Supporting Information for

Optical Detection of Interleukin-6 using Liquid Janus Emulsions using Hyperthermophilic Affinity Proteins

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1. MATERIALS AND INSTRUMENTATION

Materials. **N_3-PEG-NH_2** was synthesized according to literature procedures[1,2] using PEG 400 purchased from Alfa Aesar. Poly(ethylene glycol) methyl ether methacrylate (M_n = 500), 2,2-azobis(2-methylpropionitrile) (AIBN), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid, benzyl methacrylate, poly(ethylene glycol) methyl ether azide (M_n = 1000), Zonyl FS-300, Tween 20, sodium ascorbate, 1,4-dioxane, diethylbenzene (DEB), N,N-dimethylformamide (DMF), hexanes, tetrahydrofuran (THF), anti-mouse IgG (whole molecule)-FITC antibody, and human serum (from human male AB plasma, USA origin, sterile-filtered) were purchased from Sigma-Aldrich. Pentafluorophenyl methacrylate and 2-(trifluoromethyl)-3-ethoxydodecafluorohexane (HFE-7500) were purchased from Synquest. DBCO-amine was purchased from BroadPharm. 2-hydroxyethyl acrylate and cetyltrimethylammonium bromide (CTAB) were purchased from Alfa Aesar. 4,4'-difluorobenzophenone and dimethyl sulfoxide (DMSO) were purchased Oakwood Chemical. Mouse anti-human IL-6 capture antibody (Human IL-6 DuoSet ELISA) was purchased from R&D Systems. Recombinant human IL-6 was purchased from RayBiotech. Human CRP was purchased from Innovative Research. IL-6 monoclonal antibody (MQ2-13A5), eBioscience and goat anti-rat IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 were purchased from ThermoFisher. Azidobutyric acid NHS ester and DBCO NHS ester were purchased from Lumiprobe. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) was purchased from Ambeed. Copper sulfate was purchased from Mallinckrodt Baker, Inc. 5'-hexynyl-AAA AA-FAM-3' was purchased from Integrated DNA Technologies. PBS tablets, ethanol, and nuclease free water were purchased from VWR. Mili-Q water from Barnstead Nanopure Water System (Thermo Fisher Scientific) was used for the preparation of the buffer for the emulsion continuous phases. All reagents and solvents were used as received without further purification unless otherwise stated. Inhibitor was removed from poly(ethylene glycol) methyl ether methacrylate, pentafluorophenyl methacrylate, benzyl methacrylate, and 2-hydroxyethyl acrylate using basic alumina from Sigma-Aldrich prior to use.

Instruments. ^{1}H and ^{19}F NMR spectra were collected with Bruker Avance-III HD Nanobay (400 MHz), Bruker Avance Neo (400 MHz), Bruker Avance Neo (500 MHz), or JEOL ECZ (500 MHz) spectrometer at room temperature. Quantitative NMR was performed with 4,4'-difluoropbenzophenone as a standard. Polymer molecular weights were determined on an Agilent 1260 Infinity GPC system in THF at (0.5-1 mg/mL sample concentrations) with a polystyrene calibration standard. FTIR spectra were collected on a Thermo Scientific Nicolet 6700 FTIR spectrophotometer with a diamond crystal for ATR.

Optical images of the agglutination studies were collected using an inverted microscope equipped with an AmScope camera. Side-view images of the droplets were collected using a custom-built horizontal microscope with an Olympus 50x objective, a Thorlabs tube lens (effective focal length = 200 nm), and an Allied Vision Prosilica GT camera. The droplets were deposited into a 0.1 mm demountable quartz cuvette (Starna Cells, Inc.) and illuminated from the side using a Fiber-Lite

M1-152 lamp for bright field images with a white background. Confocal fluorescence images of the droplets were collected on a Zeiss LSM 700 Laser Scanning Confocal Microscope at 10% power. Fluorescence microscope images were taken on droplets functionalized with 0.5 and 2.8 nmol of FITC-labeled E1 and FITC-labeled E2, respectively, which were added to 20 and 30 μL of droplets in 500 μL of continuous phase, respectively.

Pendant drop tensiometry measurements were performed on a ramé-hart Model 500 Advanced Goniometer using a Hamilton 1418-01 1001LT BBL Barrel Assembly (1 mL) syringe equipped with an inverted 22G 304 SS needle (ramé-hart). The measurements were performed with 1X PBS as the continuous phase and 1 mg/mL of polymer as the disperse phase in a 20 mm open top quartz fluorometer cell (Starna Cells, Inc.). Interfacial tension measurements were taken every 10 s for 10 minutes for **PN_3-PPEG-*b*-PBn** and for <1 minute for **PPFP-PPEG-*b*-PBn** and **PDBCO-PPEG-*b*-PBn** because the droplet would detach from the needle within a minute due to the low interfacial tension. All measurements were performed in triplicate and the average values are reported.

2. ADDITIONAL EXPERIMENTAL DETAILS

Detailed Experimental Procedure for Droplet Bioconjugation. Droplets functionalized with rcSso7d-IL6 proteins purified via a Ni^{2+} resin and PEG-N_3 utilized 1 nmol of protein and 25 nmol of PEG-N_3 in 1X PBS in the bioconjugation step unless otherwise stated. Droplets functionalized with rcSso7d-IL6 proteins that underwent additional FPLC purification and PEG-N_3 utilized 0.2 nmol of protein and 25 nmol of PEG-N_3 in 1X PBS in the bioconjugation step unless otherwise stated. Droplets functionalized with anti-IL-6 antibodies utilized 0.2 nmol of antibody and 25 nmol of PEG-N_3 (when applicable) in 1X PBS in the bioconjugation step.

Droplets functionalized with DNA were fabricated as follows: 6 µL of 1 mM 5'-hexynyl-AAA AA-FAM-3' reconstituted with nuclease free water, followed by 7.5 µL of 2:1 volume ratio of 50 mM THPTA:20 mM $CuSO_4$, and 25 µL of 100 mM sodium ascorbate were added to 20 µL of droplets (with PN_3-PPEG-*b*-PBn in the disperse phase) in 500 µL of continuous phase (4:6 volume ratio of 0.1 wt% CTAB:0.1 wt% Zonyl in 1X PBS). The suspension was incubated overnight at an orbital shaker (100 rpm). After incubation, the continuous phase was solvent exchanged five times with fresh continuous phase solution to remove unreacted DNA and excess reagents.

Procedure for Response Curve Agglutination Assays. For assays included in the response curves, 475 µL of continuous phase followed by 14 µL of droplets was added to each imaging dish. To keep the surfactant concentration constant across each assay, the corresponding amount of IL-6 was added followed by a few microliters of 1X PBS. Ideal images should have droplets covering the entire window; however, as the droplets agglutinate together, there are often sparse regions in the dish. Therefore, for the overnight images, the dish is sometimes tilted slightly (<10°) to fill in the sparse regions.

Cell Culture and Protein Purification. Transformed cells were cultured in Luria Broth (LB) media with appropriate antibiotics. Cells were grown until mid-log phase (OD600 = 0.5-0.8) while shaking (220 rpm, 37 °C). Cells were induced by the addition of 60 µM IPTG and 1 mM 4AZP generated as described[3] and shaken overnight (220 rpm, 17 °C). Cells were then pelleted (4200 rpm, 20 min) and resuspended in lysis buffer (100 mM HEPES pH 8.0, 300 mM NaCl, 10 mM imidazole, 30mL/L of growth) and frozen until protein purification.

Frozen cell suspensions were thawed at room temperature. Lysis was performed via incubation with lysozyme (0.02 g/L growth) while vigorously stirring at room temperature for 30 minutes, followed by sonication (60 sec on, 60 sec off, 3 times while on ice). Lysates were clarified via centrifugation (20,000 x g, 60 min, 4 °C) followed by syringe filtration (0.22 micron). Clarified lysates were purified via gravity using HisPURE Ni^{2+}-NTA resin. After loading, resin was washed with 20x column volumes of Lysis buffer, and eluted using 3x column volumes of elution buffer (100 mM HEPES pH 8.0, 150 mM NaCl, 250 mM imidazole). Proteins were buffer exchanged into storage buffer (20 mM HEPES pH 8.0, 100 mM NaCl) via spin concentrator (Amicon Ultra

3kDa MW cutoff) and flash frozen. Protein concentration was determined by Bradford assay, and protein purity was determined via SDS-PAGE. An example SDS-PAGE gel is shown in Figure S1. Proteins were further purified for assays comparing E1/E2 to commercial antibodies via Size Exclusion Chromatography (SEC) at 4°C (HiLoad® 16/600 Superdex® 200 pg). The SEC column was equilibrated with 400 mL storage buffer at 1.5 mL/min, and proteins were loaded via syringe injection. Fractions containing E1/E2 were concentrated as previously described.

