTAL PROCEDURES

Materials and Methods. Synthesis of 5-methyl-5-benzyloxycarbonyl-1,3-trimethylene carbonate (benzyl-protected TMCC, TMCC-Bn) was carried out as previously reported.[4] 3,6-Dimethyl-1,4-dioxane-2,5-dione and 1-[3,5-bis-(trifluoromethyl)phenyl]-3-[(1*R*,2*R*)-(−)-2(dimethylamino) cyclohexyl] thiourea (Strem Chemicals, Newburyport, MA) were used as received to synthesize P(LA-*co*-TMCC).[22] Boc-NH-PEG(10K)-NHS (Rapp Polymere, Tübingen, Germany) was modified using previously reported protocols for furan and azide-functional groups[4,10] *N*,*N*′-diisopropyl carbodiimide, *N*,*N*′-diisopropylethylamine, and hydroxybenzotriazole (TRC, Toronto, ON) were used as received. Amino acid derivatives were purchased from Novabiochem (Billerica, MA). Anti-FLAG (Rabbit) antibodies were purchased from Cell Sorting (Boston, MA), anti-human IgG (Rat) FITC from Sigma-Aldrich (St. Louis, MO), and anti-rabbit (Goat) Alexa-647 from Invitrogen (Grand Island, NY). All solvents and reagents were purchased from Sigma-Aldrich and were used as received, unless otherwise noted.

The Alexa 488 C_5 maleimide was obtained from Invitrogen (Burlington, ON), the Alexa 488-dibenzylecylooctyne (DBCO) was purchased from Click Chemistry Tools (Scottsdale, AZ), and sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Rockford, IL). Trastuzumab was purchased through Hoffmann-La Roche Limited (Mississauga, ON). 5-((2-(and-3)-*S*-(Acetylmercapto)succinoyl)amino) (SAMSA) fluorescein (Invitrogen, Eugene, OR) was used to quantify the amount of SMCC that coupled to the trastuzumab. The human ovary cancer cell line SKOV-3luc was purchased from Cell Biolabs, Inc. (San Diego, CA).

The ^{1}H NMR spectra were recorded at 400 MHz at room temperature using a Varian Mercury 400 spectrometer. The chemical shifts are in ppm. The molecular weights and polydispersity of P(LA-*co*-TMCC) were measured by gel permeation chromatography (GPC) in THF relative to polystyrene standards at room temperatures on a system with two-column sets (Viscotek GMHHR-H) and a triple detector array (TDA302) at a flow rate of 0.6 mL/min. Polymeric micelle size and distribution were measured by dynamic light scattering (DLS) using the Zetasizer Nano ZS system (Malvern, UK). The NH_2-PEG-azide was characterized using the Perkin-Elmer FT-IR Spectrum 1000. The Sepharose CL-4B column was prepared by soaking the beads in distilled water overnight and packing the beads in a column (5 × 15 cm). The column was washed with distilled water for 1 h before use, and the flow rate was determined by gravity. The Sephadex G-25 column was prepared using the same method, except PBS buffer (1×, pH 7.4) was used as the eluent and the beads were packed in a column (1.5 × 15 cm). FPLC of the micelles used a Superdex 200 column. The column was washed with distilled water for 20 min and PBS buffer (1×, pH 7.4) for 20 min at a flow rate of 1.5 mL/min before use. Fluorescence measurements were completed with the Tecan Infinite M200 Pro fluorescent plate reader and absorbance was quantified using the NanoDrop Spectrophotometer.

Synthesis of NH_2-PEG-azide. As previously shown, bifunctional *tert*-butyloxycarbonyl-protected amine-PEG activated acid (BocNH-PEG-(*N*-hydroxysuccinimide)), 0.523 g, 52 μmol) was modified to incorporate a terminal azide group[10] by reactinve with 11-azido-3,6,9-trioxaundecan-1-amine (Sigma

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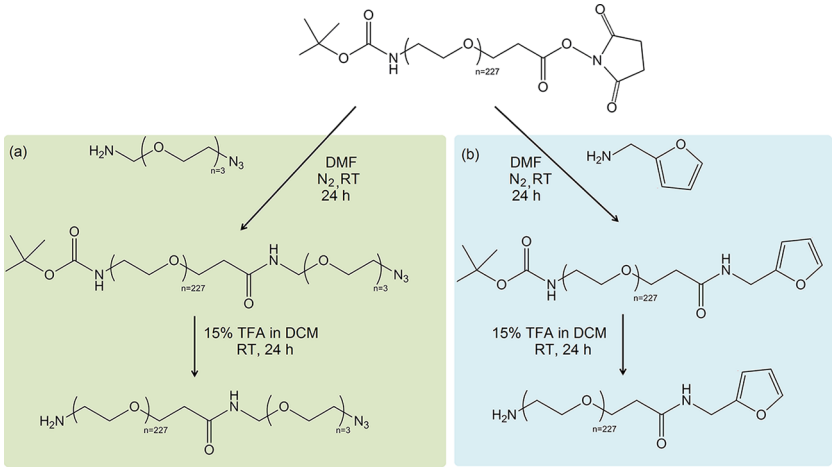
Scheme 1. Synthesis of (a) NH_2-PEG-azide and (b) NH_2-PEG-furan by Reacting NHS-Activated PEG Chains with Furfurylamine or Amine-Terminated Triethylene Glycol-Azide, Respectively, Followed by BOC Deprotection



Aldrich, 1.0 mL, 5 mmol) in DMF (Scheme 1). The final product yielded NH_2-PEG-N_3 (0.3629 g, 80.6%). The presence of the terminal azide group (2101 cm^{-1}), terminal amine (3389 cm^{-1}), C−H alkane (2888 cm^{-1}), C═O amide (1692 cm^{-1}), and C−O ether (1101 cm^{-1}) was characterized by FTIR (Supporting Information Figure S1).

