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Chemically Orthogonal Three-Patch Microparticles**

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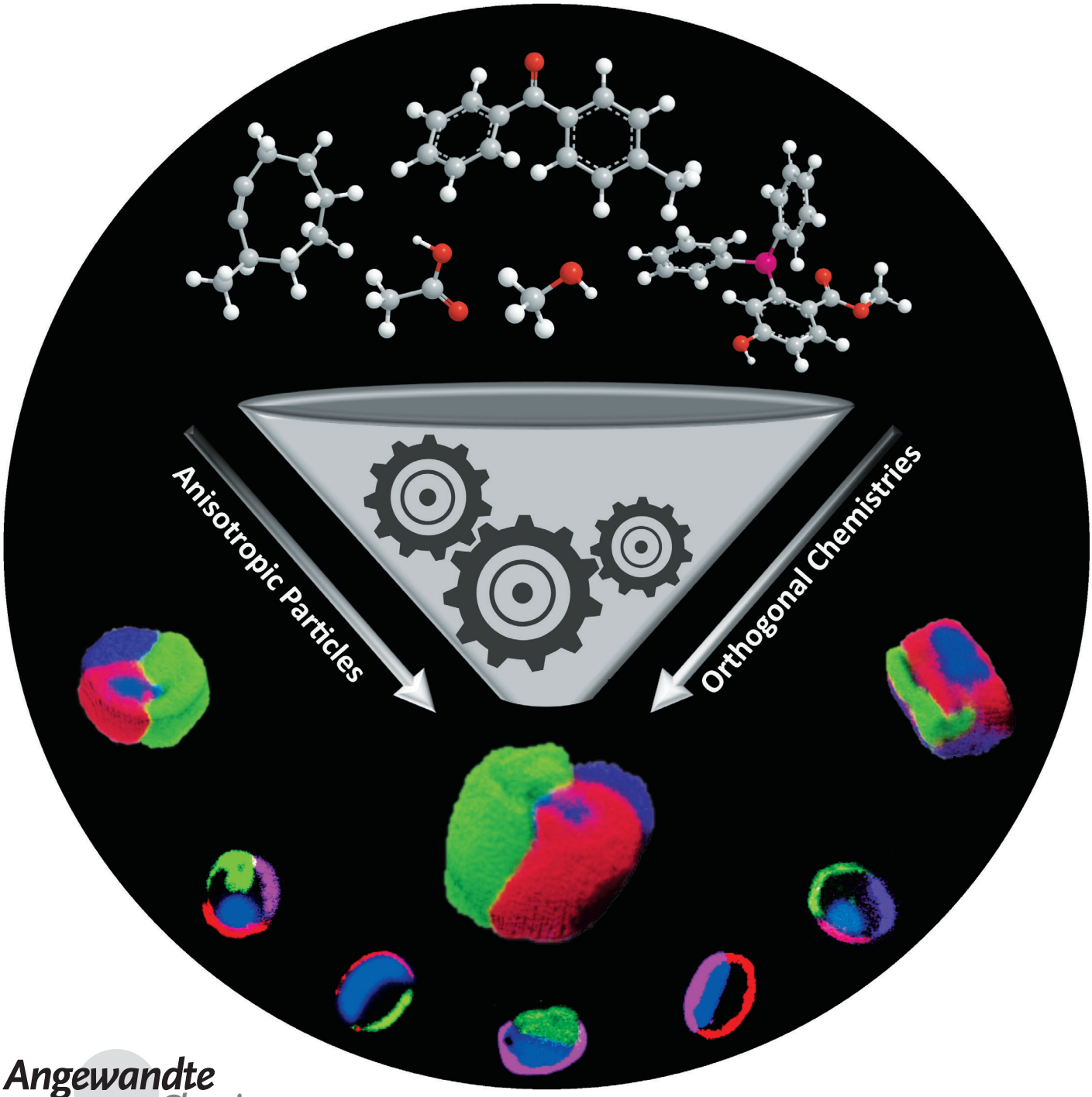