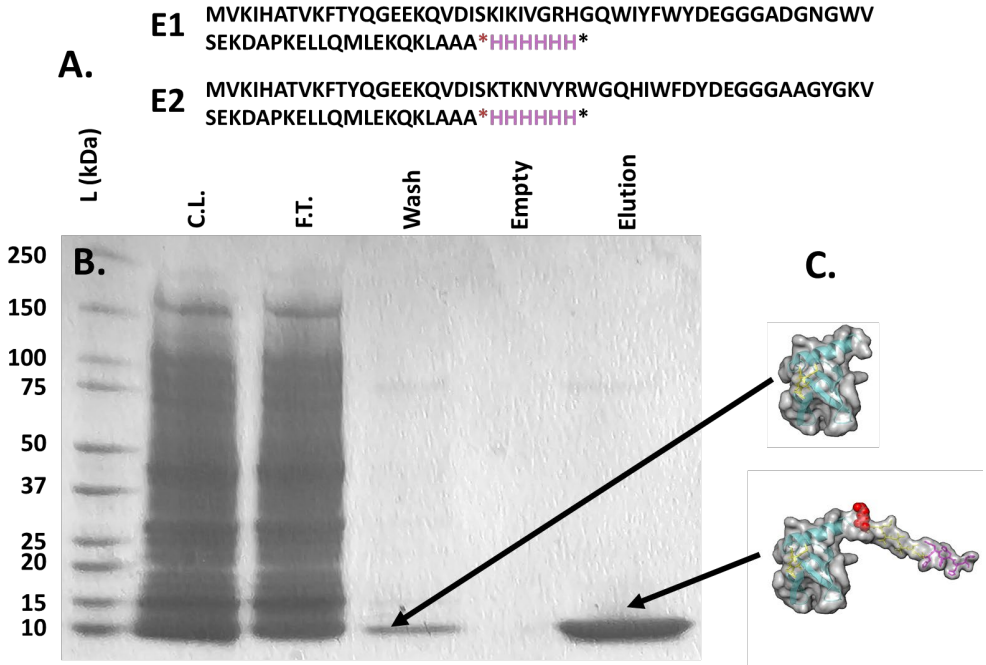


Figure S1. A. Representative sequence of E1. non-native stop codon represented as red *, native stop as black *, 6X His tag shown in purple. **B.** SDS-PAGE gels of IMAC purification of rcSso7d. Lanes contain clarified lysate (C.L), flow-through of nickel column (F.T.), wash, an empty lane, and elution of the purified protein. **C.** Structural representation of rcSsso7d synthesis projects with truncated product (top) lacking 6xHis tag (purple sticks) and full length protein with 4AZP incorporated (bottom, red balls).

***E. coli* expression plasmid design and ncAA incorporation.** Plasmids for bacterial expression were generated by Genscript. Amino acid sequences for two clones, which form a sandwich pair originally titled "clone2" (E1) and "clone8," (E2) were provided to Genscript for codon optimization, synthesis, and cloning into the bacterial expression plasmid pRSF-Duet. The original sequences were modified to include an internal amber (TAG) stop codon at the original stop codon, the short spacer LAAA, followed by a hexa-Histidine tag terminated by the ochre (TAA) stop codon. The full sequences for each clone can be found in Figure S1.

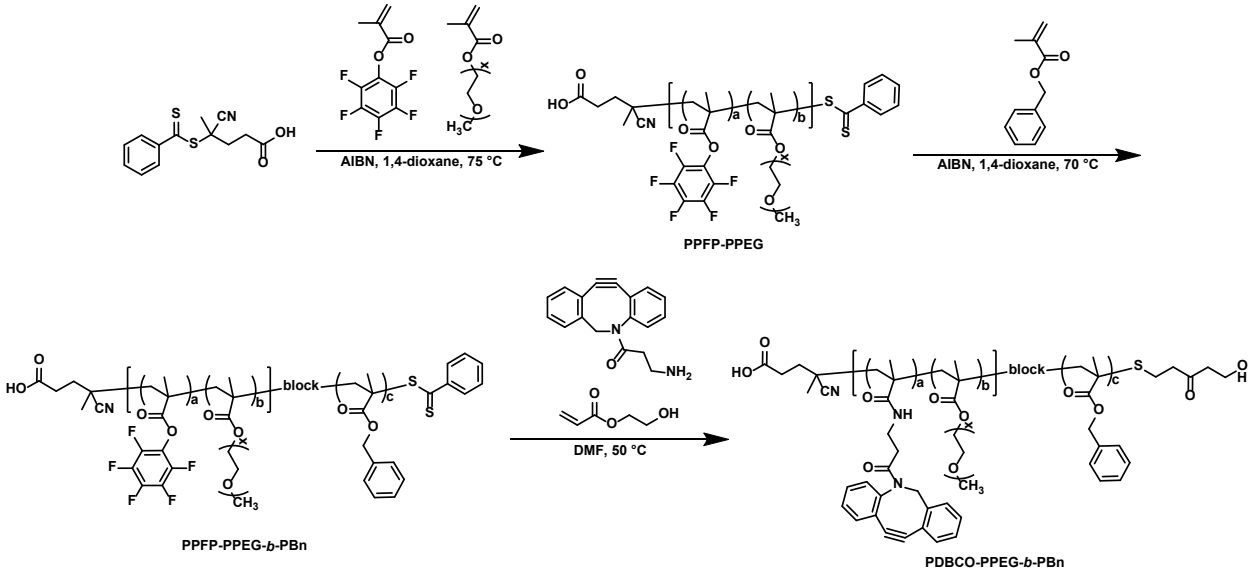
To functionalize the described rcSso7d proteins, BL21(DE3) cells were co-transformed with one of the described rcSso7d plasmids alongside the pDULE plasmid previously described,[4] which encodes an engineered tRNA that pairs with the amber stop codon and a tRNA synthetase that loads the engineered tRNA with 4-AzidoPhenylalanine (4AZP). This methodology allows the site-specific functionalization at any internal TAG stop codon of proteins with a free azide. This free azide can be used for orthogonal attachment to covalently bond 4AZP containing proteins to various substrates using alkyne azide click chemistry. Transformed cells were cultured, induced,

and lysed and the proteins were purified according to procedures stated in the experimental section. A portion of all proteins purifications was labeled with FITC NHS esters to verify attachment to droplets. To label the proteins, proteins were incubated with FITC NHS ester dye at a ratio of 7:1 (dye:theoretical lysines/protein) at room temperature while rocking for 30 minutes. Unbound dye was separated from dyed proteins via gel filtration spin columns (BioGel650).

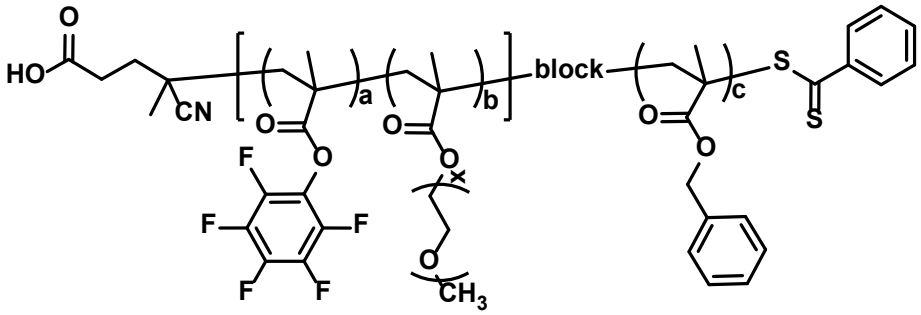
Synthesis of Azido-Modified Anti-IL-6 Antibody. IL-6 monoclonal antibody (MQ2-13A5) was buffer exchanged using a spin desalting column (Zeba Spin Desalting Columns, 7K MWCO, ThermoFisher) with 20 mM HEPES pH 8.0, 100 mM NaCl. The antibody was then concentrated using a spin concentrator (Amicon Ultra 30kDa MW cutoff, Millipore Sigma) to 10.85 μM (1.62 mg/mL); the protein concentration was determined using a Bradford assay. 26 μL of concentrated anti-IL-6 antibody, 1.4 μL of a 1.6 mM solution of azidobutyric acid NHS ester in DMSO, and an additional 1.5 μL of DMSO was combined in a microcentrifuge tube. The reaction was shaken on a tilter (lowest speed) at room temperature for ~4 h. Unreacted NHS ester was removed using a spin desalting column.

Synthesis of DBCO-Modified Anti-IL-6 Antibody. 18 μL of ~3.3 mg/mL of mouse anti-human IL-6 capture antibody dissolved in 20 mM HEPES pH 8.0, 100 mM NaCl and 2 μL of a 1.6 mM solution of DBCO-NHS in DMSO were combined in a microcentrifuge tube. The reaction was shaken on a tilter (lowest speed) at room temperature for ~4 h. Unreacted NHS ester was removed using a spin desalting column.

3. POLYMER SYNTHESIS AND CHARACTERIZATION

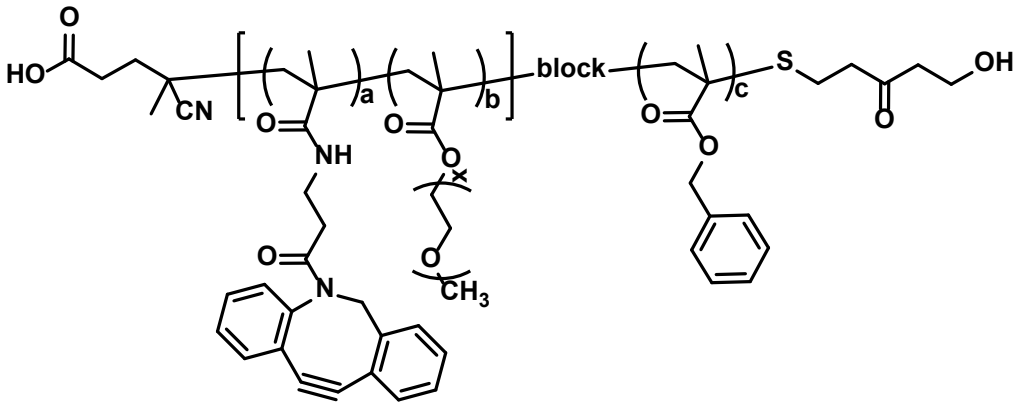


Scheme S1. Synthetic scheme of **PDBCO-PPEG-*b*-PBn**.

