Supporting information for:

Synthesis of Nanostructured and Biofunctionalized Water-in-Oil Droplets as Tools for Homing T Cells

Ilia Platzman,[§,‡] Jan-Willi Janiesch[§,‡] and Joachim Pius Spatz[§*]

Department of New Materials and Biosystems, Max Planck Institute for Intelligent Systems, Heisenbergstr. 3, Stuttgart 70569, Germany & Department of Biophysical Chemistry, University of Heidelberg, Heidelberg 69120, Germany.
E-mail: spatz@is.mpg.de

1. Surfactant Synthesis

1.1. Perfluorpolypropylene–Polyethylene glycol-Perfluorpolypropylene (PFPE-PEG-PFPE)

The synthesis of PFPE-PEG-PFPE triblock followed the procedure reported earlier[1] but with several modifications as shown in Figure 1S. The synthesis was carried out under argon atmosphere in dry THF solvent (Acros Organics, Germany) in a heated Schlenk-flask. PEG600 (600 mg, 1 mmol, molecular weight 600 g/mol, Fluka, Germany) was solved in 80 ml dry THF and cooled to -78 °C. **N**-butyl lithium (1.22 ml of a 1.6 M solution in hexane, 2 mmol, Sigma-Aldrich, Germany) was added dropwise over a period of 60 min at **-**78 °C to the PEG solution and stirred for additional 30 min at **-**78 °C. Under continuous stirring the reaction was slowly heated to room temperature followed by an additional 30 min stirring. PFPE2500-carboxylic acid (5 g, 2 mmol, molecular weight 2500g/mol, DuPont, Netherlands) was added dropwise over a period of 30 min and stirred for another 12 h. After the reaction was finished, the THF solvent with unreacted PEG was removed by separatory funnel. Two additional washing steps with dry THF solvent were used to purify the crude product from the unreacted PEG. The product was dissolved in methanol (Carl Roth GmbH, Germany) to separate it from unreacted PFPE2500-carboxylic acid. The PFPE-PEG-PFPE product soluble in the methanol was transferred to a clean flask and dried with a rotary evaporator at 40°C. After removing the methanol and drying on the vacuum line, the desired PFPE-PEG-PFPE triblock-copolymer surfactant was obtained (4.6 g, 82%). The purity of the product was analysed by NMR and IR measurements (see following sections).

NMR spectra were recorded using a Bruker DRX 500 (Bruker spectrometer with the 13C spectra being measured in the 1H-decoupled mode. Massachusetts, USA). Chemical shifts are given in ppm referenced to solvent (MeOH - D4, 22°C; δ = 3.35, 4.78 for ^{1}H, δ = 49.3 for ^{13}C). **^{1}H** NMR δ 3,63 (CH_2, s); **^{13}C** NMR δ 161.05 (Carbonyl-C); 120,2 (fluorinated C); 113,3-118,0 (fluorinated C´s); 101,0-104,2 (fluorinated C´s); 71,13 (CH_2).

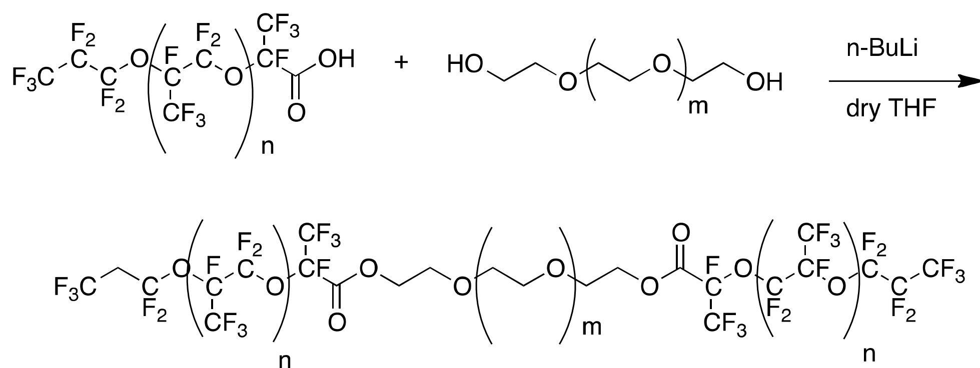

Figure 1S. Synthesis of PFPE2500-PEG600-PFPE2500 triblock-copolymer surfactants.

1.2. Gold-PEG-PFPE

Synthesis of the gold nanoparticle-linked surfactants was performed in a one step process as shown in Figure 2S. PFPE7000-carboxylic acid (2.1 mg, 0.3 µmol, molecular weight 7000 g/mol, DuPont, Netherlands) and KOH (5N, 10 µl, Sigma Aldrich, Germany) where added to the (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticle solution (2% w/w solution in water, 5 ml, Sigma Aldrich, Germany) and stirred for 1 h. The PFPE-PEG-Gold and unreacted PFPE were flocculated during the reaction. The crude product was washed three times with water to remove remaining KOH. The rest of the water was removed by freeze-drying for 24 hours. The product was dissolved in 1 ml of fluorinated oil FC-40 (Acros Organics, Germany) and filtered with a hydrophobic filter (PTFE 0,2 µm, Carl Roth GmbH, Germany) to remove unreacted, hydrophilic (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles. The desired Gold-PEG-PFPE surfactant was obtained (9.9 mg, 86%). The final product was chemically analyzed by IR and the gold nanoparticles were observed by cryo-SEM.

