Electronic Supplementary Material (ESI) for Lab on a Chip.
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Electronic Supplementary Information for

High-throughput Double Emulsion-based Microfluidic Production of Hydrogel Microspheres with Tunable Chemical Functionalities toward Biomolecular Conjugation

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1. **Raw epifluorescence micrographs CS-PEG microspheres conjugated with TCO-modified R-PE in Figure 6**

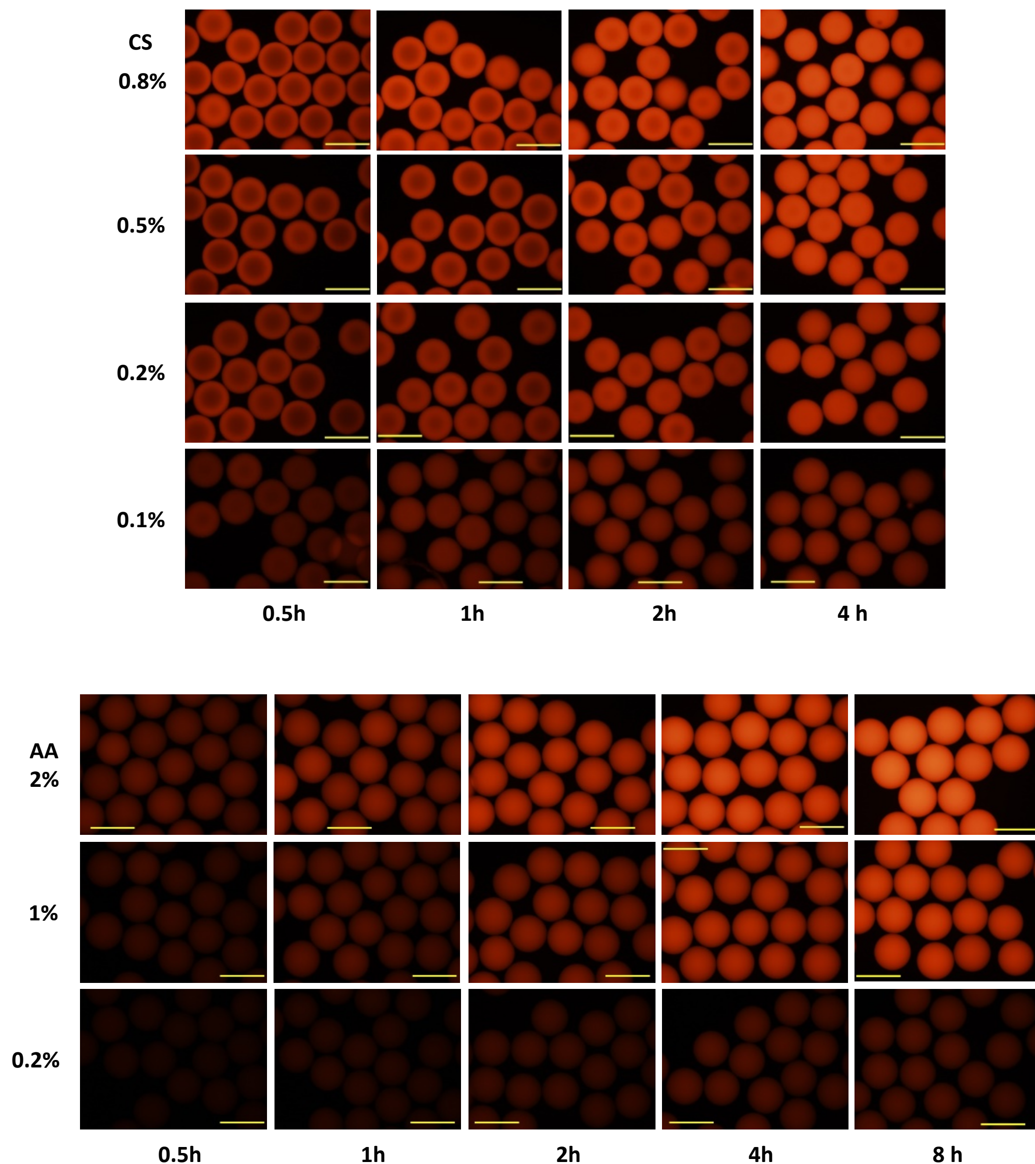

Figure S1 Raw epifluorescence micrographs of R-PE conjugation with CS-PEG and AA-PEG microspheres from 0.5 – 8 h

In Figure S1 we provide the raw epifluorescence micrographs used in Figure 6, the total fluorescence intensity plots of R-phycoerythrin (R-PE) conjugation with chitosan-poly(ethylene glycol) (CS-PEG) and acrylic acid-poly(ethylene glycol) (AA-PEG) microspheres over time. For this, we reacted 2 µM *trans*-cyclooctene (TCO)-modified R-PE with tetrazine (Tz)-activated CS-PEG microspheres prepared with varying CS content in the prepolymer solution and took samples at 0.5, 1, 2, and 4 h. We also reacted 2 µM R-PE with NHS ester-activated AA-PEG microspheres prepared with varying AA content in the prepolymer solution and took samples at 0.5, 1, 2, 4, and 8 h. The total fluorescence intensity plots were taken by multiplying the area of individual microspheres with their mean gray values (corresponding to their fluorescence intensities) of the epifluorescence micrographs using ImageJ. These total fluorescence intensity values were then subtracted by the background fluorescence and scaled to account for

differences in exposure times of the micrographs. Each data point represents the averaged total fluorescence intensity of 10-20 microspheres per condition.

