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

Supplementary Information

A Versatile Platform for Surface Modification of Microfluidic Droplets

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Experimental section

1. Materials and methods

Pluronic copolymer F127, poly(ethylene glycol) methyl ether acrylate (average M_n 480), thionyl chloride, triethylamine, bovine serum albumin (BSA), biotin, anhydrous magnesium sulfate and doxorubicin hydrochloride (DOX·HCl) were purchased from Sigma-Aldrich and used without further purification. Pyridine, methanesulfonyl chloride, ammonium hydroxide, 6-maleimidohexanoic acid, succinic anhydride, fluorescein isothiocyanate (FITC), dichloromethane, 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), *N,N′*-Dimethylformamide (DMF), and benzophenone were all ordered from Alfa Aesar. *N*-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), Texas Red-conjugated NeutrAvidin (NeutrAvidin-Texas Red, NA-TR) were obtained from ThermoFisher Scientific. Diethyl ether (Acros Organics), [5-((2-(or-3)-S-(Acetylmercapto)succinoyl)amino)fluorescein] (SAMSA fluorescein, Setareh Biotech, USA), poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning), HFE-7500 (3 M, St. Paul, MN), Pico-Surf™ 2 surfactant (Dolomite Microfluidics, Charlestown, MA) were used without further purification. DMF was purified by vacuum distillation with CaH_2. PAMAM G3 dendrimer-Cy5 conjugate (PAMAM-Cy5) was prepared as described previously.[1] Purified deionized water was prepared using a Milli-Q Plus system (Millipore Co., Billerica, MA, USA). ^{1}H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer in $CDCl_3$ and DMSO-d_6, with tetramethylsilane (TMS) as the internal standard. FT-IR spectroscopy was performed using a Perkin Elmer Spectrum Two FT-IR spectrometer equipped with a universal ATR sampling accessory (resolution: 4 cm^{-1}). The generation of microfluidic droplets was observed using an inverted microscope equipped with a digital camera (Orca Flash 4.0, Hamamatsu, Japan). Fluorescent images were captured using a Nikon Eclipse TE2000-U inverted microscope (Nikon, Tokyo, Japan).

2. Synthesis of F127-NH_2

Amine-terminated F127 (F127-NH_2) was obtained by mesylation of F127 in dichloromethane, with subsequent ammonolysis in ammonium hydroxide (**Scheme 1a**). First, F127 (151.445 g, 12.02 mmol) was added into a flask and heated to 120 °C for 4 h to remove water. After cool-down, 500 mL of anhydrous dichloromethane was added to dissolve the dried F127. Then,

triethylamine (20.0 mL, 143.49 mmol) and methanesulfonyl chloride (20.0 mL, 258.4 mmol) were added under nitrogen protection. After stirring at room temperature for 3 days, the mixture was washed with saturated sodium chloride solution five times, precipitated into an excess of cold diethyl ether three times, and dried in a vacuum oven to afford a white solid. The resulting mesylated F127 was dissolved in ammonium hydroxide and stirred for 3 days, after which the mixture was extracted with dichloromethane several times. The combined organic layers were subsequently washed with saturated sodium chloride solution, dried over anhydrous magnesium sulfate, filtered, concentrated by rotary evaporation, precipitated thrice in cold diethyl ether, and dried in vacuum oven to obtain the final product. Yield: 75%.

3. Synthesis of F127-COOH and F127-NHS

Synthesis of NHS-terminated F127 (F127-NHS) was conducted in two steps as shown in **Scheme 1b**. First, carboxyl-terminated F127 (F127-COOH) was obtained through the reaction of F127 with succinic anhydride in pyridine. Briefly, F127 (30.0 g, 4.76 mmol OH) was dissolved in pyridine (60 mL), followed by the addition of succinic anhydride (7.1478 g, 71.4 mmol). The reaction was performed at room temperature for 48 h under a dry nitrogen atmosphere. Afterwards, the reaction mixture was diluted with dichloromethane (150.0 mL) and washed with saturated sodium chloride solution three times. The organic layer was separated and dried over anhydrous magnesium sulfate overnight, filtered, and concentrated by rotary evaporation. The product (F127-COOH) was isolated by precipitation in cold diethyl ether three times and dried under vacuum. Yield: 94%.

After structural confirmation *via* NMR, the terminal carboxyl groups of F127-COOH were further activated by EDC/NHS. Briefly, F127-COOH (8.12 g, 1.27 mmol COOH) was dissolved in 70 mL of anhydrous dichloromethane, to which a mixture of EDC·HCl (741 mg, 3.86 mmol) and NHS (445 mg, 3.86 mmol) dissolved in 10 mL of dichloromethane was added. Following stirring for 24 h at room temperature, the solution was washed with saturated sodium chloride, dried over anhydrous magnesium sulfate, filtered, concentrated under vacuum and precipitated thrice in cold diethyl ether to yield the white powdered F127-NHS. Yield: 86%.

4. Synthesis of F127-MAL

Maleimide-terminated F127 (F127-MAL) was prepared by acyl-chlorination of 6-maleimidohexanoic acid into 6-maleimidohexanoic acyl chloride, followed by direct reaction with F127, to form the covalent conjugation (**Scheme 1c**).

For the first step, 6-maleimidohexanoic acyl chloride was synthesized as previously described with minor modifications.[2] Briefly, 6-maleimidohexanoic acid (500 mg, 2.37 mmol) and thionyl chloride (3.4 mL, 46.8 mmol) were added into a heat-dried flask under a dry nitrogen atmosphere. The reaction was maintained at 60 °C for 48 h. After that, the redundant thionyl chloride was removed under vacuum and the resulting yellow crude product was used without further purification.

For the second step, F127 was reacted with 6-maleimidohexanoic acyl chloride in anhydrous dichloromethane in the presence of triethylamine. Specifically, F127 (2.1945 g, 0.174 mmol) was added into a flask and heated to 120 °C for 4 h to remove water. After cool-down, 10 mL of anhydrous dichloromethane was added to dissolve the dried F127. Then, 6-maleimidohexanoic acyl chloride obtained from the first step was dissolved in 10 mL of anhydrous dichloromethane and introduced into the F127 solution, followed by the addition of triethylamine (97.1 μL, 0.696 mmol). The reaction mixture was stirred at room temperature for 24 h, and the solvent was concentrated by rotary evaporation. Finally, the product was purified by precipitation in cold diethyl ether five times and dried under vacuum. Yield: 69%.

5. Synthesis of F127-Biotin

First, biotin N-succinimidyl ester (Biotin-NHS) was synthesized according to previously published methods,[3-5] with minor modifications (**Scheme 1d**). Briefly, under argon atmosphere, a flame-dried flask was charged with biotin (20.0 g, 81.86 mmol), NHS (10.4 g, 90.36 mmol) and anhydrous DMF (200 mL). After complete dissolution, EDC·HCl (20.6 g, 107.46 mmol) was added, and the solution was stirred at room temperature for 24 h. Most of the solvent was evaporated under reduced pressure, and the residue was precipitated with an excess of cold diethyl ether. The resulting precipitate was recrystallized from isopropanol to yield a white powder.

For the synthesis of biotinylated F127 (F127-Biotin), Biotin-NHS (7.80 g, 22.85 mmol) and F127-NH_2 (36.0 g, 2.86 mmol) were dissolved in anhydrous DMF (450 mL), and the reaction mixture was stirred at room temperature for 24 h. After the solvent was concentrated under reduced pressure, the residue was precipitated in cold diethyl ether. The resulting product was subsequently dissolved in DMF, dialyzed against DMF for 2 d, and then against water for another 2 d. The final product was obtained in the form of a white power after lyophilization of the dialyzed solution. Yield: 78%.

6. Preparation of FITC-labeled BSA

FITC-labeled BSA (BSA-FITC) was prepared according to a slightly modified version of the reported procedure.[6] BSA (40.0 mg) was dissolved in 4 mL of sodium bicarbonate (0.1 M). Then, FITC (4.0 mg) dissolved in 600 μL of DMSO was added to the solution. After overnight stirring at room temperature, the resulting solution was dialyzed exhaustively against water and lyophilized to yield a yellow fluffy powder.

7. Cell culture

Human umbilical cord blood-derived endothelial progenitor cells (EPCs) were derived as previously described.[7] The collection and usage of human blood for this study were performed according to the protocol that was previously approved by the Duke University Institutional Review Board. Normal human dermal fibroblasts (NHDFs), available from neonatal foreskin, were purchased from Lonza. Human promyelocytic leukemia cell (HL-60), human bone marrow-derived mesenchymal stem cell (MSC), human intestinal cell (Caco-2), and human non-small lung cancer cell (A549) were purchased from ATCC.