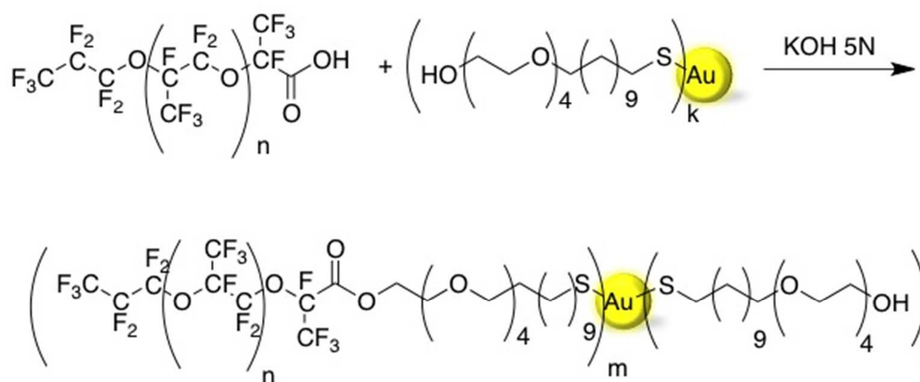

Figure 2S. Synthesis of PFPE7000-PEG-Gold diblock-copolymer surfactants.

1.3. Rhodamine B-PEG-Gold-PEG-PFPE

Synthesis of Rhodamine B-linked (RhB-linked) surfactants as shown in Figure 3S was carried out in dry THF solvent in a heated Schlenk-flask under argon atmosphere. Gold-PEG-PFPE solution (4.5 nmol, 15 µl in FC-40, see section 1.2) and dry THF (10 ml) were cooled to -78 °C. **N**-butyl lithium (30 µl of a 1.6 mM solution in hexane, 48 nmol) was added to the Gold-PEG-PFPE solution and stirred for 30 min at **-**78 °C. Under continuous stirring the reaction was slowly heated to room temperature and stirred for additional 30 min. THF solution of RhB (10 µl, 60 nmol, Sigma Aldrich, Germany) was added and stirred for another 12 h. After the reaction was finished, the THF solvent with unreacted RhB was removed by separatory funnel. Two additional washing steps with dry THF solvent were used to purify the crude product from the unreacted Rhodamine(B). The final RhB-PEG-Gold-PEG-PFPE product was dissolved in FC-40.

As a preliminary step to the RhB-PEG-Gold-PEG-PFPE synthesis, the simple reaction between the PEG(150) (molecular weight 150 g/mol, Sigma Aldrich, Germany) and RhB was made under the same conditions as mentioned in a previous paragraph. This was done in order to characterize the success of the reaction between PEG hydroxyl group and the RhB carboxylic acid group (see IR measurements in section 4.1).

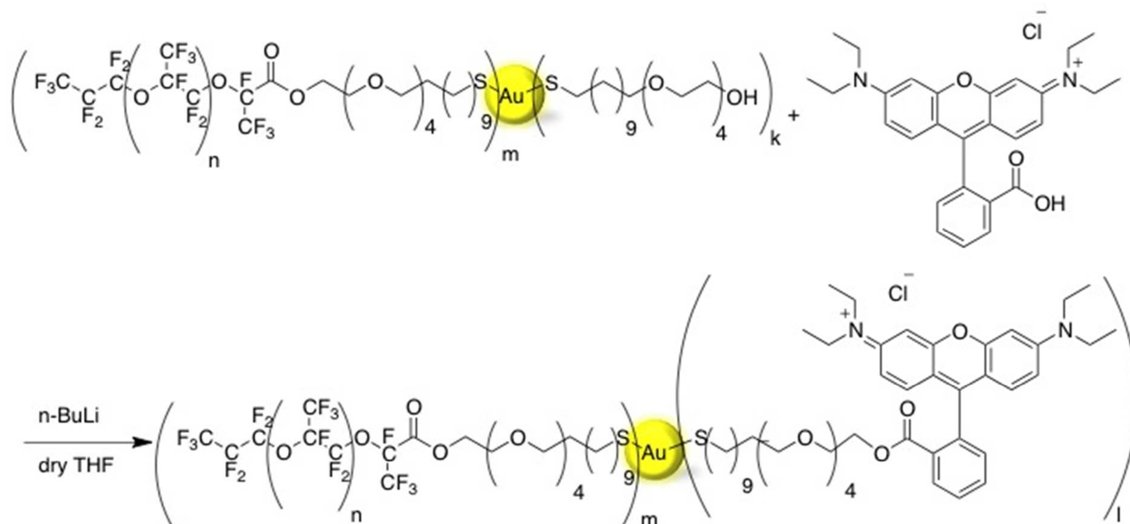

Figure 3S. Synthesis of PFPE-PEG-Gold-RhB surfactants.

