Electronic Supplementary Information

Chemically orthogonal trifunctional Janus beads by photochemical "sandwich" microcontact printing

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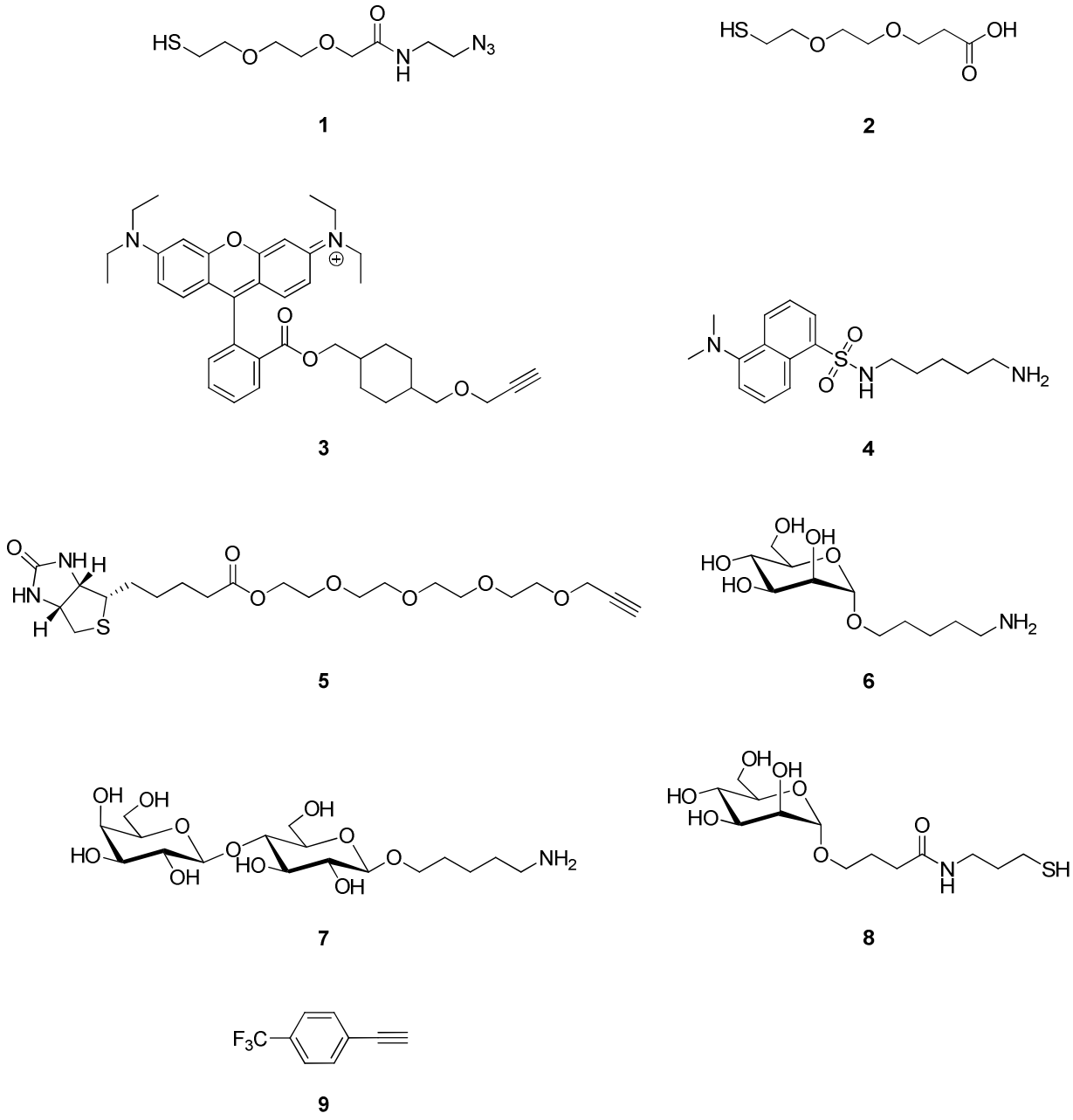
1. General methods:

