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ARTICLE

# Simultaneous Orthogonal Chemoligations on Multiresponsive Microgels

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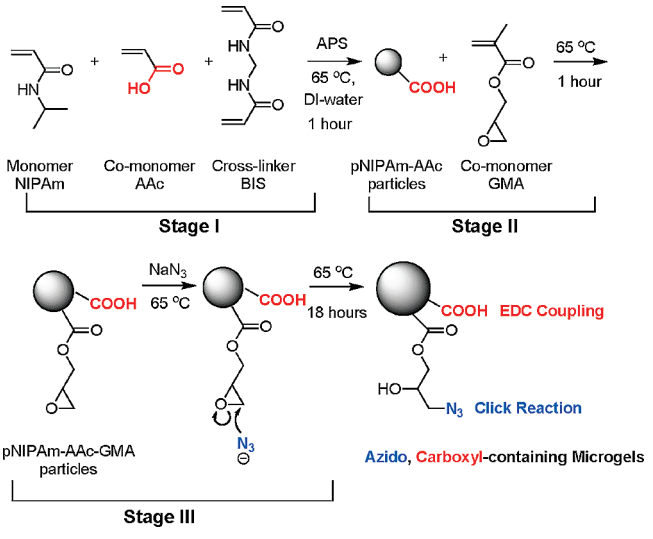
ABSTRACT: We describe the straightforward synthesis of "clickable" multiresponsive microgels containing both carboxylic acid groups and azidohydrin or terminal alkyne groups, via a one-pot multistage polymerization approach. The clickable functional groups on the microgels were confirmed by FTIR. Additionally, we simultaneously performed "click" and acid−amine coupling reactions on microgels with fluorescent dyes containing complementary functional groups. Epifluorescence microscopy was employed to confirm the coupling of those dyes to the microgels. The orthogonality of the click reaction to other functional groups such as hydroxyl, carboxylic acid, and amino groups was confirmed, suggesting the potential utility of such microgels in applications where multifunctional colloidal particles are required.

Microgels are micrometer- or submicrometer-sized polymeric networks swollen in good solvent[1,2] and are called hydrogel microparticles[3] or hydrogel microspheres[4] if the dispersant is water. Solvent occupies the majority of the volume within microgels, as the interactions between the polymer and the solvent dominate over interactions between polymeric chains.[2] However, external stimuli can trigger an abrupt shift toward the dominance of polymer−polymer over polymer−solvent interactions. A classic illustration of this effect is observed in polymers displaying a lower critical solution temperature (LCST), above which the polymer desolvates in an entropically driven phase transition.[5] The most extensively investigated thermoresponsive microgels are composed of poly(*N*-isopropylacrylamide) (pNIPAm),[6,7] which displays an LCST at ~31 °C.[2,7] Furthermore, the copolymerization of acidic or basic comonomers yields microgels that exhibit not only temperature- but also pH- and ionic strength-responsivity.[8,9] As a result of these properties, stimuli-responsive microgels have found applications as targeted drug delivery vehicles[10−12] and in diagnostics.[13] Importantly, targeted drug delivery vehicles often require the presence of multiple different chemical handles for the immobilization of both therapeutic drugs and targeting biomolecules.[14,15] However, the synthesis of such vehicles can be compromised when the targeting moieties and therapeutics possess functionalities that are cross-reactive or do not permit controlled, high-yield coupling to the microgel carrier. To circumvent this problem, the chemical handles should possess chemical orthogonality; they should be chemically inert to common functionalities and reactions in biological environments. The handles should also react rapidly with complementary functionalities under ambient conditions and in aqueous media without interference from other functional groups. Bio-orthogonal click reactions, especially Cu(I)-catalyzed azide-terminal alkyne 1,3-dipolar cycloadditions,[16] meet the above criteria and therefore represent a good choice for functionalization of microgel particles.