1.4. His6-GFP-Ni-NTA-Thiol coupling

His6-GFP (300 µL, 30 µM, 1 GFP was a gift of S. Gardia Addgene plasmid #29663; Protein was expressed in E.coli using standard protocols and purified by Ni-NTA chromatography) was mixed with $NiCl_2$ (9 µL, 100 mM, Fluka, Germany) and NTA-thiol (300 µL, 1 mM, ProChimia Surfaces Sp. z o.o., Poland) and diluted with 600 µL PBS. NTA-thiol and $NiCl_2$ were mixed by stirring for 20 minutes. EtOH was removed partly to a final volume of 50 µl by pointing a nitrogen flow on the surface of the mixture. Following this procedure the His6-GFP was added and mixed for another 1 h. PBS was added and a final GFP concentration of 8 µM was determined using extinction coefficient ε = 30000 at A_{395nm}.

1.5. Bio-functionalization of the Gold Nanoparticles

To provide cell interactions with nanostructured droplets, c(RGDfK)-PEG-(cysteine)$_3$ peptide (Peptide Speciality Laboratories GmbH, Heidelberg, Germany) was immobilized on gold nanoparticles via the thiol residues of the cysteine linker. The chemical structure of cRGD peptide with the PEG-spacer and cysteine-linker can be observed in Figure 4S. The freeze-dried Gold-PEG-PFPE copolymer from the synthesis presented in section 1.2 was dissolved in 100 µL FC-40 and then the aqueous solution of cRGD (50 µM, 100µL) was added and stirred for 1 h. To remove the unbound cRGD peptide, the crude product solution was centrifuged. The supernatant solution was removed and the precipitant was freeze-dried for 24 h to remove completely the remained water. Finally, the product was dissolved in 1 ml of fluorinated oil FC-40 (Acros Organics, Germany) and filtered with a hydrophobic filter (PTFE 0,2 µm) to remove unreacted, hydrophilic (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles and cRGD traces.

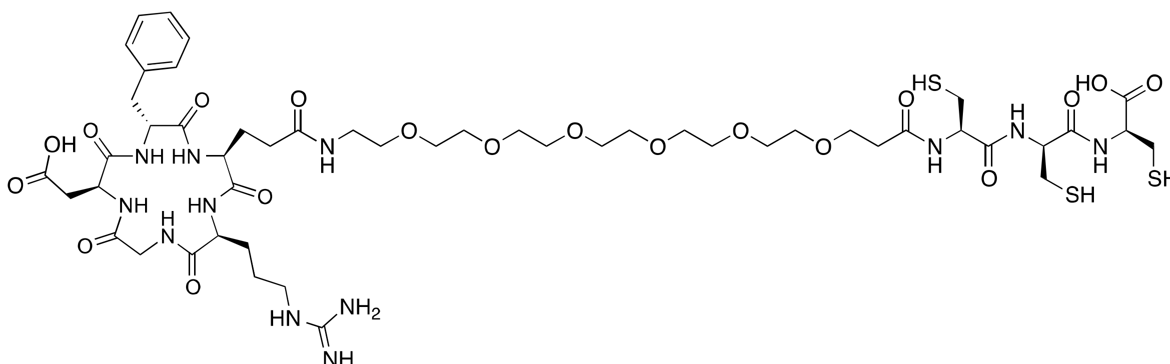

Figure 4S. Molecular structure of the c(RGDfK) peptide used within this study. The cyclically arranged amino acids are arginine (R), glycine (G) and aspartate (D). A PEG spacer (6 units) was used to bridge between the peptide and cysteine3 linker.

1.6. **Perfluoroctanyl-Triethylen glycol (PFO-PEG)**

The synthesis of 2-(2-(2-Hydroxyethoxy)ethoxy)ethyl 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctanoate shown in Figure 5S was carried out under argon atmosphere in dry THF solvent in a heated Schlenk-flask. Triethylene glycol (6.36 g, 30.6 mmol, molecular weight 208.05 g/mol Sigma-Aldrich, Germany) was added to 200 ml dry THF and cooled to -78 °C. N-butyl lithium (18.5 ml of a 1.6 M solution in hexane, 29.6 mmol) was added drop wise over a period of 60 min at -78 °C to the PEG solution and stirred for additional 30 min at -78 °C. Under continuous stirring the reaction was slowly heated to room temperature followed by an additional 30 min stirring. Pentadecafluorooctanoyl chloride (12.97 g, 30 mmol, molecular weight 432.51 g/mol, Sigma-Aldrich, Germany) was added drop wise over a period of 30 min and stirred for another 4 h. After the reaction was finished, the THF solvent with unreacted PEG was removed by separatory funnel. The crude product was washed with 10 ml H2O to remove the lithiumchlorid. Following the washing procedures the crude product was dried on the vacuum line and purified using column chromatography. The column was packed with silica gel and a mix of petroleum ether (40/60) and THF (mix 2:1) was used as mobile phase. The diblock (R_f = 0.28) as a bright yellow color product was collected and used as emulsion destabilizer (see section 3.2) and a colorless oil which is the triblock (R_f = 0.72) was stored for other experiments. The desired 2-(2-(2-Hydroxyethoxy)ethoxy)ethyl 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctanoat diblock-copolymer was obtained (9.02 g, 16.02 mmol 55%) and analysed by NMR and mass spectroscopy (MS).