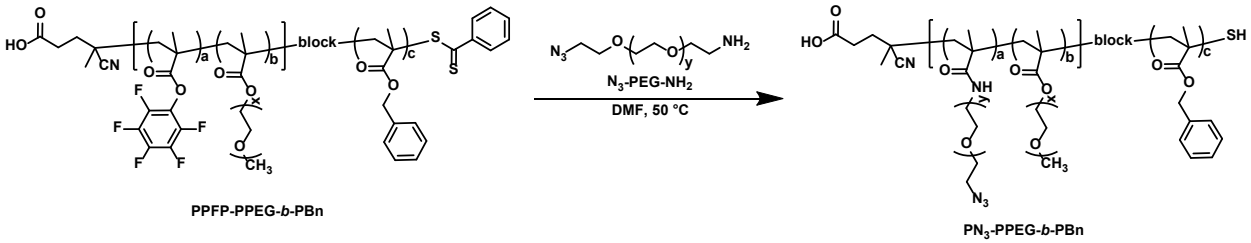


PPFP-PPEG-*b*-PBn. (1) Pentafluorophenyl methacrylate (0.99 g, 3.93 mmol), poly(ethylene glycol) methyl ether methacrylate (1.00 g, 2.01 mmol), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (0.11 g, 0.41 mmol), AIBN (8.32 mg, 0.05 mmol), and 1,4-dioxane (3 mL) were added to a 10 mL Schlenk flask. The flask was deoxygenated via three freeze-pump-thaw cycles and flushed with argon. The reaction was heated at 75 °C overnight. The reaction mixture was cooled down to room temperature and then quenched with liquid nitrogen. The mixture was precipitated into hexanes, forming a gel. The hexanes was decanted and the polymer was dried under vacuum, resulting in a pink gel, **PPFP-PPEG**, which was used without further purification. (2) **PPFP-PPEG** (0.49 g, 0.08 mmol), benzyl methacrylate (1.17 g, 6.64 mmol), AIBN (2.6 mg, 0.016 mmol), and 1,4-dioxane (3 mL) were added to a 10 mL Schlenk flask. The flask was deoxygenated via three freeze-pump-thaw cycles and flushed with argon. The reaction was heated at 70 °C for 3 days. The reaction mixture was cooled down to room temperature and then quenched with liquid nitrogen. The mixture was precipitated into hexanes. The resulting polymer was redissolved using THF and precipitated in hexanes two more times. The polymer was dried in a vacuum oven at 60 °C overnight, resulting in a pink solid (1.4 g, 84%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.41-7.19 (m, 5H; benzyl CH), 4.99-4.81 (m, 2H; benzyl CH_2), 4.33-3.96 (m, 0.2H), 3.82-3.37 (m, 2.5H), 3.37-3.24 (m, 0.2H; PEG-CH_3), 2.68-1.61 (m, 2.5H),

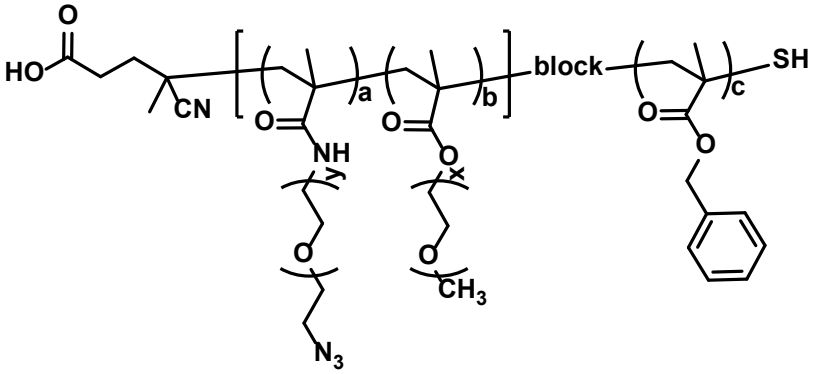
1.60-0.54 (m, 4.2H). ^{19}F NMR (376 MHz, CD_2Cl_2) δ -150.20 (1F), -151.64 (1F), -158.29 (1F), and -162.90 (2F). SEC (PS standards): M_n = 14.5 kDa, $Đ$ = 1.24.



PDBCO-PPEG-*b*-PBn. PPFP-PPEG-*b*-PBn (79.2 mg, 0.005 mmol), DBCO-amine (20.5 mg, 0.074 mmol), 2-hydroxyethylacrylate (16 µL, 0.153 mmol), and DMF (0.7 mL) were combined in a round bottom flask. The reaction mixture was heated at 50 °C overnight. Upon cooling to room temperature, the mixture was precipitated into cold ethanol. The polymer was dried under vacuum, resulting in a white solid (34 mg, 42%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ 7.44-7.18 (m, 5H, benzyl CH), 5.02-4.81 (m, 2H, benzyl CH_2), 4.28-3.99 (m, 0.1H), 3.84-3.38 (m, 1.6H), 3.38-3.28 (m, 0.1H), 2.53-0.50, 6.5H). ^{19}F NMR (471 MHz, CD_2Cl_2) δ -149.94 (1F), -151.55 (1F), -158.35 (1F), -162.85 (1F). SEC (PS standards): M_n = 16.3 kDa, $Đ$ = 1.18.



Scheme S2. Synthetic scheme of **PN$_3$-PPEG-*b*-PBn**.



PN$_3$-PPEG-*b*-PBn. PPFP-PPEG-*b*-PBn (300 mg, 0.021 mmol), **N$_3$-PEG-NH$_2$** (117 mg, 0.28 mmol), and DMF (2.4 mL) were combined in a round bottom flask. The reaction mixture was

heated at 50 °C overnight. Upon cooling to room temperature, the mixture was precipitated into cold ethanol. The resulting polymer was redissolved using THF and precipitated into cold ethanol. The polymer was dried under vacuum, resulting in an off-white solid (115 mg, 36%). ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.40-7.21 (m, 5H; benzyl CH), 5.02-4.81 (m, 2H; benzyl CH_2), 4.27-4.01 (m, 0.1H), 3.72-3.29 (m, 2.5H), 2.53-1.22 (m, 2.8H), 1.16-0.58 (m, 3.3H). SEC (PS standards): M_n = 19.4 kDa, $Đ$ = 1.23.

4. NMR & FTIR SPECTRA

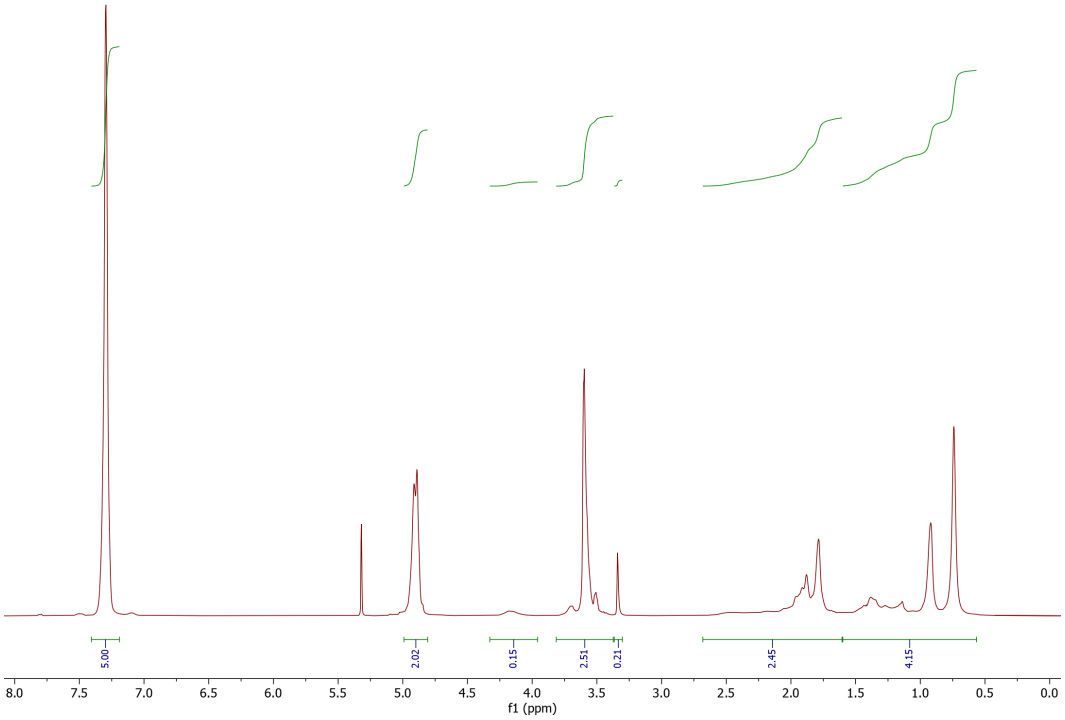


Figure S2. ^{1}H NMR spectrum (400 MHz, CD_2Cl_2) of **PPFP-PPEG-*b*-PBn**.