Abstract: *Compared to two-dimensional substrates, only a few methodologies exist for the spatially controlled decoration of three-dimensional objects, such as microparticles. Combining electrohydrodynamic co-jetting with synthetic polymer chemistry, we were able to create two- and three-patch microparticles displaying chemically orthogonal anchor groups on three distinct surface patches of the same particle. This approach takes advantage of a combination of novel chemically orthogonal polylactide-based polymers and their processing by electrohydrodynamic co-jetting to yield unprecedented multifunctional microparticles. Several micropatterned particles were fabricated displaying orthogonal click functionalities. Specifically, we demonstrate novel two- and three-patch particles. Multi-patch particles are highly sought after for their potential to present multiple distinct ligands in a directional manner. This work clearly establishes a viable route towards orthogonal reaction strategies on multivalent micropatterned particles.*

The directionally controlled presentation of chemical ligands on the surface of particles defines critical materials processes, such as three-dimensional gelation,[1] directed self-assembly,[2] or the controlled interaction of particles with biological cells.[3] While the spatially controlled presentation of chemical and biological ligands is well established for two-dimensional substrates,[4] very few methodologies exist for the spatially controlled decoration of three-dimensional objects, such as microparticles.[2f,5] Many of the patterning methods for two-dimensional substrates including photolithography, microcontact printing, dip-pen nanolithography, or block-copolymer micelle nanolithography,[4d,6] are not easily extendable to the three-dimensional surfaces of microparticles. Thus, there is a clear need for efficient microstructuring methodologies for *meso*-scale particles and some of the more promising strategies include microfluidic and microforming techniques,[5c,7] template-assisted polymerizations,[8] electrohydrodynamic co-jetting,[9] or the use of pickering emulsions.[10] With the requirement for spatially controlled immobilization of ligands also comes an increased need for the parallel immobilization of multiple, chemically distinct ligands on defined and independent interfacial patches of the same object. While most multi-ligand attachment strategies have relied on statistical distributions of ligand mixtures,[11] several studies have emphasized the need for the independent attachment of two or more ligands on the same surface by orthogonal immobilization strategies.[12] In this case, an important prerequisite is the compatibility with the biological "reaction" environment. Bertozzi et al. coined the term bio-orthogonal ligation to express the need for chemical selectivity relative to 1) the biological environment and 2) the different types of ligands to be co-presented on different surface patches.[13] Typically, different orthogonal immobilization schemes take advantage of click-type reactions and, in case of two-dimensional substrates, have been well-established,[14] including "double-click" strategies.[15] Recent work has revealed the potential of particles with multiple surface patches, however, the orthogonal functionalization of different surface locations still poses significant challenges.[16] Herein, we now report the spatially controlled immobilization of three chemically distinct patches on the same microparticle by orthogonal surface reactions. Combining electrohydrodynamic (EHD) co-jetting with synthetic polymer chemistry, we were able to create two- and three-patch microparticles displaying chemically orthogonal anchor groups on three distinct surface patches of the same particle.

As depicted in Figure 1, the preparation of multi-patch microparticles was achieved by EHD co-jetting of up to three polymer solutions in parallel. The EHD co-jetting process yielded well-defined microfibers that were subsequently sectioned into multi-patch particles.[17] In this study, we employed disk-shaped microparticles with an average diameter of 10–15 μm and an average aspect ratio of 1:2 (height:diameter). Size and shape of the microparticles can be controlled within a broad range,[17] but were ultimately selected to be in the 10–20 μm range for practical reasons, such as optimal imaging of the individual chemical surface reactions by fluorescence and Raman confocal microspectroscopy. The polymer used throughout this study was a biodegradable poly(lactide-*co*-glycolide) (PLGA) polymer. To impart chemical orthogonality, several different chemically functionalized polylactide (PLA) polymers were synthesized. The chemically orthogonal PLA derivatives can be added to the different PLGA compartments to provide chemical patches for directionally controlled surface modification. In our case, a set of five different PLA derivatives was selected for their ability to support orthogonal surface modification without cross-reactions (Figure 1).

The first step of the synthesis of the functionalized PLA derivatives **2**–**5** involved the ring-opening co-polymerization of monomer **1** and L-lactide in the melt, and subsequent palladium-catalyzed hydrogenation of the benzyl ether bonds to yield the hydroxy-modified PLA derivative **2** (Supporting Information Section 2.1). Polymer **2** was then used as the starting point for further diversification of the functionalities