EPCs were maintained in complete endothelial cell growth medium containing EBM-2 (Lonza) with EGM-2 SingleQuot Kit supplements (Lonza) and 100 U mL^{-1} Penicillin-Streptomycin (Life Technologies). Medium was changed every other day. Cells were passaged at a ratio of 1:10 upon confluence, and passage 6 was used in cell toxicity tests. NHDFs were maintained in Dulbecco's Modified Eagle's Medium (DMEM w/High Glucose, L-Glutamine, and Sodium Pyruvate, Cat. No. 11995, Life Technologies) with 10% fetal bovine serum (Life Technologies) and 100 U mL^{-1}

Penicillin-Streptomycin (Life Technologies). Cells were passaged at a ratio of 1:5 upon confluence, and passage 6 was used in cell toxicity tests. MSCs were maintained in complete mesenchymal stem cell growth medium that contains hMSC Basal Medium (Lonza) with hMSC SingleQuot Kit supplement (Lonza) and 100 U mL^{-1} Penicillin-Streptomycin (Life Technologies). Upon confluence, the MSCs were passaged at a ratio of 1:5, and passage 8 was used in cell toxicity tests. HL-60 cells were maintained in RPMI 1640 (Life Technologies) with 15% fetal bovine serum (Life Technologies) and 100 U mL^{-1} Penicillin-Streptomycin (Life Technologies). CACO-2 cells were maintained in DMEM/F12 (Life Technologies) with 15% fetal bovine serum (Life Technologies) and 100 U mL^{-1} Penicillin-Streptomycin (Life Technologies). A549 cells were maintained in DMEM (Life Technologies) with 10% fetal bovine serum (Life Technologies) and 100 U mL^{-1} Penicillin-Streptomycin (Life Technologies). All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Before each experiment, the cell density was determined using a hemocytometer.

8. Cytotoxicity assay

The cytotoxicities of F127, F127-NH_2, F127-COOH, F127-NHS, F127-MAL and F127-Biotin were evaluated by MTT assays. The cells were seeded in 96-well plates (8×10^3 cells per well for NHDF, A549, HL-60 and Caco-2 cells; 6×10^3 cells per well for EPC and MSC), with 180 μL of culture medium in each well, and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. The culture medium was replaced with 200 μL of fresh medium containing F127, F127-NH_2, F127-NHS, F127-MAL and F127-Biotin. After another 24 h of incubation, the cells were subjected to an MTT assay. The absorbency of the solution was measured with a BMG Labtech FLUOStar Optima microplate reader (BMG Labtech, Ortenberg, Germany) at 490 nm. The relative cell viability was determined by comparing the absorbance at 490 nm with the absorbance of control wells containing only cell culture medium. Data are presented as mean ± standard deviation ($n = 6$).

The cytotoxicities of HFE-7500, single emulsion and double emulsion droplets were also determined using the procedures described above.

9. Fabrication of microfluidic devices

Microfluidic chips were fabricated using slightly modified versions of the photo- and soft-lithography techniques described in our previously published work.[8-9] Briefly, the cross-channel microfluidic patterns were designed using AutoCAD (Autodesk) and printed out as transparent photomasks. The patterned mold was fabricated by depositing negative photoresist (SU-8 3000, MicroChem, Westborough, MA) onto a silicon wafer, based on the manufacturer's photo-lithography protocol. The microfluidic devices were prepared by soft-lithography with PDMS, followed by bondage to glass slides after oxygen plasma treatment. A similar hydrophilic coating procedure was performed, as described by Schneider *et al,*[10] to create a hydrophilic surface along the walls of the microchannels. In particular, the anionic acrylic acid used in the reported method was replaced by a neutral monomer, poly(ethylene glycol) methyl ether acrylate.

10. Single emulsion (SE) droplet generation

Oil-in-water (O/W) single emulsion droplets were prepared in the flow-focusing microfluidic device with hydrophilic channels (**Figures 1A and S7A**). The continuous fluid and disperse fluid (HFE-7500) were separately infused into the channels by syringe pumps (Harvard Apparatus PHD 2000), and the droplets were formed at the cross-junction. For the preparation of O/W single emulsion droplets with different functional groups on the surface, each of the following aqueous solutions were used as the continuous phase for their corresponding functional groups: 3.0 wt% F127; 3.0 wt% F127-NH_2; 3.0 wt% F127-COOH; 3.0 wt% F127-NHS; 3.0 wt% F127-MAL; 3.0 wt% F127-Biotin; 1.5 wt% F127-NHS plus 1.5 wt% F127-MAL; or 1.5 wt% F127-NHS plus 1.5 wt% F127-Biotin. The flow rates of the oil and water phases were 5 and 15 μL min^{-1}, respectively.

11. Double emulsion (DE) droplet generation

A setup of two stand-alone chips was used to prepare DE droplets.[8, 11] The water-in-oil (W/O) droplets were first generated in a hydrophobic chip and subsequently flowed through a hydrophilic chip to form the water-in-oil-in-water (W/O/W) emulsions. The inner aqueous fluid consists of 1% F127 while HFE-7500 supplemented with 1% Pico-Surf™ 2 surfactant comprises the middle phase. The outer aqueous phase consists of one of the following solutions: 3.0 wt% F127; 3.0 wt% F127-NH_2; 3.0 wt% F127-COOH; 3.0 wt% F127-NHS; 3.0 wt% F127-MAL; 3.0 wt% F127-

Biotin; 1.5 wt% F127-NHS plus 1.5 wt% F127-MAL; or 1.5 wt% F127-NHS plus 1.5 wt% F127-Biotin. The flow rates of the inner, middle and outer phases were 2, 3 and 14 μL min^{-1}, respectively.

12. Surface modification of droplets

In general, the SE and DE droplets were collected in 1.5 mL Eppendorf tubes pre-filled with 1.0 mL water, and washed 6 times with water to remove any free surfactant. Briefly, after the emulsion droplets sank to the bottom, as much of the supernatant as possible was removed (the liquid level must be higher than the droplets) without disturbing the settled droplets, and then the same volume of water was slowly added to the tube, constituting the first wash. This step was repeated five more times. All of the modification procedures were performed in a 24-well tissue culture plate. Droplets stabilized with unmodified F127 served as a control group for the various modifications. Detailed procedures were described.

12.1. F127-NH_2

The negatively charged protein BSA (isoelectric point: ~4.7) was used as a model for surface coating on the positively charged surface of droplets. The amine-functionalized droplets were transferred to an aqueous solution of BSA-FITC (50 μg mL^{-1}) and allowed to sit for 30 min, followed by thorough washing with water and examination under a fluorescence microscope.

12.2. F127-COOH

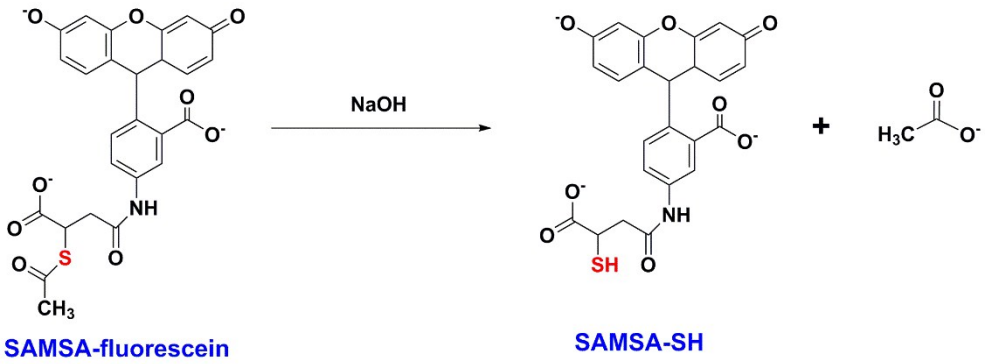
The cationic PAMAM dendrimer was used as a model for surface coating on the anionic surface of droplets. After free surfactant was removed, the carboxyl-functionalized droplets were incubated with PAMAM-Cy5 (2 μg mL^{-1}) for 1 h and washed with water before imaging.

12.3. F127-NHS

DOX with a primary amine was used as a model molecule to test the reactivity of the NHS-activated droplet surface. The droplets with reactive NHS ester were incubated with DOX solution (0.2 mg mL^{-1}, phosphate buffer, 10 mM, pH 7.4) for 3 h. Images were captured with a fluorescence microscope after removal of free DOX by washing.

12.4. F127-MAL

SAMSA fluorescein, with a protected thiol group, was used as a model molecule for surface modification of droplets *via* thiol-maleimide click reaction. SAMSA fluorescein was activated by following the reported procedures.[12, 13] Briefly, SAMSA (2.0 mg) was dissolved in 0.2 mL of sodium hydroxide solution (0.1 M) and stirred for 15 min. Afterwards, 5.6 μL of hydrochloric acid solution (3.0 M) and 50.0 μL of phosphate buffer (pH 7.0, 0.5 M) were added and stirred for another 10 min.



Scheme S1. Activation of SAMSA-fluorescein by treatment with NaOH solution.

Next, the maleimide-functionalized droplets were incubated with the activated SAMSA fluorescein (SAMSA-SH, 0.2 mg mL^{-1}) at room temperature for 2 h. After repeated washing to remove free fluorescent molecules, the droplets were imaged through a fluorescence microscope.

12.5. F127-Biotin

The biotin-functionalized droplets were added to an aqueous solution of NeutrAvidin-Texas Red (2 μg mL^{-1}, 10 mM phosphate buffer, pH 7.4). After incubation at room temperature for 30 min, the droplets were repeatedly washed to remove free NeutrAvidin. Then, the droplets were imaged under a fluorescence microscope.

12.6. F127-NHS and F127-MAL

The NHS and MAL surface-functionalized droplets were cultured with DOX (0.2 mg mL^{-1}) and SAMSA-SH (0.2 mg mL^{-1}) in phosphate buffer solution (10 mM, pH 7.4) for 3 h. The droplets were analyzed by fluorescence microscopy after the removal of free dyes through washing.