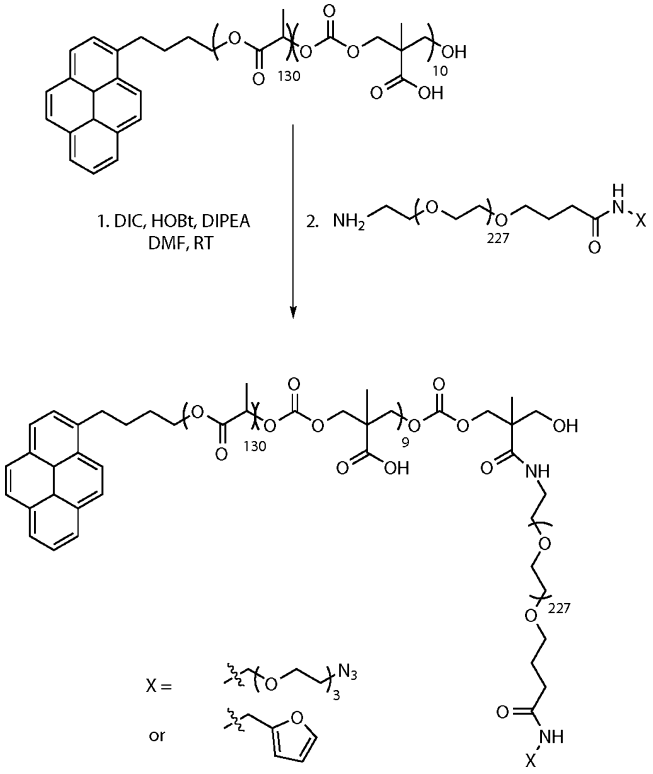
Synthesis of NH_2-PEG-furan. Similar to the procedure used to synthesize PEG-azide, BocNH-PEG-NHS (1.00 g, 0.1 mmol) and furfurylamine (1.00 mL, 11.2 mmol) were reacted to yield bocNH-PEG-furan (0.8021 g, 80.2% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.44 (s, Boc CH_3), 2.32 (t, J = 14 Hz, −C**H**$_2$−), 3.61 (m, −OC**H**$_2$C**H**$_2$−), 4.42 (s, −C**H**$_2$-furan), 6.22 (d, J = 3 Hz, furan C**H**-3), 6.30 (dd, J = 5 Hz, furan C**H**-4), and 7.34 (d, J = 3 Hz, furan C**H**-5) . For NMR spectra, see Supporting Information Figure S2. The product was deprotected with treatment of 15% trifluoroacetic acid in CH_2Cl_2 for a final yield of NH_2-PEG-N_3 (0.6614 g, 81.5%).

Grafting of PEG to P(LA-*co*-TMCC). Synthesis of P(LA-*co*-TMCC) by ring-opening polymerization has previously been shown.[22] ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.22 (br s, C**H**$_3$ from TMCC), 1.59 (m, C**H**$_3$ from LA), 4.31 (br s, C**H**$_2$ from TMCC), 5.15 (m, C**H** from LA), 7.33 (m, benzyl Ar), and 8.02 (m, pyrene). Absence of the benzyl group peaks in the ^{1}H NMR after the hydrogenolysis of P(LA-*co*-TMCC-Bn) showed full deprotection of the polymer backbone. See Supporting Information Figure S3 for ^{1}H NMR spectra.

The modified H_2N-PEG (PEG-X) was grafted onto the P(LA-*co*-TMCC) as previously shown (Scheme 2).[22] P(LA-*co*-TMCC) (100 mg, 8.3 μmol) was dissolved in 3 mL of DMF with *N,N′*-diisopropyl carbodiimide (DIC, 100 μL, 0.65 mmol), hydroxybenzotriazole (HOBt, 63.1 mg, 0.48 mmol), and *N,N′*-diisopropylethylamine (DIPEA, 0.52 mmol) and stirred for 30 min. The PEG-X (100 mg, 10 μmol) was dissolved in 3 mL of DMF and added to the P(LA-*co*-TMCC) solution dropwise. An additional 90 μL of DIPEA was added, and the mixture was sealed under argon for 2 min and left to react overnight.

Borate buffer (400 μL, 500 mM, pH 9.0) was added to the P(LA-*co*-TMCC)-*g*-PEG-X solution before dialyzing against distilled water with a 2 kDa RC membrane. The P(LA-*co*-TMCC)-*g*-PEG-X was purified using the Sepharose CL-4B column, then freeze−dried (37.5 mg, 38%). From the ^{1}H NMR spectra, it was calculated that there was an average of 0.9 PEG-azide chains per P(LA-*co*-TMCC) backbone. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.25 (br s, C**H**$_3$ from TMCC), 1.57 (m, C**H**$_3$ from LA), 3.61−3.64 (m, −OC**H**$_2$C**H**$_2$−), 4.35 (m, C**H**$_2$ from TMCC), and 5.17 (m, C**H** from LA). See Supporting Information Figure S4a for ^{1}H NMR spectra.

Scheme 2. Synthesis of P(LA-*co*-TMCC)-*g*-PEG-X by Grafting NH_2-PEG-X with DIC Chemistry[a]



[a]X represents an azide or furan functionality.