Unless otherwise noted, starting compounds and solvents were purchased from Sigma Aldrich (St. Louis, MO/USA), Thermo Fisher Scientific Inc. (Waltham, MA/USA), Merck KGaA (Darmstadt, Germany) or ABCR GmbH & Co KG (Karlsruhe, Germany) and were used as received. DMF used for reactions was always meeting SPPS grade and ethanol used for ink solution preparation was absolute standard. Inert conditions include a protective argon atmosphere created by standard Schlenk techniques. Preparative silica gel column chromatography was carried out using silica gel with 40-65 µm grain size purchased from Merck KGaA (Darmstadt, Germany). Solvents used as mobile phase in column chromatography were purified by distillation prior to use. TLC was done on silica coated aluminum sheets with added fluorescence indicator (60 F254, Merck, Darmstadt, Germany). Spots were detected by UV light (254 nm or 365 nm) and/or by oxidation using basic potassium permanganate solution. PDMS stamps were prepared using a standard method established in literature.[2] Fluorescence and light microscopy was done using a Reflected Fluorescence System CKX41 (Olympus, Shinjuku, Tokyo/Japan) in combination with a Kappa DX L-FW camera (Kappa optronics GmbH, Gleichen/Germany) and Kappa imageBase Control (version 2.7.2) as operating software. ToF-SIMS measurements were conducted at the Physikalisches Institut WWU Münster using a type IV compatible ToF-SIMS instrument equipped with a liquid metal ion gun (ION-TOF GmbH, Münster, Germany). Bi^{3+} (25 keV) was used as primary ions. The sample was imaged with a primary ion dose density of 2.2×10^{11} ions/cm^2. The beads were immobilized in a random orientation on the sample holder by double-faced Scotch tape. To reduce charging effects on the insulating sample the measurement was paused after every scan for 5s to flood the sample with low energy electrons.

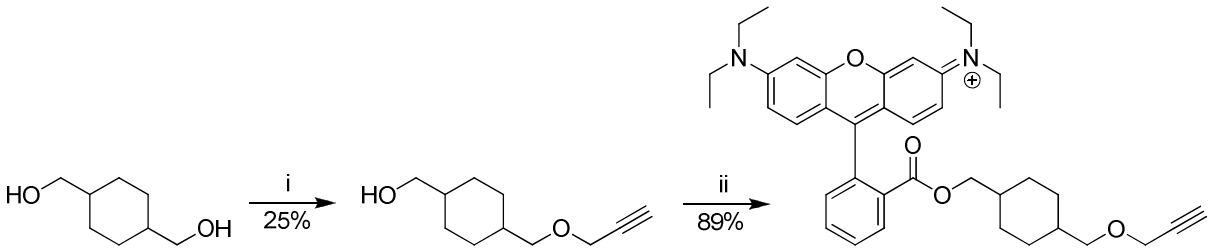
2. Synthesis:



Scheme ESI-1: Survey of used organic compounds for chemical reactions on bead surfaces.

Synthesis of heterobifunctional linker molecules **1** and **2** as well as biomolecules **5** and **8** used for protein binding studies were recently published.[1] Also the preparation of compound **6** and **7** has been published before.[2] Dansylcadaverine **4** and fluoro alkyne **9** were purchased from Sigma Aldrich and used without further purification.

Rhodamine alkyne **3** was synthesized by the following sequence:



10 **11** **3**

Scheme ESI-2: Synthesis of Rhodamine alkyne **3**; i: ^{t}BuONa, propargylbromide, THF, 12 h; ii: Rhodamine B, EDC*HCl, HOBt, NMM, DMF, 12 h.