12.7. F127-MAL and F127-Biotin

The Biotin and MAL surface-functionalized droplets were mixed with SAMSA-SH (0.2 mg mL^{-1}) and NeutrAvidin-Texas Red (2 μg mL^{-1}) in phosphate buffer (10 mM, pH 7.4). After 2 h of incubation at room temperature, the droplets were washed and imaged under a fluorescence microscope.

13. Stability of the functionalized droplets

For SE droplet preparation, the flow rates of the oil and water phases were 5 and 15 μL min^{-1}, respectively. For DE droplet preparation, the flow rates of the inner, middle and outer phases were 3, 4 and 16 μL min^{-1}, respectively. Surface modification of F127-NH_2, F127-COOH, F127-NHS, F127-MAL and F127-Biotin-stabilized SE and DE droplets was carried out according to the above protocol. The droplets were imaged under a fluorescence microscope (magnification, 100×) at different time points.

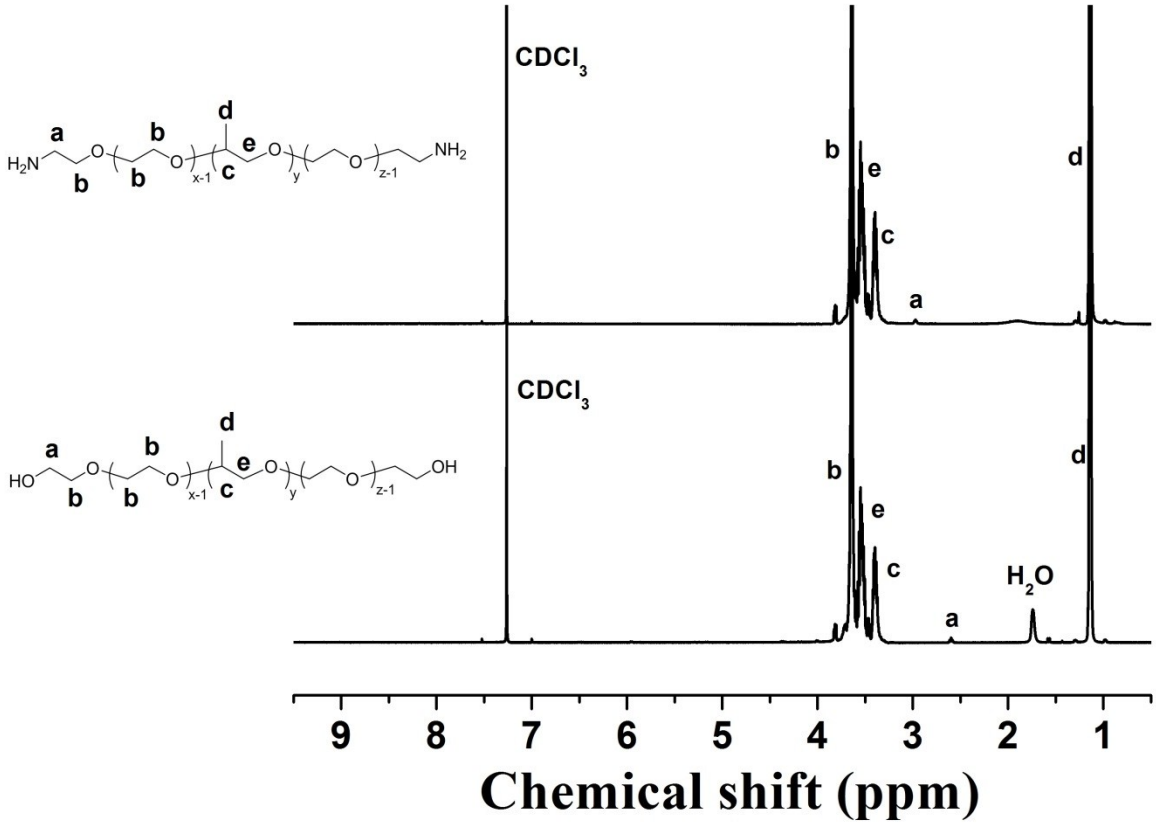


Fig. S1 ^{1}H NMR spectra of F127 and F127-NH_2 in $CDCl_3$.

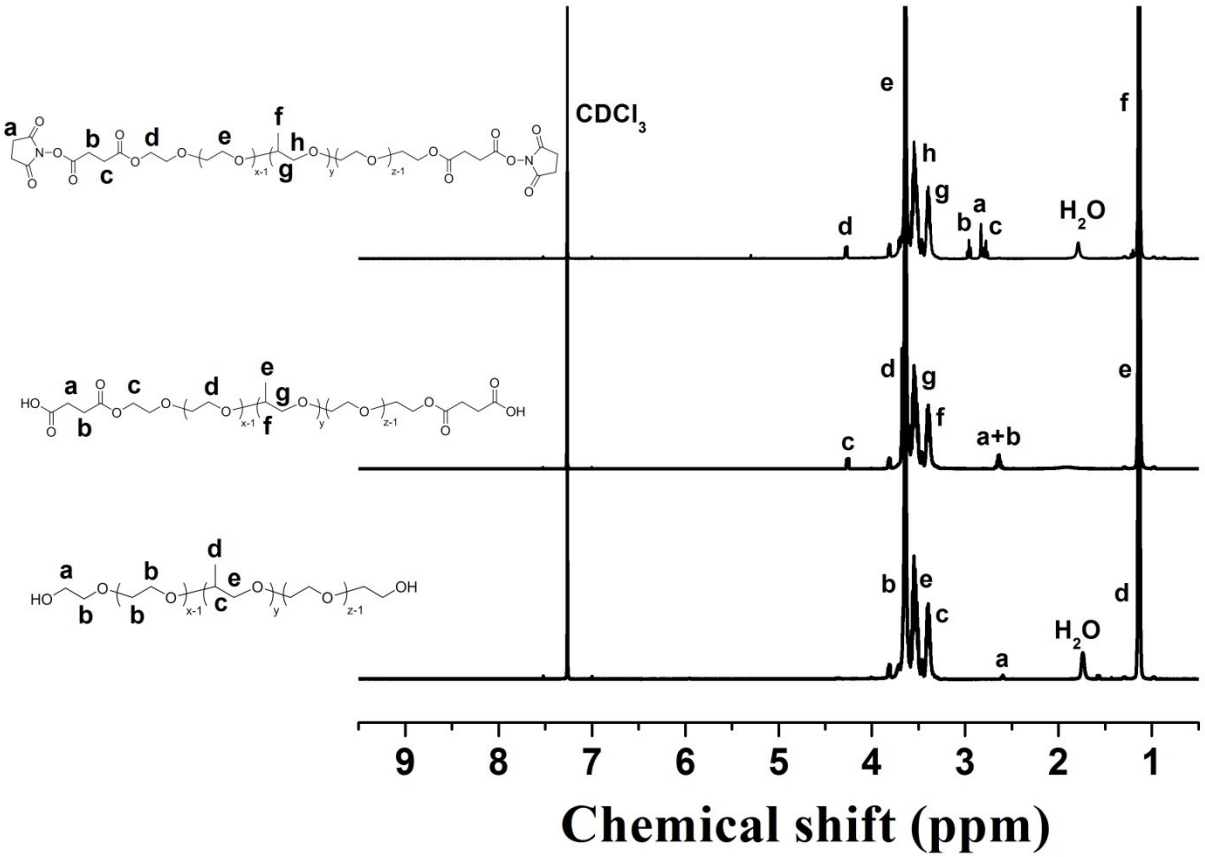


Fig. S2 ^{1}H NMR spectra of F127, F127-COOH and F127-NHS in $CDCl_3$.