The same procedure was carried out to graft PEG-furan to P(LA-*co*-TMCC). An average of 0.7 PEG-furan chains per backbone was calculated using the ^{1}H NMR spectra. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.22 (br s, C**H**$_3$ from TMCC), 1.56 (m, C**H**$_3$ from LA), 3.59−3.69 (m, −OC**H**$_2$C**H**$_2$−), 4.31 (m, C**H**$_2$ from TMCC), and 5.15 (m, C**H** from LA). See Supporting Information Figure S4b for ^{1}H NMR spectra.

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Micellization. The micelles were prepared by co-self-assembly of P(LA *co*-TMCC)-*g*-PEG-furan and P(LA-*co*-TMCC)-*g*-PEG-azide by membrane dialysis, as previously shown for single functionalized chains .[23] To form the dual-functional micelles, P(LA-*co*-TMCC)-*g*-PEG-azide (1.8 mg, 20.2 kDa) and P(LA-*co*-TMCC)-*g*-PEG-furan (2.2 mg, 18.2 kDa) were combined for an average of 80 nmol each of azide and furan functional groups. The average number of azide and furan functional groups per micelle accounted for the difference in grafting efficiency (0.9 PEG and 0.7 PEG per backbone, respectively) by adjusting the ratio of each polymer dissolved in 1 mL of DMF. Borate buffer (50 μL, 0.5 M, pH 9.0) was added dropwise to the solution, and an additional 500 μL of distilled water was added to form the micelles. The solution was dialyzed against distilled water using a dialysis membrane with MWCO of 2 kDa at room temperature overnight to form micelles. The water was changed every 2 h for the first 6 h. The micelles were filtered using a 0.45 μm nylon filter and brought to a concentration of 50 μM of polymer before characterization by DLS.

Azide-only and furan-only micelles were prepared using the same dialysis method as controls for the coupling chemistry. P(LA-*co*-TMCC)-*g*-PEG-azide (4.0 mg, 20.2 kDa) was used for the azide micelles and P(LA-*co*-TMCC)-*g*-PEG-furan (4.0 mg, 18.2 kDa) was used for the furan micelles. The average diameter was measured by DLS of the azide micelles was 102.9 nm with PDI = 0.165; the average diameter of the furan micelles was 66.43 nm with PDI = 0.218;

Synthesis of Dibenzylcyclooctyne-Modified FLAG Peptide. The FLAG peptide (DYKDDDDK) was synthesized by standard Fmoc-based solid-phase peptide chemistry on a CEM Liberty 1 microwave peptide synthesizer. The resin-bound FLAG peptide (100 mg, 1.1 meq/g, 0.11 mmol) was swollen in CH_2Cl_2 and then rinsed several times with *N,N*-diisopropylethylamine (DIPEA). Dibenzylcyclooctyne-NHS (80 mg, 0.165 mmol) was dissolved in DMF (~2 mL), added to the resin, and allowed to react overnight. The resin was washed several times with DMF and CH_2Cl_2. The product was deprotected and cleaved from the resin with treatment of 95% trifluoroacetic acid in CH_2Cl_2. Solvent was removed from the resulting product and peptide precipitated in cold Et_2O. Product was purified by HPLC and analyzed by electrospray ionization mass spectrometry (ESI+ MS). Expected 1327.5, found 1329.6 (M+2H).

Synthesis of Trastuzumab-SMCC. Trastuzumab (6.6 mg, 45.5 nmol) was dialyzed overnight (2 kDa MWCO) against Tris buffer (100 mM, pH 8.5) to remove the storage solution of sodium azide. Sulfo-SMCC (0.5 mg, 1.15 μmol) was added to the trastuzumab solution and left to react for 4 h. The SAMSA fluorescein (10 mg/mL in DMSO) was dissolved in 500 μL of NaOH (0.1 M) and incubated for 15 min at room temperature. The solution was neutralized using 7 μL of HCl (6 M) and buffered with 100 μL Na_2HPO_4 (0.5 M). From this solution, the SAMSA fluorescein (0.55 μmol, 63 μL) was mixed with trastuzumab-SMCC (55 nmol, 100 μL) and incubated at room temperature for 30 min. The mixture was dialyzed against Tris buffer overnight. A standard curve was prepared using the activated SAMSA fluorescein solution and was used to quantify the trastuzumab-SMCC at an excitation wavelength of 480 nm and emission at 530 nm. An average of 1 fluorescein molecule per antibody translated to 1 SMCC group available per antibody.

Coupling of Alexa 488-DBCO and Alexa 647-MI to Functionalized PEG-X by Click Chemistry. Coupling reactions were carried out using two modified PEG-X polymers and functionalized Alexafluor dyes. NH_2-PEG-N_3 (1.0 mg, 100 nmol) was reacted for 24 h with Alexa 488-DBCO (500 nmol) in 1 × PBS (pH 7.4). NH_2-PEG-furan (1.0 mg, 100 nmol) was reacted with in Alexa 488-C_5-MI (500 nmol) in MES buffer (100 mM, pH 5.5). Reactions were purified by dialyzing against ddH_2O (MWCO = 3500 Da) for 72 h and then characterized by FTIR and ^{1}H NMR.

Coupling of Alexa 488-Dibenzylcyclooctyne (DBCO) on Azide Micelles. P(LA-*co*-TMCC)-*g*-PEG-azide micelles (2 mg/mL, 100 nmol) were reacted for 24 h with Alexa 488-DBCO (0.5 mg/mL, 200 nmol). Using the G-25 Sephadex column, the unreacted Alexa 488-DBCO was removed.