Cyclohexane-1,4-dimethanol **10** (8.407 g; 58.295 mmol) and potassium *tert*-butoxide (1.09 g; 9.716 mmol) were dissolved in dry THF (300 mL) under argon atmosphere and stirred for 1 h. Propargylbromide was added dropwise to the suspension and the reaction mixture was stirred over night at rt. Residual solid was removed by filtration over Celite. Water was added to the solution, extraction with dichloromethane and drying over $MgSO_4$ followed. The crude product was purified by silca gel column chromatography (dichloromethane-methanol = 100:3), leading to pure alkyne **11** in 25% yield.

R_f value (DCM-MeOH = 100:3) = 0.35.

Molecular Formula (MW in g/mol): $C_{11}H_{18}O_2$ (182.259).

ESI-HRMS (m/z): Calculated for $[C_{11}H_{18}O_2Na]^+$: 205.1199; found: 205.1192.

Alcohol **11** (0.413 g; 2.264 mmol), Rhodamine B (0.723 g; 1.510 mmol), EDCI (0.434g; 2.264 mmol), HOBt (0.162 g; 1.057 mmol) and NMM (0.33 mL; 3.020 mmol) were dissolved in dry DMF (20 mL) under argon atmosphere and stirred at rt overnight. After adding water and 0.5 M HCl extraction with dichloromethane followed. The organic layer was washed with brine and dried over $MgSO_4$. The product **3** was purified by silica gel column chromatography (dichloromethane-methanol-ethyl acetate = 10:4:5) and obtained in 89% yield.

R_f value (DCM-MeOH-EA = 10:4:5) = 0.20.

Molecular Formula (MW in g/mol): $C_{39}H_{47}N_2O_4Cl$ (643.2524).

ESI-HRMS (m/z): Calculated for $[C_{39}H_{47}N_2O_4]^+$: 607.3530; found: 607.3529.

3. Selective surface reactions:

a. Alkene functionalization of polymer beads

Alkene terminated particles were derived from epoxide functionalized polymer beads[3] by reaction with allyl mercaptane. Therefore the beads were immersed into a solution of allyl mercaptane (0.1 mL, 1.2 mmol) in DMF (0.1 mL). NEt_3 was added as base to assure deprotonation of thiol groups and the solution was gently rotated using a rotary evaporator at 70°C for 24 hours under inert atmosphere. After completion of the reaction, large beads were washed with copious amounts of DMF, methanol, ethanol, DCM and DEE in a glass filter. Small alkene functionalized beads were prepared by the same procedure, only cleaning was done by five-fold sonication-centrifugation procedure using methanol as solvent and discarding the supernatant solution after each centrifugation step.

b. Microcontact printing on alkene beads

Large beads: The first stamp was loaded by applying several drops of an ethanolic solution of the respective thiol (80 mM) under addition of DMPA (40 mM) as photoinitiator radical starter. After 20 - 60 seconds, the stamp was blown dry in a stream of argon and the dried stamp was glued to a transparent objective slide connected to a mechanical, hand operated press. The second stamp was loaded accordingly with the second thiol, dried and put with its loaded surface pointing upwards onto a cut piece of a mirror. The alkene beads were then applied as a monolayer on the lower stamp being located on the mirror, which was then inserted into the press and the upper stamp was brought into conformal contact with the alkene beads. The stamp-beads-stamp sandwich, located between a transparent objective slide on top and a mirror for light reflection from beneath was then irradiated with an intense UV-LED (365 nm) for 5 minutes. Subsequently, the beads were collected in a glass filter and washed with water, ethanol and ether before being extracted in hot ethanol for at least 9 hours.

