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# Development of Droplet-Based Microfluidics for Synthetic Biology Applications

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## Abstract

Microfluidics combines principles of science and technology, and enables the user to handle, process and manipulate fluids of very small volumes. This technology permits the integration of multiple laboratory applications into one single microfabricated chip, requires minimal manual user intervention and sample consumption, and allows enhanced data analysis speed and precision. Due to these numerous advantages, the potential for this technology to be applied in fundamental biophysical and biomedical research is vast. The major aim of this thesis was to explore the capacities of microfluidics, particularly droplet-based microfluidic technology in the following topics: 1) Mimicry of the immune system cellular environment, with the ultimate goal of programing T cells for adoptive T cell therapy; 2) Bottom-up assembly of minimal synthetic cells. Towards this end, a novel approach to form gold-nanostructured and specifically biofunctionalized water-in-oil droplets was developed. This thesis highlights the advanced properties of nanostructured droplets to serve as 3D antigen presenting cell (APC) surrogates for T-cell stimulation. The combination of flexible biofunctionalization and pliable physical droplet properties work in tandem, providing a flexible and modular system that closely models in situ APC-T cell interactions. The research within this thesis focused also on the dissection of complex cellular sensory machinery implementing an automated droplet-based microfluidic approach. Towards this goal, nanostructured droplets as cell-sized compartments and droplet-based pico-injection technology were used to achieve the bottom-up assembly of the minimal number of proteins required for a "simple synthetic cell." While the applied methodology has a potential for assembly of a wide range of subcellular functional units, the focus in this thesis was on the reconstitution of the actomyosin cortex. Successful optimization of the biochemical and biophysical conditions within the droplets allowed to achieve precise control over the actin polymerization and actomyosin network organization by their linkage to the droplets periphery. These experimental steps were also necessary to generate signaling events including myosin-driven droplet migration and self-propulsion with reduced molecular complexity compared to living cells.

### Zusammenfassung

Mikrofluidik ist eine Technologie, die es ermöglicht mit sehr kleinen Flüssigkeitsvolumina zu arbeiten, diese zu untersuchen und zu manipulieren. Die Miniaturisierung erlaubt präzise und schnelle Datenanalyse, sowie die Integration unterschiedlichster Laborfunktionen auf einem wenige Zentimeter großen Chip. Die zahlreichen Vorteile der Mikrofluidik Technologie eröffnen ein weites Feld von Anwendungen in der biophysikalischen und medizinischen Forschung. Das wesentliche Ziel dieser Arbeit war es Mikrofluidiksysteme, insbesondere Tropfen basierte Mikrofluidik, für die Anwendung in den folgenden Forschungsaufgaben zu konfigurieren und zu optimieren: 1) Simulation der Zellumgebung des Immunsystems mit dem Ziel der Programmierung von T-Zellen für die adoptive T-Zelltherapie. 2) Systematischer Aufbau und Untersuchung minimaler synthetischer Zellen. Hierzu wurde eine neue Methode zur Erzeugung gold-nanostrukturierter und spezifisch biofunktionalisierter Wasser-in-Öl-Tropfen entwickelt. Diese Arbeit demonstriert das Potential von gold-nanostrukturierten Tropfen als synthetische dreidimensionale antigenpräsentierende Zellen (APCs) in der T-Zellstimulation zu fungieren. Die Verbindung von präziser Biofunktionalisierung so wie die steuerbaren physikalischen Tropfeneigenschaften bilden ein flexibles und modulares System, das die natürliche APC-T-Zell Wechselwirkung realitätsnah nachbildet. Des Weiteren wurden komplexe zelluläre sensorische Mechanismen des Aktomyosin-Kortex untersucht. Hierzu wurden gold-nanostrukurierte Tropfen als Zellbehältnisse verwendet und Pikoinjektions-Technologie zur nachträglichen Tropfenmanipulation angewandt, um einfache "synthetische Zellen" mit der minimal benötigten Anzahl an Proteinen zu erzeugen. Diese Methode ermöglicht die Erzeugung eines weiten Spektrums von subzellulären Funktionseinheiten, die dann isoliert untersucht werden können. In dieser Arbeit lag der Fokus auf der Nachbildung des Aktomyosin-Kortex. Die Verbindung des Aktins zur Tropfenperipherie sowie die Optimierung der biochemischen und biophysikalischen Bedingungen im Tropfen erlauben die Aktinpolymerisation und die Organisation des Aktin-Netzwerks gezielt zu steuern. Diese experimentellen Schritte waren notwendig, um Signalereignisse einschließlich Myosin-getriebener Tröpfchenmigration und Eigenantrieb mit eingeschränkter molekularer Komplexität im Vergleich zu lebenden Zellen zu erzeugen.

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# Abbreviations

$2\mathrm{D}$	two-dimensional
3D	three-dimensional
ASB	angle selective backscattered
$\mathbf{AC}$	alternating current
ACT	adoptive T cell therapy
AFM	atomic force microscopy
APC	antigen presenting cell
ATP	adenosine triphosphate
ATR-IR	attenuated total reflectance-infrared
B.C.	before Christ
CCD	charge-coupled device
CMC	critical micelle concentration
conc.	concentration
cond.	conditions
m cRGD	cyclic arginylglycylaspartic acid
δ	deformation
D	diameter
DC	direct current
De	Dean flow
DMEM	eagle's minimal essential medium
DMF	dimethylformamide
DMSO	Dimethyl sulfoxide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	$1, 2- {\rm dioleoyl-sn-glycero-3-phosphoethanolamine}$
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
DTT	Dithiothreitol

#### $\operatorname{Contents}$

EGTA	ethylene glycol tetraacetic acid
EtOH	ethanol
F-actin	filamentous actin
FBS	fetal bovine serum
FCS	fluorescence correlation spectroscopy
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
FT-IR	fourier transform infrared
G-actin	globular actin
GFP	green fluorescent protein
$\mathbf{GNp}$	goldnanoparticle
GTP	guanosine-5'-triphosphate
$\operatorname{GUV}$	giant unilamellar vesicles
HEPES	eq:4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLB	hydrophilic-lipophilic balance
Hz	hertz
IFT	interfacial tension
ITO	indium tin oxide
i.e.	id est
IS	immunological synapse
$\mathbf{L}$	liter
FE-SEM	field emission electron microscope
$\mathrm{LogD}$	distribution coefficient
KBr	potassium bromide
KCl	potassium chloride
m	meter
$\mathbf{M}$	mole
MHC	major histocompatibility complex
MHz	mega hertz
min	minutes
$\mathbf{MS}$	mass spectroscopy
$\mathbf{M}\mathbf{W}$	molecular weight

Ν	newton
$N_2$	nitogen
N-WASP	neural wiskott-aldrich syndrome protein
$N_A$	avogadro constant
NA	numerical aperture
NHS	N-hydroxysuccinimide
nm	nano meter
NMR	nuclear-magnetic-resonance
NTA	nitrilotriacetic acid
Pa	pascal
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
PFO-PEG	perfluoroctanyl-Triethylen glycol
PFPE	perfluorpolypropylene
PIP2	phosphatidylinositol 4,5-bisphosphate
PIPES	$piperazine-N, N-bis (2-ethane sulfonic \ acid)$
$\mathrm{pL}$	pico liter
$\mathbf{pN}$	pico newton
PTFE	polytetrafluorethylen
R	gas constant
R	electrical resistance
$\mathbf{RhB}$	rhodamine B
Rf	retardation factor
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
$\mathbf{SE}$	secondary electron
$\mathbf{SE}$	succinimidyl ester
sec	seconds
SLB	supported lipid bilayer
SMAC	supra-molecular activation cluster
STED	stimulated emission depletion microscopy

#### Contents

Surfactants	surface active agents
Т	temperature
TCR	T cell receptor
Th1	type 1 helper cell
THF	tetrahydrofuran
TRI2500	PFPE(2500)- PEG(600)-PFPE(2500)
<b>TRI7000</b>	PFPE(7000)-PEG(1400)- PFPE(7000)
TRIS	tris(hydroxymethyl)aminomethane
TRITC	rhodamine
$\mathbf{UV}/\mathbf{VIS}$	ultraviolet-visible
$\mu$	micro
V	volt
$\mathbf{v}/\mathbf{v}$	volume fraction
W	watt
$\mathbf{w}/\mathbf{w}$	mass fraction
W/O/W	water-oil-water

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## I. Introduction

### 1. Microfluidics

Microfluidics is a science and technology of systems that enable the user to handle, process and manipulate fluids of very small volumes - down to less than picoliters - via micro channels with diameters ranging from 0.1 to 100 µm.<sup>[1]</sup> Microfluidics permit the integration of multiple laboratory functions into one single micro-fabricated chip with an average dimension of few centimeters. This technology profits from minimized manual intervention by the user, increased analysis speed, high data precision, less sample consumption and minimal exposure to hazardous materials. Due to these numerous advantages, their is great potential for this technology to be applied in biological, <sup>[2,3,4]</sup> chemical<sup>[5,6]</sup> and biomedical<sup>[7]</sup> context. Despite the progress made in recent years, it still seems that this technology remains in its first development stages.<sup>[8]</sup> To move it to the next stage, it has to become successfully adapted to solve "real" end-user problems, rather than remain a field based on proof-of-concept experiments.

The major aim of the research within this thesis was to bridge the gap between the development of microfluidics, particularly droplet-based microfluidic technology, and its application in fundamental biophysical and biomedical research with the goal to analyze the reconstitution of actomyosin cortex units. Specifically, droplet-based microfluidics was explored in the following topics: 1) Bottom-up assembly of minimal synthetic cells, with the goal of actomyosin cortex constitution; and 2) mimicry of the immune system cellular environment, with the goal of programing T cells.

### 1.1. Droplet Based Microfluidics

Micro-emulsions can offer various possibilities for chemical and biological research. The potential of water-in-oil micro-emulsion droplets has already been described in 1954 by

#### 1. Microfluidics

Joshua Lederberg.<sup>[9]</sup> Since successful implementation of microfluidic technology toward controlled micro-emulsion creation - droplet-based microfluidics have emerged as new scientific and technological field of research.<sup>[10]</sup>

Most droplet-based microfluidic devices are produced by standard photolithography and the following softlithography methods in which polydimethylsiloxane (PDMS) is cast over the wafer with desired positive relief (details Section III.2).<sup>[1,11]</sup> PDMS is the most common material in microfluidic biological applications due to its low price, high transparency (down to 280 nm), good permeability to gases and biocompatibility.<sup>[12]</sup>

Using flexible microfluidic devices, the formation speed and diameter size of monodisperse droplets can be controlled during their creation: the first may be varied from a slow driping to over 1 MHz (mega hertz),<sup>[13]</sup> while the latter is variable upwards of 1 µm (difference in diameter < 1%).<sup>[14,15]</sup> The droplet diameter is controlled mostly by the channel dimensions but can also be regulated by flow rates at the flow focusing junction where the droplets are generated (detailed information see Section III.2.3). In the flow-focussing junction an aqueous phase is cut by the stream of the oil phase (from both sides) and droplets are generated by self-assembled accretion of surfactants at the interface between water and oil. Figure I.1 shows three structures of the most common types of flowfocusing junctions for droplets production. Following optimization, crossroad junction structure (Figure I.1.C) was discovered as the most efficient for stable droplet production. Therefore, in this research only the crossroad junction structure was used for droplet production.



Figure I.1.: Representative structures of junctions in which the droplets are generated A) T-junctions, B) Y-junctions and C) crossroad flow-focusing. Blue and red channels indicate aqueous and oil phases, respectively.

Besides the flow-focusing junction many other droplet-based microfluidic units have been designed and implemented to manipulate and to analyze the droplets content (Figure I.2). The most commonly used are:

- A) Pico-injection unit (Figure I.2 A), in which an alternating electric field induces surfactant poration and allows well-controlled injection of aqueous phase into the droplets;<sup>[16]</sup>
- B) Droplet storage chambers (Figure I.2 B), where the droplets can be stored in specially designed PDMS niches connected via narrow channels, which is mainly used for time-lapses high-throughout analysis of the droplets content;<sup>[17]</sup>
- C) Droplets sorter unit (Figure I.2 C), in which an electric field is triggered, based on the microscopic readout of the droplet content (i.e., the expression level of specific markers) and deflect the droplets to the left arm of a Y-junction due to dielectrophoretic force, otherwise the droplets will flow into the wider right arm, due to lower hydraulic resistance;<sup>[18]</sup>
- D) Mixing unit (Figure I.2 D), in which zig-zag channels are used to mix fluids inside the droplets;<sup>[19]</sup>
- E) Deformation chambers unit (Figure I.2 E). which offers a possibility to investigate the dynamic surfactants accretion on the oil-water interface;<sup>[20]</sup>
- F) Spiral inlet unit (Figure I.2 F), which is required for the Dean flow (De) regime, allowing the separation of cells or particles by an inertial lift and viscous drag force.<sup>[21]</sup>

Upon interest, these operating units can be integrated together into one single device or composed for special experimental needs.