As a control, the reaction was repeated with Alexa 488-DBCO where the Alexa 488-DBCO (200 nmol) was quenched by incubation with 11-azido-3,6,9-trioxaundecan-1-amine (400 μL, 2 mmol) prior to the click chemistry reaction. The micelles were purified using the same procedure with a size exclusion column.

Coupling of Alexa 488-Maleimide (MI) on Furan Micelles. P(LA-*co*-TMCC)-*g*-PEG-furan micelles (2 mg/mL, 50 nmol) were reacted for 24 h with Alexa 488-C_5-MI (2 mg/mL, 100 nmol) in MES buffer (100 mM, pH 5.5, 100 μL). Using the G-25 Sephadex column, the unreacted Alexa 488-MI was separated. Fractions containing the micelles were detected using Bradford reagent and quantified by absorbance at 260 nm. Fluorescence was used to quantify the Alexa 488 that conjugated to the micelle.

Controls were prepared by quenching the Alexa 488-MI (100 nmol) with furfurylamine (10 μmol) and cysteine (10 μmol) in MES buffer (100 mM, pH 5.5, 100 μL) before the coupling reaction. To fully quench the maleimide with the cysteine, tris(2-carboxyethyl)phosphine) (80 μmol) was added for the 48 h reaction. The same purification procedure was followed to remove the unreacted dye.

The Diels–Alder (DA) reaction was characterized by reacting NH_2-PEG-furan (100 nmol) with Alexa 647-MI (150 nmol) overnight. The solution was dialyzed to remove the unreacted Alexa 647-MI with 2 kDa MWCO dialysis tubing in distilled water. The sample was lyophilized before forming a KBr pellet.

Coupling of Alexa 488-DBCO and Alexa 647-MI on Dual-Functional Micelles by Click Chemistry. P(LA-*co*-TMCC)-*g*-PEG micelles with azide and furan functional groups (50 μM) were sequentially reacted with maleimide and dibenzylcyclooctyne-functional moieties. First, Alexa 647-MI (100 nmol) was added at room temperature with MES buffer (100 mM, pH 5.5) for 24 h. The solution was dialyzed overnight against PBS buffer (1×, pH 7.4) using 2 kDa MWCO dialysis tubing. The buffer was changed every 2 h for the first 6 h. Then, the solution was transferred to a glass vial to react with the Alexa 488-DBCO (100 nmol) at room temperature for 24 h. The reacted micelles were purified using size exclusion chromatography on a Sephadex G-25 column with PBS buffer (1×, pH 7.4) as the eluent. Fractions were collected in 1 mL volumes for a total of 20 fractions. Fluorescence of the Alexa 647 (ex. 640 nm, em. 675 nm) and Alexa 488 (ex. 490 nm, em. 525 nm) were used to quantify the conjugation efficiency. The concentrations of the micelles were measured by absorbance at 260 nm. Controls were repeated by quenching either the Alexa 647-MI or Alexa 488-DBCO with 3 orders of magnitude excess

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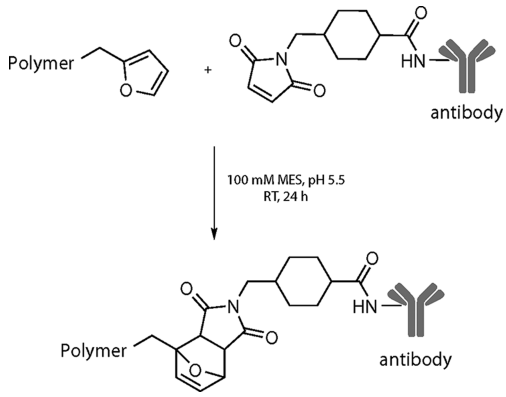
of furfurylamine or 11-azido-3,6,9-trioxaundecan-1-amine, respectively, before reacting with the micelles. See Supporting Information Figure S5 for fluorescence and absorbance measurements from the size exclusion column.

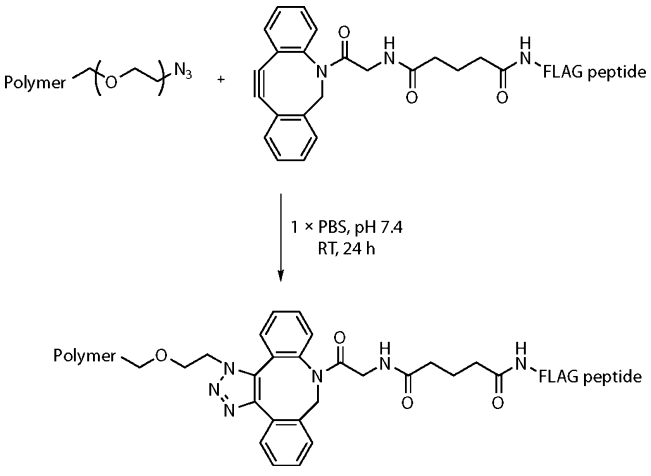
Coupling of FLAG-DBCO and Trastuzumab-SMCC on Dual-Functional Micelles by Click Chemistry. The dual-functional micelles were prepared using the same procedure as described above (see Micellization). As with the Alexa dyes, the trastuzumab-SMCC and FLAG-DBCO were conjugated sequentially. First, trastuzumab-SMCC (100 nmol) was added to dual functional micelles at room temperature with MES buffer (100 mM, pH 5.5) and reacted for 24 h (Scheme 3). The

Scheme 3. Diels-Alder Reaction between Furan-Modified Polymer and Maleimide-Modified Trastuzumab



solution was dialyzed overnight against PBS buffer (1×, pH 7.4) using 2 kDa MWCO dialysis tubing. The buffer was changed every 2 h for the first 6 h. Then, the solution reacted with FLAG-DBCO (100 nmol) at room temperature for 24 h (Scheme 4). The reacted micelles were purified using size

Scheme 4. Strain-Promoted Alkyne-Azide (SPAAC) Reaction between Azide-Modified Polymer and Cyclooctyne-Modified FLAG Peptide



exclusion chromatography on a Sephadex G-25 column by FPLC (GE Healthcare AKTA Purifier) with 1× PBS as the eluent, monitoring absorbance at 215 and 280 nm.