Small beads: In principle the procedure of printing was the same for small as for large beads, only some modification as a result of the different size had to be made. The first stamp was loaded as described above and glued to the objective slide. The second stamp was also loaded, dried and then used to pick up a monolayer of small beads from another objective slide. Before, the small beads had to be separated from clusters, build during synthesis and solution based modification. Therefore a small amount of clustered beads was applied on an objective slide and gently grounded with a coverslip. After picking up the beads form the “dusty monolayer”, the second stamp was placed on the mirror piece and inserted into the press with its bead-loaded side facing upwards. The first stamp was brought into conformal contact with the beads and the system was irradiated for 5 minutes with the UV-LED. Cleaning was done by a repeated sonication/centrifugation procedure in methanol.

c. Peptide coupling reaction on chemically orthogonal bead surfaces

Direct peptide coupling: Peptide coupling of amino-terminated compounds to surface-bound acid groups was done using a solution of the amino compound (0.2 M) in DMF (0.1 mL) under addition of O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyl uronium tetrafluoroborate (TBTU, 0.16 M, 5.1 mg, $1.6*10^{-5}$ mol) as coupling agent and diisopropylethylamine (DIPEA, 0.2 M, 2.6 mg, $2*10^{-5}$ mol) as base. The beads, bearing the orthogonal functionalities on their surface were put into the solution and agitated by gentle rotation at a rotary evaporator for 5 hours at room temperature. After completion of the reaction, the samples were collected in a glass filter and washed with DMF, water, ethanol, DCM and DEE before being extracted in hot ethanol for 9 hours. Small beads were cleaned by a dispersion/centrifugation procedure.

Aminolysis via active ester formation: Active esters were formed from surface-bound acid patches by immersion in a solution of dicyclohexylcarbodiimide (DCC, 1 M, 103 mg, $5*10^{-4}$mol), N-hydroxysuccinimide (1 M, 58 mg, $5*10^{-4}$ mol) in DMF (SPPS grade, 0.5 mL; a very small amount of acetic acid was added to catch residual amines). The solution was stirred at room temperature for 5 minutes before the beads were added. Subsequently, the suspension was rotated for one hour at room temperature. Afterwards the beads were filtrated and washed with DMF (SPPS grade), DCM (p.a.) and DEE before they were quickly transferred to a solution of the respective amino-compound (10 mM) in DMF (SPPS grade, 0.1 mL) containing NEt_3 (0.3 µL) as base and agitated using a rotary evaporator (20 rpm) at room temperature overnight. The obtained functionalized beads were filtrated and washed with DMF (SPPS grade), methanol (abs.), ethanol (abs.), DCM (p.a.) and DEE before they were extracted in hot ethanol for 9 hours.

d. CuAAC reaction on chemically orthogonal bead surfaces

For CuAAC on surface-bound azides a solution of the respective alkyne (10 mM) and a catalytic amount of tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMF (0.1 mL) was prepared and degassed by bubbling argon for 15 minutes. Then the beads and 2-3 drops of a Cu(I) solution (5 mg $CuSO_4*5H_2O$ and 20 mg sodium ascorbate dissolved in 1 mL H_2O) were added and the suspension was agitated at 70°C overnight using a rotary evaporator. Then the beads were collected in a glass filter and washed with DMF, water, ethanol and DEE before they were extracted in hot ethanol for 9 hours. Small beads were cleaned by a dispersion/centrifugation procedure.

e. Thiol-ene immobilization of mannose thiol

Mannose thiol **8** was used to verify remaining alkene functional groups at the bead surface after printing of hetero bifunctional linkers **1** and **2**. Therefore, a drop of neat mannose thiol, containing 2 wt% of DMPA was applied onto the beads on an objective slide and mixed thoroughly. Irradiation for 10 minutes linked the carbohydrate ligand chemically on the polymer surface via thiol-ene reaction.

4. Protein binding on surfaces:

Chemically orthogonal polymer beads bearing at least one carbohydrate ligand on one pole were incubated with fluorescently labeled proteins. Therefore the well-known binding affinities of different lectins were used. In the case of *α*-mannoside ligands concanavalin A (ConA) was used as specifically binding protein, in the case of *ß*-lactoside ligands the affinity for peanut agglutinin (PNA) was exploited whereas biotin ligands are known to strongly bind streptavidin. If only one carbohydrate ligand was present at the bead surface, only one protein was used for incubation. However, if two different carbohydrate ligands were present on opposing sides of the beads, a mixture of two different proteins in one solution was employed for incubation. This emphasizes the specifity of the carbohydrate-lectin recognition process when compared to sequential protein incubation.