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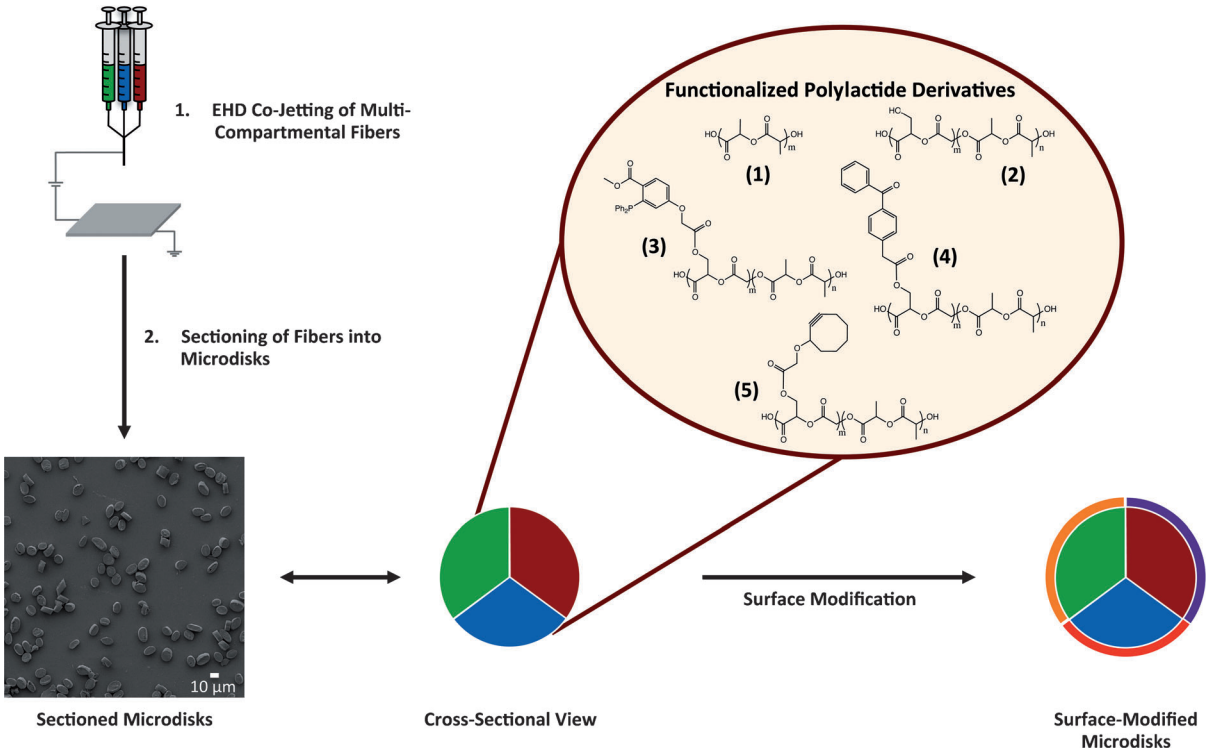

Figure 1. Fabrication of microparticles with orthogonally functionalized interfacial patches by EHD co-jetting. Functionalized polylactide derivatives (**1**–**5**) are incorporated into different jetting solutions, leading to compartmentalized fibers, which can be sectioned into the corresponding microdisks. Each compartment surface establishes a unique surface patch that is then selectively modified by orthogonal reactions.

using a number of post-polymerization modifications: 1) Reaction with 3-(diphenylphosphino)-4-(methoxycarbonyl) benzoic acid with *N*,*N*′-dicyclohexylcarbodiimide (DCC)/4-dimethylaminopyridine (DMAP) yielded the PLA derivative **3** for subsequent Staudinger Reaction. 2) Alternatively, polymer **2** was converted with 2-(4-benzoylphenyl) acetic acid and DCC/DMAP under dry conditions to yield the photoreactive PLA derivative **4**. 3) Polymer **5**, a cyclooctyne-modified polylactide, was derived from polymer **2** by straightforward conversion with 2-(cyclooct-2-yn-l-yloxy) acetic acid and DCC/DMAP (Supporting Information Section 2.2).

In general, we found a satisfying compatibility of the PLA derivatives with the PLGA base polymer: For concentrations of up to 50% of the PLA additives, no adverse effect on the electrohydrodynamic co-jetting process was observed—independent of the chemical nature of the PLA derivative that was added to the jetting solutions. On the other hand, preliminary immobilization experiments demonstrated that PLA concentrations above 20% were adequate to ensure effective surface coupling of model ligands (Supporting Information Section 2.3).