Figure I.2.: Summary of the most common droplet-based microfluidic units. Representative images of microfluidic units: (A) pico injection, (B) droplet storage, (C) droplet sorting, (D) mixing the content in droplets, (E) droplet deformation and (F) spiral device.

The soft and dynamic nature of biocompatible droplets, their ultra-fast generation and well-controlled environment add up to a system that combines many necessary functions of micro-compartments for various biochemical and biomedical applications. Therefore, droplet-based microfluidic systems are commonly implemented as screening tools for high throughput chemical and biological analysis.<sup>[22]</sup> They may home cells<sup>[4,23]</sup> and are used to create biocompatible gels and polymer particles.<sup>[24]</sup> Moreover, droplet-based microfluidics have been implemented as containers for PCR (polymerase chain reaction)<sup>[25]</sup> analysis and for *in vitro* transcription and translation applications.<sup>[26]</sup>

### 1.2. Surfactants

Surfactants (surface active agents) are amphiphilic molecules, which are part of many products used in our daily life e.g. shampoo, washing powder or cosmetics. The oldest known surfactant is soap that was used already 2500 years B.C. in Egypt.<sup>[27]</sup> Surfactants consist of hydrophobic and hydrophilic parts, variable in their number, length, molecular weights and functional groups (Figure I.3).



Figure I.3.: Schematic representation of a diblock and triblock surfactant

The hydrophobic part consists mainly of hydrocarbon or (per)fluorinated-carbon chains and is selected according to the chemical nature of the carrier oil. In general fluoro-based surfactants are considered to be more effective in stabilizing droplets than hydrocarbon surfactants with the same chain length and hydrophilic head groups.<sup>[28,29]</sup> This can be explained by the larger difference in energy for transferring a -CF<sub>2</sub>- group from bulk to micellar state in comparison to  $-CH_2$ - groups.<sup>[29]</sup> As hydrophilic head groups many polar and non polar molecules have been tested.<sup>[30]</sup> In biological applications the most common hydrophilic units are composed of polyethylene glycol (PEG) groups. PEG molecules create a passivation layer and therefore minimize unspecific depositions of proteins from e.g. the cell media or buffer to droplet's inner surface.<sup>[15,30]</sup> Chemically modified surfactants (see detailed description in Section III.5 and IV.4) can also act as defined adhesion structures on the inner surface of the droplet.<sup>[4,31]</sup>

During droplet formation surfactants are organized by self-assembly at the oil-water interface and decrease the interfacial tension by creation of a dense monolayer. This monolayer also serves as a steric barrier against droplet coalescence.<sup>[32]</sup> Surfactant shielding efficiency is mostly determined by the structure, length and chemical composition of the hydrophobic part. To asses quantitatively the surfactant's ability to form stable emulsion, the hydrophilic-lipophilic balance (HLB) has to be evaluated:<sup>[33,34,35]</sup>

$$HLB = 20 \frac{H_{\rm w}}{H_{\rm w} + L_{\rm w}}$$

Where  $H_w$  and  $L_w$  are the molecular weights of the hydrophilic and hydrophobic parts respectively. The HLB values are in the range of 0 to 20, where 0 represents a hydrophobic and 20 a hydrophilic molecule.<sup>[28]</sup> For the formation of stable water-in-oil emulsion droplets with surfactants dissolved in the oil phase the HLB-value for the surfactant needs to be adjusted between 1 to 10.<sup>[29]</sup>

An additional factor to be considered for stable droplets creation is the critical micelle concentration (CMC), which represents the lowest concentration of a surfactant at which micelles are created.<sup>[36]</sup> Below the CMC, surfactants are dissolved in solution as monomers, above the CMC the surfactants form aggregates like micelles or vesicles depending on their hydrophobic to hydrophilic ratio and their structure, while the concentration of free surfactants remains constant.<sup>[27]</sup> Micelles have no effect on the interfacial tension. Typical CMC values for water-soluble surfactants at room temperature are in the range of 10<sup>-1</sup> to 10<sup>-5</sup> M.<sup>[36]</sup> The CMC value is affected by surfactant-dependent factors, for example its constitution (diblock, triblock or else) and the structure of its hydrophilic and hydrophobic moieties, but also by the temperature and the type of solvent (polar/apolar). Very stable droplets can be obtained with surfactants having large fluorocarbon hydrophobic residues. These surfactants closely overlap and form a dense layer with a thickness of about 10 nm to 50 nm.<sup>[15]</sup> However, if the surfactants molecular weight exceed 20000 dalton their adsorption to the resultant droplet's interphase will be affected by slow diffusion in the oil phase.<sup>[15]</sup> Therefore, it is important to consider the kinetics and diffusion rates of surfactants as they have to accrete sufficiently at the generated droplet's interface prior the droplets gets in contact with other droplets. Table I.1 presents the summary of the physicochemical properties of hydrophobic and hydrophilic parts of the surfactant on the CMC values.<sup>[37,38,39,40,41,42]</sup>

In this research the effectiveness of surfactants to reduce the interfacial tension as a function of the concentration was assessed by two interfacial tension measurement methods: static mode, using pendant drop method as described in Section III.1.2, and dynamic mode, using a droplet deformation microfluidic device (Section III.1.3).

	CMC increase	CMC decrease
Hydrophobic portion	<ul><li>Trifluormethylene groups</li><li>Branches</li><li>Polar groups (O or OH)</li><li>Doublebonds</li></ul>	<ul><li>Perfluorinated compounds</li><li>Large number of C-atoms</li></ul>
Hydrophilic portion	<ul><li>Several headgroups</li><li>Ionic groups</li><li>Polar groups in the middle of the surfactant</li></ul>	<ul> <li>Polar organic groups</li> <li>e.g. OH or NH<sub>3</sub></li> <li>Several binding sites for counterions</li> </ul>

Table I.1.: Summary of the physicochemical properties of the surfactants that influence the CMC-values

In this thesis droplet-based microfluidics was explored in the following topics: 1) mimicry of the immune system cellular environment, with the goal of programing T cells; and 2) Bottom-up assembly of minimal synthetic cells, with the goal of actomyosin cortex constitution

## 2. Artificial Biointerfaces for Immunological Applications

In immunology the two main players are antigen-presenting cells (APCs) and T lymphocytes (T stands here for thymus, the organ where T cells mature). APCs are pivotal for the recognition and capturing of disease-causing antigens and their presentation to lymphocytes in lymphoid organs.<sup>[43,44,45]</sup> T cells respond to the presented antigens and perform actions that play a crucial role in orchestrating the body's adaptive and innate immune system response .<sup>[46,47,48,49,50]</sup> In patients who suffer from life-threatening diseases like cancer, HIV, or autoimmune disorders the clinically beneficial T cell types and/or APCs are found in low frequencies. To increase the disease-fighting capacity of a patient's T cells a new type of therapeutic strategy, the adoptive T cell therapy (ACT), has recently been developed.<sup>[46,47,48,49,50]</sup> ACT aims to generate a large pool of engineered T cells resulting in a potent immune response specific to antigens on the disease-causing cells. However, despite the beneficial impact of engineered T cells, the road towards broadly applicable ACT is still long and winding. Many clinical trials have been largely disappointing because of a lack of highly proliferative engineered T cells, whether naive or early memory phenotypes.<sup>[51]</sup> Activation, ex vivo expansion and differentiation of T cells into an early memory subset are central steps in an effective ACT and represent a challenge as it remains difficult to simulate the intimate cellular interactions between T cells and APCs during their contact in peripheral lymphoid organs.<sup>[52,53]</sup>

### 2.1. Immunological Synapse

The fate of T cells is not solely regulated by the presence of certain molecules on the surface of APCs but also by their density and spatial distribution on the micro- and nanometric scales.<sup>[44,54]</sup> Moreover, mechanical properties of APCs and force-dependent conformational changes during the formation of an immunological synapse (IS; a highly organized supramolecular complex at the APC-T cell interface, see Figure I.4) play a crucial role in T cell fate regulation.<sup>[43,55]</sup>



Figure I.4.: APC T Cell interaction (A) Schematic representation of the direct interaction between a T cell and an APC.An ideal APC surrogate must present recognition/stimulation signals delivered through a TCR during interaction with the appropriate pMHC complex or antibodies (antiCD3), which bypasses signals that normally must be provided from the natural antigen-presenting cell through close cell-cell contact: 1) antigen recognition signal, 2) co-stimulation signal (provided mainly by B7 proteins, CD80 and CD86, as well as antibodies (antiCD28) provided upon interaction with CD28 and adhesion molecules such as ICAM-1). The third signal, termed cytokine priming, influences T cell activation, proliferation and differentiation. (B) Crosssection representation of the IS. Signaling molecules are organized into the supra-molecular activation clusters (SMACs) by actin-dependent motility: the central SMAC (cSMAC, yellow) is enriched with stimulation and co-stimulation complexes, the peripheral SMAC (pSMAC, orange) is enriched with adhesion complexes, and the distal SMAC (dSMAC, purple) is enriched with large and bulky molecules such as CD43, CD44 and CD45. Adapted with permission from Ref.<sup>[43]</sup> Copyright 2013, Israel Journal of Chemistry.

In this research the primary goal was to design and to implement nanopatterned and specifically biofunctionalized water-in-oil droplets as APC surrogates for immunological applications, with the intense to develop and implement antigen-presenting interfaces that represent flexible and modular systems that closely model *in situ* APC-T cell interactions.

A critical event in the initiation of the adaptive immune response is the activation of T lymphocytes. This step is mediated by antigenic substances (short peptide fragments of 10-15 amino acids), which are presented by major histocompatibility complex (MHC) molecules on the APC membrane and recognized by T cell receptors (TCRs).<sup>[56]</sup> The generation of productive T cell responses, such as proliferation and differentiation, requires signal mediation by the TCR, but also the engagement of additional co-stimulatory signals and adhesion complexes in the IS.<sup>[43]</sup>

The hallmark trait of an IS is the dynamically coordinated formation of micrometerscale spatial patterns of cellular surface molecules, the so-called supra-molecular activation clusters (SMACs) (see figure I.4). Real-time imaging of T cell-APC interactions *in vivo*<sup>[57]</sup> and *in vitro*<sup>[58]</sup> showed that synapse formation starts with TCRs-pMHC microclusters at



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### 2.2. State of the Art Antigen-Presenting Interfaces

During the past twenty years much effort has been concentrated on the development of 2D planar APC analogues as summarized in details in Platzman et al.<sup>[43]</sup> The most common technology was based on supported lipid bilavers (SLBs), which provide a suitable model system for mimicking the cell membrane (reduced from three to two dimensions), because the lateral mobility of lipids and proteins partially resembles the *in vivo* situation.<sup>[60,61]</sup> SLBs containing fluorescent-labeled APC ligands enabled the discovery of the IS, and have since then been heralded as a powerful model for investigating the dynamically coordinated SMACs formation.<sup>[62,63]</sup> Moreover, to better mimic membrane properties in vivo, different semiconductor fabrication strategies have been implemented to define spatial constraints within SLBs in order to alter the mobile fraction and the diffusion of incorporated proteins.<sup>[64,65,66]</sup> For example, TCR signaling can be controlled by maintaining TCR in the periphery and preventing its dynamic recruitment to the center of the synapse. by means of continuous linear barriers that increase TCR-associated phosphorylation, as well as intracellular Ca<sup>2+</sup> levels (Figure I.5 A).<sup>[66]</sup> Furthermore, DeMond et al.<sup>[64]</sup> used electron-beam to create molecular mazes composed of short, periodic linear fences. It was observed that TCR clusters were deflected by maze fences, when they encounter them, but continued to move parallel to the fences, at speeds that scale with the relative angle of motion to the preferred centripetal direction. Moreover, this study revealed that a frictional drag force determined by the coupling chemistry (e.g., via talin) to the dynamic actin cytoskeleton, drives the movement of protein microclusters.<sup>[67]</sup>



Figure I.5.: Schematic representation of supported lipid bilayers in defined compartments and T cell proliferation on soft substrates A) Schematic view of a patterned SLB surface and the hindered organization of SMACs. Adapted with permission from Ref.<sup>[43]</sup> Copyright 2013, Israel Journal of Chemistry. B) Rigidity-dependent activation of mouse naive CD4+ T cells presented by IL-2 secretion and cell attachment characterizations 6 hours after seeding. Adapted with permission from Ref.<sup>[68]</sup> Copyright 2012, The Journal of Immunology.