The copper(I)-catalyzed azide−alkyne 1,3-dipolar cycloaddition proposed by Sharpless, Kolb, and Finn[17−19] is an excellent example of click chemistry,[17] a versatile synthetic toolbox for fields such as molecular biology,[20] bioconjugate synthesis,[21] drug discovery,[19] organic synthesis,[22−24] and macromolecular and material science.[23,25−27] Click reactions are by definition easy and robust to perform, generate products in high yield with little or no byproducts, and tolerate $O_2$, $H_2O$, or even physiological conditions.[17] Furthermore, due to the very narrow reaction profile of azides and alkynes in biological environments, Cu(I)-catalyzed azide−alkyne cycloaddition is also bio-orthogonal, as no known reactions occur between azido or alkynyl groups and the common functional groups found in biomacromolecules. Whereas metal-free click reactions have recently been developed,[16,28] Cu(I)-catalyzed azide-terminal alkyne cycloaddition is most extensively investigated reaction for bio-orthogonal conjugation of macromolecules.[26,27] In addition to dendrimers[22] and polymers,[23,26,29] this click reaction has also been executed on the microgel particles[30−32] and biological nanoparticles.[33,34] Hawker and Wooley prepared shell-cross-linked (SCK) nanoparticles via a click reaction.[30] The Frechet group developed a

**Scheme 1. One-Pot, Three-Stage Synthesis of Azido- and Carboxylic Acid-Containing, Multiresponsive, Clickable Microgels**

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**Scheme 2. One-Pot, Two-Stage Synthesis of Alkynyl- and Carboxylic Acid-Containing Microgels**

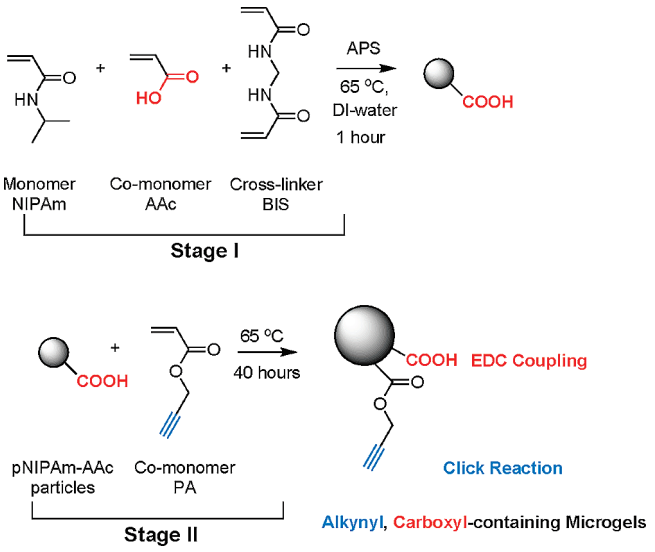
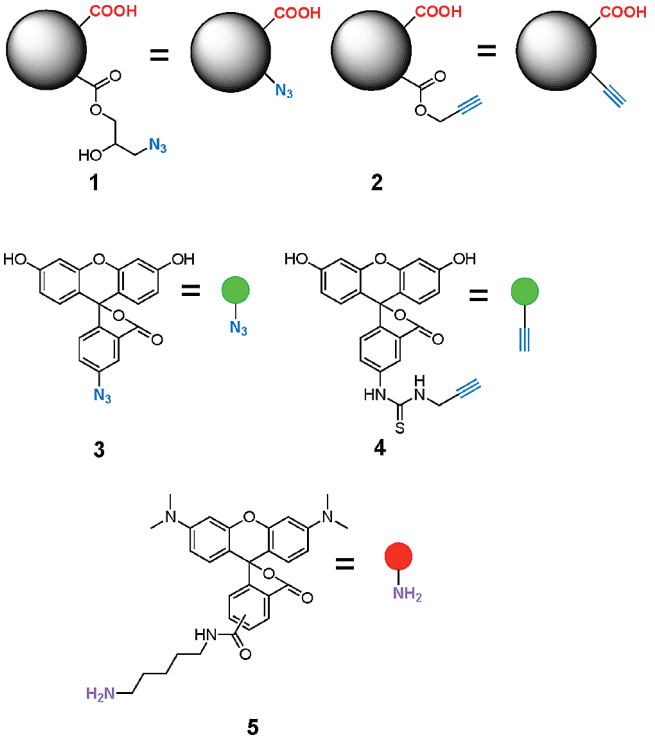