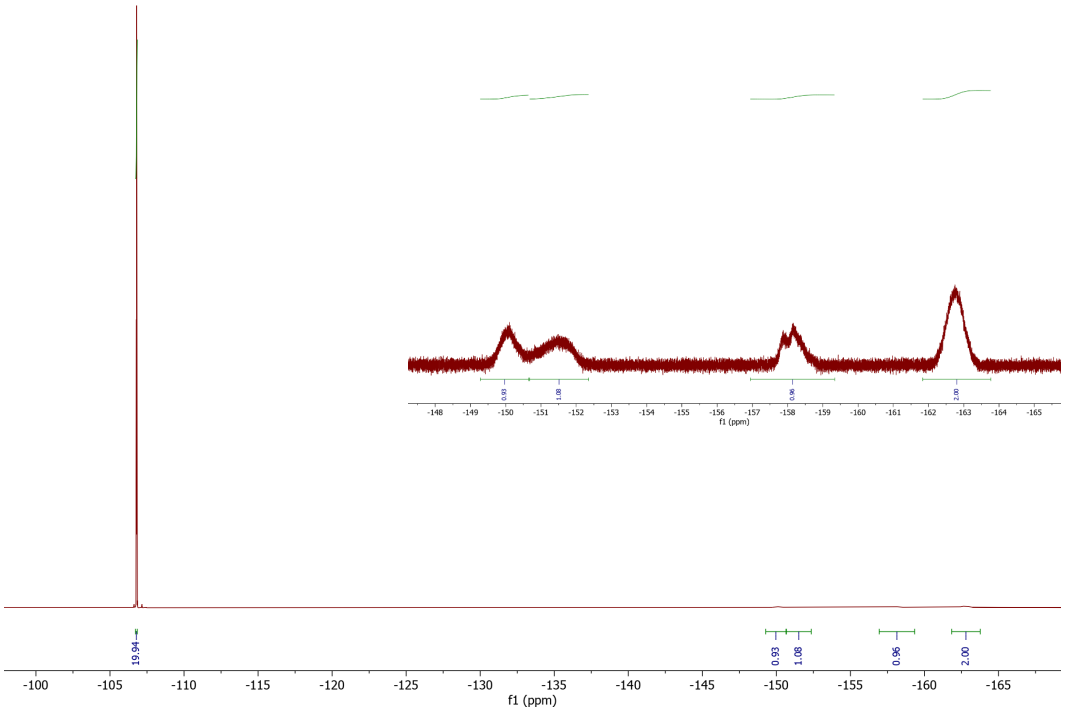


Figure S3. ^{19}F NMR spectrum (471 MHz, CD_2Cl_2) of **PPFP-PPEG-*b*-PBn** with 4,4'-difluorobenzophenone as a standard for quantitative NMR. There are ~6.5 pentafluorophenyl units per polymer.

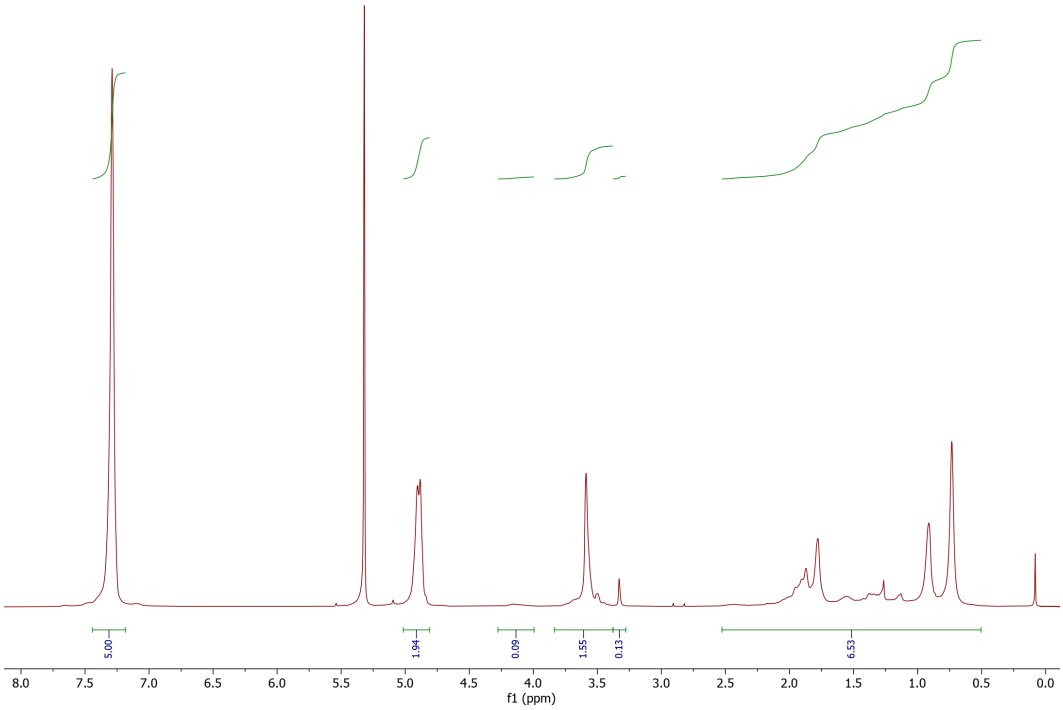


Figure S4. ^{1}H NMR spectrum (400 MHz, CD_2Cl_2) of **PDBCO-PPEG-*b*-PBn** with trace DMF.

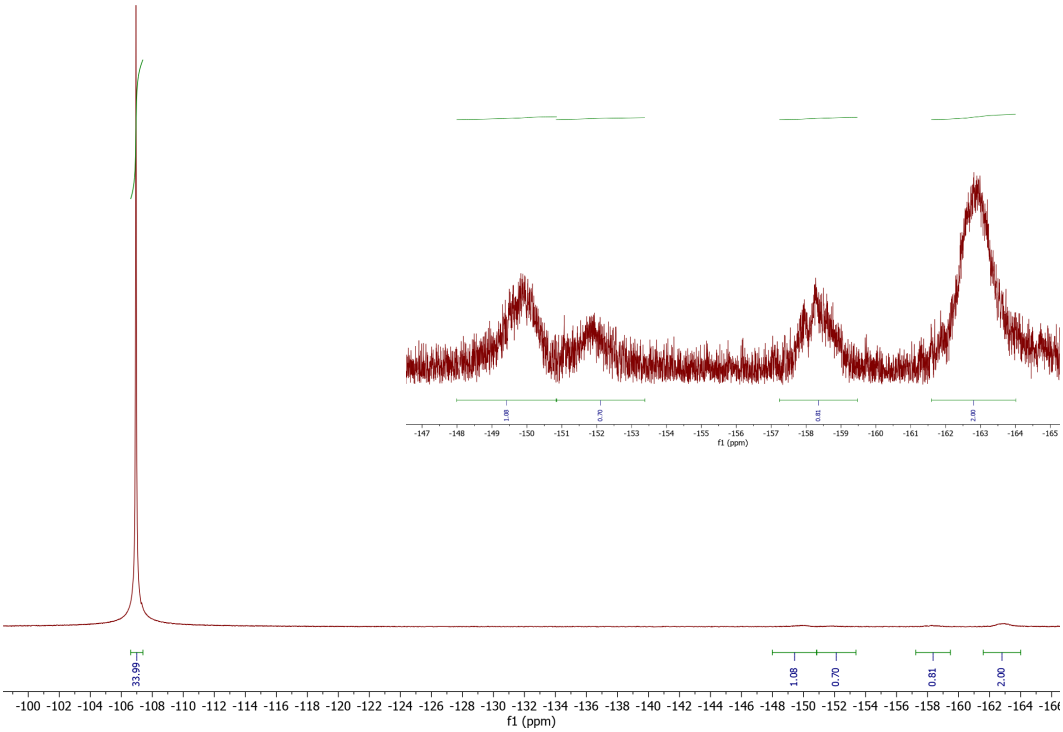


Figure S5. ^{19}F NMR spectrum (471 MHz, CD_2Cl_2) of **PDBCO-PPEG-*b*-PBn** with 4,4'-difluorobenzophenone as a standard for quantitative NMR. ~80% of the pentafluorophenyl subunits were substituted.

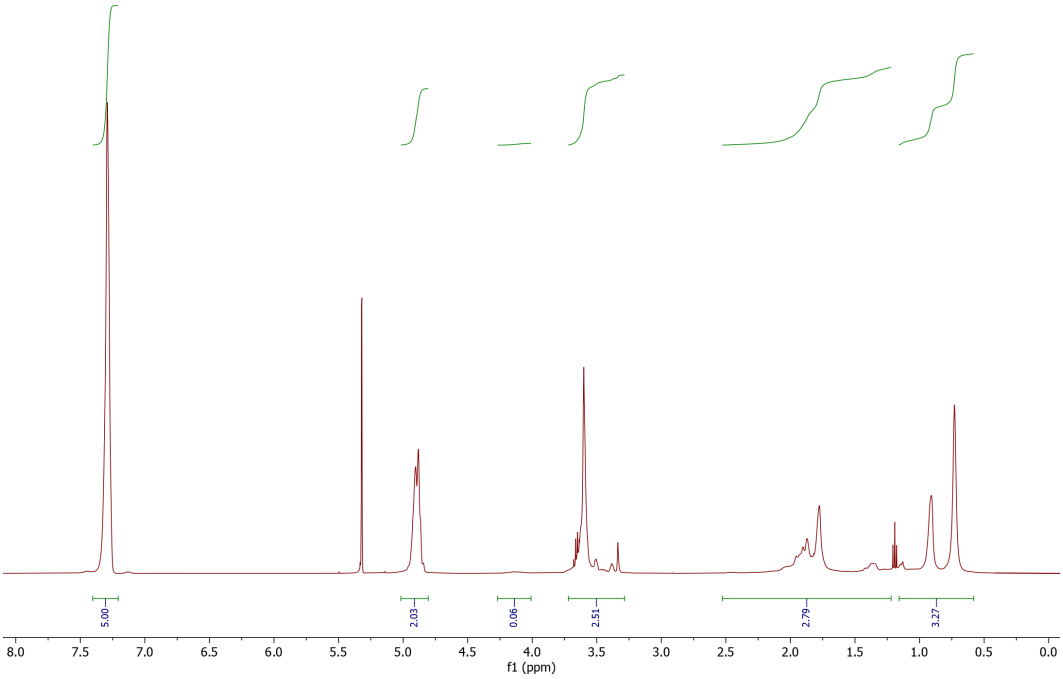


Figure S6. ^{1}H NMR spectrum (500 MHz, CD_2Cl_2) of **PPFP-PPEG-*b*-PBn** with trace ethanol.