As previously mentioned, IS formation upon interaction with APCs is also characterized by a dynamic cytoskeleton, and adhesion proteins (e.g. integrin - LFA-1) interactions. This suggests the involvement of mechanical forces in T cell activation. That is supported by recent studies which showed that TCRs are sensitive to forces in the pN range.<sup>[69,70]</sup> This indicates the important role of cytoskeleton contractility in APCs during T cell activation.<sup>[69,70]</sup> Unlike cellular membranes, SLBs do not allow any pushing or pulling force due to the supported rigid surfaces. Therefore, considering that T cells are unlikely to encounter stimulatory surfaces with the stiffness of glass in *vivo* – much effort has been put into the development of biocompatible APC supports that more closely mimic the physiological elasticity ranges found in living organisms.

In attempt to provide answers to the question if T cells can sense the stiffness of the antigen presenting surfaces O'Connor et al.<sup>[68]</sup> explored the impact of PDMS substrates stiffness ( $E_Y < 2$  MPa) on the *ex vivo* activation, proliferation and differentiation of the primary human T cells (Figure I.5 B). It was shown that soft substrates (Young's modulus -  $E_Y < 100$  kPa) on average support a 4-fold increase in overall T cell expansion and greater IL-2 secretion than stiffer ( $E_Y = 2$  MPa) substrates. Moreover, CD4<sup>+</sup> T cells expanding on soft substrates yield in an average 3-fold greater proportion of IFN-producing type 1 helper (Th1) T cells, indicating that naive T cells expanding on soft substrates following adoptive therapy in cancer.<sup>[71]</sup> These

results depicted the important and afore unrecognized influence of the APC surrogate mechanical properties on T cell proliferation and differentiation. In the case of clinicalgrade culture systems for *in vitro* T cell expansion such as bead-based APC analogs (see the following paragraph), these findings could contribute to more rigorous material selection, with an eye to mechanical property control.

Monodisperse, cell-sized polystyrene or magnetic iron oxide rigid beads endowed with stimulatory and co-stimulatory antibodies were qualified as clinical-grade APCs systems for T cell expansion in adoptive therapy (Figure I.6 A).<sup>[43]</sup> These beads have been highly useful to define the optimal size of artificial 3D APCs as well as to determine the spatial requirements and the minimal number of receptors  $(60 \,\mu\text{m}^{-2})$  to stimulate T cells efficiently.<sup>[72,73]</sup> Bead-based APC surrogate systems provide good control over T cell activation and differentiation, but the approach has several limitations. The ability of these systems to serve as an optimal APC analogues is mainly hindered by their inability to dynamically remodel their protein composition, which is in stark contrast to the natural IS. Additionally, the rigid nature of the bead material limits mechanosensing, which was shown to be crucial for effective T cell activation control.



Figure I.6.: Schematic representation of T cell bead interactions and experimental results of bead mediated T cell proliferation in  $\mu$ well arrays A) Schematic sketch of A) latex and B) magnetic bead-based APCs. Adapted with permission from Ref.<sup>[43]</sup> Copyright 2013, Israel Journal of Chemistry. B) Representative images of microwell-arrays with trapped beads and T cells. Adapted with permission from Ref.<sup>[74]</sup> Copyright 2012, Lab on a Chip.

So far most T cell experiments described in literature were performed in suspension mode. Unfortunately, this approach limits long-term dynamic analysis and control over T cell processes using live-cell imaging. Firstly, primary naive T cells are non-adherent and become highly motile after stimulation - this means they escape rapidly out of the field of view. Secondly, T cell differentiation is a relatively slow process (several days). Thirdly, cell cytokine secretions become diluted. Lastly, T cell activation is considered to be a stochastic process. Therefore, analyzing individual T cells and their most proximal environment brings new insights of T cell response.<sup>[75]</sup> Recently, an experimental platform which combines microwell arrays and bead-based APC,<sup>[74]</sup> as shown in Figure I.6, was used for long-term monitoring of individual T cells. However, while this system was able to provide a more controlled environment for long-term monitoring it failed to contribute to a better design of synthetic 3D APC systems.

In this thesis a new APC analogue was designed overcoming the described drawbacks by combining the afore mentioned advantages of 2D and 3D systems namely:<sup>[4]</sup>

- Control over identity and quantity of bio-ligands
- Dynamic organization of bio-ligands
- Allow T cells to exert forces in all three dimensions
- A well defined environment for long-term (up to 3 days) monitoring of individual T cells over the course of their proliferation and differentiation.

### 3. Bottom-Up Assembly of Minimal Synthetic Cells

"What I cannot create, I do not understand." This quote by the American physicist Richard Feynman can be also applied to biology. This way of thinking constituted a trigger or, in other words, a catalyzer for a new biological field of research – i.e. synthetic biology.<sup>[76]</sup> Traditionally, in biological research a complex bio-relevant matter is observed and examined as it is normally found in nature. Therefore, a biological research generally suffers from the fact that the systems to be technologically explored, such as tissues or cells exhibit an enormous complexity. In an attempt to overcome this complexity, synthetic biology thrives not only to observe the biological matter but also to generate it by means of simple man-made devices that can be externally controlled to allow a bottom-up assembly.<sup>[77,78]</sup> The aim of synthetic biology is not to replace the traditional biological research, but to contribute synergistically by the implementation of the mechanical insights gathered over decades of research in molecular biology to provide biomimetic systems for better understanding of biological properties.

In this thesis, the primary goal was to explore the possibilities of droplet-based mi-

crofluidics in the engineering of biomimetic micro-compartments for a bottom-up assembly of sub-cellular units *in vitro*. Towards this end, water-in-oil nanostructured emulsion droplets were used as cell-size compartments to achieve the functional bottom-up assembly of the minimal number of proteins required for reconstitution of the actomyosin cortex.

### 3.1. Formation of Biomimetic Micro-Compartments

The main distinguishing feature of eukaryote cells is further compartmentalization – the presence of lipid-membrane-enclosed compartments within the cell in which specific metabolic activities take place. Therefore, in synthetic biology, especially in bottom-up approach, it is imperative to create stable and well defined cell-sized compartments. An important property of compartments is to provide boundaries in order to avoid diffusion and dilution of biological components. Another important task of compartments is to provide a connection with its surrounding. While keeping essential intercompartmental functions, this connection can be provided by selective in and out material and/or signal transport via biochemistry on the compartment interface. There are three main types of functional micro-compartments in synthetic biology: 1) vesicles (lipid membrane-based micro-compartments); 2) polymersomes (polymer-made synthetic membranes) and; 3) droplets (water-in-oil emulsion micro-compartments).

### 3.2. Vesicles

Lipid membrane-based vesicles are the most traditional compartments in synthetic biology. These compartments provide a suitable model system for mimicking the cell membrane because the mobility of lipids and incorporated proteins closest resembles the *in vivo* situation.<sup>[79]</sup> Nowadays, the most common method for the preparation of giant unilamellar vesicles (GUVs) is electroformation (Figure I.7)<sup>[80,81]</sup> or spontaneous swelling e.g. hydrogels.

This method uses a thin lipid film on electrodes by deposition of a lipid solution and evaporation of the organic solvent under a flow of nitrogen to inhibit lipid oxidation. Glass slides coated with indium tin oxide (ITO), a transparent semiconductor, or platinum wires are usually used as electrodes for the application of direct current (DC) or alternating current (AC) electric fields. It was shown, that the upper boundaries for the AC electrical field's frequency and strength are 10 kHz and 10Vmm<sup>-1</sup> respectively.<sup>[82]</sup> Despite the fact
several theories tried to explain the growth of GUVs under electrical fields the exact mechanism of GUVs generation is still unclear.<sup>[80,83,84]</sup>



Figure I.7.: Schematic representation of GUVs creation by electroformation Illustration of the electroswelling device consisting of two ITO-coated glasses, each connected with a copper electrode and isolated to the other ITO glass to crate an else cortical field for electroswelling.

To achieve a higher yield of GUVs formation, electroformation method was combined with the addition of high concentration of sodium ions. This is in contrast to the cytosol of animal and human cells which exhibit a relatively small concentration of sodium and a relatively large concentration of potassium.<sup>[85,86]</sup> Therefore, current studies elucidate the mechanical properties of lipid vesicles exposed to physiological buffers with high potassium concentration, which has a strong effect on the kinetics of enzymatic reactions and filament polymerization.

In general, lipid vesicles demonstrate the potential advantages of using fluid membrane compartments for synthetic biology applications. Nevertheless, several general challenges for using GUVs for these types of applications exist. Mechanical instability of GUVs is considered to be a main challenge in utilization of this micro-compartment system. Above 1 µm the mechanical stability of GUVs decreases with increasing size.<sup>[87]</sup> Furthermore, the limited chemical stability of GUVs caused by oxidation processes of unsaturated fatty acids and the hydrolysis of ester bonds are another drawback of this system, unless antioxidants and chelating agents are added.<sup>[87]</sup> Moreover, the functional and controllable delivery of protein complexes into the GUVs is time consuming, especially when their stability is low.<sup>[88]</sup> Another drawback of this system is the uncontrolled orientation of incorporated proteins. All this has led to research on other kinds of artificial micro-compartments that possess advantages similar to those of lipid vesicles but have greater stability.

#### 3.3. Polymersomes

Vesicles made of amphiphilic copolymers have been used in synthetic biology applications due to their increased stability, rigidity of their membranes and increased lifetime.<sup>[89,90]</sup> In contrast to lipid vesicles, where lipid properties manipulation is limited, the thickness, bending and stretching moduli, and the permeability of the polymeric membrane can be easily tuned by changing the blockcopolymer molecular properties. In context of controlled orientation of incorporated proteins polymersomes have also several advantages in comparison to lipid vesicles. Directed insertion of membrane proteins into the synthetic membrane can be achieved by orientation control of membrane proteins via tethering to functional groups and/or antibodies.<sup>[91,92]</sup>

Nowadays, the most common methods for the preparation of the cell-sized polymersomes are electroformation (similar to lipid vesicles formation, see Section I.3.2)<sup>[80,93]</sup> and double emulsion microfluidics as described in Figure I.8.<sup>[94]</sup> Polymersomes production via electroformation generate polymersomes with poor control of size distribution and a low efficiency of encapsulation as already discussed in the case of GUVs.<sup>[94]</sup> Narrow polymersomes size distribution can be achieved by microfluidic approach using mixtures of chloroform and hexane as hydrophobic phase which allows quick evaporation and the creation of polymersomes with a double layer of polymers in the periphery. Nevertheless, encapsulation and further manipulation of protein containing polymersomes still represent big challenges.<sup>[95]</sup>



Figure I.8.: Schematic representation of double emuslion polymersome productrion Illustration of a microfluidic capillary device for W/O/W-double emulsion droplets stabilized by polymersomes. These devices consist of pointed cylindrical capillaries in a square capillary. With the first cylindrical capillary the solution for encapsulation is injected into the oil phase and the double emulsion droplets are collected by the second capillary.

These drawbacks are mainly due to complications related to controlled permeability of the traditional polymersomes<sup>[96]</sup> and due to the lack of technological means that allow precise and efficient injection of different biological components into polymersomes. To overcome these obstacles, a new highly innovative and creative approach in bottom-up synthetic biology had to be created, as described in the following section.

#### 3.4. Droplets

Microfluidic block copolymers-stabilized water-in-oil droplets can also be classified as polymersomes, but due to the continuous oil phase they are considered in the scientific community as emulsion droplets. The advantages of water-in-oil emulsion droplets for biological applications have been described in details in Section I.1.1. In the context of bottom-up assembly of artificial cells droplet-based compartment systems represent the advantages of the polymersomes, but can also be easily adapted to the droplet-based microfluidic technologies for precise delivery of bio-relevant ingredients. The continuous oil phase can play an important role in preserving the droplet material content (see Section IV.3). Moreover, a continuous oil phase is required to control an injection (details see Section I.1.1) of precise amounts of high-value biomaterials exclusively into the droplets Figure I.9.



Figure I.9.: A droplet based pico-injection unit. (A) Shows a junction to control the spacing between the droplets through addition of oil via the second oil channel. Figure (B) shows a injection channel and the electrodes of an alternating electric field with 30kHz and 600V used to reduce the stability of the surfactants in the droplet periphery for an aqueous injection of other cellular components (injection volume can range between 2 to 100 pL dependend on the applied pressure) from a pico-injection channel.

Due to the variety of required cellular components in the droplets the microfluidic device with small and compact electric systems was integrated to apply electric fields in microchannels. These electrical fields allow the destabilization (poration) of surfactants and lipid bilayers towards controlled fusion (pico-injection) of solutions containing different cellular components into the droplets. Figure I.9 (A) shows how the spacing between the droplets carrying different cellular components is controlled through addition of oil via the second oil channel. Figure I.9 (B) the injection unite where, an alternating electric field with 30kHz and 600V reduces the stability of the surfactants in the droplet periphery for an aqueous injection of other cellular components (injection volume can range between 2 to 100 pL depending on the applied pressure) from a pico-injection channel.