Cell Culture and In Vitro Assessment of Dual Functionalized Micelle Uptake. SKOV-3luc cells were cultured in McCoy's 5A media containing 10% FBS, 10 UI/mL penicillin, and 10 μg/mL streptomycin under standard culture conditions (37 °C, 5% CO_2, 100% humidity). Cells were seeded in 16-well chamber slides at 10 000 cells/well and allowed to adhere for 22 h. Dual functionalized micelles having both trastuzumab and FLAG peptides (100 μL, 100 nM trastuzumab) were added. Cells were supplemented with an additional 200 μL of media and incubated for 3 h. Cells were fixed in 4% paraformaldehyde before staining and analysis. Cells were incubated with 1% BSA in PBS with 0.05% Tween 20 (PBST) for 30 min to block unspecific binding of the antibodies. Using immunofluorescence, the FLAG peptide was labeled with anti-FLAG (Rabbit) antibody (Cell Sorting) followed by anti-Rabbit (Goat) Alexa-647 (Invitrogen). Trastuzumab was labeled with anti-human IgG (Rat) FITC (Sigma-Aldrich).

Cells were then incubated with the primary antibody (anti-FLAG or anti-human IgG) diluted in 1% BSA in PBST for 1 h at room temperature. Secondary antibodies were incubated in the same manner. Cells were then washed several times with PBS and mounted with medium containing DAPI. Images were collected by confocal microscopy (Olympus FV1000)—for DAPI, excitation at 405 nm, emission at 460; for FITC, excitation at 485 nm, emission at 520 nm; for Alexa-647, excitation at 650 nm, emission at 675 nm.

RESULTS

Synthesis of P(LA-*co*-TMCC)-*g*-PEG-X. The number average molecular weight and polydispersity of P(LA-*co*-TMCC) was determined by GPC to be 11.2 kDa and 1.12, respectively. On the basis of ^{1}H NMR integrated peak areas for TMCC methylene (4.35 ppm) and LA methyl (5.15 ppm), the molar percentage of TMCC in the copolymer was determined to be 7.1%. The copolymer composition was calculated using the ^{1}H NMR spectra based on the integrated peak areas of LA methyl groups and TMCC methylene groups relative to the single pyrene initiator group (8.02 ppm) present on each polymer chain: $P(LA)_{130}$-*co*-$(TMCC)_{10}$. These data are consistent with the GPC measurement.

Using DIC chemistry, we grafted NH_2-PEG-azide or NH_2-PEG-furan onto the carboxylic acid groups present on the TMCC segments of the copolymer. We estimated the ratio of grafted PEG to P(LA-*co*-TMCC) by comparing the integrated peak areas of the PEG methylene (3.61−3.64 ppm) to the TMCC methylene (4.35 ppm). On average, there was approximately 0.9 PEG-azide or 0.7 PEG-furan chains grafted to every P(LA-*co*-TMCC) backbone. The number average molar mass of P(LA-*co*-TMCC)-*g*-PEG was calculated according to eq 1:

$$M_{n(P(LA-co-TMCC)-g-PEG)} = M_{n(backbone)} + (M_{n(PEG)} \times \#\text{of PEG per copolymer backbone}) \quad (1)$$

Thus, the $P(LA_{130}$-*co*-$TMCC_{10})$-*g*-$PEG_{10\ kDa}$-azide had a number average molar mass of 20.2 kDa and the $P(LA_{130}$-*co*-$TMCC_{10})$-*g*-$PEG_{10\ kDa}$-furan had a slightly smaller number average molar mass of 18.2 kDa.

Estimation of the Aggregation Number of Micelles. The micelles formed from P(LA-*co*-TMCC)-*g*-PEG-X have an average diameter of 96.2 nm and distribution is 0.166 as determined by dynamic light scattering. The aggregation number (N_{agg}) and constant k for these micelles was estimated