**Chart 1. Azido- and Alkynyl-Containing Microgels and the Corresponding Functionalized Fluorophores**



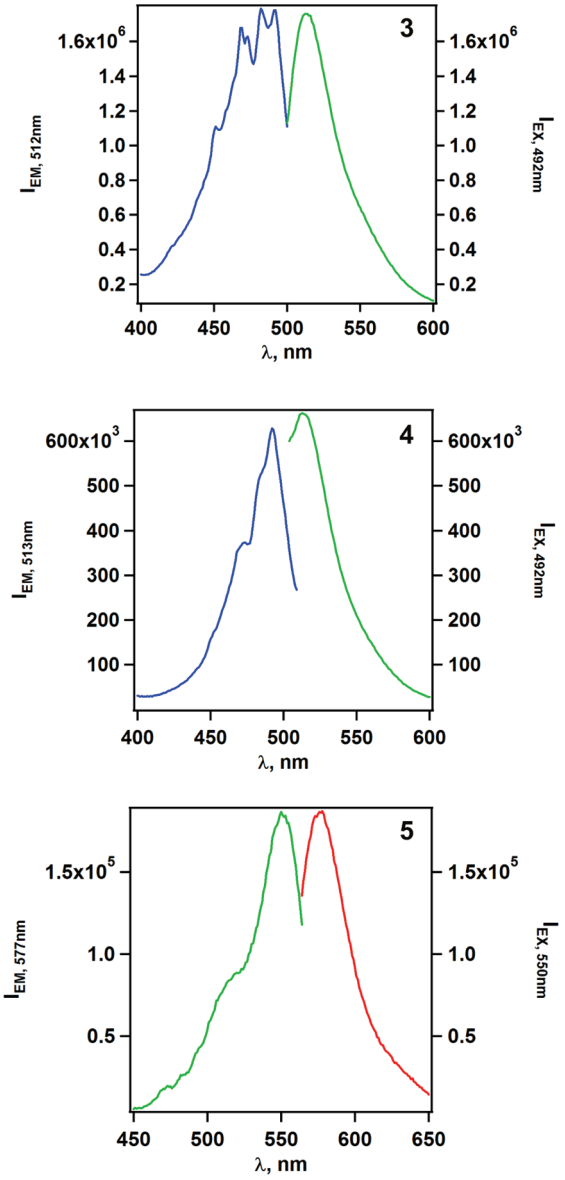




**Figure 1.** Fluorescence spectra of **3**, **4**, and **5** (marked on the top right corner of each panel). The left axes and curves correspond to the excitation spectra; the right axes and curves are the emission spectra. All spectra were measured at 1 $\mu$M in pH 5.0 aqueous buffer.

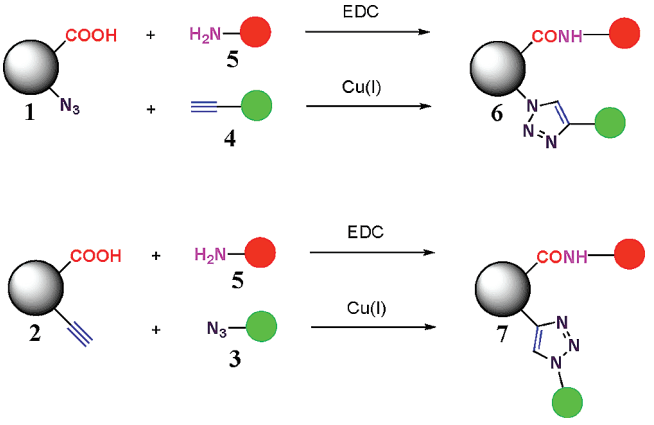
"two-step" polymerization−azidation process to fabricate azido-containing polymer beads for HPLC separation.[31] The Foulger group has prepared alkynyl-containing latex particles to stabilize colloidal crystals.[32] Recently, Liu's group prepared thermoresponsive core/shell polyionic microgel via click cross-linking ionomer complexes.[35] However, none of these papers demonstrated azido-containing microgel particles in a "one-pot" polymerization process. In the present study, we demonstrate a one-pot, multifeed synthesis of microgel particles with either azido or terminal alkynyl groups on poly(*N*-isopropylacrylamide-*co*-acrylic acid) (pNIPAm−AAc) microgel particles. Furthermore, the click reaction between microgel-based azide and terminal alkyne groups with alkynyl- and azido-containg fluorophores confirms the validity of the click reaction on microgel carriers. Additionally, the demonstration of a simultaneous click reaction and a 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) coupling reaction[36] confirms the orthogonality of click reaction to functionalities such as hydroxyl, carboxyl, and amino groups.