NMR spectra were recorded using a Bruker DRX 500 (Bruker spectrometer with the 13C spectra being measured in the 1H-decoupled mode. Massachusetts, USA). Chemical shifts are given in ppm referenced to solvent ($CDCl_3$, 22°C ; δ = 37.26 for ^{1}H, δ = 77,16 for ^{13}C) ^{1}H NMR δ = 4.48 (tr, 2 H, **CH2**, C9); 3.74 (tr, 2 H, **CH2**, C10); 3.66 (tr, 2 H, **CH2**, C13/14); 3.61 (s, 4 H, **CH2**, C11, 12); 3.54 (tr, 2 H, **CH2**, C13/14); 1.37 (s, 1H, OH); ^{13}C NMR δ = 158.3 (tr, **COOR**, C8); 117.0 (trq, **CF**3 , C1); 114-105 (m, **CF**2 , C2-7); 72.5(C13/14); 70.7(C11/12); 70.2(C11/12); 68.1(C10); 67.1(C9); 61.6 (C13/14)

MS was performed in the MS-department in the organic-chemical institute Heidelberg by Frau A. Seith, Herrn Dr. J. Gross and Herrn N. Nieth, using a Jeol JMS-700 (JEOL GmbH, Germany) with an VG ZAB-F (Pfeiffer Vacuum GmbH, Germany) MS (ESI): m/z (%) = 546.9 (43%) [M+H]+; 563.9 (100 %) [M+NH4]+

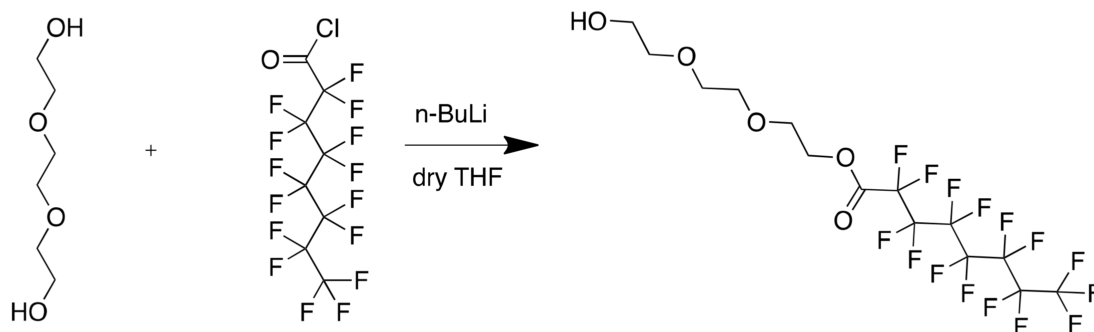

Figure 5S. Synthesis of PFO-PEG diblock-copolymer surfactants.

2. Microfluidic Device, Droplet Creation, Collection and Storage

A droplet-based microfluidic device, made of PDMS (Sylgard 184, Dow Corning, Michigan, USA) was prepared by standard photo- and soft-lithography methods[2] as described previously.[3] To create water in oil emulsion droplets at a rate of 1kHz the flow rates were adjusted to 100 µL/h for the aqueous phase (cell culture media and cells) and 200 µL/h for the oil phase, which contained the surfactant mixture of PFPE-PEG-PFPE and PFPE-PEG-Gold with different concentration ratios. Figure 6S (A, B) shows an example for a microfluidic device and the flow junction in which droplets are created. The flows were controlled with precision syringe pumps (Aladdin-1000, World Precision Instruments, USA) connected to the inlets of the microfluidic device via polyethylene-tubes (inner diameter 0.3 mm, Portex Fine Bore, Smiths Medical Instruments Ltd., USA). Droplets were collected with a pipette tip at the outlet of the microfluidic device and stored in an analysis chamber[3] for further analysis and characterization, as shown in Figure 6S (C, D).