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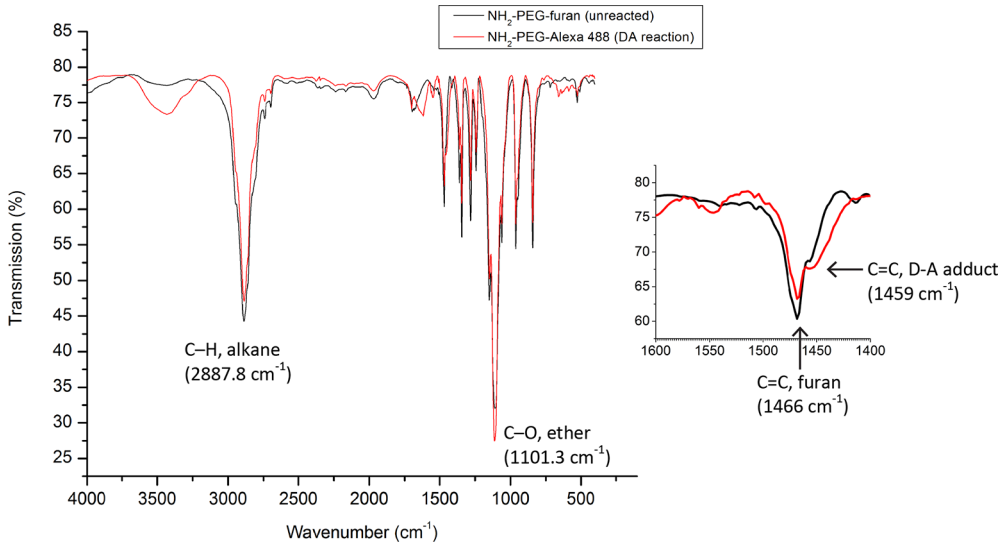

Figure 2. FTIR spectra for the SPAAC reaction between NH_2-PEG-N_3 and Alexa 488-dibenzyl cyclooctyne. The decrease in the azide peak (2109 cm^{-1}) of NH_2-PEG-N_3 after reaction with Alexa 488-dibenzyl cyclooctyne demonstrates the successful coupling reaction.

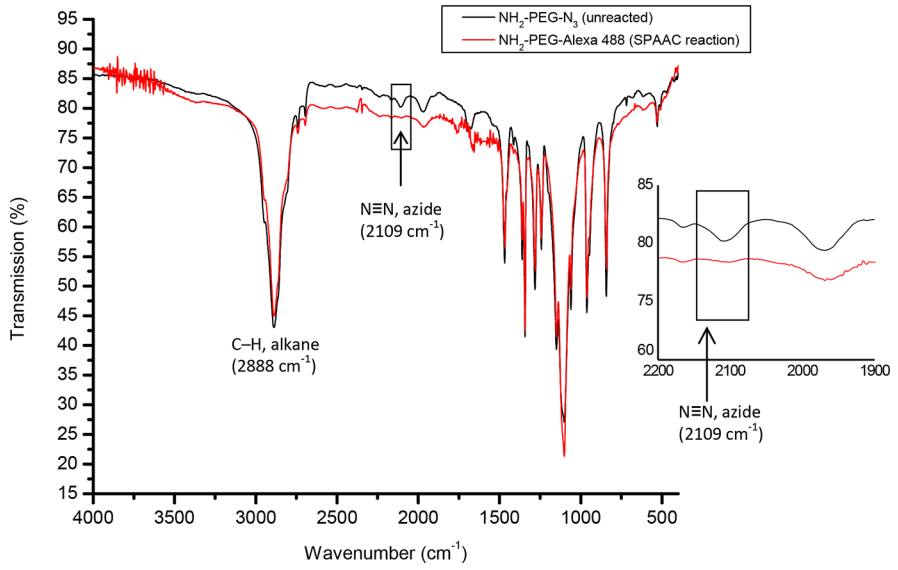

Figure 3. DA Reaction of NH_2-PEG-Furan with Alexa-647-MI. The decrease of the characteristic peaks of maleimide C═C (1466 cm^{-1}) and the appearance of the C═C Diels–Alder adduct peak (1459 cm^{-1}) indicate the successful reaction.

as previously described,[10] according to eq 2, where k is a scaling factor between the radius of the polymeric micelle, $R_{micelle}$, and the aggregation number. For this micelle, k = 0.38 chains/nm^2.

$$N_{agg} = kR_{micelle}^{2} \quad (2)$$

For these micelles, with average diameter of 96.2 nm, the aggregation number is approximately 3500. Micelles synthesized here have a lower average number of chains aggregated compared to similar, previously reported micelles ((6.5 ± 0.4) × 10^3 polymer chains)[10] likely due to relatively fewer TMCC groups in this copolymer.

Coupling of Alexa 488-DBCO and Alexa 647-MI to Functionalized PEG by Click Chemistry. We first verified the coupling reactions in our system using two functionalized dye molecules, Alexa 488-DBCO and Alexa 647-MI, as surrogate ligands for the trastuzumab FLAG peptide and, respectively. To characterize the SPAAC reaction, NH_2-PEG-N_3 (100 nmol) was reacted with Alexa 488-DBCO (500 nmol) overnight and characterized by FTIR (Figure 2). The presence of the terminal azide group (2109 cm^{-1}) in NH_2-PEG-N_3 decreased after reaction with 488-DBCO, demonstrating the success of this reaction.

To verify the Diels–Alder click reaction, NH_2-PEG-furan was reacted with Alexa 647-MI. The characteristic FTIR peaks of furan C═C (1466 cm^{-1}) in the unreacted NH_2-PEG-furan decreased after conjugation with Alexa-647-MI. In addition, the characteristic C═C Diels–Alder adduct peak (1459 cm^{-1}) was present after conjugation, demonstrating the DA reaction (Figure 3). The reaction was further characterized by ^{1}H NMR showing expected peaks for conjugated product: δ 1.10 (m), 1.88 (t) (CH_2 from Alexa-488 MI linker), 3.59–3.69 (m), ($-OCH_2CH_2-$ from PEG) (Supporting Information Figure S5).