This thesis comprises a pioneering attempt to dissect the complex cellular sensory machinery by means of an automated droplet-based microfluidic approach. Towards this end, water-in-oil nanostructured emulsion droplets (see Section IV.4) as cell-size compartments were used in combination with pico-injection technology to achieve a functional bottom-up assembly of a minimal number of proteins required for "droplet-based minimal synthetic cells". While the applied methodology allows for the assembly of a wide range of subcellular functional units, the main focus here was on actin cytoskeleton filament organization.

## 4. In Vitro Reconstitution of Actin Cytoskeleton network

The filamentous actin (F-actin) is one of the three main constituents of eukaryotic cytoskeleton. In vivo it is formed by the assembly of actin monomer units into filaments that can be dynamically organized in a variety of ways, via recruitment of more than 130 different accessory proteins.<sup>[97]</sup> Some of these proteins affect F-actin assembly and disassembly rates, others, like molecular motors, cross-linkers and nucleators generate mechanical forces, crosslink, bundle and branch the filaments. These dynamic processes can selforganize the filaments into a variety of sub-cellular structures such as contractile rings,<sup>[98]</sup> protrusions<sup>[99]</sup> and the actin cortex.<sup>[100,101]</sup> This biochemical dynamic organization is in a close interplay with the cellular biophysical processes. Therefore, actin cytoskeleton affects many cellular processes, including, but not limited to, cell division, force generation, polarization, intracellular transport and motility.<sup>[102,103,104]</sup> This broad range of actin-mediated processes also underscores their central physiological role, as well as their involvement in a wide variety of disease states.<sup>[105,106,107]</sup> Although attaining a fundamental characterization of how macroscopic cytoskeletal dynamics emerge from its molecular constituents is a compelling goal, little understanding has yet been accomplished, mainly due to the extensive complexity of the cytoskeleton network and difficulties inherent in studies *in vivo*.

Engineering of biomimetic systems for controlled bottom-up reconstitution of subcellular units *in virto* has become an important strategy for dissecting complex actincytoskeleton systems. In principle, efficient biomimetic systems should allow full control of the constituents for studying the effect of specific changes in molecular composition, and their biophysical properties have to resemble as close as possible *in vivo* conditions.<sup>[108]</sup> In living cells, a part of the actin cytoskeleton is coupled to membranes and thereby provides structural integrity. Therefore, while engineering biomimetic systems, a particular focus has to be on coupling of reconstituted actin filaments to synthetic membranes.<sup>[109,110]</sup>

The most known biomimetic system for in vitro actin-based motility and cytoskeletal organization in bulk is a supported lipid bilayer (SLB). SLBs are planar continuous fluid films of uniform density that form by the spontaneous fusion and self-assembly of liposomes and/or proteoliposomes on clean silicon oxide surfaces. The experimental data concerning the formation of the SLBs and their chemical biofunctionalization has been summarized and reported in detail previously.<sup>[61,111,112]</sup> SLBs provide a good model system for mimicking the cell membrane (reduced from three to two dimensions) because the lateral mobility of lipids and proteins partially resembles the situation in vivo. Furthermore, high-resolution imaging and manipulation techniques, including laser ablation and fluorescence recovery after photobleaching (FRAP),<sup>[113,114]</sup>fluorescence correlation spectroscopy (FCS),<sup>[115,116]</sup> stimulated emission depletion microscopy (STED),<sup>[117,118]</sup> and atomic force microscopy (AFM)<sup>[119,120]</sup> can easily be applied on the flat geometry of SLB systems for actin cytoskeleton investigation. During the last fifteen years, several groups developed biochemical methods to couple between the actin and the SLBs.<sup>[121,122,123,124,125]</sup> The basic strategies to couple actin to SLBs in vitro were based on cell-derived or recombinant transmembrane proteins such as ponticulin that anchor the actin network to the cell membrane and serve as nucleation sites for actin assembly.<sup>[121,126,127]</sup> Another strategy is based on addition of phosphatidylinositol 4,5-bisphosphate (PIP2) lipids that serve as anchoring points for N-WASP actin nucleators.<sup>[128,129]</sup> For example, Lee et al.<sup>[125]</sup> used PIP2 containing SLB system to generate a thin dendritic actin layer on the surface of the membrane, and by addition of frog egg extract to the membrane actin was able to reconstitute long filopodia-like actin structures. Other studies added G-actin or F-actin to ponticulin-containing SLBs and created cortex-like actin structures with the average actin layer thickness of 15 nm as revealed by AFM measurements.<sup>[122]</sup>

Together, these studies point to a powerful role of SLBs in modulation of spatial dynamic organization of actin filaments. However, unlike in biological membranes, the planar bilayers do not allow pulling of the proteins and instead are rigid in the vertical dimensions due to the trapping at the glass surface. Therefore, the ability of actomyosin cortex to exert forces in all three directions is restricted in studies on supported rigid surfaces. Moreover, to emulate cortical dynamics it is essential not only to incorporate actin turnover dynamics to soft interfaces, but also reproduce a cell-like geometry.

Lipid vesicles or droplets can overcome the aforementioned limitations due to their 3D cell-like geometry and soft interfaces. For example, Liu et al.<sup>[110]</sup> used PIP2 containing GUVs to show that elastic interactions between the synthetic lipid vesicle membrane and reconstituted actin cytoskeleton can cooperate without accessory proteins to induce the formation of actin filament protrusions (Figure I.10).



Figure I.10.: Role of membrane in formation of thin actin filament protrusions. A model of membraneinduced formation of actin filaments. Left: Small, local deformations of the membrane induced by actin filament polymerization against the membrane (black arrows).

Merging attractions to create a larger deformation (curly brackets). Middle: Deformations that fail to create a larger deformation (grey arrows). Right: After bundling of filaments to overcome the resistance of the membrane. Actin filaments bundle and can elongate without further physical constraint. Adapted with permission from Ref.<sup>[110]</sup> Copyright 2008, Nature Physics.

Model of membrane-induced formation of a thin actin filament protrusion. Left: Smallamplitude local deformations of the membrane arise as actin filaments polymerize against the membrane (black arrows). Deformations that are within the range of attraction are able to merge to create a larger deformation that gathers extra filaments (curly brackets). Middle: Deformations that fail to gather extra filaments are stalled and diminish (grey arrows). Right: After bundling enough filaments to overcome the membrane resistance to tube formation, a 'proto-filopodium' can elongate without further physical constraint.

This work highlighted the mutual influence of the membrane on the actin cytoskeleton and vice versa. To mimic better the physiological conditions of actin–lipid membrane coupling, Merkle et al.<sup>[130]</sup> used porcine lipid extract to generate GUVs containing actin and spectrin/ankyrin actin anchoring proteins. F-actin was found in contact with the interior walls of GUVs, indicating an efficiency of membrane anchoring protein complexes. Other studies examined morphological response of the actin networks (without linkage to GUVs periphery) as a function of accessory proteins, such as crosslinkers or F-actin depolymerizing agents. It was shown that the formation of randomly linked networks to ring-like structures depends not only on different ratios of accessory proteins but also on temperature and ion concentration.<sup>[131]</sup> Moreover, it was shown that by only changing the dimensions (i.e. diameter) of the GUVs and keeping constant the biochemical and biophysical conditions of an encapsulated actin system, one can easily control the morphology of actin network structures.<sup>[132]</sup> However, it must be noted here that these studies were performed with the actin concentration that is fifty times lower in comparison to intracellular actin concentration.

One of the major drawbacks of GUVs system in the case of actin cytoskeleton investigation is that the lipid membrane is destroyed by high concentrations of salts and actin during an electroformation. To partially overcome this obstacle, new methods for GUVs formation have been developed. These methods include: 1) dried hybrid lipid-agarose films,<sup>[133]</sup> 2) water-in-oil lipid monolayer droplets as precursors for GUVs formation,<sup>[134]</sup> and 3) double emulsion microfluidics (see Section I.3.3).<sup>[95]</sup> For example, Tsai et al.<sup>[133]</sup> implemented recently the dried hybrid lipid-agarose films<sup>[133]</sup> method to reconstitute the actomyosin cortex within the GUVs. To test whether myosin-driven contractility can cause actin membrane rupture, biotinylated lipids were used to anchor biotinylated actin via streptavidin linkage. An important outcome of this study was that at the high myosin/actin ratios (e.g. 1/50 and 1/100) motor-driven forces can rapture actin-membrane attachments and no cortex structure can be observed. In a recent study Miyazaki et al.<sup>[98]</sup> used water-in-oil lipid monolayer droplets as cell-sized compartments to

demonstrate that actin filaments could assemble into a single ring-shaped bundle without spatial regulatory signals. On the basis of droplet size and geometry dependencies the authors proposed a confinement-induced assembly mechanism, which indicates a direct contribution of cell rounding to contractile ring assembly. Another important outcome of this study was that in addition to droplet size and geometry dependencies, myosin concentration and its state (dimer or oligomer, controlled by KCl concentration)<sup>[135]</sup> can regulate both actin ring assembly and its contraction. Based on the results the authors proposed that myosin dimers promote the cytokinetic ring assembly, and myosin oligomers could switch the ring assembly phase to the contraction phase and induce the complete contraction.

Despite the fact that new methods for GUVs formation have improved the concentration related limitation in the field of actin reconstitution studies, the mechanical instability of the cell-sized GUVs and a lack of technological means for their controlled manipulation still represent major drawbacks of this system. Therefore, to overcome stability issues, polymer-stabilized water-in-oil emulsion droplets become an alternative microcompartment system for actin reconstitution studies. Recently Pinot et al.<sup>[136]</sup> reconstituted a dynamic, flowing F-actin network using *Xenopus* meiotic extracts (these extracts ostensibly represent a full array of *in vivo* actin regulatory proteins) artificially confined within the polymer-stabilized emulsion droplets. In addition, the droplets periphery was functionalized to recruit actin nucleation promoting factors. To examine how confinement induces the generation of symmetry breaking and F-actin flow, the authors applied biochemical perturbations of actin polymerization dynamics by cytochalasin D or phalloidin or affected the filament nucleation growth by addition of suppressor of G-protein-coupled cyclic-AMP receptor (SCAR) domains that stimulate bulk nucleation or inhibited the activity of myosin II. The authors showed that perturbation of actin polymerization or filament nucleation growth lead to the inhibition of F-actin flow. On the other hand, inhibition of myosin activity did not significantly affect F-actin flow dynamics in the droplet confinement. Based on these observations the authors highlighted the primary role of actin polymerization dynamics and nucleation in generation of actin flow. More recently, Enas Abu Shah and Kinneret Keren used similar polymer stabilized water-in-oil droplets to reconstitute actomyosin cortex from *Xenopus* laevis egg cytoplasmic extracts supplemented with labeled actin.<sup>[137]</sup> In contrast to previous studies they showed that symmetry breaking is induced by myosin-driven cortical actin flow and sufficient network connectivity, but does not depend on pre-patterned localization of actin nucleators. Moreover, it was shown that the dynamics of artificial cortices is temperature-dependent – a shift by a few degrees leads to a change in behavior, ranging from homogenous cortices at high temperature (30 °C) to asymmetric actin caps at low temperature (20 °C).

The controversy in the results can be related to the fact that these studies used cell extracts to reconstitute the actomyosin cortex within the droplets. Cell extracts reflect better the cellular conditions, whereas the molecular composition is much less controlled than in purified systems. Therefore, in this thesis the actomyosin cortex has been reconstituted from purified proteins. Towards this end, water-in-oil nano-structured droplets as cell-size compartments and integrated microfluidic picoinjection technology were used to achieve the functional bottom-up assembly of the minimal number of proteins required for "synthetic actomyosin cortices".

# II. Motivation

Cellular interactions with the extracellular matrix or other cells are involved in nearly every cellular event. These responses, in turn, affect almost all facets of cell's life, including, but not limited to, directional migration, cell proliferation, differentiation, survival and gene expression. This broad range of adhesion/interaction-mediated processes also underscores their central physiological roles, as well as their involvement in a wide variety of disease states. Although attaining a fundamental characterization of these cellular functions is a compelling goal, little understanding has yet been accomplished, mainly due to the extensive complexity of these processes. Therefore, engineering of biomimetic systems for controlled manipulation of individual cells or subcellular units *in vitro* has become an important strategy, particularly in biomedical applications.

In this thesis the primary objective was to dissect complex cellular sensory machinery by means of an automated, high-throughput droplet-based microfluidic technology. Specifically, the research was focused on the capacities of this technology in the following topics: 1) Mimicry of the immune system cellular environment for adoptive T cell therapy; 2) bottom-up assembly of "droplet-based synthetic cells" capable to self-assemble different sub-cellular functions, and, as a consequence, to generate signaling events including actomyosin cortex organization, migration and self-propelling.