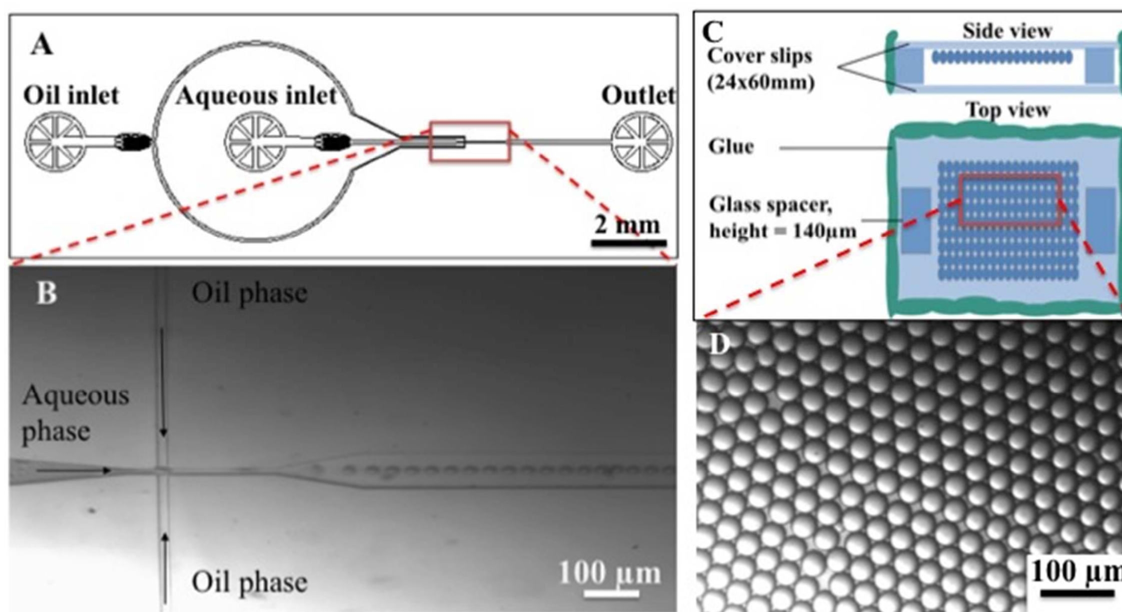

Figure 6S. (A) Representation of the droplet-based microfluidic device. (B) Shows the flow-focusing junction with 20 µm width channels, in which the droplets are generated and flow to the outlet. (C) Schematic representation of an analysis chamber in which the droplets are stored for further analysis and characterization. (D) Representative phase contrast image of the 30 µm diameter droplets in an analysis chamber.

3. Cells

3.1. Adhesion Experiments

The human acute T cell leukemia cell line Jurkat E6.1 clone was purchased from ATCC (American Type Culture Collection, Manassas, USA) and maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS (Invitrogen, Darmstadt, Germany or Sigma-Aldrich, Taufkirchen, Germany) and 1% (v/v) penicillin/streptomycin (Gibco, Darmstadt, Germany) at 37 °C and 5% CO_2.

For adhesion experiments, Jurkat E6.1 cells (6 x 10^6 cells) were suspended in adhesion medium (RPMI 1640, 2 mM L-glutamine, 1% (v/v) penicillin/ streptomycin, 1 % FBS). This medium containing cells was used as an aqueous phase for droplets creation. The droplets with cells were imaged using an AxioVert 40 CFL microscope (Zeiss, Jena, Germany).

3.2. Cell Recovery and Live/Dead Staining

Recovery of encapsulated cells and live/dead staining followed the procedure reported earlier[4] but with several modifications. From each experiment, 1 ml of the resulting droplets emulsion was splitted equally between five (1 ml) falcons and incubated within the incubator condition ($37^{o}C$, 5% CO_2). Following different incubation times (1, 2, 3, 4 and 5 days), the emulsion was broken by the addition of 5 % v/v PFO-PEG surfactants, which used as an emulsion destabilizer (see section 1.6) and the supernatant was transferred into a 15 ml centrifuge tube. The cells were washed with 10 ml PBS and centrifuged to remove serum esterase activity. Following washing step, 100 µL of cells-containing buffer solution was added to the microplate reader flat-bottom well where the total capacity is 250-300 µL. The cells were treated for 45 min at room temperature with the 100 µL of the live/dead staining solution, containing 2 µM calcein AM and 4 µM Ethidium homodimer-1 (Live/Dead Viability/Cytotoxicity Kit for mammalian cells, Invitrogen Kit L-3224). After staining the percentage of live cells was calculated from the fluorescence measurements using the microplate reader.

4. Characterization and Analysis

4.1. Infrared (IR) Measurements

IR measurements were performed to confirm the success of the surfactant syntheses and the purity of the products (see sections 1.1 and 1.2). The measurements were conducted on a Nicolet Nexus 870 Fourier transform infrared spectrophotometer (Thermo Electron GmbH, Dreieich, Germany).

Figure 7S (A) shows the representative IR spectra of the PFPE2500-carboxylic acid reactant and the triblock surfactant product (PFPE2500-PEG600-PFPE2500). This figure presents seven major bands at 1705, 1775, 2883, 2934, 2920, 3031 and 3550 cm^{-1}. The band at 1775 cm^{-1} is attributed to a stretching mode of the (C=O) bond of the PFPE-carboxylic acid which is strongly blue-shifted (by ~ 50 cm^{-1}) due to the electronegative fluor-atoms in alpha position to the carboxylic group.[5] The same blue-shift of the carboxylic (C=O) band was observed previously in the studies measuring the IR spectrum of the trifluoroacetic acid.[6,7] The bands which appear in the PFPE spectrum in the region 2800-3600 cm^{-1} are assigned to the different (OH) vibrations. Namely, to the asymmetric stretching ν_{as}(OH), to symmetric stretching ν_{s}(OH) and to subbands complex, corresponded to ν(C-O) + ν_{b}(OH) at 3550, 3031 and 2934 cm^{-1}, respectively.[8] The bands at 1705, 2883 and 2920 cm^{-1} are assigned to the PFPE-PEG-PFPE product. The bands at 2883 and 2920 cm^{-1} are attributed to symmetric and asymmetric stretching modes of the PEG (C-H) groups, respectively.[5] The band at 1705 cm^{-1} attributed to the ester (C=O) stretching mode. Figure 7S B shows the IR spectra of the PFPE7000-carboxylic acid as an educt used to synthesize the PFPE7000-PEG-Gold surfactant product. The spectra in Figure 7S (B) show similar seven major bands at 1695, 1775, 2850, 2934, 2950, 3031 and 3500 cm^{-1}. These bands are attributed to the same vibrations modes, as previously described. The lower intensity of the PFPE-PEG-Gold product bands in the Figure 7S (B) in comparison to the PFPE-PEG-PFPE product as presented in Figure 7S (A) is attributed to much lower concentration of the gold-linked surfactants (3 µM *VS.* 20 mM).