To achieve the azido-containing microgel synthesis, we used glycidyl methacrylate as precursor comonomer in one-pot, multifeed copolymerization with *in situ* ring-opening conversion of the epoxy group to an azidohydrin.[37,38] Although the conversion of glycidyl methacrylate (GMA) to 3-azido-2-hydroxypropyl methacrylate (AzHPMA) has been reported previously, those approaches required either a polymeric phase-transfer catalyst in water[39] or samarium chloride hexahydrate catalyst in DMF.[40] In our approach, no specific phase-transfer catalyst or organic solvent was used for azidation of the microgels during polymerization. Furthermore, the one-pot synthesis of azido-containing clickable microgels eliminated the complications involved with synthesis and purification of AzHPMA.[39,40] Compared with seed-feed two-step polymerization our group has used previously

for the synthesis of core−shell microgel particles,[41] one-pot multifeed polymerization is a much more straightforward technique, which still produces particles with a flexible control of functionality distribution.[42] The one-pot, three-stage copolymerization of NIPAm, AAc, and GMA with $NaN_3$ is shown in Scheme 1 (see Supporting Information for synthetic protocols). For all schemes below, carboxylic acid groups for EDC coupling are denoted by red, whereas azido and alkynyl groups for the click reactions are denoted by purple and blue, respectively.

Scheme 1 contains three synthetic stages in a multifeed polymerization. Stage I consists of the copolymerization of NIPAm, AAc, and *N,N′*-methylenebis(acrylamide) (BIS) initiated by ammonium persulfate (APS) in deionized (DI) water at 65 °C for an hour. Stage II consists of the addition of GMA to the microgel copolymerization mixture. The late addition of GMA was performed to circumvent phase segregation of hydrophobic GMA into the core of pNIPAm-based microgels.[43] At this stage, residual NIPAm, AAc, and BIS copolymerized with the added GMA, suggesting that the GMA or derived AzPMA units are distributed close to the surface of microgel particles, which should facilitate subsequent bioconjugation. After another hour of copolymerization, stage III was started with the addition of $NaN_3$ to the copolymerization mixture for the azidation of the GMA moieties *in situ*. Note that the amount of $NaN_3$ added was divided into several portions to avoid an abrupt ionic strength increase, which induces the rapid coagulation of microgels at elevated temperature. The *in situ* azidation of GMA did not inhibit the copolymerization process if the $NaN_3$ was added 2 h after initiation. Under these conditions, it is not known whether the azidation occurs before or after GMA copolymerization with NIPAm or AAc, although given the late addition of $NaN_3$, it is likely that the majority of the azidohydrin formation occurs after GMA incorporation into the microgel. The detailed mechanism of this polymerization is currently under further investigation in our group.