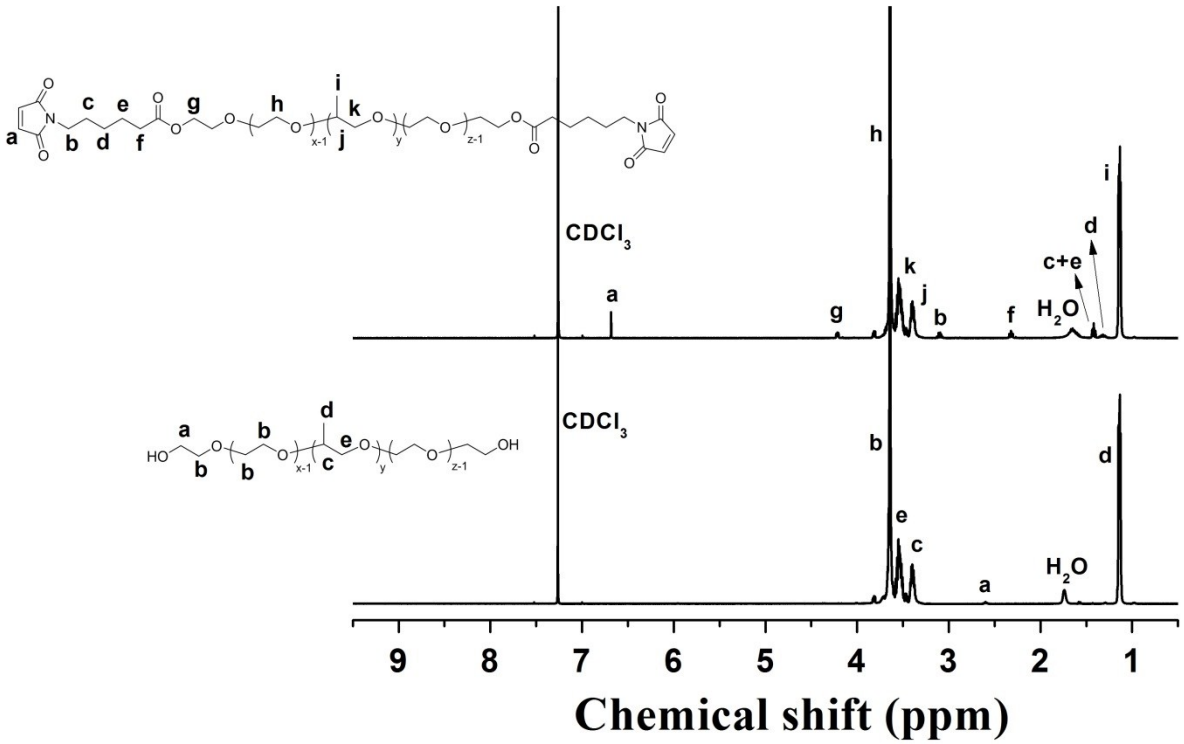


Fig. S3 ^{1}H NMR spectra of F127 and F127-MAL in $CDCl_3$.

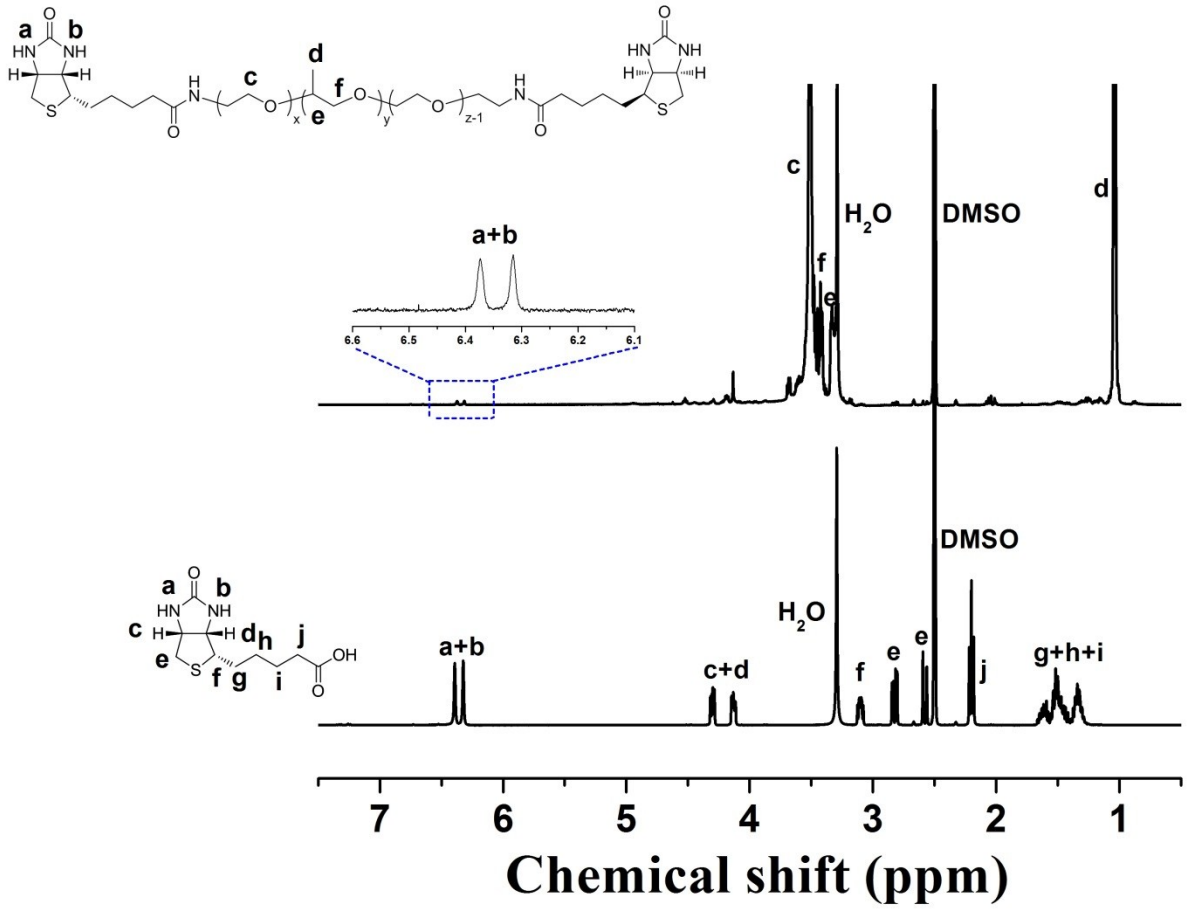


Fig. S4 ^{1}H NMR spectra of biotin and F127-Biotin in DMSO-d_6.

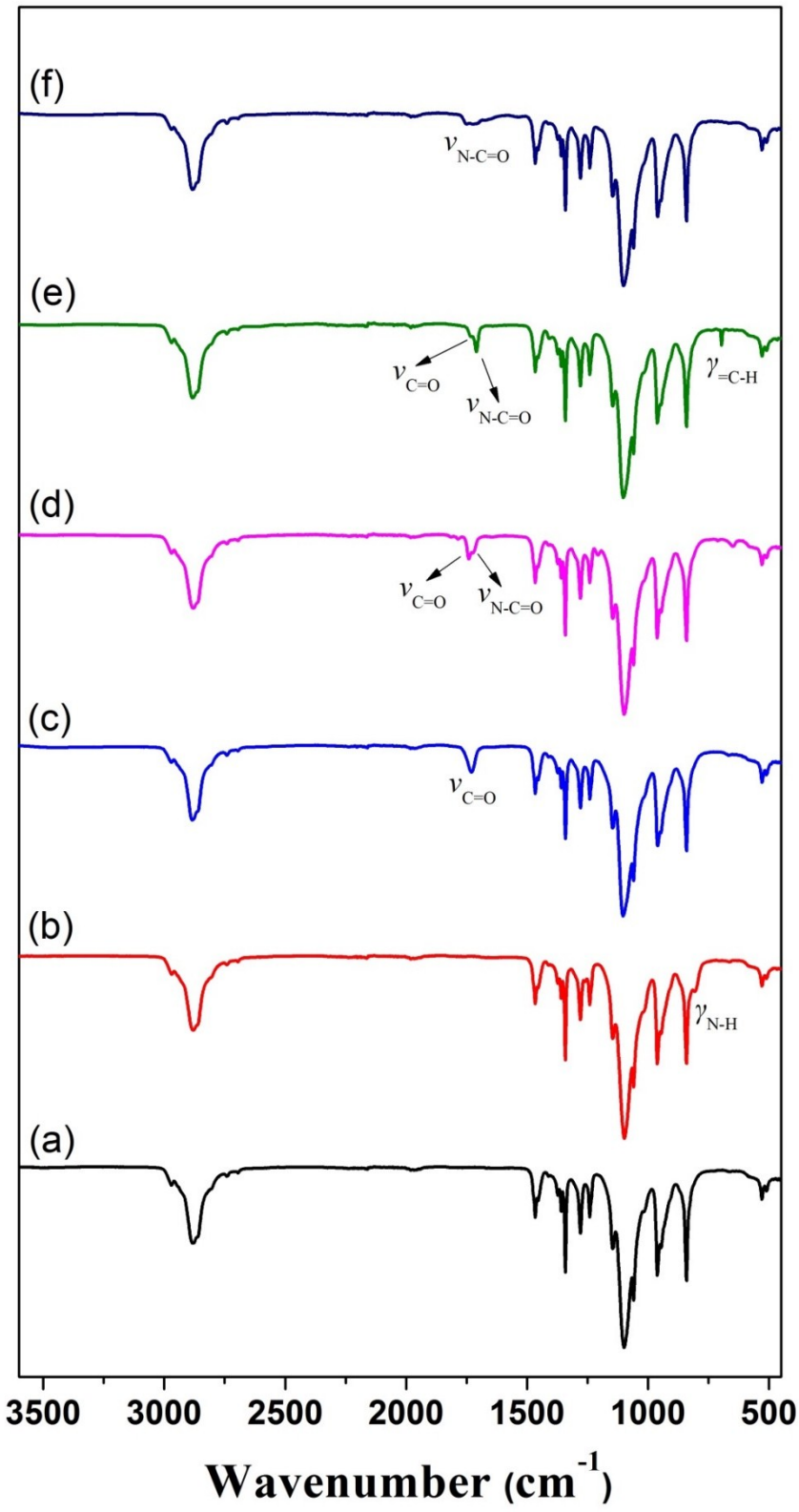


Fig. S5 FT-IR spectra obtained for (a) F127, (b) F127-NH_2, (c) F127-COOH, (d) F127-NHS, (e) F127-MAL, and (f) F127-Biotin.