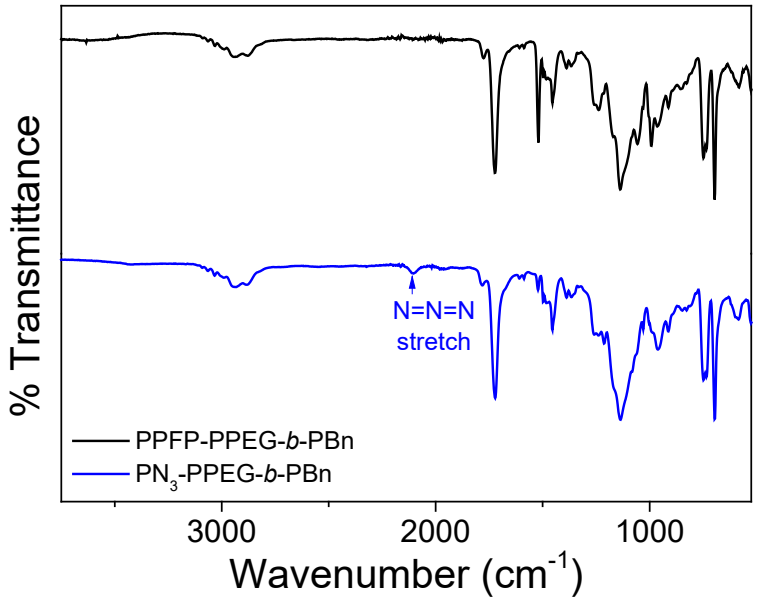


Figure S7. FTIR spectrum of **PPFP-PPEG-*b*-PBn** (black) and **PN_3-PPEG-*b*-PBn** (blue). There is a clear azide stretch in the **PN_3-PPEG-*b*-PBn** spectrum that is not present in **PPFP-PPEG-*b*-PBn** spectrum.

5. SUPPLEMENTARY OPTICAL IMAGES & SCHEMES

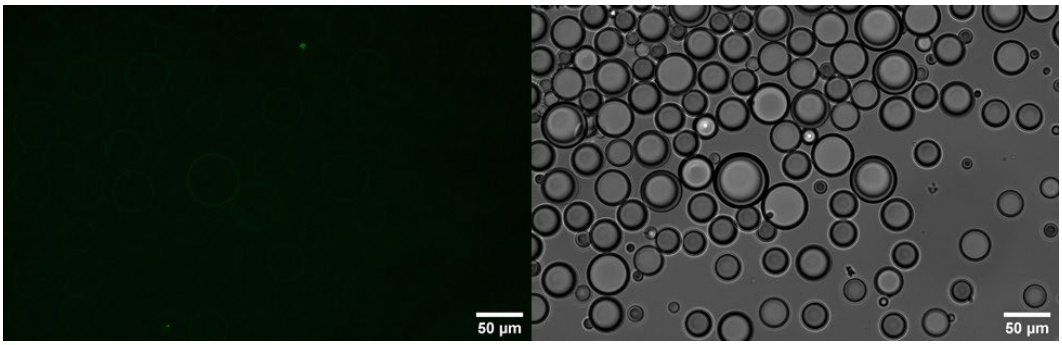


Figure S8. Fluorescence microscopy images of N_3-functionalized droplets after attempted bioconjugation with FITC-labeled E2. 2.94 nmol of FITC-labeled E2 was added to 30 µL of droplets.

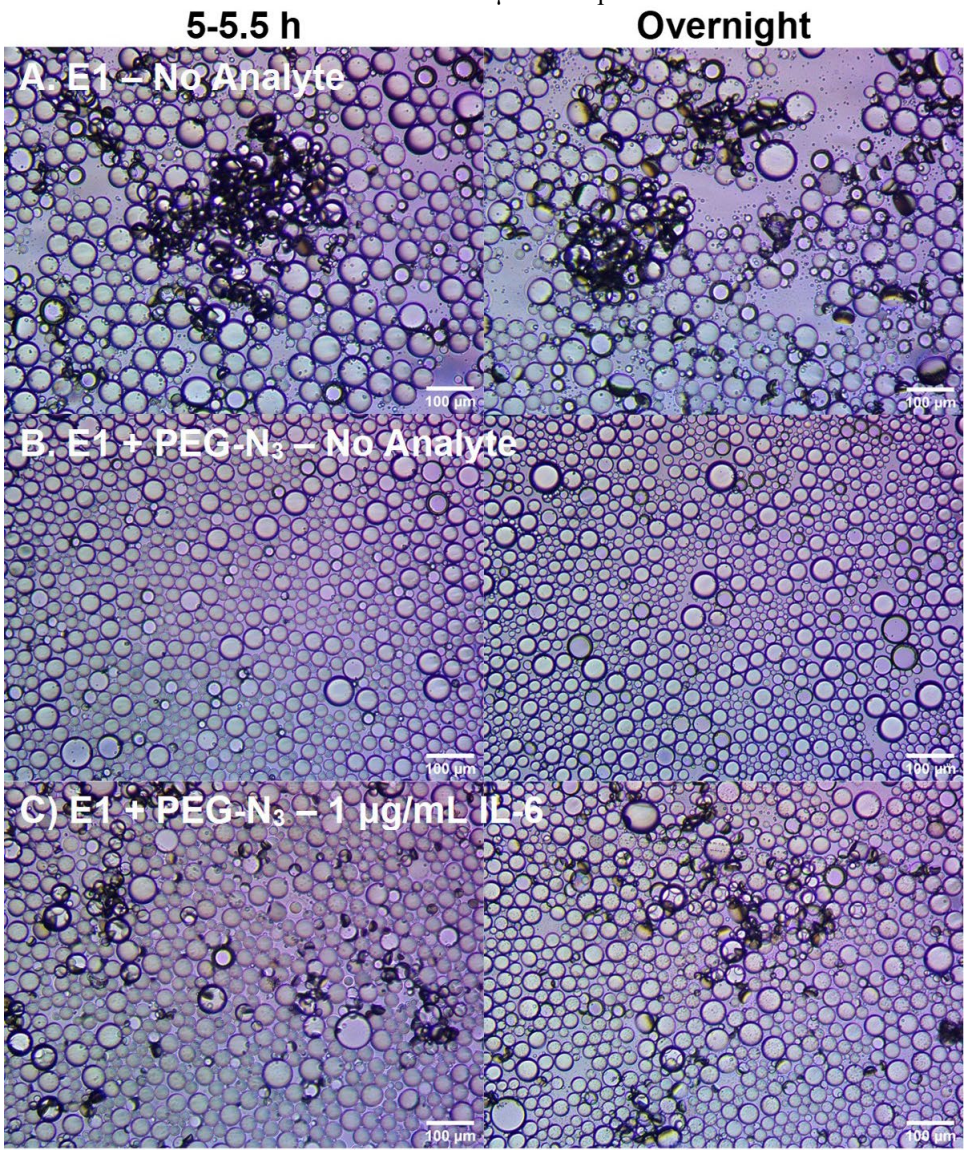


Figure S9. Inverted microscope images of **A.** E1-functionalized droplets with no analyte, **B.** E1+PEG-functionalized droplets with no analyte, and C) E1+PEG-functionalized droplets with 1 µg/mL of IL-6. ~8.9 nmol of E1 (purified via Ni^{2+} resin) was added to 30 µL of droplets during the bioconjugation step. For the E1+PEG-functionalized droplets, an additional 3 nmol of PEG-N_3 was added to the droplets.