**Scheme 3. One-Pot Click and EDC Coupling on Microgels**




Similarly, we also used propargyl acrylate (PA) as comonomer in a one-pot, two-stage, multifeed approach for the preparation of alkynyl-containing microgels, which is shown in Scheme 2 (see Supporting Information for synthetic protocols). To avoid phase separation of PA into the microgel core, we also delayed the addition of PA until 1 h after the initiation. Note that the reaction time required for high conversion is longer for PA copolymerizations, which is likely due to its relatively slow propagation rate.[32]

The azido and alkynyl groups in the resultant copolymer microgels were confirmed via FTIR,[29] giving strong IR absorptions at 2105 and 2125 $cm^{-1}$, respectively (Supporting Information, Figure S1). The existence of carboxylic acid group was confirmed by pH responsivity of microgels in aqueous buffer (Supporting Information Figure S3). The thermoresponsivity of the microgels (Supporting Information, Figure S4) was essentially identical to that of a pNIPAm homopolymer microgel under these conditions, where the molar ratio in the monomer feed solution was 90:5:5 NIPAm:AAc:GMA/PA.

To examine the click reaction between microgels and fluorescent dyes, the clickable fluorophores, fluorescein propargyl thiourea (FPTU) and 5-azidofluorescein (AzF), were synthesized and purified in our laboratory using published procedures (see Supporting Information for synthetic details). Furthermore, to test the orthogonality of click reaction to EDC coupling between carboxylic acid groups on microgels and fluorescent dyes with amino groups, tetramethylrhodamine-5,6-carboxamide cadaverine (TMRC) was used. The chemical functionalities on the two clickable microgels and the structures of the fluorophores are shown in Chart 1.

The fluorescence spectra of fluorophores **3**−**5** (1 $\mu$M in pH 5.0 buffer) are shown in Figure 1. The azido-containing fluorescent dye **3** (AzF) shows a structured excitation spectrum with a

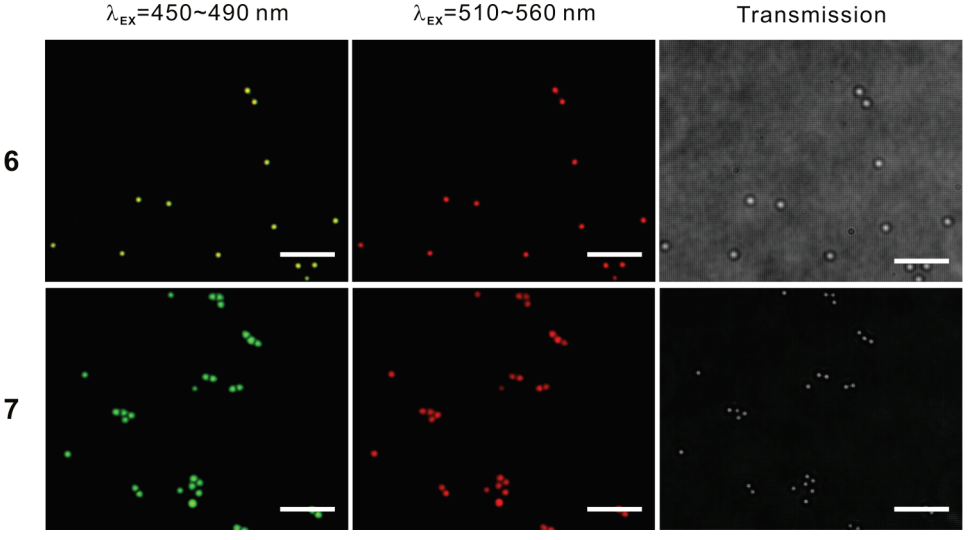



**Figure 2.** Top row: microscopy images of microgel **6**. Bottom row: microscopy images of microgel **7**. Images in the left and central columns show fluorescence of the microgels excited by irradiation of 450−490 and 510−560 nm, respectively. Transmission microscopy images are displayed in the right column. Scale bar = 5 $\mu$m.