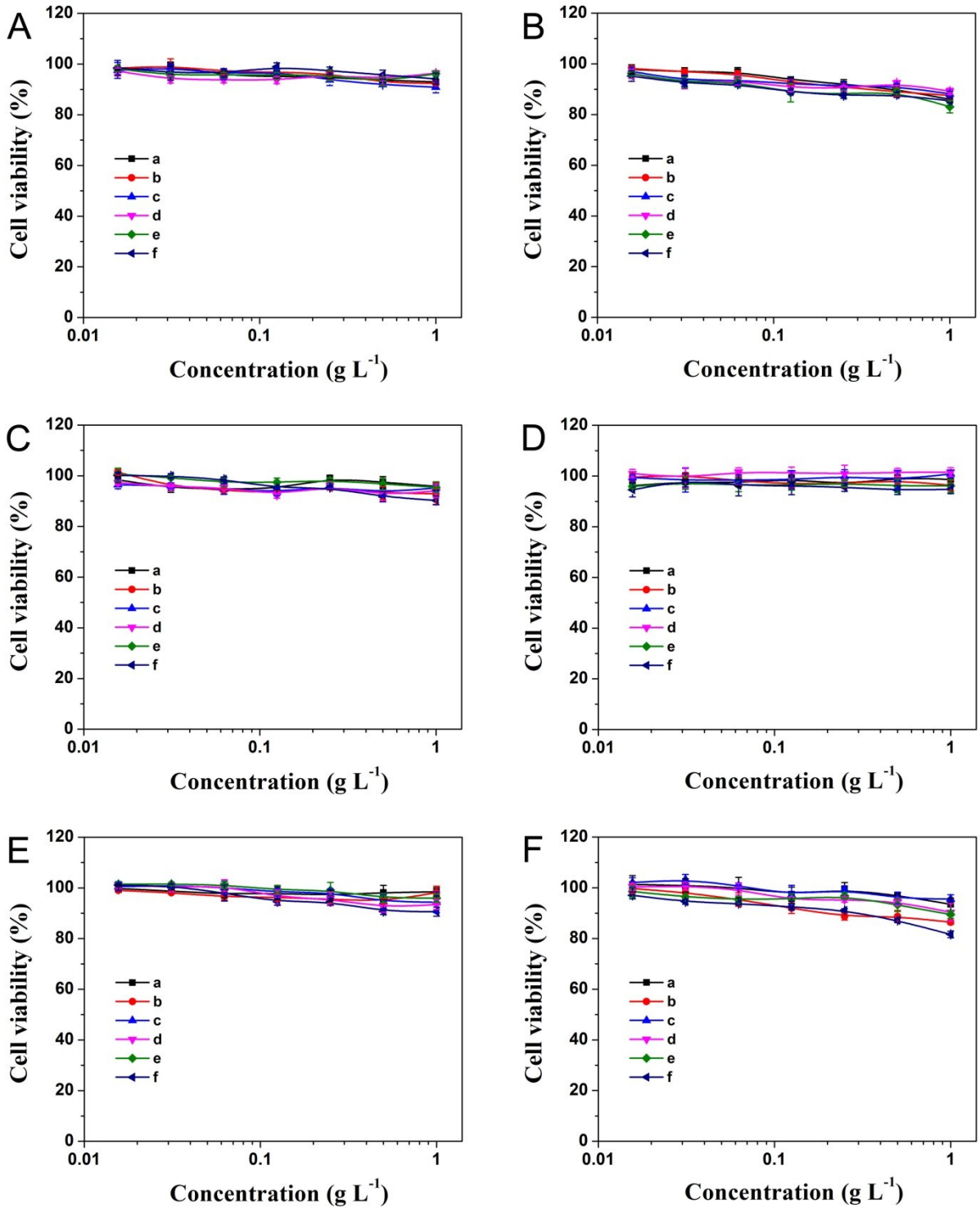


Fig. S6 *In vitro* cytotoxicity of (a) F127, (b) F127-NH_2, (c) F127-COOH, (d) F127-NHS, (e) F127-MAL, and (f) F127-Biotin against (A) MSC, (B) EPC, (C) NHDF, (D) Caco-2, (E) HL-60, and (F) A549 cells.

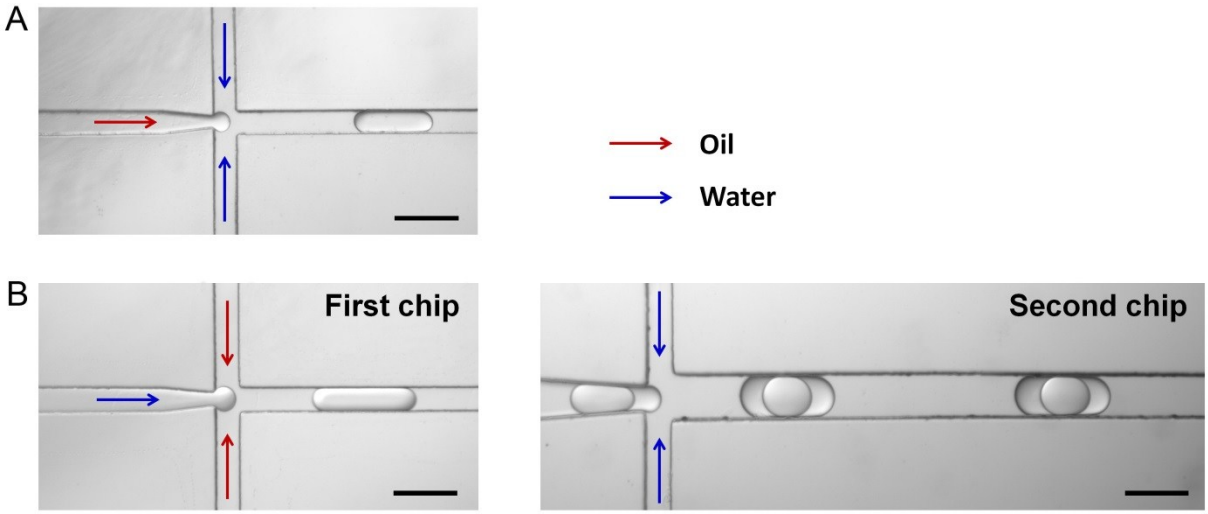


Fig. S7 Microfluidic preparation of (A) oil-in-water single emulsions and (B) water-in-oil-in-water double emulsions. The scale bars represent 300 μm. The heights of the chips are 80 and 180 μm, respectively.

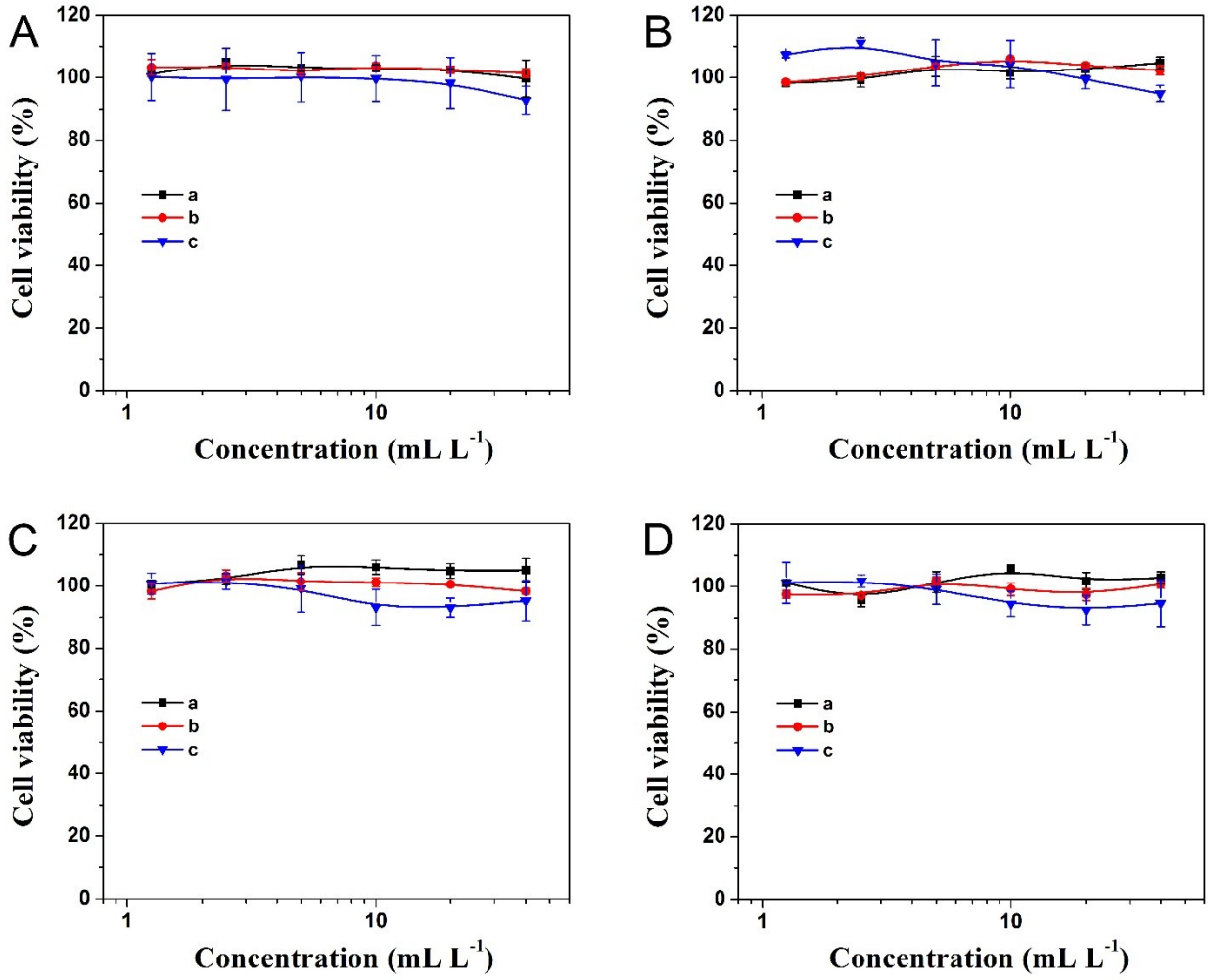


Fig. S8 *In vitro* cytotoxicity of (a) HFE-7500 oil, (b) single emulsion, and (c) double emulsion against (A) MSC, (B) EPC, (C) Caco-2, and (D) A549 cells.