We then created a group of bicompartmental microparticles, which presented a single hemispheric patch selectively displaying only one of the functional anchor groups. In this case, the second hemisphere was composed of the PLGA base polymer only, and served as an internal reference for the surface reactions. Figure 2 depicts three different particle architectures. These architectures allowed for spatially controlled modification of one hemisphere only. The reference patch showed only very low levels of non-specific ligand adsorption. In Figure 2A, PLGA particles with one hemispheric surface patch containing polymer **3** (with green dye) was treated with azide-PEG-Biotin (PEG: polyethylene glycol) by Staudinger ligation. The biotin was then labeled with TRITC-Streptavidin (red dye) for imaging purposes. Coexistence of the red and green fluorescence in the CLSM images (Figure 2A3) confirms the spatially controlled surface modification of the microparticles. Similarly, Figure 2B displays particles with polymer **4** in the blue hemisphere only. As found for all PLA derivatives, the functional polymer is restricted to only one hemisphere of the microparticles (Figure 2B1) underpinning the high degree of patchiness obtained by the EHD co-jetting process. The microparticles were subsequently incubated with a protein, Bovine Serum Albumin (BSA) tetramethylrhodamine (red dye), and exposed to UV light at 365 nm to initiate the photoimmobilization of the BSA to the reactive surface patch (Figure 2B2). The high degree of selectivity of the photoimmobilization reaction is confirmed by the spatially controlled surface binding of the protein, as depicted in the overlay images of Figure 2B3. Similarly, the copper-free click chemistry of bicompartmental microparticles using polymer **5** was successfully carried out, as verified by CLSM analysis (Figure 2C). In this case, the unreactive PLGA hemisphere was labeled with a green fluorescence dye loaded in the bulk of the compartment, whereas the azide-reactive compartment

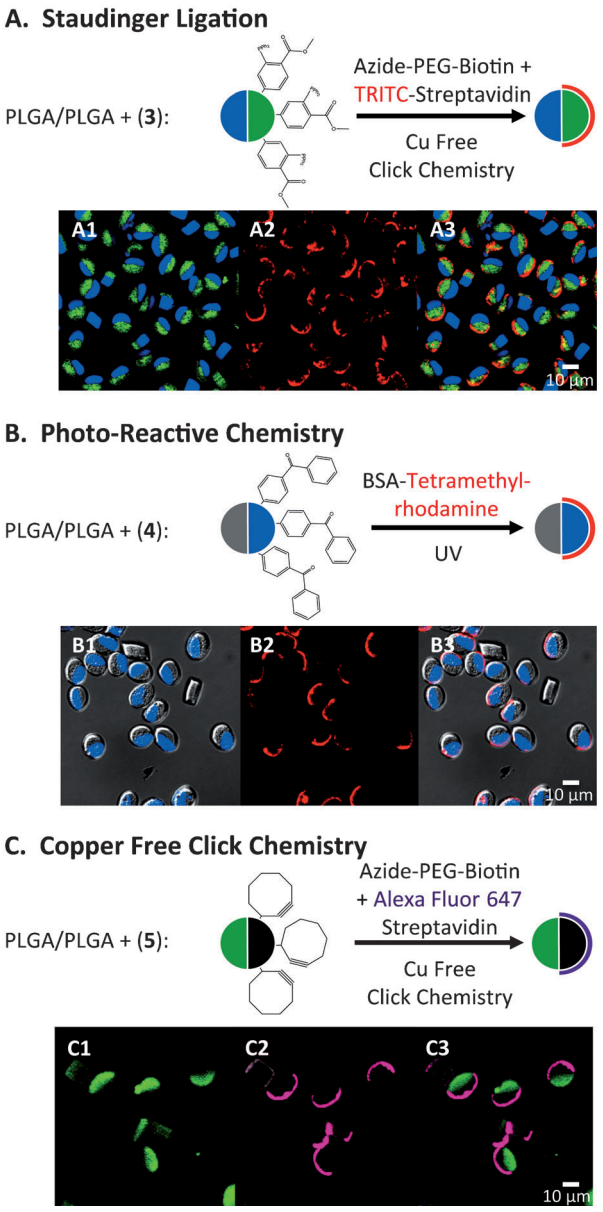

Figure 2. Selective surface modification of microparticles containing three different orthogonally functionalized PLA derivatives exclusively present in one hemisphere. In (A)–(C), microparticles displaying polymers **3** to **5** in one hemisphere only are selectively surface functionalized through Staudinger ligation (A), photo-immobilization (B), and alkyne/azide click chemistry (C). CLSM images show the spatioselective nature of the surface modifications. See text for details.