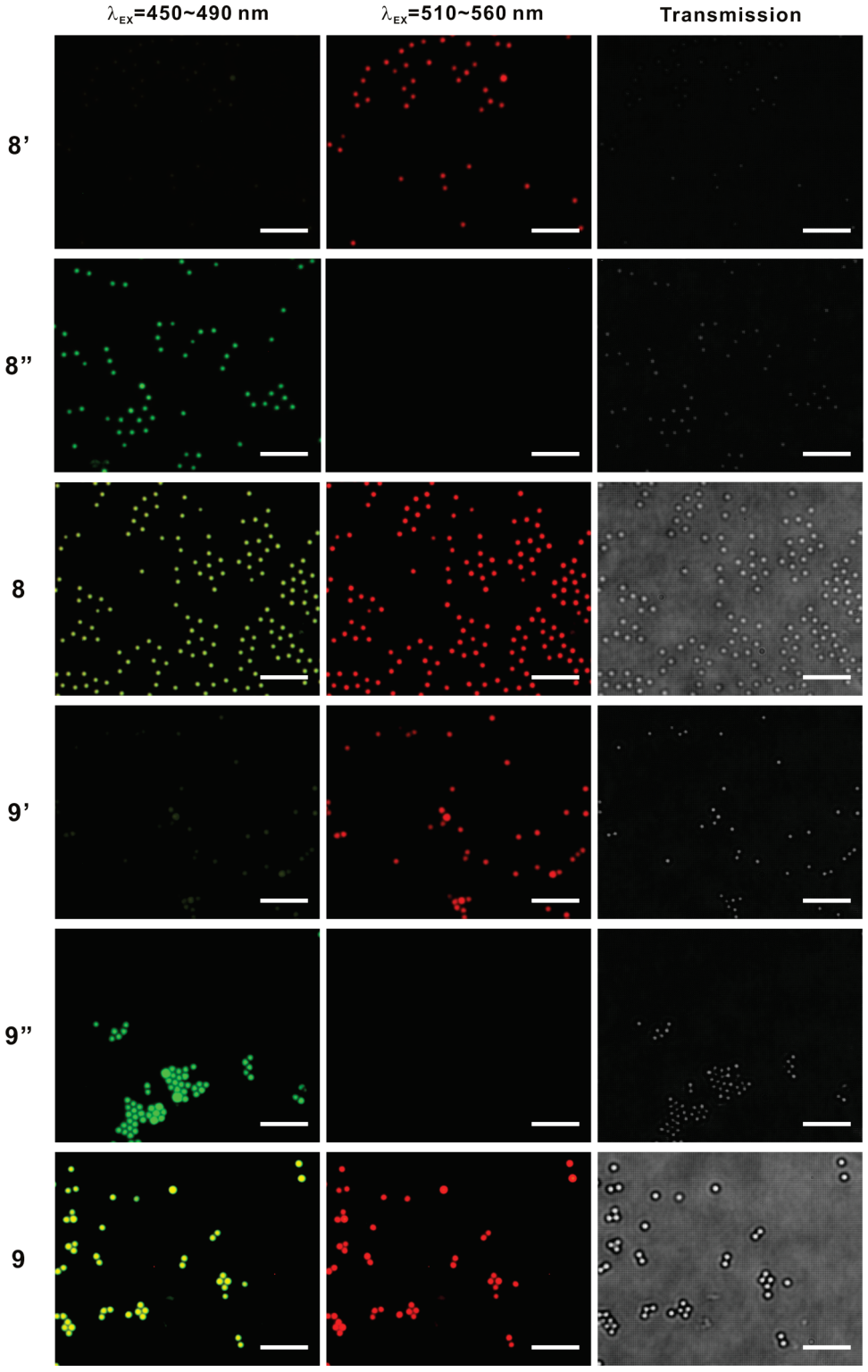
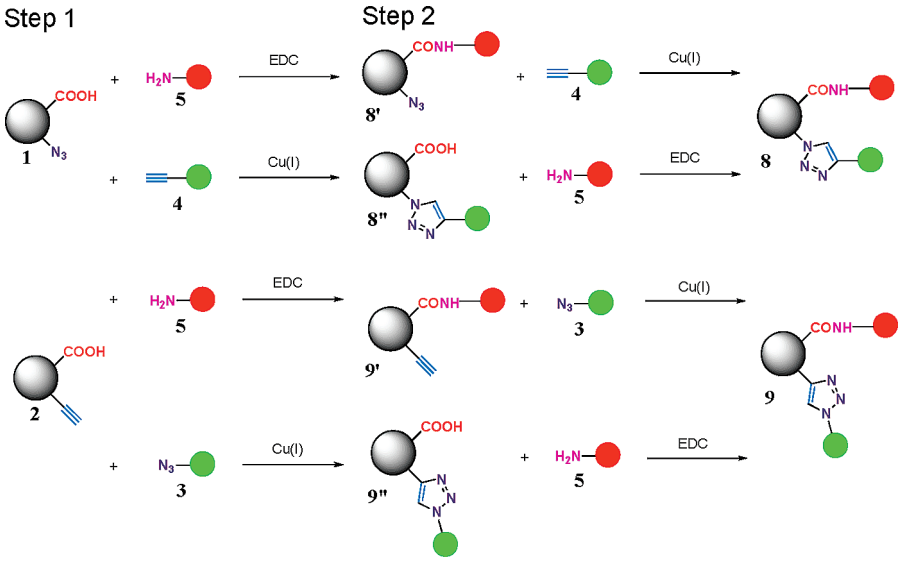