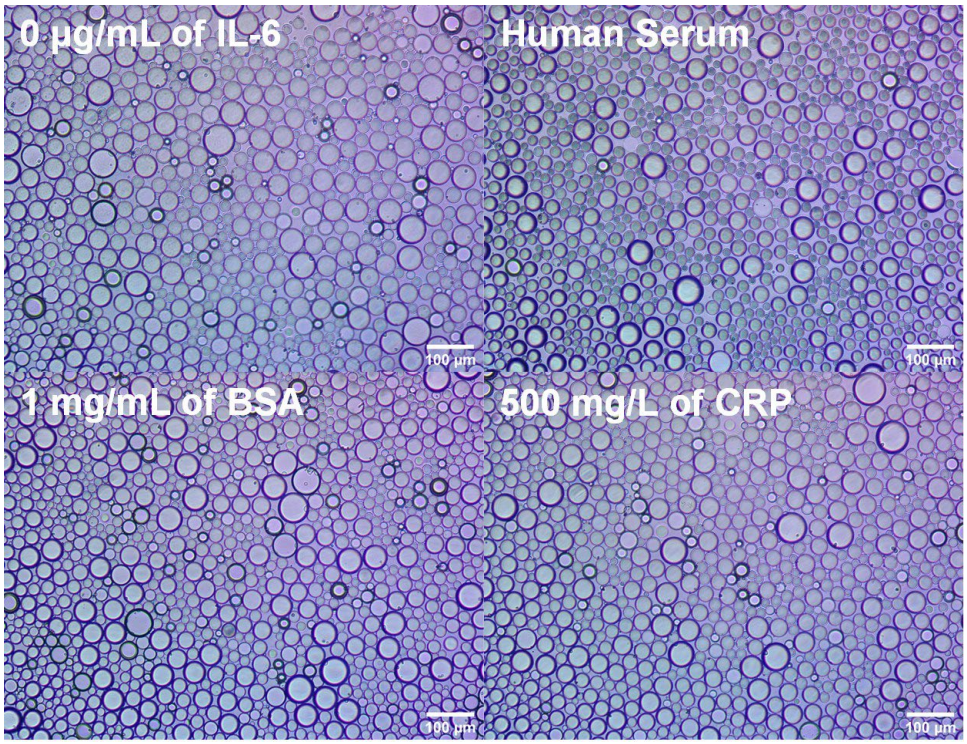
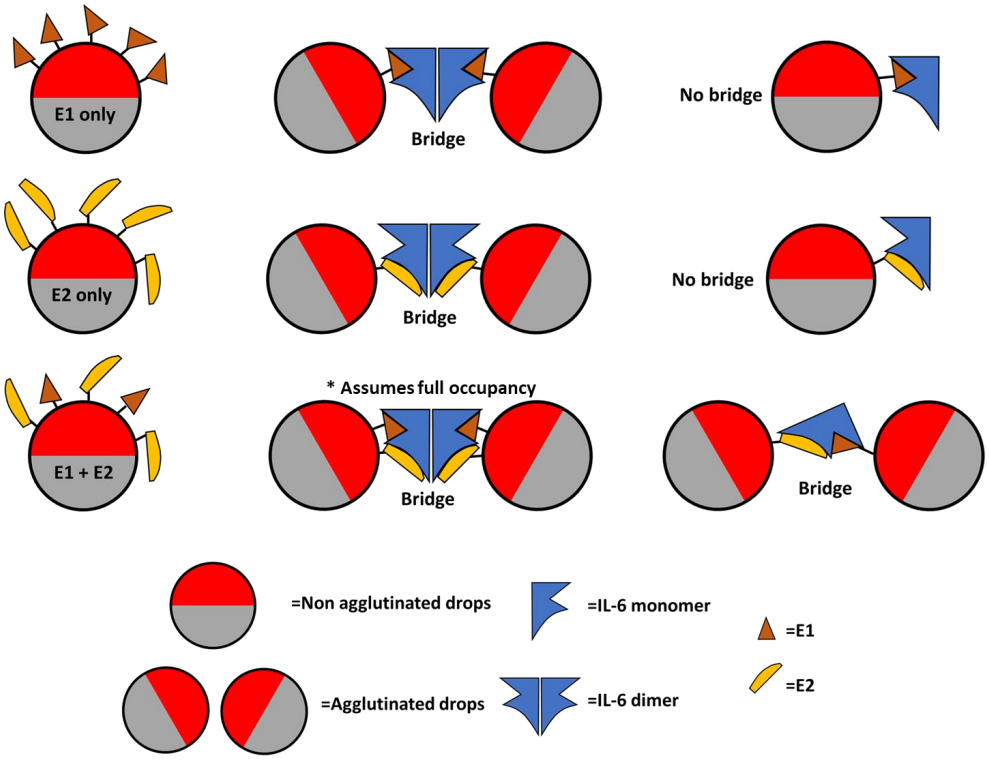


Figure S10. Inverted microscope images of 1:1 E1+PEG- and E2+PEG-functionalized droplets incubated in different conditions overnight (0 µg/mL of IL-6, human serum, 1 mg/mL of BSA, 500 mg/L of CRP, and 10 µg/mL of IL-6). Droplets functionalized with proteins purified via Ni^{2+} resin and subsequent size exclusion chromatography are shown.

Potential Modes of Binding



Scheme S3. Representative image of potential binding schemes of singly (E1/E2 only) functionalized droplets which can only productively bind the dimer of IL-6 on both sides resulting in a bridge between droplets, and doubly (E1+E2) functionalized droplets which can productively bind both the dimer and monomer to promote agglutination. * indicates that a productive bridge could be formed with only one binder on each side of IL-6, but can potentially be bound by four total binders.

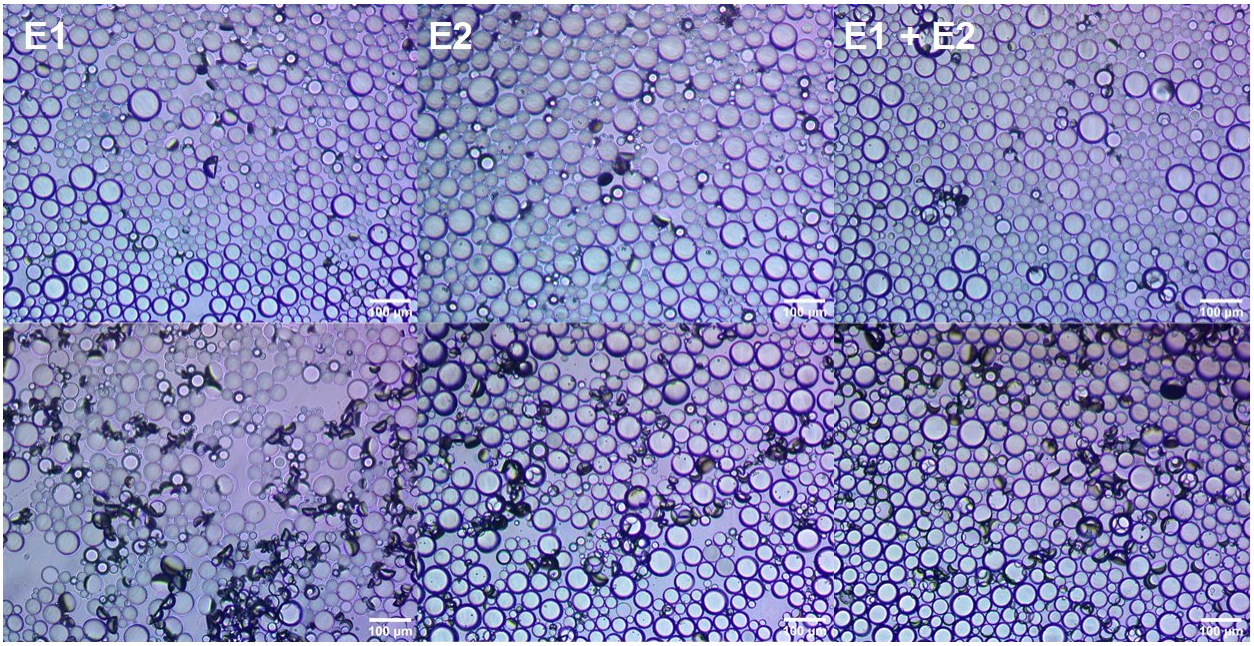


Figure S11. Inverted microscope images of rcSso7d-IL6+PEG-functionalized droplets incubated with 10 µg/mL of IL-6 in 1X PBS. Agglutination assays after 1 h and overnight incubation are shown in the first and second rows, respectively.

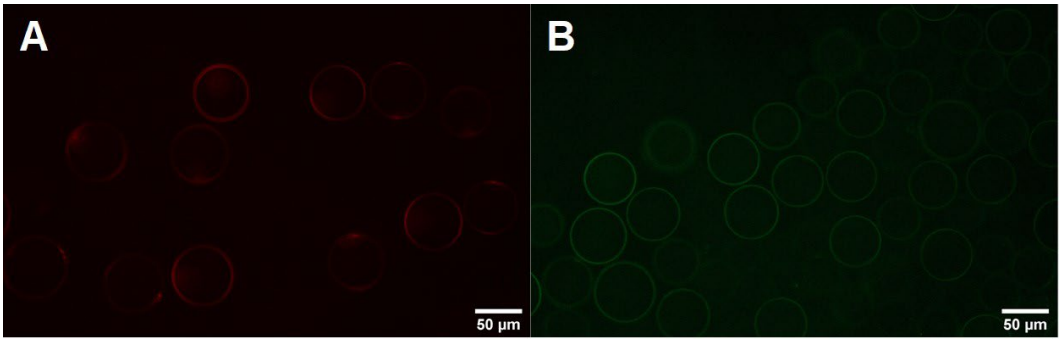


Figure S12. Fluorescence microscopy images of A) antibody-functionalized droplets (ThermoFisher) after incubation with anti-rat secondary antibody (Alexa Fluor 594) and B) antibody-functionalized droplets (R&D Systems) after incubation with anti-mouse secondary antibody (FITC).

6. IMAGE ANALYSIS

Image Analysis with ImageJ. Each RGB image was split into its red, green, and blue components. Pixel counting was performed via the built-in histogram function on the blue channel image; the blue channel was chosen because the droplet outlines were less intense compared to the original image and the red and green component images (Figure S13).