Coupling of Alexa 488-DBCO and Alexa 647-MI on Dual-Functional Micelles by Click Chemistry. In order to verify the specificity of the two click reactions with our

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polymeric micelles, we tested multiple combinations of reactants and controls (Table 1). The first reaction, (a),

Table 1. Dual-Functional Micelle Reactions with Maleimide- and Dibenzylcyclooctyne-Functional Moieties (R_1 = Alexa 647; R_2 = Alexa 488)[a]

	average R_1-MI/micelle	average R_2-DBCO/micelle
(a) Micelles + R_1-MI + R_2-DBCO	88 ± 11	270 ± 28
(b) Micelles + R_1-MI (quenched) + R_2-DBCO (quenched)	11 ± 7	18 ± 18
(c) Micelles + R_1-MI + R_2-DBCO (quenched)	110 ± 42	14 ± 11
(d) Micelles + R_1-MI (quenched) + R_2-DBCO	18 ± 11	510 ± 7

[a]The average numbers of each moiety bound to a dual-functional micelles with equal furan and azide groups are tabulated (n = 4; data presented as mean ± standard deviation).

showed efficient binding from sequential click reactions between nanoparticle micelles and Alexa 647-MI followed by Alexa 488-DBCO. Three controls demonstrated the selectivity of the DA and SPAAC reactions: the functional groups of either the Alexa 647-MI or Alexa 488-DBCO, or both, were quenched prior to the click chemical reaction with dual functionalized micelles that had an equal number of azide and furan groups.

For one control (b), both dye molecules were quenched prior to the double click reaction. The results confirmed that there was little nonspecific binding between the dyes and micelles. When only the DBCO was quenched (c), we observed the selective reactivity between the maleimide moiety and available furan groups on the micelle: the average number of Alexa 647-MI groups that bound to each micelle was similar to reaction (a). By contrast, when only the maleimide was quenched (d), the number of Alexa 488-DBCO which bound to the micelle was greater than reaction (a), where the available furan sites were occupied by the Alexa 647-MI, indicating that the DBCO reacted with both azide and furan groups.

We confirmed that the conjugations were due to DA and SPAAC reactions with two additional control reactions: (1) between a furan-only micelle and Alexa 647-MI; and (2) between an azide-only micelle and Alexa 488-DBCO. The DA reaction with the furan micelle and Alexa 488-MI showed an average of 1 dye for every 83 polymer chains (i.e., 1:83 dye/polymer). When the Alexa 488-MI was first quenched with either furfurylamine or cysteine prior to reaction with furan-micelles, the average number of dyes per polymer decreased significantly to 1:1200 dye/polymer chains for furfurylamine prequenched controls and 1:8200 dye/polymer chains for cysteine prequenched controls. These demonstrate that there is little nonspecific binding between Alexa 488 and our polymeric micelles, which is consistent with previous reports of the DA reaction between furan and maleimide groups, as confirmed by FTIR.[24]

For the SPAAC reaction with the azide micelle and Alexa 488-DBCO, we detected an average of 1 dye for every 13 polymer chains (i.e., 1:13 dye/polymer). When the DBCO was first quenched with excess 11-azido-3,6,9-trioxaundecan-1-amine, the number of dyes per polymer dropped to an average of 1:200 dye/polymer chains, demonstrating that there was little nonspecific binding between Alexa 488 and the polymeric micelles.

Coupling of FLAG-DBCO and Trastuzumab-SMCC on Dual-Functional Micelles by Click Chemistry. To demonstrate the functional utility of a dual-labeled polymeric micelle, we performed sequential click reactions with the SMCC-functionalized antibody (trastuzumab-SMCC) and DBCO-functionalized peptide (FLAG-DBCO). Trastuzumab is a humanized antibody that binds human epidermal growth factor receptor 2 (HER2). A FLAG peptide was selected as a simple, model peptide sequence that can be recognized by an antibody. We chose SKOV-3luc cells, a human ovarian cancer cell line that overexpresses HER2, as a target to verify cell binding of the dual-functionalized micelles, through anti-HER2 and HER2 interactions. After the double-click reaction described above, micelle constructs were separated from free, unreacted ligands by size-exclusion FPLC. Micelle elution peaks were completely resolved from unreacted material (Figure 4).

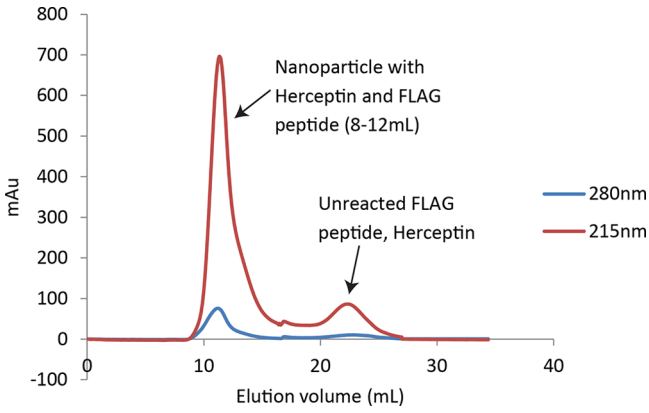

Figure 4. FPLC purification of nanoparticles with trastuzumab and FLAG peptide. Nanoparticles conjugated with trastuzumab and FLAG were separated from the unreacted moieties. Fractions between 8 and 12 mL were incubated with SKOV-3luc cells to observe cell uptake.