As described in details in the previous section, over the past decade water-in-oil emulsion droplets created in droplet-based microfluidic devices have become widely used as a screening tool for biological and chemical applications. The soft and dynamic nature of these biocompatible droplets, their ultra-fast generation and well-controlled environment add up to a system that combines the necessary biophysical functions of micro-compartments for synthetic biology applications. To generate antigen-presenting cell surrogates for T cell stimulation and to create "synthetic cells" using a bottom-up approach the aim of this research was to further optimize the biophysical properties and to provide droplets with required active biochemical functions. In the immunological application the goal was to generate a novel antigen-presenting interface that closely mimics *in vivo* conditions. The intention was to create a system that offers: control over identity and quantity of bio-ligands; dynamic organization of bio-ligands; T cells to exert forces in 3D; a defined environment for long-term (3 days) monitoring of individual T cells over the course of their proliferation and differentiation.

In an attempt to dissect the complex cellular sensory machinery by means of an automated, high-throughput droplet-based microfluidic technology, the research aimed to apply the functionalized droplets as cell-sized micro-compartments for a bottom-up assembly of "minimal cells". In this regards, particular attention was on achieving precise control over the reconstitution of the actomyosin cortex, with the following analysis of its dynamics.

# III. Materials and Methods

## 1. Surfactants

Surfactants were synthesized as described in chapter IV section 1 using perfluorpolypropylenecarboxylic acid 2500 and 7000 molecular weight (PFPE-acid, DuPont, Netherlands) and polyethylenglycol 600 and 14000 g/mol (PEG, Fluka, Germany). A representative sketch of a micro droplet with gold-nanoparticle surfactants and triblock surfactants is presented in figure III.1.



Figure III.1.: Schematic representation of a nano-structured surfactant-stabilized droplet water-in-oil emulsion droplet. The structures of the triblock-copolymer surfactant, which are essential for long-term stability, and the diblock gold-linked surfactant required for the biofunctialization are presented.

All solvents used for synthesis were purchased from Sigma-Aldrich in dry state and stored with molecular sieve, under nitrogen or argon atmosphere. Reactions with watersensitive substances were performed in heated flasks under nitrogen atmosphere. Successful surfactant synthesis and their purity were confirmed using Fourier transform infrared (FT-IR) and nuclear-magnetic-resonance (NMR) spectroscopies. Following synthesis the surfactants were dissolved in Fluorinert<sup>®</sup>-FC40 (1.85 g cm<sup>-3</sup>, Iolitec, Germany) and filtered with 0.2 µm PTFE-filters (Rotilobo<sup>®</sup>-Syringe-filter, Carl Roth GmbH, Germany) to obtain final concentrations of 0.5 to 50 mM for stabilizing surfactants and up to 300 µM for gold-linked surfactants.

#### 1.1. Chemical characterization of synthesized surfactants

#### 1.1.1. FT-IR Analysis

IR measurements were conducted on a Nicolet 6700 Nexus infrared spectrophotometer (Thermo Electron GmbH, Dreieich, Germany) equipped with a DTGS detector. A demountable path-length cell for liquid FTIR (Thermo Scientific, USA) with KBr glasses and FC-40<sup>®</sup> perflourinated oil as solvent was used for all measurements. An average of 100 scans per analysis was collected with a resolution of 2 cm<sup>-1</sup>.

#### 1.1.2. NMR Analysis

<sup>1</sup>H and <sup>13</sup>C spectra were recorded using Bruker Avance III 600 with sample changer (B-ACS 60) und cryo-unit, magnet: Bruker Ultrashield plus, 14.1 Tesla, test frequency <sup>1</sup>H: 600.13 MHz, probehead: 5 mm QNPCryoProbeTM with ATMA (automatic tuning and matching), inner coil 15N/13C/31P, Z- Gradient, temperature Range -10°C to 80°C.

#### 1.2. Interfacial Tension Measurements (IFT)

OAC (Data Physics, USA) tensiometer with 90° turned CCD-camera and pendant drop method was used to measure the surface tension of surfactant stabilizing liquids. Laplace-Young was selected as a fitting-method.<sup>[138]</sup> Oil phase (Fluorinert<sup>®</sup> FC-40, Iolitec, Germany) and aqueous phase densities were set to  $1.9 \,\mathrm{g}\,\mathrm{cm}^{-3}$  and  $0.99 \,\mathrm{g}\,\mathrm{cm}^{-3}$ , respectively. As aqueous phase 3 ml type 1 (ISO 3696) water was used in a glass cuvette. The oil phase contained the sample surfactant diluted in FC-40. Prior to measurement samples were drawn into 1 ml syringes and attached to the metal bracket via  $0.4 \,\mathrm{mm} \times 20 \,\mathrm{mm}\,\mathrm{BL/LB}$  cannula and PTFE-tubing ( $\emptyset_{\mathrm{in}} 0.32 \,\mathrm{mm}$ ,  $\emptyset_{\mathrm{out}} 0.78 \,\mathrm{mm}$ ). The test cannula ( $0.8 \,\mathrm{mm} \times 22 \,\mathrm{mm}\,\mathrm{blunt/}$  dull) was attached to the metal bracket and lowered into the aqueous phase. Drops were

created manually using the syringe. All dilution series were tested starting from the lowest concentration to reduce the effect of cross contamination. When switching to the next higher concentration, the tubing device plus test cannula and metal bracket were washed with 100 µl of the next sample and incubated 30 min to equilibrate inner surfaces. Before each measurement cuvettes were washed with 96% EtOH, rinsed with acetone, N<sub>2</sub>-dried, washed with type 1 (ISO 3696) water and again filled with 3 ml type 1 water. Before measuring a new dilution series metal brackets and cuvette were ultra-sonicated 30 min in a 2:1 mixture of Extran<sup>®</sup> (Merck Millipore, Germany) and type 1 water (ISO 3696) at room temperature, thoroughly washed with type 1 water and again ultra-sonicated 30 min in type 1 water. Metal brackets were furthermore ultra-sonicated 30 min in 99.9% MeOH, rinsed with acetone and N<sub>2</sub>-dried. Before attaching the metal brackets to the tubing device the inner surface of the test cannula was rinsed with 1 ml FC-40. Droplet interfacial tension (IFT [mN m<sup>-1</sup>]) was analyzed over time. Once IFT did not show any change over a course of 1000 s the measurement was stopped and IFT<sub>final</sub> was collected for each concentration C.

The exponential decay of IFT<sub>final</sub> =  $\gamma = \alpha \cdot e^{-\beta C} + \eta$ , with surfactant concentration C, was fitted to the data where  $\eta$  is IFT after reaching CMC (critical micelle concentration),  $\alpha$  is the surface pressure after reaching the CMC.<sup>[33]</sup> Therefore  $\alpha + \eta$  is the IFT of the oil phase without surfactant.  $\beta$  is a factor, which indicates the slope of the IFT. All parameters were calculated using the Gauss-Newton Algorithm.

$$\gamma_{CMC} = \eta$$
$$\Pi_{CMC} = \alpha$$
$$CMC = e\frac{1}{\beta}$$

From the Gibbs-Helmholtz equation the surface excess  $\Gamma = -\frac{1}{2.303RT} \cdot \frac{d\gamma}{dlgC}$  with the gas constant R and temperature T was derived. The area each surfactant molecule occupies on the surface is  $a [\mathring{A}^2]$  with Avogadro constant  $N_A$ .

$$\Gamma = \frac{\alpha}{RTe}$$
$$a = \frac{1}{N_A \Gamma}$$

For each surfactant all three parameters  $\alpha$ ,  $\beta$  and  $\eta$  were calculated for each dilution series. All analysis was done with R (RStudio, Version 0.98.1103 – © 2009-2014 RStudio, Inc., USA) (script in appendix).

#### 1.3. Droplet Deformation Analysis

Droplet deformation microfluidic analysis as described by Brosseau<sup>[139]</sup> was implemented for dynamic IFT characterization. Figure III.2 shows images of the flow cell in which the droplets deformation occured by a sudden change in a flow regime. To detect the deformation, high speed videos were recorded with 16000 frames per second (Phantom v7.3, Vision Research, USA).



Figure III.2.: Droplet deformation in flow cell chamber Time series from left to right depicting droplet deformation due to different flow rates as a reason of a sudden change in flow rate.

ImageJ was used to extract droplet shapes from the videos. Evaluation is displayed in figure III.3. First, a time average of the video was calculated and subtracted from the raw video. For the result (-avg) a threshold based on the video histogram was calculated using Otsu's method.<sup>[140]</sup> This method divides a histogram into two partitions by minimizing the variance within each partition. The *Particle Analyzer* Plugin (ImageJ) was used to extract shapes from the resulting (thresholded) binary video. Recognized shapes were constrained to closed shapes of  $1500 \text{ px}^2$  to  $5000 \text{ px}^2$  in size. Displays of extracted shapes showed that only droplets not touching the walls of the flow cell were recognized.



Figure III.3.: Droplets shape evaluation from deformation recordings Using ImageJ a Z-average of the raw videos was calculated and subtracted from the raw video. For the result (-avg) a threshold for the video histogram using Otsu's method was calculated. This binary video (thresholded) was then analyzed with the *Particle Analyzer*-Plugin and shapes were recognized (*shape*).

Shape information such as Feret's diameter, minimum Feret's diameter and slice were saved and further analyzed with  $\mathbb{R}(\text{software})$  (detailed analysis program script in appendix). Deformation  $\delta$  for each shape was calculated with  $\delta = \frac{Feret - Feret_{min}}{Feret + Feret_{min}}$ .<sup>[139]</sup> Figure III.4 shows the deformation of each droplet entering the flow cell chamber. Total of fifty frames (3 ms) were necessary for each droplet to reach the maximum in deformation and to become round again. For all experiments only the entry region into the chamber was recorded to exclude outgoing droplets. For calculating local maxima deformation values were compared to at least 31 neighbouring values (red in figure III.3).



Figure III.4.: Representative analyses of droplets deformations as obtained in the deformation flow cell chamber. Calculated deformation of water droplets over time [slice] in FC40 with 2.5 m M of 60% Tri-7000-PEG-1400 and 40% Di-7500-750-O-Me recorded in the last flow cell chamber. Local maxima are displayed in red.

## 2. Microfluidic Devices, and Droplet Production

All microfluidic devices used in this research were made of PDMS (Sylgard 184, Dow Corning, USA) and prepared by standard photo- and soft-lithography methods.<sup>[141]</sup> Microfluidicdevices were designed using QCAD-pro (version 3.11.3, RibbonSoft GmbH, Germany). The designs were printed with a high resolution printer (JD Photo-Tools, Lancs, UK) on to a transparency (film mask). In case the resolution of less than 10 µm was required reactive ion etching was used to create a chromium mask. The slides were used as a mask in a photolithography process to obtain positive relief via photolithography. PDMS (Sylgard 184, Dow Croning Corporation, USA) was used to produce via soft-lithography the microfluidic device from the positive relief.

#### 2.1. Photolithography

Photolithography was used to create the positive relief with structure heights ranging from 10 to 100  $\mu m$  using negative SU-8-25 photoresist on silicon wafer.

Photoresist thickness	$25 \mu { m m}$
Drying oven	180°C (30 min)
Dispense of Pothoresist	$500~\mathrm{rpm}~5~\mathrm{s}$
Tapering of Photoresist	$2000~\mathrm{rpm}~30~\mathrm{s}$
Prebaking 1	3 min at 65 $^{\circ}\mathrm{C}$
Prebaking 2	7 min at 95 $^{\circ}\mathrm{C}$
Exposure	7 s
Post baking 1	1 min at 65 $^{\circ}\mathrm{C}$
Postbaking 2	3 min at 95 $^{\circ}\mathrm{C}$
Developping	$2 \min$

Table III.1.: Optimized photolithography experimental parameters to obtain 25 µm channel height structures with the negative SU-8-25 photoresist.

To ensure the quality of microfluidic structures the production was carried out under clean room conditions (class 100). First the silicon wafers were dried for 30 min at 180°C to drive off any moisture that may be present on the wafer's surface. Afterwards, the wafer was covered with 1 ml photoresist by spin coating (table III.1). After spin-coating the wafers were prebaked for 3 min at 65°C and 7 min at 95°C to remove air bubbles and to drive off excess of photoresist solvent. The photoresist was cured 7 seconds with UV-light and baked again at 65°C and 95°C to solidity. The remaining photoresist was removed by Developer MR-700 (10 ml, microchem, Germany). The final structures were analyzed with a profilometer (Veeco, Dektak 8 Advanced development, USA).



Figure III.5.: PDMS flow cell production via photo- and softlithography methods. A silicon wafer coated with photoresist is exposed with UV light. Negative photoresist which is, exposed to UV-light is polymerized and following the development step form rectangular structures as a positive relief. Unexposed photoresist is removed and the wafer is doused with PDMS. After the polymerization, the PDMS is pealed of the wafer and after a plasma treatment covalently bound to a glass cover-slip.