2. Negative controls for one-pot assembly in Figure 7

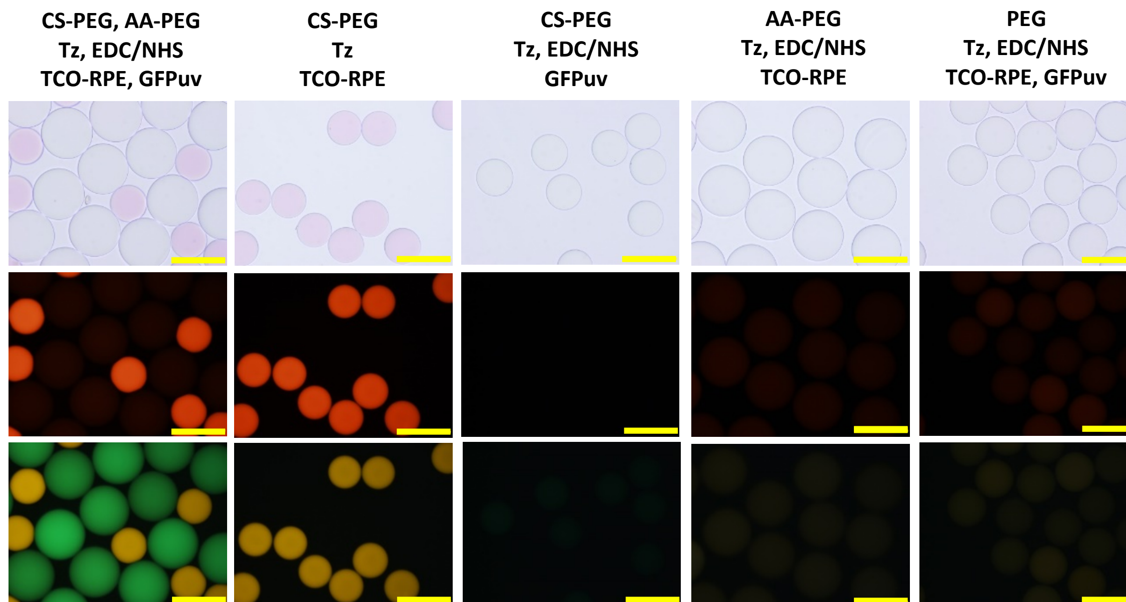

Figure S2: Brightfield (top row) and epifluorescence (middle and bottom row) micrographs of one-pot reaction of green fluorescent protein (GFPuv) and TCO-modified R-PE with CS-PEG and AA-PEG microspheres and negative controls.

In Figure S2, we provide further detailed negative control results for the orthogonal one-pot conjugation of two model fluorescent proteins R-PE and GFPuv with CS-PEG and AA-PEG microspheres using the Tz-TCO and EDC/NHS chemistries respectively and simple size-based encoding, shown in Figure 7. For this, we placed both 140 µm diameter CS-PEG microspheres and 200 µm diameter AA-PEG microspheres in one pot and added Tz-PEG_5-NHS ester (reactive only to the primary amines in CS-PEG microspheres), as shown in the schematic diagram of Figure 7a. After washing excess Tz-PEG_5-NHS ester, we then added EDC/NHS to the solution, which should react with the carboxylates in the AA-PEG microspheres. Upon washing away excess EDC/NHS, we then simultaneously added both GFPuv and TCO-modified R-PE of varying concentrations to the microsphere solution for 1 h to allow for the covalent coupling of TCO-modified R-PE to the CS-PEG microspheres and GFPuv to the AA-PEG microspheres.

As shown in the epifluorescence micrographs in the first column of Figure S2, only Tz-activated CS-PEG microspheres fluoresce brightly red under red filter (second row), while only NHS ester-activated AA-PEG microspheres fluoresce brightly green under UV filter (third row), indicating that TCO-modified R-PE is selectively coupled with the Tz groups in the CS-PEG microspheres, while GFPuv is selectively coupled with the NHS ester groups in the AA-PEG microspheres. We attribute the orange color of the CS-PEG microspheres in the UV filter to be the result of the high molar extinction coefficient of the R-PE (1.96 x 10^6 M^{-1} cm^{-1})[1] and the spectral overlap between the red and the UV filter set. The orange color is not likely the result of GFPuv fluorescence, as we see the same intensity of orange color in the UV filter when Tz-activated CS-PEG microspheres are conjugated with only the TCO-modified R-PE (second column). Tz-activated CS-PEG microspheres conjugated with GFPuv displayed minimal fluorescence (third column), further indicating that GFPuv does not readily react with the Tz groups in CS-PEG

microspheres and that EDC/NHS does not readily convert the amine functional groups into NHS esters. Next, as shown in the fourth column of Figure S2, AA-PEG microspheres reacted with Tz and then EDC/NHS display minimal fluorescence upon conjugation with the TCO-modified R-PE, indicating that the TCO-modified R-PEs do not readily react with the NHS ester groups in the AA-PEG microspheres. Finally, PEG microspheres without functional groups reacted with Tz and EDC/NHS display minimal fluorescence upon conjugation with both TCO-modified R-PE and GFPuv, indicating that the microspheres exhibit minimal non-specific binding, due to the non-fouling properties of PEG[2]. In summary, Figure S2 provides further evidence of the orthogonality as well as the selectivity of the two reactions that enables one-pot conjugation of two different proteins with respective hydrogel microspheres.

References

1. M.-H. Yu, A. N. Glazer, K. G. Spencer and J. A. West, *Plant Physiol.*, 1981, **68**, 482-488.
2. R. Langer and D. A. Tirrell, *Nature*, 2004, **428**, 487-492.