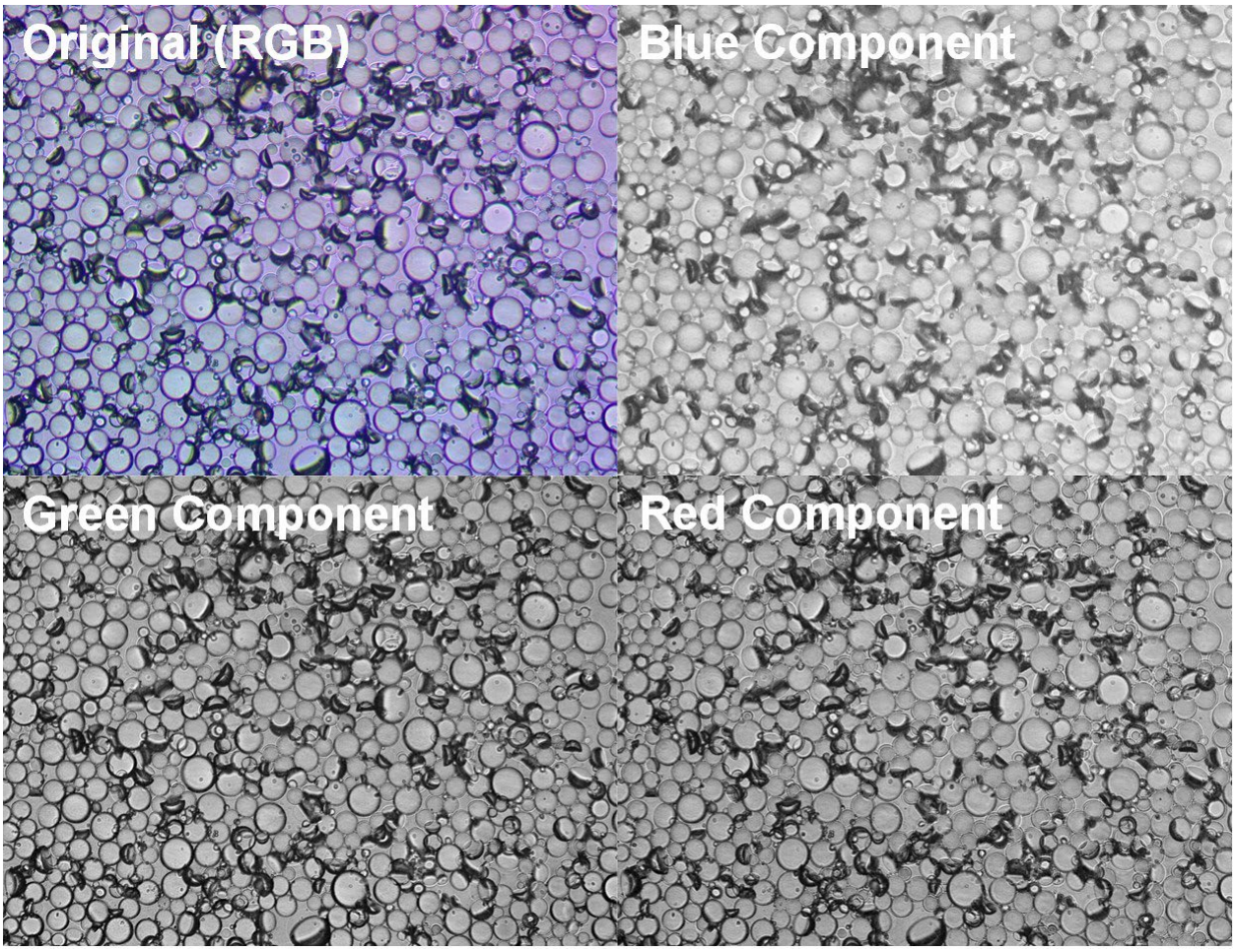


Figure S13. Inverted microscope images of rcSso7d-IL6+PEG-functionalized droplets incubated overnight with 10 µg/mL of IL-6 in 1X PBS.

For each assay, the pixel threshold for agglutination and baseline correction was chosen as follows: the histogram function was applied to each 0 h image to determine the average and standard deviation. These values were tabulated and the value two standard deviations below the average ('thresh') was determined for each 0 h image. The threshold for that particular assay is the mean of the 'thresh' value for each 0 h image of a particular dataset. To determine the baseline correction value, the counts between 0 (darkest pixel) and the threshold were summed and divided by the total number of pixels. The average of all these values was used as the baseline correction for that specific assay. Images with different lighting that causes different hues and shadows and multilayers of droplets were not included in this process.

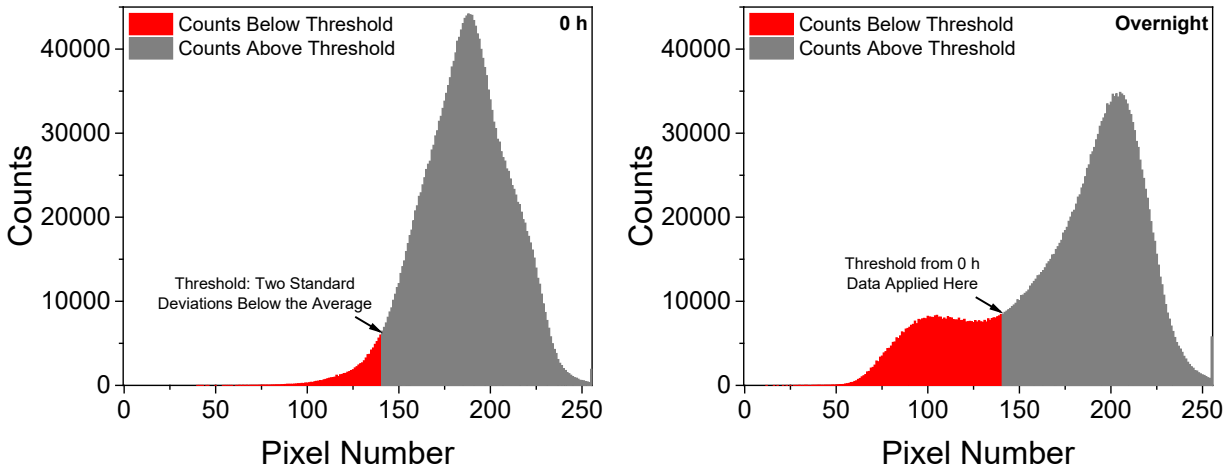


Figure S14. Example histograms of the pixels from the blue component of microscope images collected at 0 h (left) and overnight (right). Pixels below the threshold are shaded in red. The threshold is determined by tabulating the value two standard deviations below the average for each 0 h image and then averaging the set of values. In this example, the threshold is pixel number 140.

To determine the percent agglutination for a particular assay, the histogram function was applied to each image taken at a specific time point. For each image, the number of pixels below the threshold were summed and divided by the total number of pixels. The following images were not included in the final set of images: (1) images with distorted droplets such as large monophasic droplets (Figure S15A), (2) images with sparse amounts of droplets (Figure S15B), (3) images with lighting artifacts such as shadows and different color profiles/hues (Figures S15C and S15D), and (4) images with foreign objects (Figure S15E). The top quintile of images or the top three images with the most agglutination, whichever was greater, were used for the final response curve.

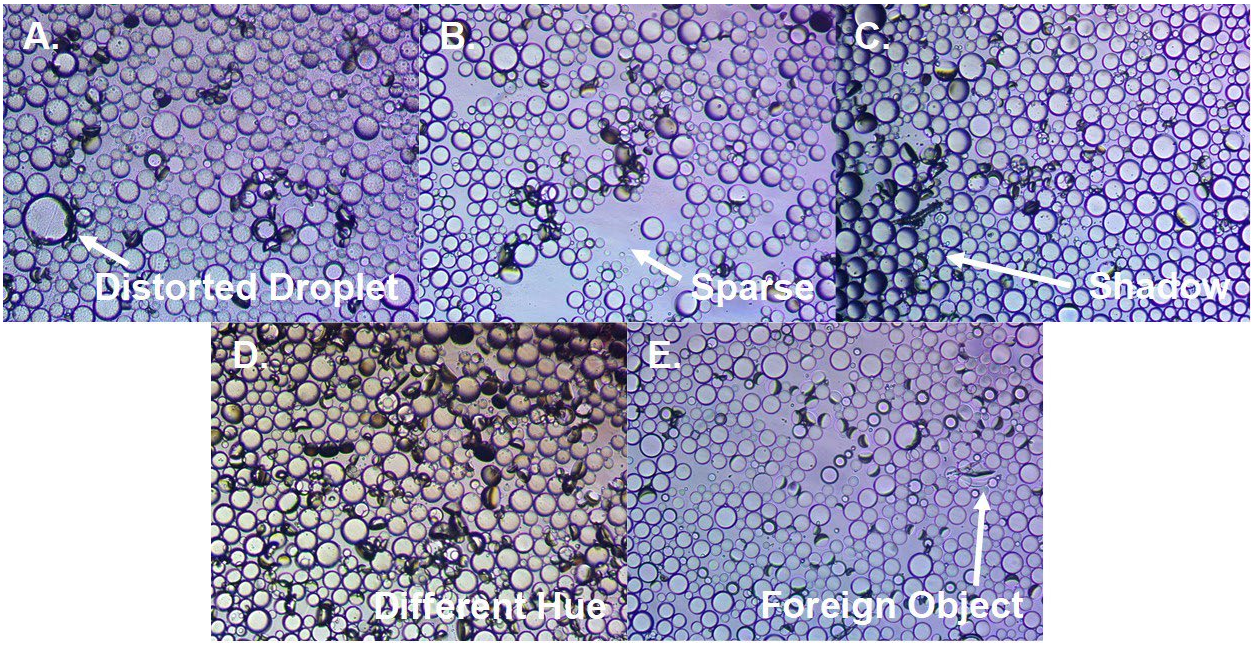


Figure S15. Types of images not included in the set of images: **A.** images with distorted droplets; **B.** images with sparse amounts of droplets; **C.** images with shadows; **D.** images that are different, often pinkish, hue; and **E.** images with foreign objects.