#### 2.2. Softlithography

The manufacturing process starts with the production of the PDMS elastomer by mixing the polymerization catalyst (cross-linking oligomers contain at least 3 silicon hydride bonds each) with the oligomer (siloxane base oligomers contain vinyl groups) in a 1:10 (w/w) ratio. Following mixing, the polymer was degassed under vacuum in a desiccator for 25 min, cast over the wafer and cured at 65°C for 2 hours in the oven. After curing, the devices were pealed carefully from the wafer. A puncher (0,5 mm diameter, Ted Pella Inc., USA) was used to create inlet- and outlet-holes. The devices were cleaned with ethanol. Prior to sealing the PDMS devices on the 24 x 60 mm coverslips (Carl Roth, Germany), the glasses were cleaned with 5% Extran®-solution and twice with pure water, each for 15 min in the ultra sonic bath. To improve the sealing the coverslips and the PDMS devices were activated with oxygen plasma (Plasma-Gerät PVA TePla100, Feldkirchen, Germany) (0.5 mbar, 150 W, 15 sec).<sup>[31,142]</sup> After activation the devices were pressed on the coverslips and heated 1 hour at 65°C to enhance the sealing stability.

Ombrello<sup>®</sup> (unknown substance, Motono<sup>®</sup>, Germany) or SigmaCote<sup>®</sup> (chlorinated organopolysiloxane in heptane, Sigma-Aldrich, Germany) was used to passivate the channels with a hydrophobic layer. To test the efficiency of hydrophobic coating the PDMS devices were treated with Ombrello<sup>®</sup> or SigmaCote<sup>®</sup> and incubated for 10 min, washed with water and dried with N<sub>2</sub>, or, as control, only washed and dried with N<sub>2</sub>. After the first measurement all samples were kept in ultra pure water till the second and third analysis after 1 and 15 hours, respectively.



Figure III.6.: Contact angle measurements of glass and PDMS Contact angle measurements on (A) glass and (B) PDMS. Contact angle measured immediately after production, 1 hour and 15 hours later, respectively. Not treated surfaces (red), Ombrello coated surfaces (green) and SigmaCote coated surfaces (blue).

Dynamic contact angel measurements were preformed with a contact angle system OCA (dataphysics, Germany) in Sessile Drop Mode with a Tangent-Fitting. The results are presented in Figure III.6 and show no significant differences between SigmaCote<sup>®</sup> and Ombrello<sup>®</sup> but a stable passivation of the treated surfaces. The values of non-passivated PDMS devices fitted the values reported earlier.<sup>[143]</sup>

#### 2.3. Droplet Production and Storage

All microfluidic devices used in this study were made of PDMS and prepared by photoand soft-lithography methods<sup>[141]</sup> as described in Section 2.1 and 2.2.<sup>[4,23]</sup> Mono-disperse water-in-oil emulsion droplets with diameters ranging from 10 µm to 100 µm were created with production rates ranging from 0.1 - 30 kHz by controlling the flow rates of the aqueous phase and the oil phase from 20 to 900 µL h<sup>-1</sup> and 50 to 1500 µL h<sup>-1</sup> respectively. All surfactants were dissolved in FC-40 oil at concentrations, ranging from 0.5 to 50 mM. For experiments with gold-nanostructured droplets the stabilization surfactants were mixed with 0.1 µ M to 100 µ M gold-linked surfactants (Section IV.1.3).

For the droplet production the microfluidic devices were connected to 1 ml syringes via PTFE-tubing ( $\emptyset_{in}0.3 \,\mathrm{mm}, \, \emptyset_{out}0.6 \,\mathrm{mm}$ ) and cannula ( $0.4 \,\mathrm{mm} \times 20 \,\mathrm{mm} \,\mathrm{BL/LB}$ ) (Portex Fine Bore, Smiths Medical Instruments Ltd., USA). For aqueous volumes of less than 200 µ L 0.3 ml syringes (BD Micro- fine<sup>TM</sup> Demi, Becton, Dickson and Company, USA) were used. Flow rates were controlled precisely with syringe pumps (Aladdin-1000, World Precision Instruments, USA). Figure III.7 (A and B) show a microfluidic device and the flow focusing junction where droplets are generated. In the flow focusing junction the aqueous phase is "cut" of by the oil phase and droplets are generated by self-assembly. Following the droplets production droplets were collected with a PTFE-tubing in an Eppendorf tube (for bigger volumes) or via a pipette tip at the outlet of the microfluidic device (small volumes; 10-50 µL) and transferred immediately to an analysis chamber (figure III.7 C and D) for further analysis and characterization. The glass slides, used for the analysis chamber were cleaned with Extran and water as described in Section III.2.2. To provide spacing between the coverslips two stripes of double faced sticky tape  $(\text{thickness} = 80 \,\mu\,\text{M}, \text{Tesa}, \text{Germany})$  were glued to the edges of the longer sides of a  $24 \ge 60 \text{ mm}$  coverslip (#1, Carl Roth, Germany) as shown in figure III.7 C. Droplets were injected into the chamber which was wetted by  $2\mu L$  of FC-40 oil before, and a A 24 x 24 mm glass (#1) was glued on top. The chamber was filled completely with FC-40 oil containing the same surfactant concentration as used for droplet creation. To avoid evaporation the sides of the chamber were sealed by two-component glue (Twinsil, Picodent GmbH, Germany). Droplets stored in these chambers remained stable for up to several month.<sup>[4,144]</sup>



Figure III.7.: Droplet production and storage for analysis. (A) Droplet-based microfluidic device with red and blue colourings indicating oil and aqueous inlets, respectively. (B) Insight of a flow-focusing junction where oil (red) and aqueous (blue) phases meet at a 20 µm wide nozzle and droplets are generated. (C) Illustration of an analysis chamber to store droplets for analysis and characterization. (D) Phase contrast image of 40 µm diameter droplets in an analysis chamber.

#### 2.3.1. Different Microfluidic Operation Units

For various research purposes different microfluidic units were designed by using a QCADprogram. Most of the designs were adapted from literature and optimized for our needs. In the following paragraphs three main designs are described.

**Deformation Measurements** A droplet deformation device (figure III.8.A) was designed<sup>[20]</sup> and implemented for dynamic physical analysis of surfactants and proteins accretion and actin polymerization in microfludic droplets. The results achieved by this device are presented in Section IV.2.2. In general, two aqueous inlets (blue) and two



Figure III.8.: Representative schematics and images of different droplet-based microfluidic units. (A) Representation of droplet deformation devices and a magnified area of the deformation chambers. (B) Schematic representation of spiral device for single cell encapsulation and a magnified area of dropspot chamber. (C) Picoinjection device for controlled introduction of different bioactive components into droplets. The inset represents an example for the injection where colored liquids were used.

oil inlets (red) for droplet production and droplet spacing, respectively, were used to adjust the flow rates for high throught droplet deformation analysis, allowing high-speed recording. Installation and recording was done as described in subsection 2.3 (*Droplet production and Storage*) and section 3 (*High-speed microscopy*). Flow rates were set constant:  $50 \,\mu l \,h^{-1}$  aqueous phase (blue),  $170 \,\mu l \,h^{-1}$  oil phase (red) and  $220 \,\mu l \,h^{-1}$  spacing oil (red) (see figure III.8A).

Single cell encapsulation Single cell encapsulation and analysis experiments were performed with spiral devices as shown in figure III.8 B. This device allows Dean flow-based (De) separation of cells or particles by an inertial lift and viscous drag force and can be calculated with the presented formula where  $\rho$  is the density of the fluid, V the axial velocity scale,  $\mu$  is the dynamic viscosity, d is the channel diameter for non circular channels and R is the curvature of the channel.<sup>[145,146,147]</sup>

$$De = \frac{\rho V d}{\mu} \cdot (\frac{d}{R})^{\frac{1}{2}}$$

The inertial force and the dean flow (created due to the spiral design) forces cells to line up on the inner wall with equidistant spacing.<sup>[147]</sup> Flow rates were set to 700 to 900  $\mu$ l h<sup>-1</sup> for the aqueous phase (blue), containing media (RPMI 1604) and cells (2,5 x 10<sup>-7</sup> cells/ml) and 1200 to 1500  $\mu$ l h<sup>-1</sup> for the oil phase (red). Droplets were analyzed in drop spot chambers<sup>[17]</sup> for up to 4 days. As shown in figure III.8 the dropspot chamber consists of round niches connected by narrow channels. In the absence of flow droplets are stored in the round niches.

**Picoinjection** In the pico injection device an alternating electrical field induces surfactant poration and allows injection of aqueous phase that contains bio-relevant material.<sup>[16]</sup> The experiments were performed with a flow controller system (MFCS-EZ Microfluidic flow control system, Fluigent, France) with droplet rates of about 100-500 droplets per second. Figure III.8 C shows the device where droplets were introduced into the picoinjection device at the inlet and spaced with FC-40 oil (red) containing the same surfactant concentration as used for the produced droplets (N<sub>2</sub> pressures 100-400 mbar). The N<sub>2</sub> pressure in the picoinjection channel was 20-250 mbar (blue). Micro indium tin electrodes were placed at the proximity to the injection channel. To produce the electrode the indium tin was melted at 60°C on a heat plate and injected into the electrode channels. Injection occurred only with an applied electrical field (30 KHz, 400 V). A representative picoinjetion time-lapse is shown in figure III.8 C2. Droplets where collected with a pipette tip at the outlet or analyzed directly in the drop-spot chamber.

## 3. Droplet Content Analysis

**Confocal Microscopy** For confocal experiments an Axiovert 200M inverted confocal Laser-Scanning-Microscope (Zeiss, Germany) using  $63 \times$  magnification water-immersions-objective (LUMFI, NA = 1.1, working distance 1.5 mm; Olympus, Japan) and an argon laser (458, 488 and 514 nm, Lasos LGK-7812 ML-4, Zeiss, Germany) was used. Images were taken with the image acquisition software Pascal 5 (Zeiss, Germany).

**Fluorescence Microscopy** Fluorescence intensity experiments were performed with an upright Leica DM6000B epifluorescence microscope (Leica Microsystems, Germany) at 25°C. The droplets were excited via an external alignment free mercury metal halide lame (EL6000, Leica Microsystems, Germany) equiped with liquid light guide and observed with a 20x air objective (HC PLAN APO 20x/0.7; Leica Microsystems, Germany). The field of sample excitation was limited using a rectangular aperture in the optical pathway. Images were taken with the image acquisition LAS AF software (Leica Microsystems, Germany) with 1392 x 1040 pixels (1 pix = 0.46 µm) resolution.

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, Germany). Top-view cryo-SEM imaging was performed under low temperature conditions ( $T_{op} = -115 \pm 5^{\circ}C$ ) and working distances of 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  $(5\,\mu\text{L})$  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, Liechtenstein) preparation device via an evacuated liquid nitrogencooled shuttle BAL-TECH VLC 100 (BAL-TEC AG, 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. 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 that no fixation and no cryoprotection chemicals were used during the droplets preparation for cryo observation.

**High-speed microscopy** High speed videos were recorded with a Phantom v7.3 (Vision Research, USA) CCD camera using the Observer.Z1 inverted microscope (Zeiss, Germany) with  $40 \times$  magnification. Videos for droplet deformation experiments and single cell encapsulation experiments were recorded in bright-field with 60 µs exposure time and 16 000 pps with a resolution of  $256 \times 256$  pixels. Videos for pico-injection experiments



Figure III.9.: Representative cryo-SEM micrograph of freeze fractured microdroplets (A) Cryo-SEM micrograph with low magnification of freeze fractured microdroplets. The droplets were fractured by the cooled knife in the preparation device chamber in the 10<sup>-6</sup> - 10<sup>-7</sup> mbar vacuum chamber at -160 °C. (B) Representative cryo-SEM micrograph of the fractured droplets obtained with higher magnification. (C) Following the water sublimation in the preparation chamber a Cryo-SEM micrograph with a single fractured droplet is presented.

were recorded in bright-field with 60  $\mu s$  exposure time and 16 000 pps with a resolution of  $256 \times 512.$ 

# 4. Retention Analysis of Fluorophores and Biomolecules in Droplets

#### 4.1. Fluorophores

ATTO 488 N-Hydroxysuccinimidyl ester (SE), ATTO 495 SE, ATTO 520 SE, ATTO 532 SE, ATTO 565 SE, ATTO 590 SE, ATTO 647N SE and ATTO 655 SE were purchased from ATTO-TEC GmbH (Siegen, Germany). Alexa Fluor<sup>®</sup> 488 C5 Maleimide (M), Alexa Fluor<sup>®</sup> 532 C5 M and Alexa Fluor<sup>®</sup> 647 SE were purchased from Thermo Fisher Scientific (Schwerte, Germany).