In general, carbohydrate patterned beads were first incubated in a *bovine serum albumine* (BSA) solution (3 wt% in PBS buffer) overnight in order to minimize unspecific protein adsorption. Afterwards the beads were collected in a glass filter and placed into a solution of the respective specifically binding protein(s) (solutions in HEPES buffer (20 mM) under addition of 1 mM $CaCl_2$, 0.15 M NaCl and 1 mM $MnCl_2$); PNA and ConA: 10 µg protein/mL, 200 µL, streptavidin: 6 µg protein/mL, 200 µL) and BSA (3 wt%, 200 µL) and gently agitated using a rotary evaporator for two hours at room temperature. The beads were then carefully rinsed with PBS buffer and investigated by fluorescence microscopy.

5. Additional experiments:

a. Exclusive binding of fluorophores to one surface functionality

In order to check if each functionality on printed beads is selectively and exclusively addressable, they were derivatized by reaction with functionalized fluorophores. Azide functional groups were selectively coupled with Rhodamine alkyne **3** *via* CuAAC on both large (Figure ESI-1 a) and small beads (Figure ESI-1 c). Independently using a second batch of particles, acid functions were selectively coupled with dansylcadaverine by peptide coupling. This was done on large (Figure ESI-1 b) as well as on small beads (Figure ESI-1 d). Superimposed fluorescence images of sequential derivatization of both, azide and acid patches can be found in the main body of the text (Figure 1).

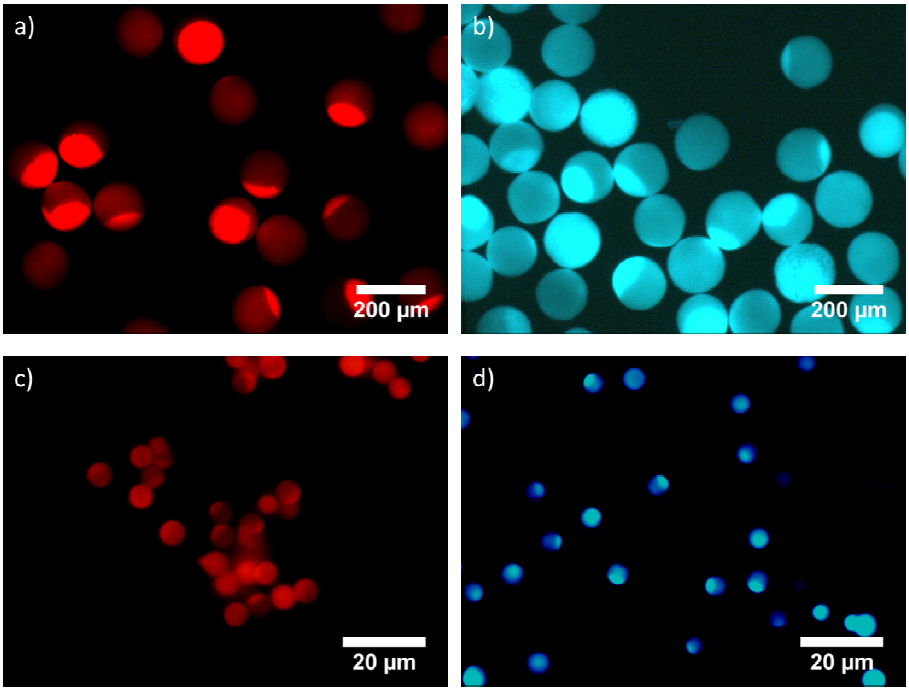


Figure ESI-1: Selective reaction of azide patches with rhodamine alkyne *via* CuAAC on large (a) and on small (c) alkene beads and selective derivatization of acid patches with dansyl cadaverine via peptide coupling on large (b) and small beads (d).