that contained polymer **5** was non-fluorescent (Figure 2 C1). The spatially controlled surface modification of these patchy microparticles with azide-PEG-biotin and Alexa Fluor 647 Streptavidin (magenta dye) was confirmed by selective surface binding, as shown in the overlay images of Figure 2 C3. We further conducted spatially controlled surface modification of two-patch particles with one functionalized patch using both polymers **1** and **2** with analogue results (Supporting Information 2.3-4). In summary, this initial immobilization studies indicated two important findings: 1) The addition of functionalized PLA derivatives into one jetting stream resulted in well-defined patchy microparticles and 2) the selected surface chemistries are fully orthogonal to the base polymer (PLGA), as shown by the fact that only negligibly low levels of non-specific binding were observed on the reference hemisphere.

Encouraged by these initial data, we conducted a second immobilization study that included two different functionalized PLA derivatives in two separate hemispheres of the same particle. For example, Figure 3 displays the CLSM, Raman characterization, and surface functionalization of these two-patch particles containing the two functional polymers **1** and **4** in separate compartments. To confirm that polymers **1** and **4** were indeed localized in different hemispheres, rather than mixed throughout the entire particle, Raman confocal microspectroscopy was employed. This method can be used to gain chemical information of two- and three-dimensional substrates and has been used to characterize Janus particles.[9b] In this case, microparticles containing a low-molecular-weight PLGA polymer with a higher number of free carboxylic acid end groups in one hemisphere and a mixture of PLGA and polymer **4** in the second hemisphere were imaged by Raman microspectroscopy (Figure 3 A). In Figure 3 A1, the two Raman spectra that were obtained from the two different hemispheres are shown. The spectrum obtained on the PLGA hemisphere is shown in red, while the spectrum obtained on the hemisphere displaying polymer **4** is indicated by a yellow color. In addition to the expected PLGA bands, the yellow spectrum further revealed characteristic bands indicative of polymer **4**. Specifically, two additional bands at 1610 and 1660 cm^{-1} signify the presence of benzophenone groups (Supporting Information Section 2.5). Therefore, the Raman maps shown in Figure 3 A3–A5 unambiguously confirm that the PLGA base polymer is present in both hemispheres (red), whereas the PLA derivative **4** is restricted to one side only (yellow). The two hemispheres were allowed to react with BSA tetramethylrhodamine (red dye) in the presence of UV light (first step) and amine-PEG-FITC (green dye) and EDC/sulfo-NHS (second step). The hemisphere containing polymer **4** autofluoresces in the blue channel (Figure 3 B3) to further distinguish the two compartments. Subsequent analysis with confocal laser scanning microscopy (CLSM) confirmed successful, yet highly selective surface modification of both surface patches (Figure 3 B2–B3). Taken together, the Raman microspectroscopic analysis and CLSM support the successful orthogonal surface reactions on these two-patch microparticles.

Beyond this particular combination of functionalized PLA derivatives, a number of different orthogonally substituted microparticles were prepared and selectively surface-modified, further elevating the potential scope of this novel approach. As depicted in Supporting Information Section 2.6, two-patch microparticles containing polymer **2** (with blue dye) and **5** (black, no dyes) in different hemispheres were prepared and the selective surface functionalization was demonstrated as well. Further expanding on the complexity of possible multifunctional particles, we adapted our EHD co-jetting strategy to create three-patch microparticles. These particles, shown in Figure 4, contained patches displaying three different functionalized PLA derivatives, that is,