**Figure 3.** The top three rows show microscopy images of microgels obtained by either first EDC coupling of azide/carboxylic acid microgel **1** with amino-containing fluorophore **5** (**8′**, row 1) followed by clicking with alkynyl-containing fluorophore **4** (**8**, row 3) or first clicking with **4** (**8″**, row 2) followed by EDC coupling with **5** (not shown, similar to **8**). The bottom three rows show microscopy images of microgels obtained by either first EDC coupling alkyne/carboxylic acid microgel **2** with amino-containing fluorophore **5** (**9′,** row 4) followed by clicking with azido-containing fluorophore **3** (**9**, row 6) or first clicking with **3** (**9″**, row 5) followed by EDC coupling with **5** (not shown, similar to **9**). The left and middle columns are fluorescence microscopy images obtained by the excitation of 450−490 and 510−560 nm, respectively. The right column contains bright-field transmission images. Scale bar = 5 $\mu$m.

maximum absorption at 492 nm. The excitation spectrum of the alkynyl-containing dye **4** (FPTU) shows two absorption peaks (470, 492 nm) with a maximum absorption at 492 nm. The excitation spectrum of the amine-containing dye **5** (TMRC) shows a shoulder at 520 nm and a maximum peak at 550 nm. In contrast to excitation spectra, the emission spectra of three fluorophores are quite simple. Fluorophores **3**, **4**, and **5** show maximum fluorescence emission at 512, 513, and 577 nm,

**Scheme 4. Two-Step Coupling to Microgels 1 and 2**




respectively. It is clear that the fluorescence emission of **3** and **4** partially overlap with the excitation spectrum of **5**, which might lead to fluorescence resonance energy transfer (FRET) from **3** or **4** to **5**. Indeed, some results below are suggestive of some degree of FRET. These effects have not yet been quantified but are under further study in our laboratories.

Epifluorescence microscopy was used to determine if click reactions or EDC coupling occurs between functional microgels and fluorophores with complementary functionalities. The click reaction between azide microgel **1** and alkyne fluorophore **4** was performed with Cu(I) catalysis in aqueous media at ambient temperature. The EDC coupling between the carboxylic acid groups on microgel **1** with amine-containing fluorophore **5** was performed simultaneously with the click reaction to test coupling orthogonality. Similarly, the click reaction of alkyne microgel **2** with azide fluorophore **3** with Cu(I) catalysis and EDC coupling of microgel **2** with fluorophore **5** were also performed simultaneously. All microgel−fluorophore ligations were allowed to proceed in pH 5.0 aqueous buffer at ambient temperature. The one-pot click reaction + EDC coupling between microgel **1** and dyes **4** and **5** or between microgel **2** and dyes **3** and **5** is shown in Scheme 3.

The one-pot, simultaneous ligation of **1** with **4** and **5** gave rise to microgel **6**. Similarly, the one-pot, simultaneous ligation microgel **2** with **3** and **5** gave rise to microgel **7**. After reaction with the fluorophores, the resultant microgels were cleaned extensively by a centrifugation−redispersion procedure to remove residual Cu(I) species and nonspecifically adsorbed fluorophores (details in Supporting Information). After purification, microgel dispersions of **6** and **7** were dried on clean glass slides. The fluorescence microscopy images of microgels **6** and **7** are shown in Figure 2; note that for microgel **6** excitation of **4** yields a somewhat yellow emission color, suggesting either simultaneous excitation of **5** or FRET is occurring in this case.