b. ToF-SIMS analysis of functionalized beads

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used to analyze bifunctional Janus beads that were reacted with fluorinated alkyne **9** by CuAAC (Figure ESI-2). The modified azide area was identified by the formation of CN^- ions in this area. The orthogonal reaction with the alkyne **9** was verified by the formation of F^- ions within the same area. The total ion image in figure ESI-2 shows that the topography of the spherical sample has an influence on the lateral intensity distribution. The comparison of the F- and CN- images with the total ion image, as well as the distribution of F- normalized to the total ion signal, show that F- and CN- distribution are not caused by the topographical effect.

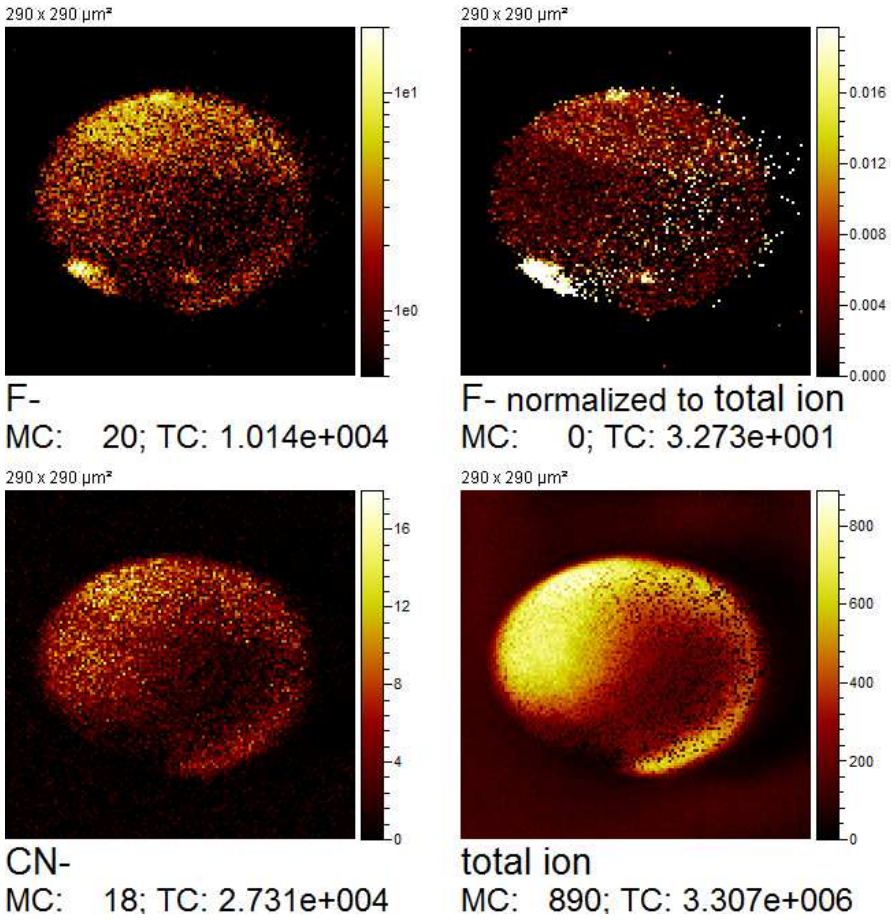


Figure ESI-2: ToF-SIMS analysis of a trifunctional Janus particle that was modified by reaction with the fluorinated alkyne **9**. The formation of F^- anions demonstrated the immobilization of the molecule within the areas, that were decorated with the nitrogen containing azide thiol linker **1**. The presence of the linker could be visualized by the detection of CN^- ions.

c. Immobilization of biological ligands

Additionally, each of the established functionalities of azide and acid patches after printing were selectively brought to reaction with alkyne and amine bioligands using CuAAC and peptide coupling, respectively. Azide groups were coupled with biotin alkyne **5** by CuAAC, which could then direct the immobilization of Dylight 405-labeled streptavidin to the modified areas (Figure ESI- a). Accordingly, acid patches were reacted with amino-mannoside **6** and subsequently incubated in fluorescein-labeled ConA, resulting in preferential adsorption of the protein on only one of the two patches (Figure ESI- b).