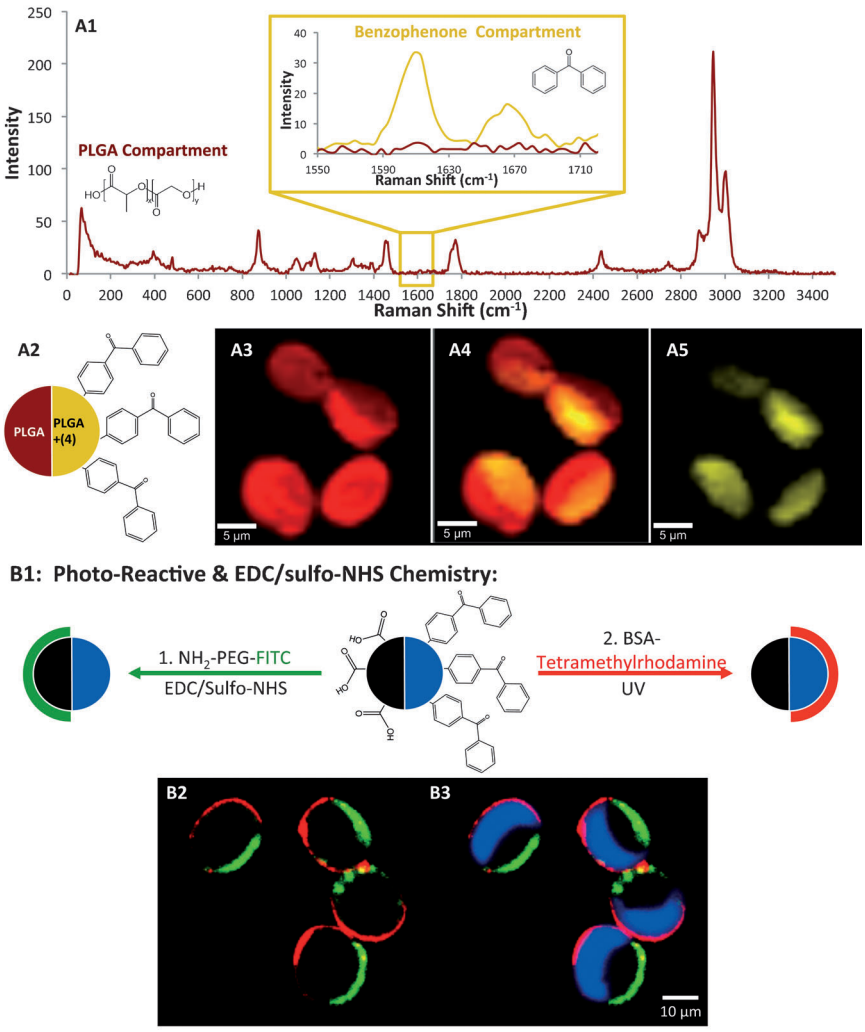

Figure 3. Selective surface modification of two-patch microparticles. Microparticles with chemically orthogonal polymers **1** and **4** in different hemispheres were characterized through Raman microspectroscopy (Figure 3 A), selectively surface-modified, and characterized using CLSM imaging (Figure 3 B). See text for details.

polymers **1**, **4**, and **5**. As noted above, it is important to ensure that the functionalized polymers are indeed localized in specific surface patches prior to conducting orthogonal surface modification. In fact, the defined localization of the reactive polymer species is an essential prerequisite for spatially controlled surface modification. Figure 4 A displays the results of the Raman microspectroscopic analysis of three-patch particles. The Raman spectra obtained from the three patches displayed characteristic bands of the PLGA base polymer (blue). In addition, characteristic bands of polymer **4** at 1610 and 1660 cm^{-1} for benzophenone (black), and at 2200 cm^{-1} for the cyclooctyne of polymer **5** (red) were located in different surface regions, as shown in Figure 4 A1 (Supporting Information 2.7.) The two-dimensional reconstruction of the Raman spectra displays three distinct patches on these microparticles. The PLGA polymer is detected in the entire particle (blue, Figure 4 A3), while polymer **4** (white, Figure 4 A4) and polymer **5** (red, Figure 4 A5) are each restricted to two different surface hemispheres. We further conducted a series of orthogonal surface reactions on these microparticles and analyzed them by fluorescence confocal microscopy. The schematic for the sequential chemistries and the results obtained by CLSM imaging are shown in Figure 4 B1 and 4 B2, respectively. In this case, the blue patch, containing polymer **4**, was functionalized by photochemical attachment of BSA tetramethylrhodamine (red dye), while the selective conjugation of azide-PEG-FITC (green dye) was restricted to the second surface patch that contained polymer **5**. Efficient binding was achieved through click chemistry with the cyclooctyne groups of polymer **5**. The third compartment, containing polymer **1**, was treated with amine-PEG-Biotin through EDC/sulfo-NHS chemistry and labeled with Alexa Fluor 647 Streptavidin (magenta dye) for CLSM imaging.