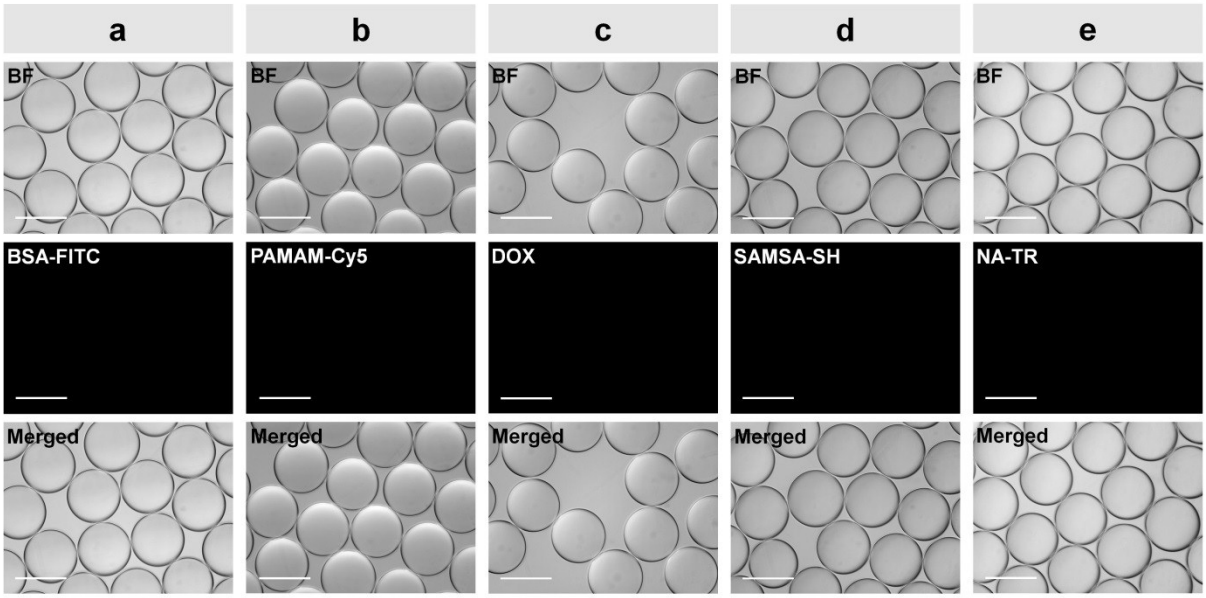


Fig. S9 Microscopy images of F127-stabilized single emulsion droplets, after incubation with (a) BSA-FITC, (b) PAMAM-Cy5, (c) DOX, (d) SAMSA-SH, and (e) NA-TR. The scale bars represent 200 μm.

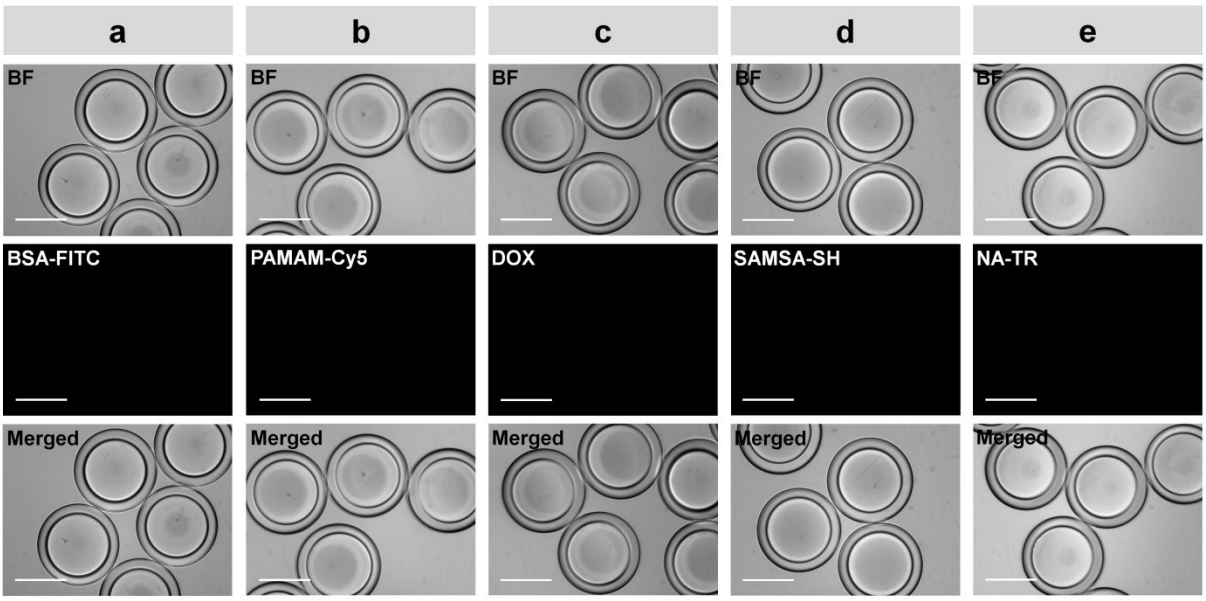


Fig. S10 Microscopy images of F127-stabilized double emulsion droplets, after incubation with (a) BSA-FITC, (b) PAMAM-Cy5, (c) DOX, (d) SAMSA-SH, and (e) NA-TR. The scale bars represent 200 μm.

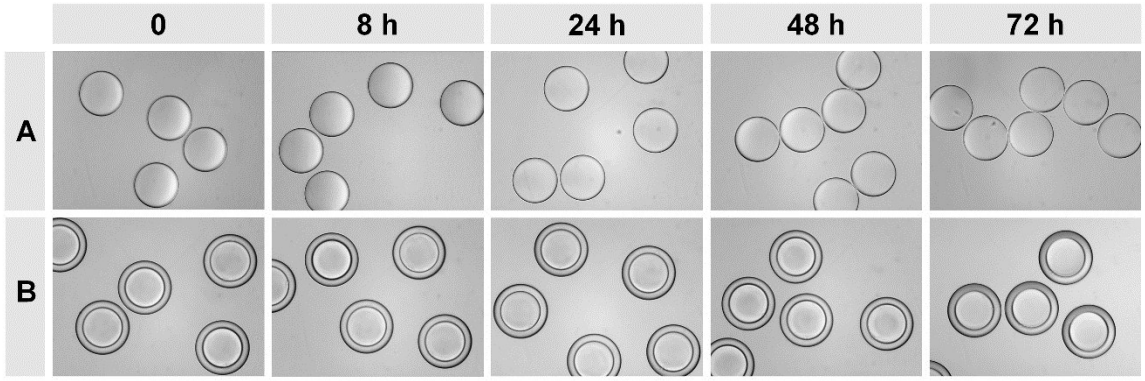


Fig. S11 Stability of F127-stabilized SE and DE droplets.