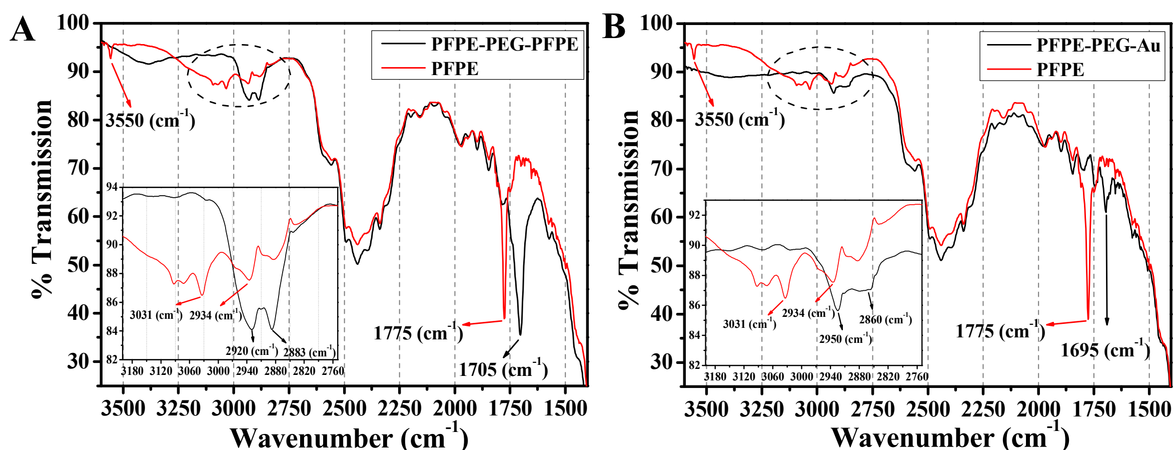

Figure 7S. IR spectra of the reactants and the products of the surfactant syntheses. (A) Comparison between the PFPE2500-carboxylic acid (20 mM) as a reactant and the PFPE2500-PEG600-PFPE2500 triblock product (20 mM) as described in section 1.1. (B) Comparison between PFPE7000-carboxylic acid as a reactant (20 mM) and the PFPE7000-PEG-Gold diblock product (30 µM) as described in section 1.2. The insets show the enlarged spectra area from 2750 to 3200 cm^{-1}. For all measurements FC-40 oil was used as a solvent.

To verify the success of the RhB linked surfactants synthesis (see section 1.3), we used the information obtained by attenuated total reflectance technique used in conjunction with IR spectroscopy (ATR-IR) (Smart Orbit accessory, Thermo Electron GmbH, Dreieich, Germany). Figure 8S shows representative ATR-IR spectra of pure RhB reactant and of the PEG(150)-RhB product powders. This figure presents ten major bands at 1558, 1571, 1587, 1611, 1640, 1646, 1707, 1751, 2887 and 2929 cm^{-1}. The bands at 1571, 1587 and 1646 cm^{-1} are attributed to a stretching modes of the RhB aromatic conjugated (C=C) bonds.[5,9,10] The band at 1692 cm^{-1} is attributed to the stretching mode of the RhB carboxylic acid (C=O) bond.[5,11] The bands at 1558, 1611 and 1640 cm^{-1} are attributed to a stretching modes of the PEG-Rhodamine aromatic conjugated (C=C) bonds.[8,9] The band at 1751 cm^{-1} is attributed to the ester (C=O) stretching mode[3] and the bands at 2887 and 2929 cm^{-1} are attributed to symmetric and asymmetric stretching modes of the PEG (C-H) groups, respectively.[5]

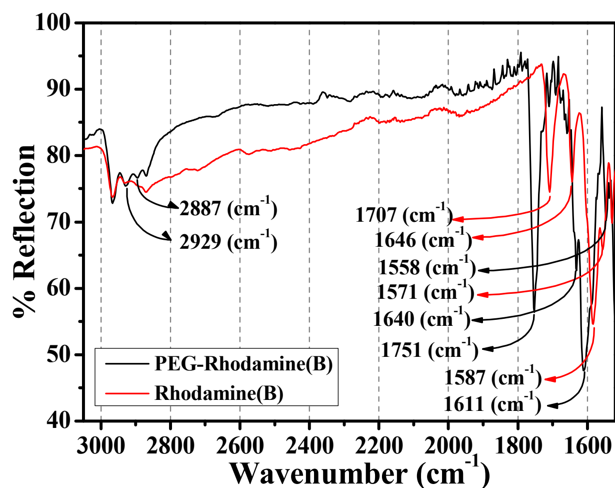

Figure 8S. ATR-IR spectra of the pure RhB powder and of the PEG-RhB product powder.