The pure fluorophores were dissolved in anhydrous DMSO at a concentration of 1 mM and stored at -20°C. In retention experiments the fluorophores were further diluted with each buffer and both cell culture media (see next Section 4.2) to a final concentration of  $5 \,\mu\text{M}$  and used as an aqueous phase for droplets creation.

The hydrophilicity of the fluorophores was analyzed by calculating the distribution coefficient, LogD, which is a measure of the expected ratio of the sum of concentrations of all forms of the fluorophore (ionized plus un-ionized) in water and in a non-polar solvent (octanol). Negative LogD values correspond to hydrophilic fluorophores, positive values to hydrophobic ones. The calculations for the fluorophores with a hydrolyzed succinimidyl ester and Saleimide reactive groups at pH 7.4 were performed by MarvinSketch version 14.10.20.0 (Chem Axon, Cambridge, MA), using the dye structures (see Appendix). Three different calculation methods were used (VG, KLOP, PHYS) and weighted equally. Tautomerization and resonance were considered and electrolyte concentrations were adapted according to LogD (PBS): 141 mM Cl<sup>-</sup>, 141 mM Na<sup>+</sup> & K<sup>+</sup>, pH7.4 and LogD (TRIS): 156 mM Cl<sup>-</sup>, 150 mM Na<sup>+</sup> & K<sup>+</sup>, pH7.7.<sup>[144]</sup>

Fluorophore	$\mathrm{LogD}$	$\mathrm{LogD}$	$\lambda_{\mathbf{abs}}$	$\lambda_{\mathbf{fl}}$	$\mathbf{M}\mathbf{W}$	$\eta_{\mathbf{fl}}$
	PBS	TRIS	[nm]	[nm]	[g/mol]	
Alexa 488 M	-9.47	-9.61	494	517	721	0.92
Alexa 532 M	-4.43	-4.54	530	555	813	0.61
Alexa 647 M	-7.75	-7.75	651	671	1250	0.33
ATTO $488 \text{ SE}$	-7.60	-7.60	501	523	981	0.80
ATTO $495 \text{ SE}$	-0.81	-0.71	495	527	549	0.20
ATTO $520 \text{ SE}$	0.59	0.45	516	538	564	0.90
ATTO 532 SE	-4.49	-5.30	532	553	1082	0.90
ATTO 565 SE	-0.18	-0.18	563	592	708	0.90
ATTO 590 SE	1.56	1.56	594	624	788	0.80
ATTO $633 \text{ SE}$	n.a.	n.a.	629	657	749	0.64
ATTO 647N SE	4.47	4.47	644	669	843	0.65
ATTO $655 \text{ SE}$	-0.61	-1.46	663	684	887	0.30

Table III.2.: Photophysical characteristics and calculated LogD values of the investigated fluorophores.

 $\lambda_{abs}$ : maximum absorption wavelength

 $\lambda_{\rm fl}$ : maximum emission wavelength

MW: Molecular weight

 $\eta_{\mathrm{fl}}$ : Quantum yield: ratio of photons emitted and photons absorbed

#### 4.2. Buffers and Cell Culture Media

All buffers used for retention experiments were prepared with ultra-pure (type 1 water,  $R \ge 18 M\Omega/cm$ , Millipore) water and stored at 4°C. The pH values were adjusted with 1M HCl and 1M KOH solutions. Following buffers were prepared and used in retention experiments:

- 1) PBS pH 7.4 was prepared using PBS tablets according to standard protocols (Sigma –Aldrich, Germany);
- 2) TRIS buffer: 50 mM TRIS, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, pH 7,4;
- 3) 30 mM HEPES, pH 7.4;
- 4) PIPES buffer consisted of 20 mM PIPES, 7.25 mM MgCl<sub>2</sub>, 1 mM EGTA, 3 mM GTP, 1 mM 2-mercaptoethanol, 50 mM KCl, 1 μM paclitaxel, 31 mM glucose, 1 mg/ml glucose oxidase and 0.5 mg/ml catalase, 0.25 mg/ml beta-casein, pH 6.8.<sup>[144]</sup>

Two different cell culture media were used:

- 1) DMEM (Life technologies, USA) containing 4,5 g/l D-glucose, 1 mM L-glutamine, and 1% (v/v) penicillin/streptomycin (Gibco, Darmstadt, Germany);
- 2) RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS (Invitrogen, Darmstadt, Germany) and 1% (v/v) penicillin/streptomycin (Gibco, Darmstadt, Germany).

#### 4.3. Labelled Proteins

ATTO 647N-labeled streptavidin was purchased from Sigma–Aldrich, Germany. Alexa Fluor<sup>®</sup> 488 goat anti-mouse IgG and Alexa Fluor<sup>®</sup> 647 goat anti-mouse IgG were purchased from Thermo Fisher Scientific (Germany). Sheep anti-mouse antibodies were labeled with ATTO 532 SE, ATTO 565 SE, ATTO 590 SE and ATTO 647N SE according to the protocol described in the following section.

Stock solutions of labelled streptavidin and antibodies were diluted with TRIS buffer to final concentrations of 200 nM.

**Antibody and Tubulin Labeling** Anti-mouse antibodies were labeled with ATTO NHS dyes following the protocols from ATTOtec (https://www.atto-tec.com) with some modifications. The fluorophores were dissolved in DMF (Sigma-Aldrich, Germany) in a concentration of 10 mg/ml and used in a 15- to 20-fold molar excess. The AffiniPure Sheep anti-mouse (c = 2,4 mg/ml, IgG, Dianova, Germany) was mixed with 10% (v/v) of 1 M NaHCO<sub>3</sub> (pH 8,3 – 9,0) and stirred at RT. The fluorophore was added dropwise under constant stirring, the solution was protected from light and stirred for one hour at room temperature (RT). The crude product was purified by gel filtration with a Sephadex G25 (PD-10) gel filter (GE Healthcare GmbH, Solingen, Germany), equilibrated with 30 ml PBS pH 6.5. Following purification step, 5 antibody-dye fractions each 0.46 µL were collected and the protein content was determined with a Bradford Assay. The degree of labeling of 6 was determined by UV/VIS (Lambda 25 UV/VIS Spectrometer, PerkinElmer Precisely, USA). The labeled antibodies were stored at 20 °C.<sup>[144]</sup>

Tubulin was kindly provided by Thomas Surrey (Francis Crick Institute, London, United Kingdom) and was purified from pig brain as previously described.<sup>[148]</sup> Tubulin was labeled with ATTO 633-SE or ATTO 488-SE according to protocols described in Hyman et. al..<sup>[149]</sup> Tubulin concentrations were measured using UV spectroscopy ( $\lambda =$ 280 nm;  $\epsilon = 115000 \text{ M}^{-1}\text{cm}^{-1}$ ); they are expressed as tubulin heterodimer concentrations. The labelling ratio was determined by UV spectroscopy, using the appropriate extinction coefficients at 280 nm and at the absorbance maximum for the dyes. Labeling ratios of tubulin dimers labeled with ATTO 633 and ATTO 488 were 0.75 and 0.65 dye molecules per tubular dimer respectively.<sup>[144]</sup>

#### 4.4. Fluorophore Retention Analysis

Fluorophore retention was analyzed with brightfield and fluorescence images taken every 15 seconds for a period of 10 minutes and an additional image was taken 24 hours after droplet production. Images were taken with a DM6000B epifluorescence microscope as described in Section 3. The fluorescence images analysis was accomplished using Fiji (ImageJ 2.0.0-rc-14, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014) program.

Illumination times were adjusted within 10 to 50 ms to obtain the same fluorescence intensity value  $I_0$  for each tested buffer-dye-combination prior to droplet production. These illumination times were used in further experiments to analyze the droplets within the analysis chamber. Fluorescence intensities  $I_{t0}$  and  $I_{t24}$  correspond to the values obtained immediately and 24 hours after droplet insertion in the storage device, respectively. Fluorophore retention was categorized in three different groups according to Figure 4.4:



Figure III.10.: Characterization criteria for fluorophore retention in droplets

Figure III.11 shows the fluorescence intensities  $I_{t0}$  of droplets produced with three different buffers and different fluorophores and representing the three different categories of fluophore retention.<sup>[144]</sup>



Figure III.11.: Flurophore retention characterization (A, B and C) show droplets produced with 10 mM TRI7000 surfactant, containing ATTO 633 in TRIS (A), ATTO 655 in RPMI (B) and Alexa 488 in TRIS (C). The droplets were analyzed immediately after production (Intensity I<sub>t0</sub>). Upper panel: Selected fluorescence images demonstrating different fluorescence retention in droplets. Middle panel: Intensity profiles of cross-sections marked in upper panel. Lower panel: Red, yellow and green blocks illustrate the color marking used in all tables.

# Biofunctionalization of Gold-Nanostrucured Droplets

Gold-linked surfactants (see Section IV.1.3) were used in combination with triblock surfactants to generate droplets with gold nanoparticles on it's inner periphery as anchoring points for following biofunctionallization. Two chemical approaches (see following Sections) were used to achieve stable biofunctionalization.

# 5.1. NTA-Thiol coupling to Gold Nanpoarticles (One step Approach)

His6-GFP (300 µl, 300 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<sub>2</sub> (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<sub>2</sub> 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  $\epsilon = 30000$  at 395nm.

For T Cell experiments coupling procedure followed the steps as shown in GFP coupling.  $25 \,\mu$ l His-MHC-Alexa 568- and -ICAM-1 (each  $25 \,\mu$ M) were mixed with NiCl<sub>2</sub> (1  $\mu$ l) and NTA-thiol (30  $\mu$ l, 1 mM) and diluted with 500  $\mu$ l RPMI 1604 media resulting in a final concentration of 1  $\mu$ M.

#### 5.2. RGD Biofunctionalization via Thiol Gold Interaction

To provide cells the ability to interact with the nanostructured droplet periphery, c(RGDfK)-PEG-(cysteine)<sub>3</sub> peptides (Peptide Speciality Laboratories GmbH, Heidelberg, Germany) were immobilized on gold nanoparticles via the thiol residues of the cysteine linker. The chemical structure of cRGD peptide with a PEG-spacer and a cysteine-linker is illustrated in figure III.12. Freeze-dried Gold-PEG-PFPE copolymers synthesized as described in Section IV 1.3 were dissolved in 100 µL FC-40 and mixed with aqueous solution of cRGD (50 µM, 100 µL) and stirred for 1 h. The crude product solution was then centrifuged

#### 6. Cell experiments

and the supernatant with unbound cRGD peptides were removed. The precipitant was freeze-dried for at least 24 h to remove remained water completely. Afterwards, the dried product was dissolved in 1 ml of fluorinated oil FC-40 (Acros Organics, Germany) and filtered with a hydrophobic filter (PTFE  $0.2 \,\mu\text{m}$ ) to remove unreacted, hydrophilic (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles and cRGD traces. The obtained surfactant was used in different concentrations for cell adhesion experiments.<sup>[4]</sup>



Figure III.12.: Chemical structure of the c(RGDfK) peptide 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 the 3 cysteine-linkers.

#### 5.3. Rhodamine functionalization (Two step Approach)

Gold nanoparticle functionalization with rhodamine involved two steps. First the synthesis of PFPE-PEG-Gold surfactants linked to rhodamine B (RhB) (see Section IV.1.4) followed by droplet creation using a mixture of TRI2500 (20 mM, PFPE2500-PEG600-PFPE2500, synthesis described in Section IV.) and RhB-PEG-Gold-PEG-PFPE (5  $\mu$ M) surfactants as an oil phase, while PBS was used as an aqueous phase.<sup>[4]</sup>

## 6. Cell experiments

Mercaptoundecyl-tetra(ethylene glycol) functionalized gold nanoparticles and cRGD were coupled in advance. The obtained surfactant was used in different concentrations for cell adhesion experiments.<sup>[4]</sup>

#### 6.1. Cell Culture

All cells used were cultured in RPMI 1640 media supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS, Invitrogen, Darmstadt, Germany or Sigma-Aldrich, Taufkirchen, Germany) and 1% (v/v) penicillin/streptomycin (Gibco, Darmstadt, Germany) and cultured in an incubator (Thermo Scientific, USA) at 37 °C and 5%  $CO_2$ .

**T-Cells (cell line Jurkat E6.1)** The human acute T cell leukemia cell line Jurkat E6.1 clone was purchased from ATCC (American Type Culture Collection, Manassas, USA). The cells were split every two days for not more than 20 passages. Vials that were frozen again were taken after max three times splitting. For adhesion experiments, Jurkat E6.1 cells ( $200 \,\mu$  L,  $2 \, x \, 10^7$  cells/ml) were suspended in adhesion medium (RPMI 1640, 2 mM L-glutamine, 1% (v/v) penicillin/ streptomycin, 1 % FBS) and used as an aqueous phase for droplet creation.