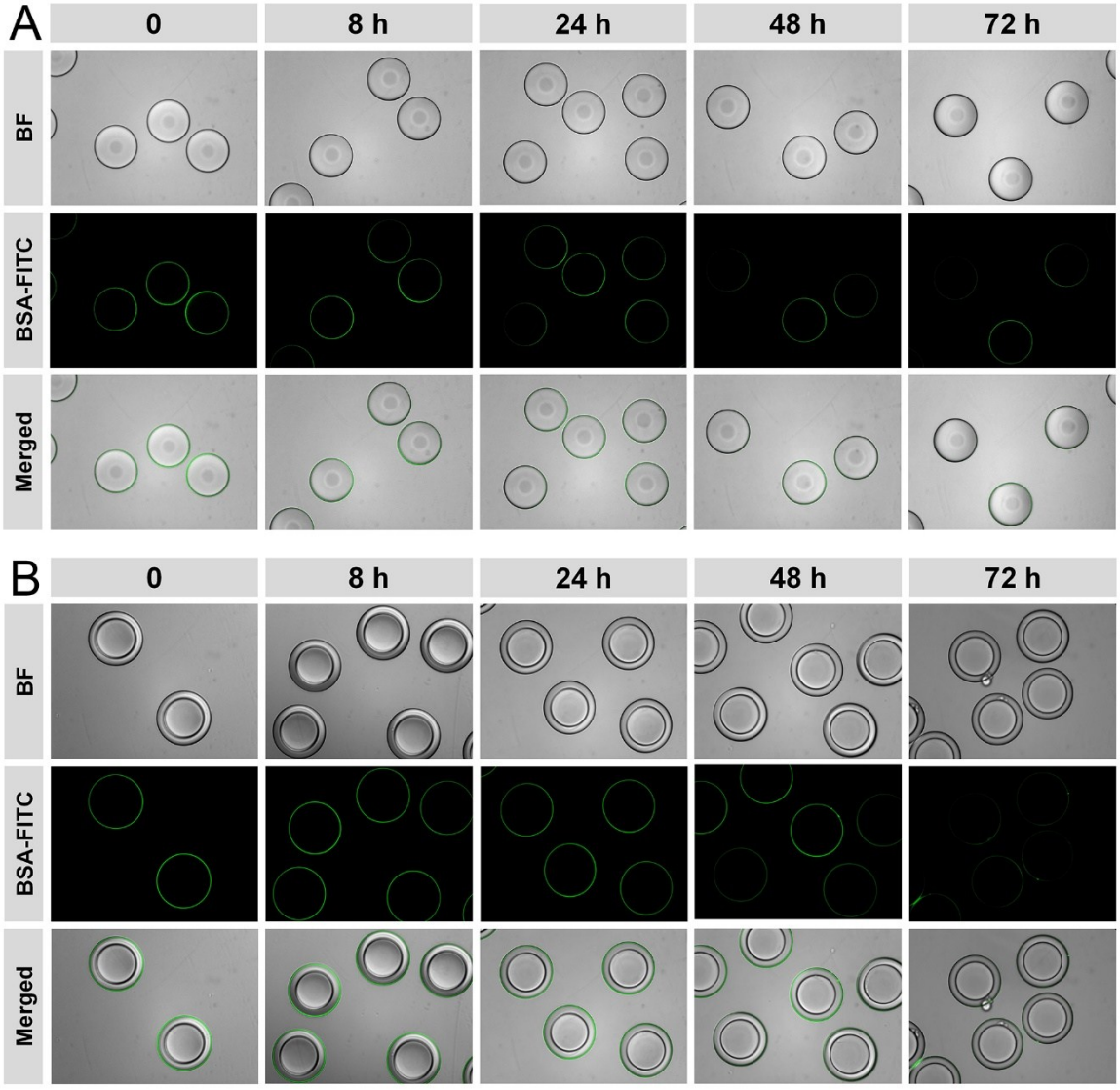


Fig. S12 Stability of F127-NH_2-functionalized (A) SE and (B) DE droplets after coating with (a) BSA-FITC *via* electrostatic adsorption.

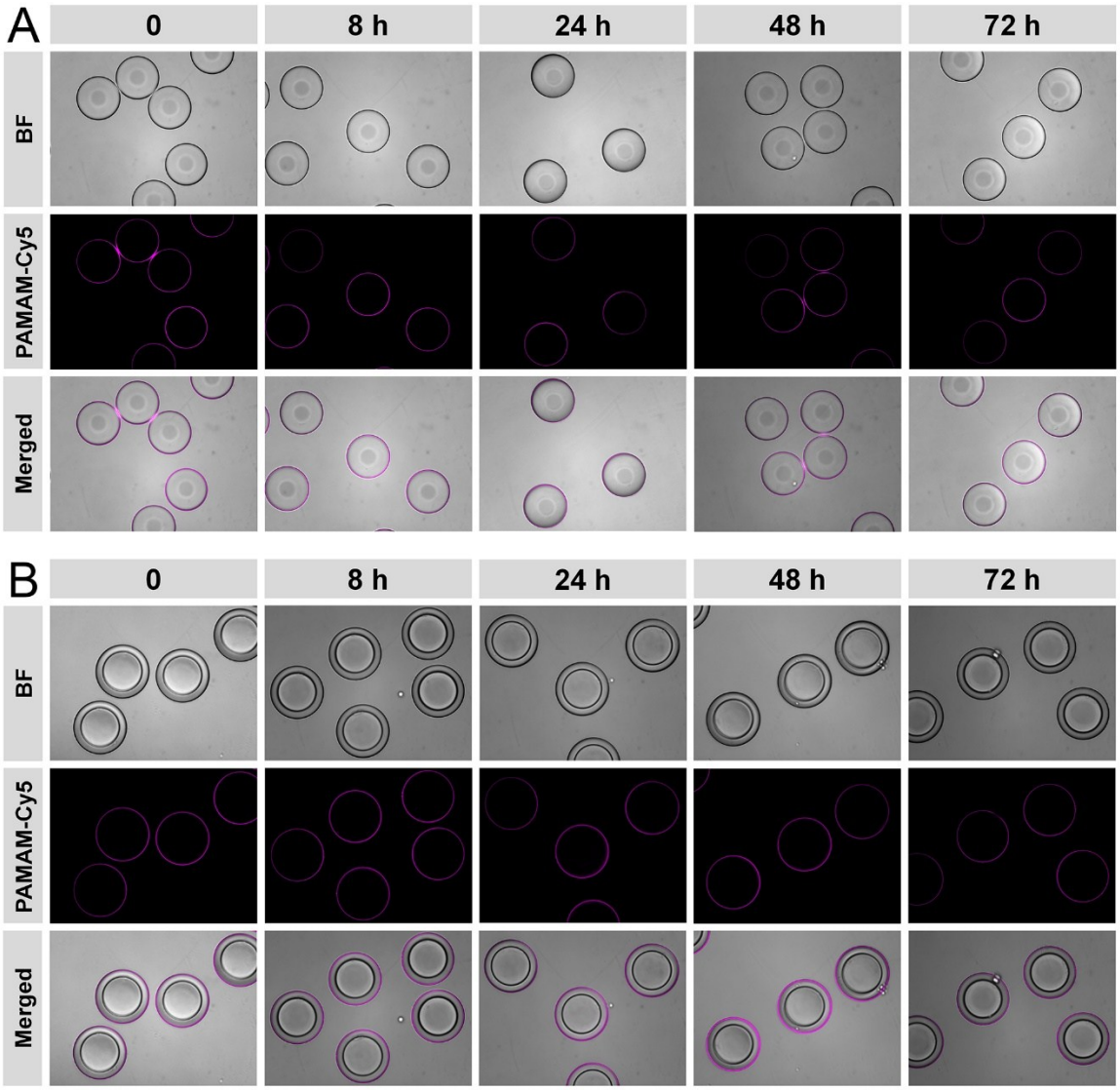


Fig. S13 Stability of F127-COOH-functionalized (A) SE and (B) DE droplets after coating with PAMAM-Cy5 *via* electrostatic adsorption,

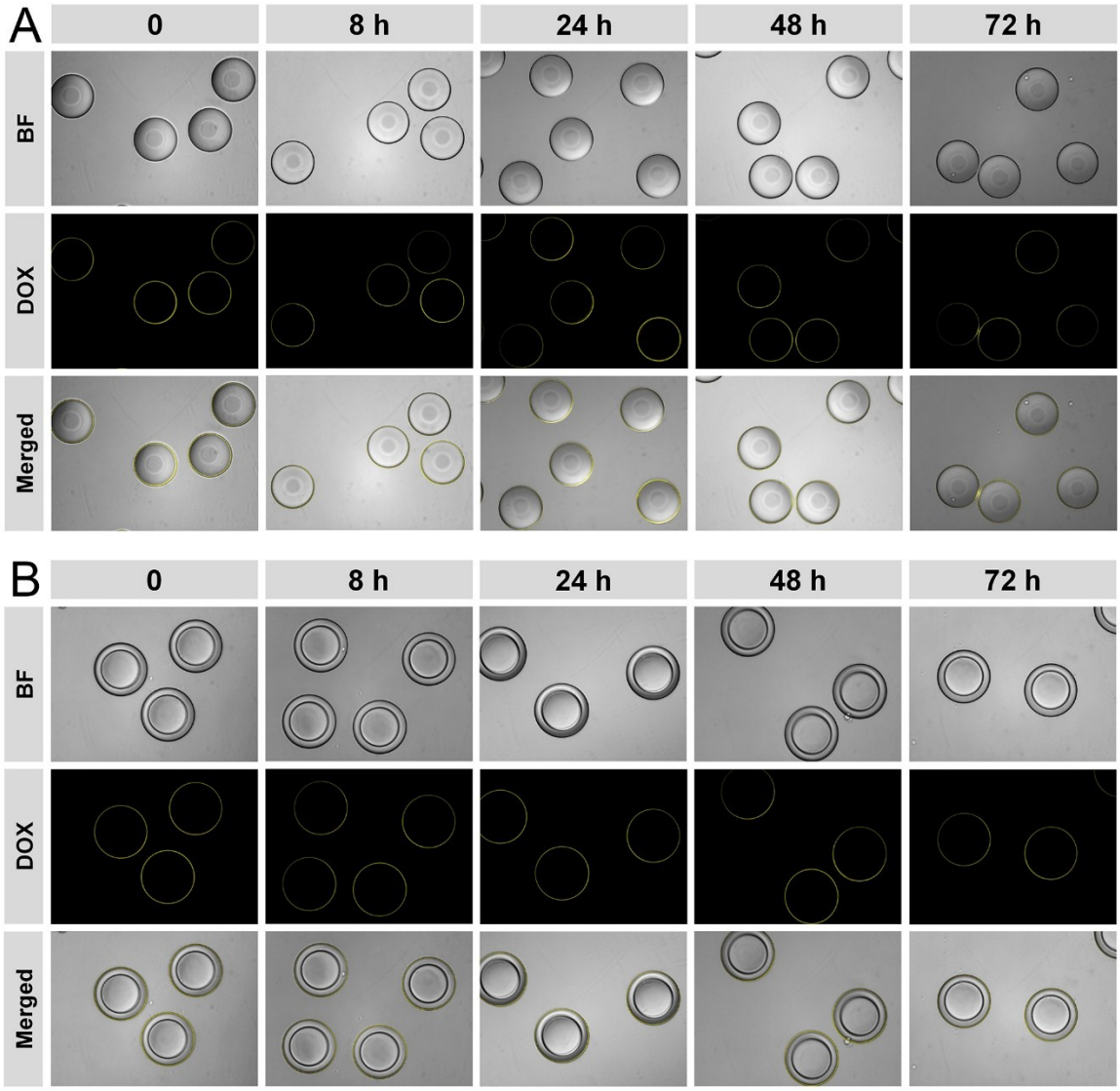


Fig. S14 Stability of F127-NHS-functionalized (A) SE and (B) DE droplets after modification with DOX *via* covalent conjugation.