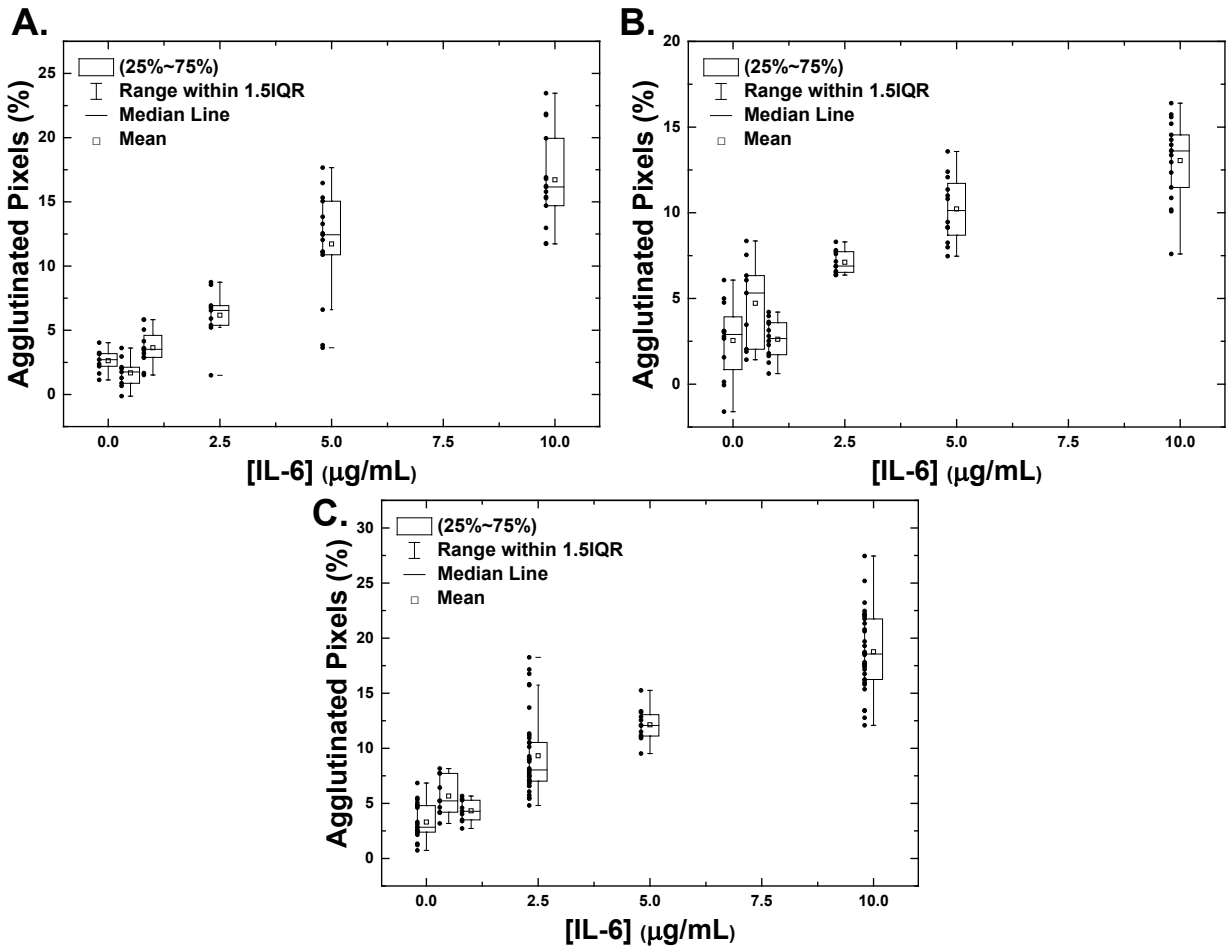


Figure S16. Box plots of the percentage of agglutinated pixels after overnight incubation with IL-6. Assays were performed with **A.** E1+PEG-functionzlied, **B.** E2+PEG-functionalized, and **C.** 1:1 E1+PEG- and E2+PEG-functionalized droplets. Data shown here were used to generate the response curves.

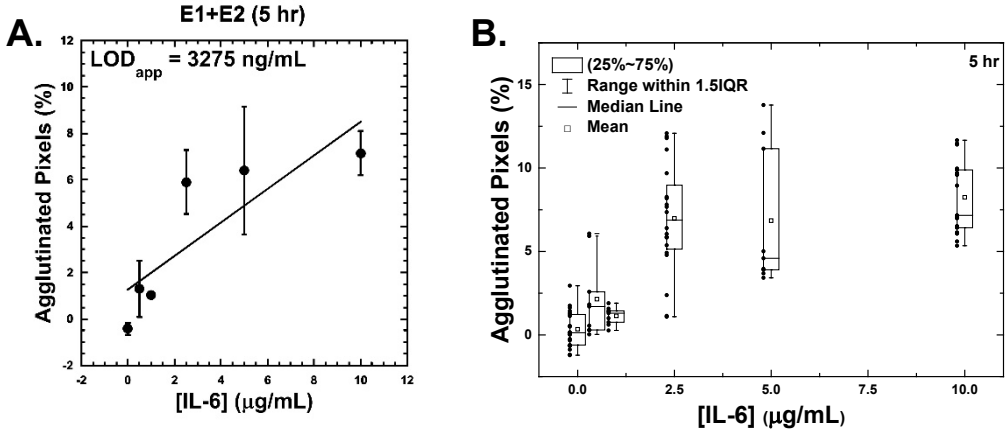


Figure S17. A. Dose response curves for 1:1 E1+PEG- and E2+PEG-functionalized Janus droplet after incubation with IL-6 for 5 hours. Data are fit to linear responses and standard errors are reported. Data represent $n \geq 3$ replicates. **B.** Box plot of the percentage of agglutinated pixels after incubation with IL-6 for 5 hours. Data shown here were used to generate the response curves.

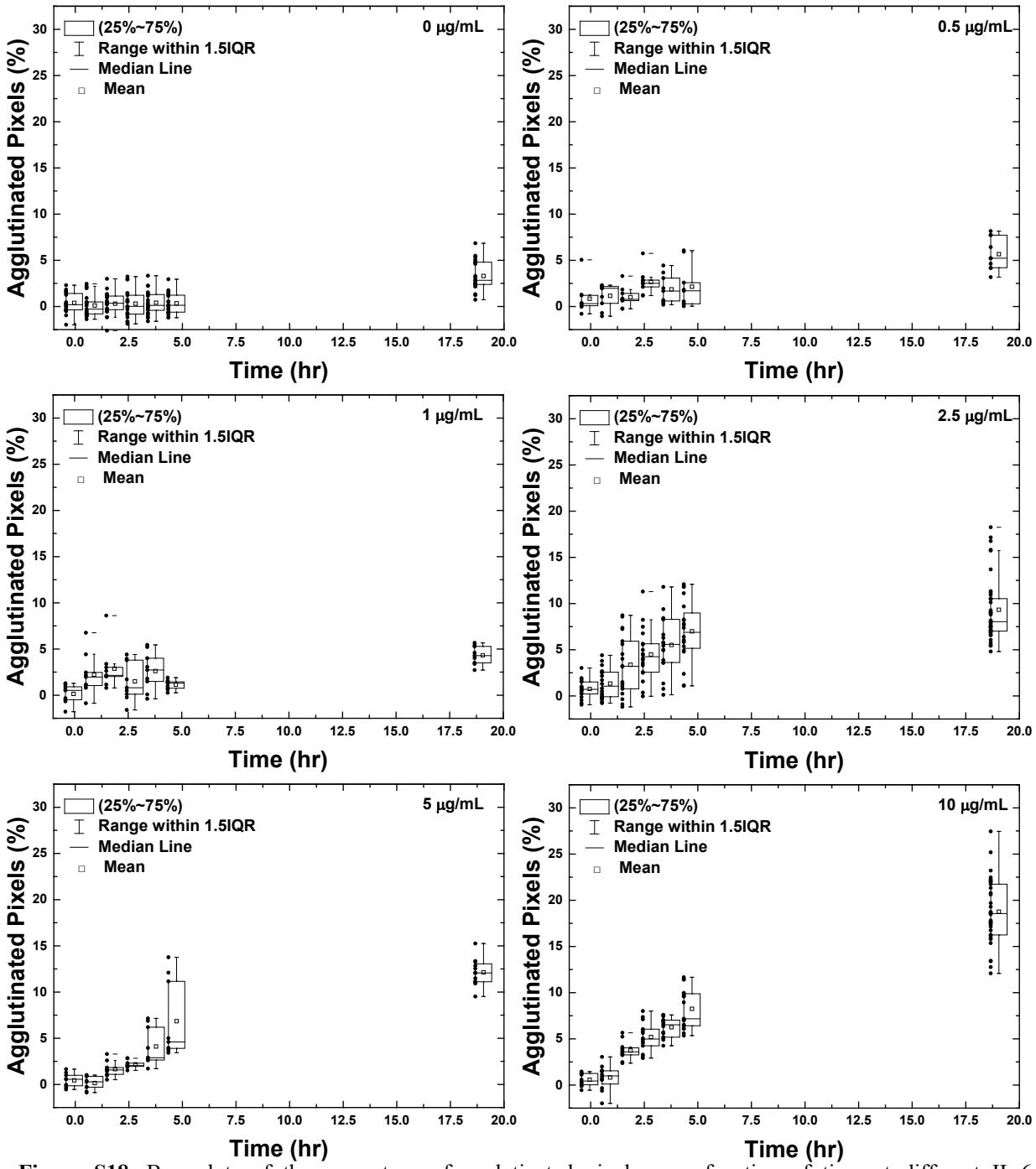


Figure S18. Box plots of the percentage of agglutinated pixels as a function of time at different IL-6 concentrations. Assays were performed with 1:1 E1+PEG- and E2+PEG-functionalized droplets. Data shown here were used to generate the kinetic plots.

7. REFERENCES

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