4.2. Cryo Scanning Electron Microscopy (cryo-SEM)

A Zeiss Ultra 55 field emission electron microscope (FE-SEM) equipped with in-lens, secondary electron (SE) and angle selective backscattered electron (ASB) detectors was used for image acquisition (Zeiss SMT, Oberkochen, Germany). Top-view cryo-SEM imaging was performed under low temperature conditions (Top = -115 ± 5°C) and working distances between 3 to 5 mm. Low acceleration voltages of 1-1.5 kV were used due to the low conductivity of the investigated samples. Signals were detected by the in-lens detector. Emulsion droplets solution (5 μl) was dropped on 0.8 mm diameter gold specimen carriers assembled on a freeze fracture holder (BAL-TEC AG, Balzers, Liechtenstein) and immersed immediately in liquid nitrogen. After vitrification in liquid nitrogen, the droplets were transferred to a BAL-TECH MED 020 (BAL-TEC AG, Balzers, Liechtenstein) preparation device via an evacuated liquid nitrogen-cooled shuttle BAL-TECH VLC 100 (BAL-TEC AG, Balzers, Liechtenstein). For freeze-fracture cryo observations the droplets were fractured in the 10^{-6} - 10^{-7} mbar vacuum chamber at -160 °C with a cooled knife. Figure 9S shows the representative cryo-SEM micrographs, obtained with different magnifications of the fractured droplets. After fracturing, the stage was heated to -90 °C and kept in the vacuum for 30 min in order to allow water in the fractured droplets to sublimate. For cryo-SEM, the samples were transferred immediately to the SEM chamber via an evacuated liquid nitrogen cooled shuttle.

It is worth mentioning here that no fixation and no cryo-protection chemicals were used during the droplets preparation for cryo observation. We are aware that lack of these steps would lead to possible morphological modifications of the cell surfaces. In case of Jurkat E6.1 this will lead to loss of microvilli surface structure.

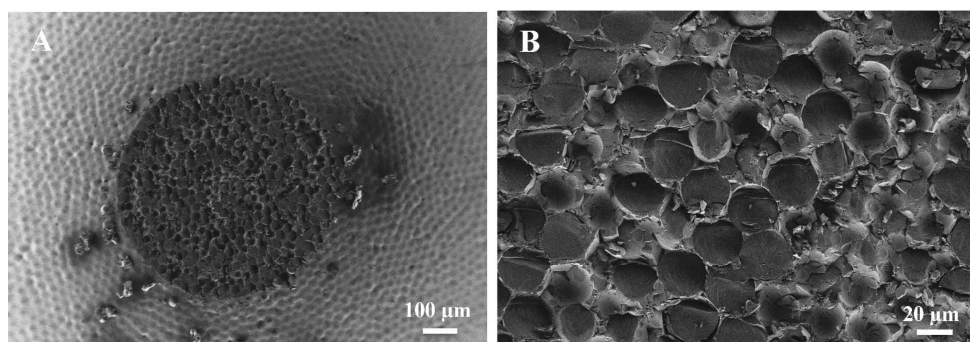

Figure 9S. (A) Representative cryo-SEM micrographs of freeze fractured droplets. The droplets were fractured by the cooled knife in the preparation device chamber, in the 10^{-6} - 10^{-7} mbar vacuum chamber at -160 °C. (B) Shows cryo-SEM micrograph of the fractured droplets obtained with higher magnification.

To present the comparison to non-gold nanostructured droplets, figure 10S show representative cryo-SEM micrographs, obtained with different magnifications of the droplets created without gold-linked surfactants.

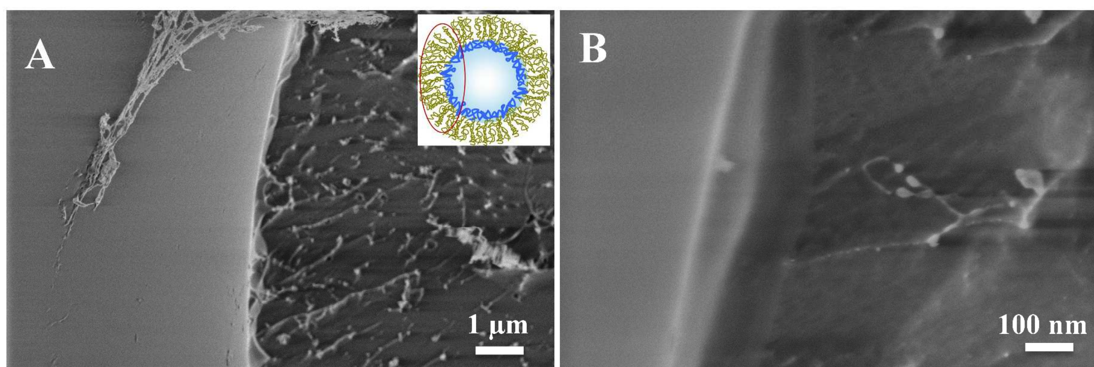

Figure. 10S. Representative cryo-SEM micrographs of the freeze fractured nanostructured droplets, obtained with different magnifications. The droplets were created using 20 mM PFPE-PEG-PFPE triblock surfactant. In sake of clarity, the insets show the schematic representation of the droplet and the area of observation (not to scale).