Figure 4 B3 shows the three-dimensional reconstruction of the microparticles on the basis of the fluorescence images collected in the *z*-direction. The particles were imaged in the *xy*-plane with a step size of 250 nm and were then reconstructed to display the three-dimensional structure and characteristics of the patchy microparticles. We note that these results correspond well with the cross-sectional view (Figure 4 B3, right image).

In conclusion, we have demonstrated an approach towards microparticles with fully orthogonal surface patches that takes advantage of a combination of novel chemically orthogonal polylactide-based polymers and their processing by electrohydrodynamic co-jetting to yield unprecedented multifunctional microparticles. These and other microstructured particles are highly sought after for their potential to present multiple distinct ligands in a directional manner. Applications may range from novel gels and particle self-assembly to use as carriers for cancer therapies with synergistic targeting effects from complementary ligands. For many of the above-mentioned applications, smaller particles are required. While designed as a proof-of-concept study to establish the feasibility of orthogonal reaction strategies on multivalent particles, future work will need to focus on refining critical particle characteristics, such as size or shape, while maintaining the same precision with respect to compartmentalization and spatially controlled surface modification. However, we have already shown that simpler versions of multi-patch particles can be made as small as 200 nm.[18] It is thus plausible that future work can access

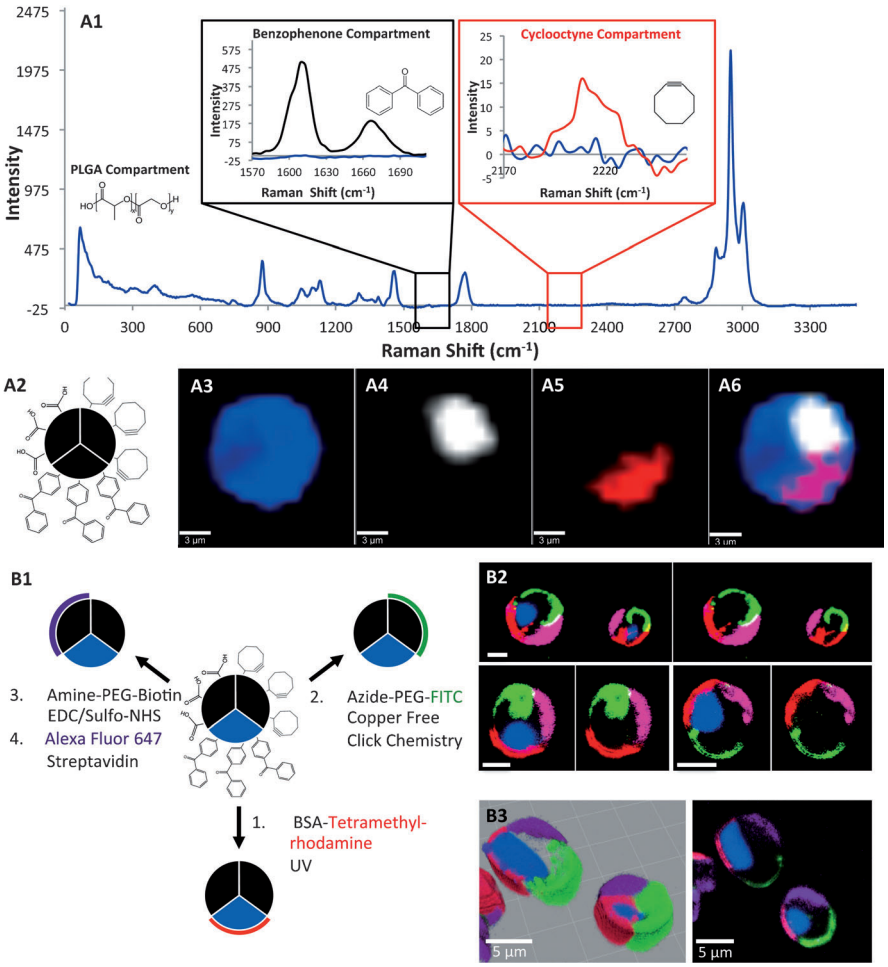

Figure 4. Characterization and selective surface functionalization of three-patch microparticles. Particles containing polymers **1**, **4**, and **5** in separate surface patches were characterized using Raman microspectroscopy (A). The surface of each patch was then selectively modified using orthogonal chemistries and analyzed through CLSM imaging (B). Unless noted, all scale bars are 5 µm. See text for details.

similar size ranges with the type of orthogonal multi-patch particles described in this study.

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