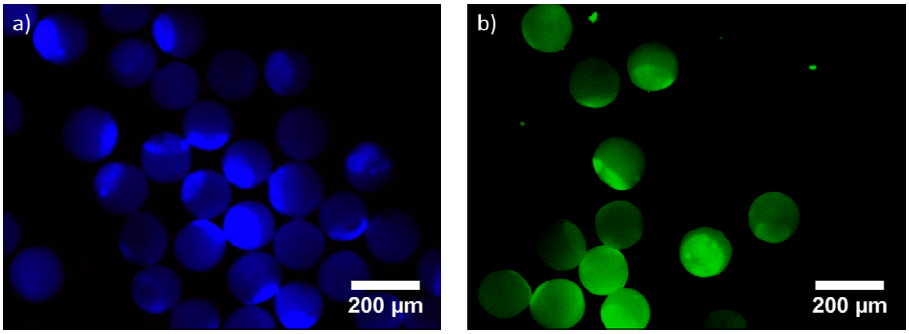


Figure ESI-3: Fluorescence microscopy on Dylight 405 labeled streptavidin bound to exclusively modified azide patches with biotin alkyne (a) and fluorescein-labeled ConA bound preferentially to amino mannoside derivatized acid patches (b).

d. Reaction of residual alkenes with mannose thiol

Remaining alkene functions after printing of the heterobifunctional linker molecules **1** and **2** and immobilization of rhodamine alkyne and dansylcadaverine were verified by reaction with mannoside thiol **8** (see ESI-section 2.5) and subsequent binding of fluorescein-labeled ConA. Due to the highly porous nature of the used polymer particles,[4] the thiol did not exclusively react at the alkene groups between the already modified poles of the bead surface, but also penetrated the polymer architecture. This resulted in a homogeneous carbohydrate ligation, which is confirmed by homogeneous fluorescence observed after incubation of the mannose functionalized polymer beads in fluorescein-labeled ConA solution (Figure ESI- b). Brightly fluorescing poles are ascribed to the rhodamine attached to azide groups, as this fluorophore also emits in the green wavelength range.

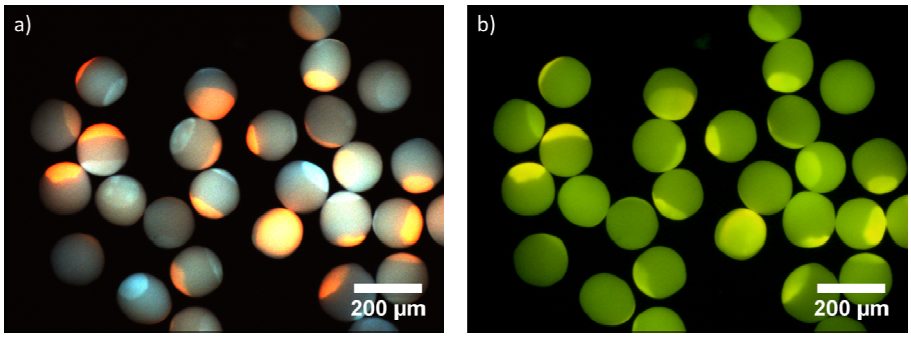


Figure ESI-4: Verification of remaining surface alkene groups by neat reaction with mannoside thiol and subsequent binding of fluorescein-labeled ConA (fluorescence microscopy image, b) on chemically orthogonal beads being derivatized additionally with rhodamine alkyne and dansyl cadaverine; superimposed fluorescence microscopy image (a). Brightly fluorescing poles in image b are due to additional rhodamine emission under use of a blue excitation filter in the fluorescence microscope.

6. References:

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