To compare with the simultaneous ligation method, two-step ligations of microgels **1** and **2** were also performed. Azido- and carboxylic acid-containing microgel **1** can either first click with **4** followed by EDC coupling with **5** or first EDC couple with **5** followed by clicking with **4**. Alkynyl- and carboxylic acid-containing microgel **2** can also click with **3** and EDC couple with **5** in a similar manner. The two-step chemoligations of fluorophores on multiresponsive clickable microgels are illustrated in Scheme 4.

The fluorescently labeled microgels produced in steps 1 (**8′**, **8″**, **9′**, and **9″**) and 2 (**8** and **9**) were purified for fluorescence microscopy. Similar purification protocols were used for microgels after ligation in each step (details are shown in the Supporting Information). After purification, microgel dispersions **8** and **9** were coated and dried on the cleaned glass slides. The fluorescence microscopy images of microgels **8** and **9** are shown in Figure 3. By comparing the fluorescence microscopy images of microgels after one-pot simultaneous ligation and two-step ligation, it is believed that the catalytic activity of Cu(I) are not significantly impaired by EDC and vice versa.[44,45] Additionally, after coupling of **5**, the fluorescence observed under blue irradiation (450−490 nm) changes from green to yellow, again suggesting either direct excitation of **5** at this wavelength or that fluorescein-to-rhodamine FRET is occurring. Since the leftmost panels in columns 1 and 4 show some faint evidence of fluorescence, which is likely due to direct excitation of **5**, it is likely that the strong yellow emission observed for **8** and **9** under blue excitation is due to a combination of direct excitation of **5** and FRET. As discussed above, these phenomena are currently being investigated in our group.

To further illustrate the fidelity of these coupling reactions, control experiments were designed to examine the reactivity of azido/alkynyl groups with amino groups and the orthogonality of click reactions to EDC coupling (detailed experimental design and characterizations are documented in the Supporting Information, Figure S5 and Schemes S1 and S2). The control experiments confirmed that the click reaction is orthogonal to other functional groups such as hydroxyl, carboxyl, and amino groups. However, some small amount of cross-reactivity is apparently observed during EDC coupling of microgel **11** (azidohydrin-modified) and fluorophore **5** (control experiment II.B in the Supporting Information). It is likely that this arises from coupling between the alcohol on the azidohydrin and the carboxylic acid moiety[46,47] on the fluorophore (tautomer of the lactone ring[48]). Despite this slight complication, all other negative controls fail to produce coupled fluorophores, illustrating the robustness of the method for producing multifunctional microgels for orthogonal chemoligations.

To summarize, we have prepared azido- and alkynyl-containing multiresponsive microgel particles in a one-pot, multifeed protocol. Furthermore, the simultaneous ligation of the microgels with fluorophores containing complementary functionalities via both click chemistry and EDC coupling was demonstrated using fluorescence microscopy. A wide range of control experiments illustrate the excellent specificity of the coupling reactions,

suggesting that such particles may be useful for synthesis of bioconjugates not typically attainable using reactions that rely exclusively on biologically prevalent functional groups.

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**Supporting Information Available:** Experimental descriptions, one-pot, multifeed synthetic protocols and FTIR of microgels **1** and **2**, $^1$H NMR of fluorophores **3** and **4**, fluorescence spectra of fluorophores **3**, **4**, and **5**, one-pot simultaneous click reaction and EDC coupling of microgels **1** and **2** with fluorophores **3**, **4**, and **5** with complementary functionalities, two-step sequential ligation of microgels **1** and **2** via click reaction and EDC coupling with fluorophores **3**, **4**, and **5**, synthetic protocols of clickable microgels for control experiments, control experiment design and implementation, fluorescence microscopy images of control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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