4.3. Fluorescence Microscopy

Fluorescence, confocal microscopy was performed with a Zeiss Axiovert 200M Laser-Scanning-Microscope (Zeiss, Jena, Germany) using a 60x water-immersions-objective (LUMFI, NA = 1.1, working distance 1.5 mm; Olympus, Tokyo, Japan) and an argon laser (458, 488 and 514 nm, Lasos LGK-7812 ML-4, Jena, Germany). Images with 1024x1024 pixels (6.4 µs/pixel) were taken with the image acquisition software Pascal 5 (Zeiss, Jena, Germany).

4.3.1. His6-GFP-linked Gold Nanostructured Droplets

Freshly prepared His6-GFP-Ni-NTA-Thiol in PBS solution (see section 1.4) was used as an aqueous phase to create droplets in a microfluidic device. Two types of droplets were created: the first contained only PFPE-PEG-PFPE (20mM) triblock-copolymer surfactants, and the second type contained PFPE-PEG-PFPE (20 mM) and Gold-PEG-PFPE (3µM) surfactants in an oil phase. The droplets were created, collected and stored for analysis as described previously in section 2.

Figure 11S A and B show fluorescent images of the non-gold nanostructured droplets and nanostructured droplets where the His6GFP was used without NTA-thiol linker taken one day after creation, respectively. It can be seen that the fluorescence intensity is distributed equally inside the droplets.

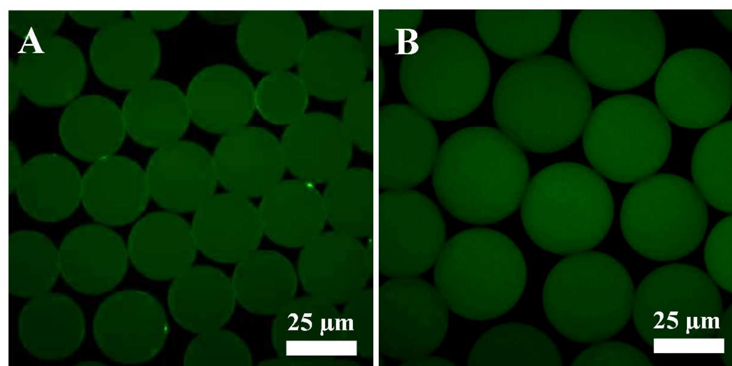

Figure. 11S. A and B show fluorescent images of the of the non-gold nanostructured droplets and nanostructured droplets where the His6GFP was used without NTA-thiol linker, taken one day after creation, respectively. All images have the same intensity scale.

4.3.2. RhB-linked Nanostructured Droplets

To create RhB-linked nanostructured droplets, the mixture of PFPE-PEG-PFPE (20 mM) and RhB-PEG-Gold-PEG-PFPE (30 μM) surfactants was used as an oil phase, while PBS was used as an aqueous phase. The droplets were produced, collected and stored for analysis as previously described in section 2.

Figure 12S shows the distribution of the florescence signal in the RhB-linked nanostructured droplets. Droplets were analyzed in an analysis chamber three days after creation and the appearance of the RhB along the droplets height was assessed by confocal microscopy. Stack 1 represents the top of the droplets, stack 12 the bottom. As can be seen, the fluorescent signal is distributed equally at the droplet periphery.

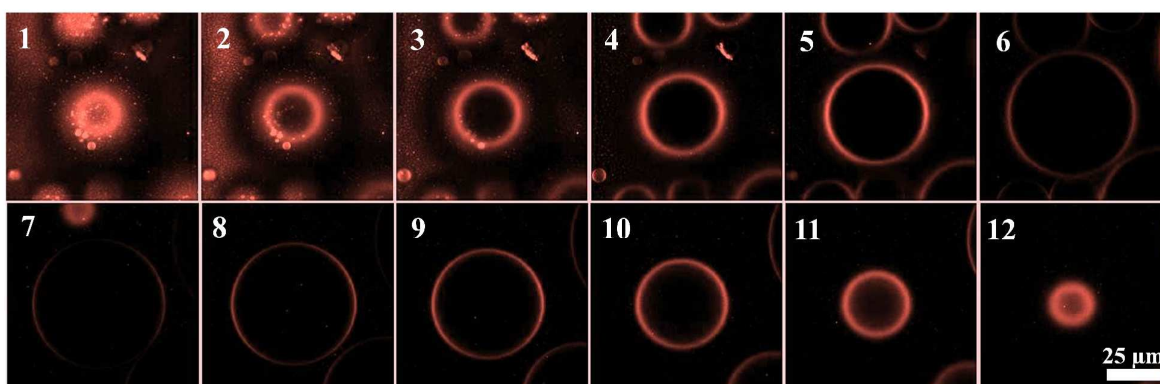

Figure 12S. A representative distribution of the fluorescence signal along the RhB-linked nanostructured droplet height, three days after creation. Stack 1 represents the top of the droplet, stack 12 the bottom. All images have the same intensity scale.

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