Cells were treated with fluorescently labeled antibodies against trastuzumab/FLAG to reveal the cellular localization of the antibody and peptide. Fluorescence images show trastuzumab and FLAG colocalized on the cell surface and inside the cells (Figure 5). Cells treated with nonfunctionalized micelles showed no uptake (Supporting Information Figure S6), thereby demonstrating receptor-mediated cell targeting. If trastuzumab was denatured or was not accessible on the micelle surface, no binding would occur. Likewise, positive staining for the FLAG peptide indicated that the peptide remained intact and accessible on the micelle surface. Since there is, on average, less than one functional group available on each polymer chain, if the micelles were to dissociate, the FLAG peptides would not colocalize with trastuzumab-labeled micelles.

DISCUSSION

Polymeric nanoparticle micelle chemical modifications are limited to aqueous reaction conditions, yet many of these involve cytotoxic catalysts or result in undesirable side reactions.[13] The dual functional micelles synthesized herein provide a platform for facile orthogonal conjugation chemistry, resulting in clean and versatile ligand modification. By forming the micelles prior to the conjugation reaction and using aqueous-based chemistry, we are able to preserve the native structure and activity of the ligands and micelles.

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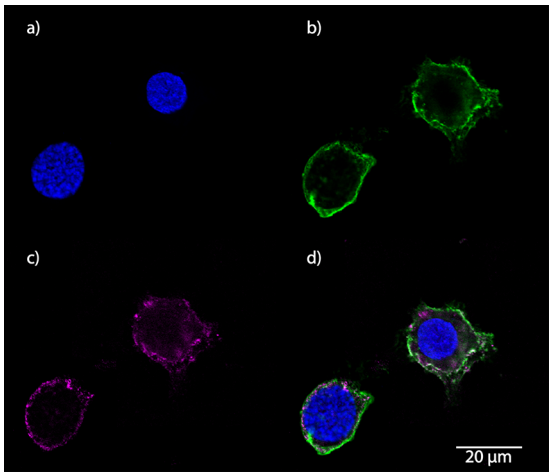

Figure 5. Confocal images of SKOV-3luc cells. Immunofluorescence used to detect trastuzumab and the FLAG peptide. (a) Cell nuclei are stained with DAPI (blue); (b) trastuzumab was labeled with a fluorescent antihuman IgG (488-green); (c) FLAG peptide was labeled with a fluorescent anti-FLAG IgG (647-purple); and (d) overlay of all channels showed cell nuclei surrounded by trastuzumab/FLAG-tagged micelles.

Interestingly, we observed a difference in the reactivity of Diels–Alder and azide–alkyne click chemistries. When the dual functionalized micelles were reacted sequentially with MI and then DBCO, more DBCO conjugated to the micelle than MI. This was also observed for micelles with a single functional group: the SPAAC reaction between the DBCO and azide functionalized micelles was more efficient than the DA reaction between the MI and furan micelle. The difference in conjugation levels may stem from faster reaction rates of SPAAC compared to DA.[25] The expanding library of cyclooctyne and alkene congeners may provide reagents with even faster, more effective reactions.[26]

Using either click reaction, not all functional groups on the micelle were reacted—only approximately 7.7% of azide groups and 1.2% of furan groups reacted on average. Although the functional groups are on the PEG termini, they are not all accessible at the micelle surface for reaction, possibly because the terminal groups are buried within the flexible PEG chain. Importantly, the click conjugated ligands remain active to facilitate interactions with cell surface receptors and antibody probes.

For the double labeling to be most effective, the DA reaction had to be completed prior to the SPAAC reaction because the DBCO was able to bind to both azide and furan whereas maleimide only reacted with furan (see Supporting Information Table S1 for a summary of possible reactions). Notwithstanding this requirement for the sequential DA and then SPAAC reactions, significantly, both reactions were carried out in the same vessel and only one purification step was required after modification.

The use of two reactive groups improves control of the chemical modifications and composition. In current micelle systems, multiple ligands are bound using a single reactive group; as such, the reactions are competitive. In contrast, with dual functional micelles, each ligand reacts independently with a specific receptor, thereby eliminating competition. In addition, multiple functionalities allow for the most effective reaction partners to be selected, based on the properties of each ligand.

Nanoparticle micelles have shown potential as drug delivery vehicles due to their tunable physical properties. One of the challenges with modifying micelles is controlling surface chemistry while maintaining micelle stability.[27] In our system, the aqueous-based click reactions are completed after the micelles have formed, preserving the assembly of the micelles and the bioactivity of the conjugated ligands. The approach of coupling multiple ligands onto a single micelle in a simple manner provides the versatility needed to achieve a number of emerging goals in drug delivery: selective cellular and subcellular targeting, cell targeting with therapeutic delivery, or facilitated codelivery of therapeutic agents with diagnostic probes.

CONCLUSIONS

Using two independent, sequential click reactions, we were able to prepare dual-functional micelles. Sequential reactions with dibenzylcyclooctyne- and maleimide-Alexa dyes allowed us to quantify the conjugation reactions and demonstrate the specificity between the DA and SPAAC reactions. To further demonstrate applicability of the dual-functional micelle, we conjugated trastuzumab antibodies and FLAG peptides onto the self-assembled, polymeric micelles by DA and SPAAC reactions, respectively. The ability to selectively bind two different moieties onto the same micelle results in a platform for future combination strategies, such as multifunctional micelles of therapeutic moieties, diagnostic agents, targeting ligands, and cell penetrating/trafficking peptides.

ASSOCIATED CONTENT

Supporting Information

FTIR and NMR spectra, fluorescence and absorbance measurements, table summarizing possible reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DA, Diels–Alder; DBCO, dibenzylcyclooctyne; CuAAC, copper catalyzed azide–alkyne cycloaddition; SPAAC, strain promoted azide alkyne cyclooaddition; MI, maleimide

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