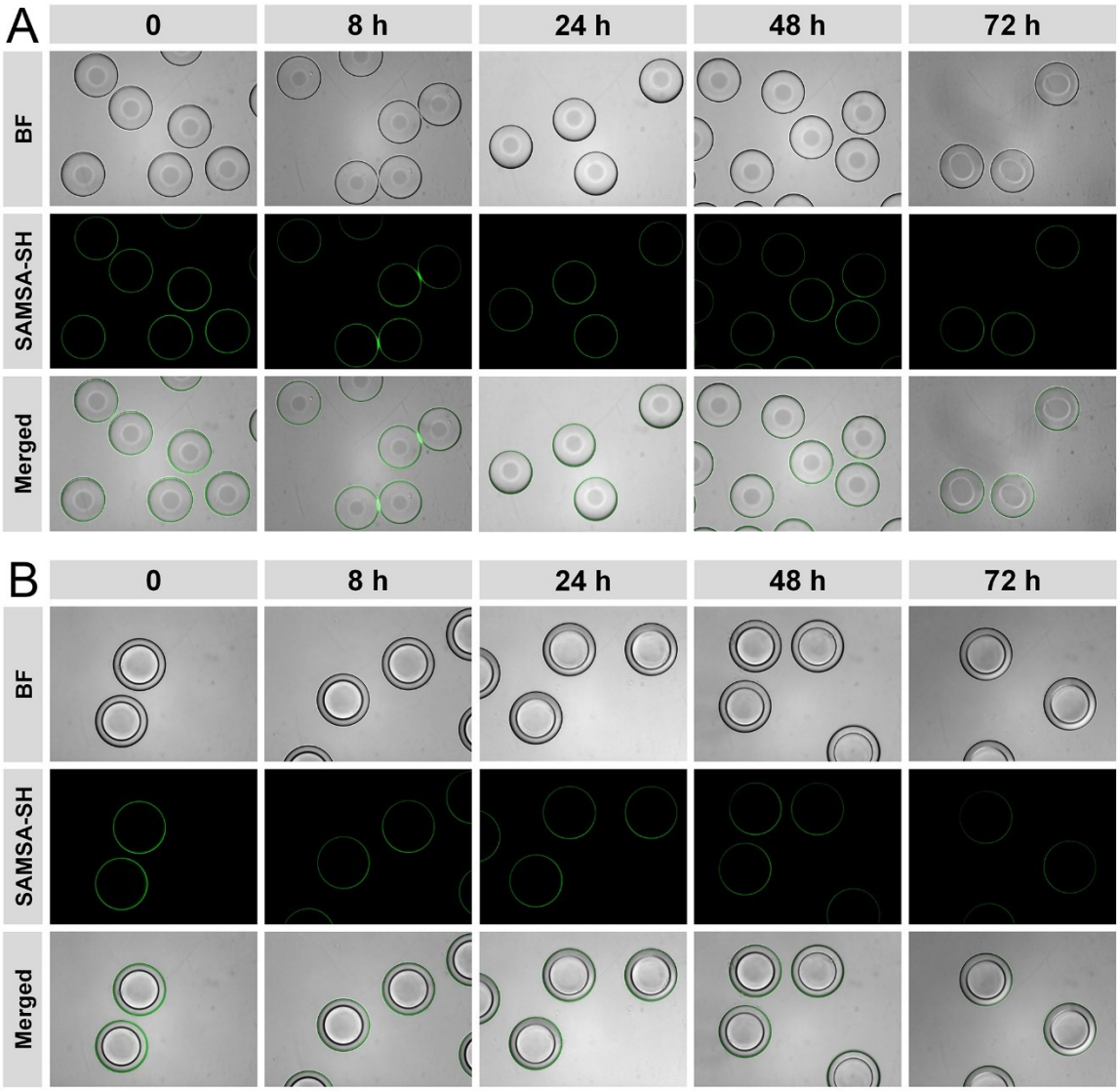


Fig. S15 Stability of F127-MAL-functionalized (A) SE and (B) DE droplets after modification with SAMSA-SH *via* covalent conjugation.

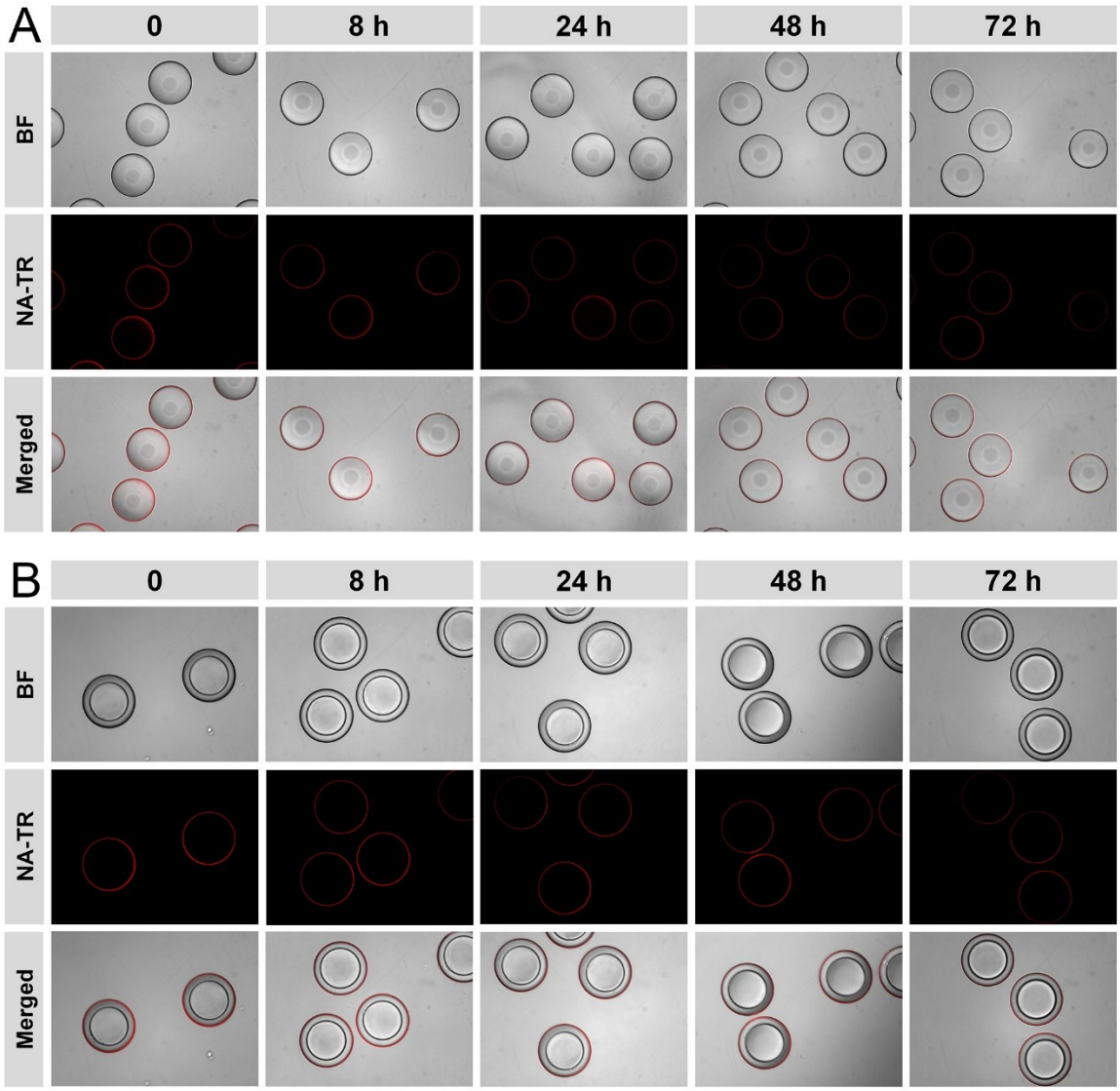


Fig. S16 Stability of F127-Biotin-functionalized (A) SE and (B) DE droplets after linkage with NA-TR *via* site-specific avidin-biotin interaction.

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