**T-Cells (Primary mouse T-cells)** Preparation of primary T cell blasts and TCR transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Cells were deep-frozen and shipped on dry ice to the laboratory where experiments were performed. Upon arrival cells were immediately transferred to a liquid nitrogen storage tank.

Cell culture steps were performed under a sterile flow hood (Heraeus Kendro; VWR International GmbH, Germany). For adhesion experiments, cells were thawed 24 h prior to the experiment. Vials contained 1.0 ml cell suspension with approximately 5.0 x  $10^6$  cells that were thawed in a water bath at 37°C. Cells were resuspended in 10 ml medium (RPMI 1640 with phenol red and glutamine) supplemented with 10% FBS and 1% penicillin/streptomycin in 15 ml falcons, centrifuged, re-suspended in 5 ml complete medium and cultured in conventional cell culture flasks in an incubator at 37°C and 5% CO<sub>2</sub>. After 24 h cell suspension was centrifuged, resuspended in complete medium and used for droplet adhesion experiments. For droplet production and analysis a microscope equipped with incubation chamber (37°C and 5% CO<sub>2</sub>) was used.

#### 6.2. Cell Recovery and Live/Dead Staining

Cell recovery and live/dead staining followed the procedures reported earlier<sup>[150]</sup> but with several modifications.<sup>[4]</sup> The cell containing emulsion was split equally (200  $\mu$ L each) to

five (1 ml) falcons (caps with punched holes) and incubated in cell culture conditions (37°C, 5% CO<sub>2</sub>). By the addition of 5 % v/v PFO-PEG surfactants the emulsions were broken after 1, 2, 3, 4 and 5 days respectively. The supernatant (cell containing media) was transferred into a 15 ml centrifuge tube. The cells were washed with 10 ml PBS. After the washing 100 µL of cells-containing buffer solution were added to a micro-plate reader flat-bottom well which had a total capacity of  $200 - 300 \,\mu$ L. These cells were incubated for 45 min at room temperature with 100 µL of the live/dead staining solution containing 2 µM calcein and 4 µM Ethidium homodimer-1 (Live/Dead Viability/Cytotoxicity Kit for mammalian cells, Invitrogen Kit L-3224). After the staining procedure the percentage of alive cells was calculated from the fluorescence measurements using the micro-plate reader reader.<sup>[4]</sup>

## 7. Actomyosin Experiments

Actin and Myosin were purchased from Cytoskeleton, Inc.(USA) or purified by Christine Mollenhauer (technical assistant, MPI, department Spatz) by following the standard protocols.<sup>[151,152]</sup> Actin was purified from rabbit skeletal muscle acetone powders by modifications of Spudich and Watt.<sup>[151]</sup> In general the method uses the ability of actin to polymerize at 50 mM KCl and 2 mM MgCl<sub>2</sub>, and depolymerize when KCl and MgCl<sub>2</sub> are removed by dialysis with a G-buffer without MgCl<sub>2</sub>, containing: 2 mM TRIS/HCl pH 8, CaCl<sub>2</sub> 0.2 mM, NaN<sub>3</sub> 0.005%, ATP 0.2 mM (Cytoskeleton Inc., USA), DTT 0.2 mM. Final purification was preformed by gel filtration with Ge-column Hi Load 26/60 Superdex 200 prep grade (General Electric, USA). Freshly produced actin was stored on ice and used within 2 weeks, or aliquoted, shock freezed and stored at -80 °C.

Myosin II was isolated from rabbit skeletal muscle according to the purification protocols from Sarkis et al..<sup>[152]</sup> In general, to remove unwanted proteins, the method uses the isoelectric point of myosin II to isolate it from all other muscle proteins by centrifugation and by salting out the desired myosin II. Freshly purified myosin II was kept on ice and was used within 4 weeks, or was aliquoted, shock freezed and stored at -80°C.

In all the experimental conditions (see table III.3) actin concentration of  $10 \,\mu$ M in Gbuffer was used to form actin cytoskeleton networks. Moreover, the optimal ATP concentration was found to be in the range from 0.4 to 1.2 mM. To keep the ATP concentration steady over the time of the experiment an ATP recovery buffer containing creatine phosphate 50 mM mixed with creatine kinase solution (5 mg/ml) (details Section III.7.1) was used. Additionally, a solution of oxygen scavenger, containing glucose oxidase 0.1 mg/ml, catalase 0.018 mg/ml and glucose 5 mg/ml was added in oder to minimize bleaching and oxidation.

To detect the dynamics of actin cytoskeleton formation 1% of Alexa Fluor 488-labeled actin (Life Technologies, Germany) (conditions 3-7, see table III.3) or 2 % of phalloidin-FITC (Sigma-Aldrich, Germany) (conditions 1-2, see table III.3) were mixed with non-labeled actin. In the experiments where no phallodin was used fascin (Novus Biologicals, USA) (conditions 4-8, see table III.3) as a cross-linking protein was added at a concentration of  $0.5 \,\mu$ M if not stated differently.

In experiments where myosin II was used (conditions 2,3,6,7, see table III.3) the KCl level for myosin II cluster length was controlled by mixing G-buffer and D-buffer at different ratios (detailed composition Section III.7.1). All solution mixtures were prepared on ice, myosin solutions were incubated for 20 minutes before droplet production to allow the myosin II to aggregate to the desired cluster length (related to the KCl concentration).<sup>[135]</sup> To inhibit myosin II activity (conditions 7, see tabel III.3) blebbistatin (0.1 mM, Sigma-Aldrich, germany) was used. In this experimental condition the myosin II solution was incubated a second time for 30 min before the solution was used for droplet production. All experiments were preformed at room temperature (24°C).

Experimental condition	1	2	3	4	5	6	7
Biochemistry							
Actin	x	х	х	х	х	х	х
Myosin		х			х	х	х
Phalloidin	x	х					
labeled Actin			х	х	х	х	х
Fascin			х	х	х	х	х
PEG				х		х	х
Blebbistatin							х

Table III.3.: Overview of protein used in different experimental conditions in an attempt of actin and actomyosin network reconstitution.

Table III.4.: Detailed experimental descriptions of buffers and proteins as used in different experimental conditions.

Experimental condition	Biochemical content
1	Pure G-buffer, $(10\mu\text{M})$ actin, $(0.2\mu\text{M})$ phalloidin,
	$20 \text{ mM MgCl}_2, 0.5 \text{ mM ATP}, \text{ oxidation-buffer}$
2	Cond. $1 + D$ -buffer with final KCl conc. of 50 mM,
	$(0.5\mu M)$ myosin II
3	Pure G-buffer, $(10\mu\text{M})$ actin, $(0.1\mu\text{M})$ labeled
	actin, $(0.5\mu\text{M})$ fascin
4	Cond. $3 + 2.5 \text{ w/w} \% \text{ PEG}$
5	Cond. 4 + D-buffer with final KCl conc. of 50 mM
	$(0.5\mu M)$ myosin II
6	Cond. 5 + D-buffer with final KCl conc. of 50 mM
	$(0.5\mu M)$ myosin II
7	Cond. $6 + (10-100 \mu\text{M})$ blebbistatin

#### 7.1. Buffers

Buffers used for actin and actomyosin experiments were prepared with ultra-pure water ( $\geq 18 \text{ M}\Omega/\text{cm}$ , Millipore) and stored at 4 °C. The pH-values were adjusted with HCl and KOH solutions. Following buffers were used:

- 1) G-Buffer: 2.0 mM TRIS pH 7.4, 0.2 mM CaCl<sub>2</sub>, 20 mM MgCl<sub>2</sub>, 0.2 mM ATP, 0,005 % NaN<sub>3</sub>, 0.2 mM DTT;
- 2) D-Buffer: 50 mM  $\mathrm{K_{2}HPO_{4}}$  pH 6.5, 600 mM KCl;
- 3) Dilution buffer: 50 mM  $K_2HPO_4$  pH 6.5;

- 4) ATP recovery buffer (a): HEPES 10 mM, DABCO 0.12 mM, KCl 0.13 mM, MgCl<sub>2</sub>, BSA 0.1%, Creatinephosphate 5 mM;
- 5) ATP recovery buffer (b): 0.02 % DTT, Creatine kinase 0.5 mg/ml;
- 6) Anti-oxidation buffer: 50 mM HEPES pH 7.4, 50 mM KCl, 1 mM ATP, 0.5 mg/ml BSA, 10 mM DTT, 3 mg/ml glucose, 0.1 mg/ml glucose-oxidase, 0.02 mg/ml glucose-catalase;

#### 7.2. Actin Experimental Analysis

Investigation of actin experiments was preformed in analysis chambers (see Section III.2.3). It is worth mentioning here that external flows could be excluded due to the completely sealed observation system. Actin network dynamics and actomyosin motion was analyzed using confocal and fluorescence microscopy using inverted microscopes as described in detail in Section III.3. Time-lapse analyses were preformed over periods of 30 to 60 minutes.

#### 7.3. Motion Analysis of Actomyosin Droplets with Beads

To quantify if actomyosin activity in the droplets also affected the surfactant layer and by this the whole droplet, a new bead-linked surfactant was synthesized. Towards this end, a PEG passivated polystyrene bead (d= 2 µm) as the hydrophilic head group<sup>[153]</sup> was chemically linked to a hydrophobic PFPE tail (see Section IV.1.6). Successful synthesis was confirmed by FT-IR- and microscopy-analysis, revealing successful chemical linkage of the beads to the PFPE molecules and the incorporation of this bead-linked surfactant to the periphery of the droplet, respectively. A track-mate plug-in (Fiji, Image J) was used to evaluate the motion. Data was plotted with R software (script in appendix).

In actomyosin experiments this new surfactant was added to the FC-40 oil phase which contained triblock surfactant and gold-nanoparticle surfactant.
# IV. Results and Discussions

## 1. Surfactant Synthesis and Analysis

## 1.1. Perfluoropolypropylene - Polyethylene glycol -Perfluoropolypropylene (PFPE-PEG-PFPE) Triblock-Copolymer Surfactants

This Section describes the general strategy of synthesis PFPE-PEG-PFPE triblock-copolymer surfactants. Three different triblock surfactants were synthesized in this thesis: a PFPE(2500)-PEG(600)-PFPE(2500), a PFPE(7000)-PEG(600)-PFPE(7000) and a PFPE(7000)-PEG(1400)-PFPE(7000). In all synthesis reactions 1 mmol of PEG interacted with 2 mmol of perfluoropolypropylene-caboxylic acid.

The synthesis of PFPE-PEG-PFPE triblock-copolymers followed the general procedure reported earlier<sup>[15]</sup> but with several modifications as shown in Figure IV.1.<sup>[4,144]</sup> The synthesis was carried out under argon atmosphere in super dry THF solvent (Acros Organics, Germany) in a heated Schlenk flask. PEG powder was solved in 80 ml super dry THF to a final concentration of 1 mmol and cooled to -78 °C by dry ice in isopropanol. 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. PFPE-carboxylic acid (2 mmol) was added dropwise over a period of 30 min and stirred for another 12 h. THF solvent with unreacted PEG was removed by separatory funnel. Two additional washing steps with super 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 PFPE-carboxylic acid. The PFPE2500-PEG600-PFPE2500 was completely soluble in MeOH whereas the other two triblocks were only soluble in small concentrations. The PFPE-PEG-PFPE product was dissolved in the methanol, 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. 4.61 g - 82%, 12.18 g - 87% and 11.53 -79%, of PFPE(2500)-PEG(600)-PFPE(2500), PFPE(7000)-PEG(1400)-PFPE(7000) and PFPE(7000)-PEG(600)-PFPE(7000) triblock-copolymer surfactants were obtained, respectively. The purity of the product was analysed by NMR and FT-IR measurements (see following Section).



Figure IV.1.: Synthesis schematics of PFPE-PEG-PFPE triblock-copolymer surfactants

#### 1.1.1. NMR and FT-IR Analysis (PFPE-PEG-PFPE)

#### NMR

NMR data was only obtained for the triblock (PFPE2500-PEG600-PFPE2500) due to the small solubility of the other two synthesized surfactants in MeOH-D<sub>4</sub>. Chemical shifts are given in ppm referenced to solvent (MeOH - D<sub>4</sub>, 22°C;  $\delta = 3.35$ , 4.78 for <sup>1</sup>H,  $\delta = 49.3$ for 13C). <sup>1</sup>H NMR  $\delta$  3,63 (CH<sub>2</sub>, s); <sup>13</sup>C NMR  $\delta$  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<sub>2</sub>).

#### FT-IR

Figure IV.2 shows a representative FT-IR spectra of the PFPE2500-carboxylic acid reactant and the (PFPE2500-PEG600-PFPE2500) triblock surfactant product. Spectra of PFPE7000-carboxylic acid reactant and (PFPE7000-PEG1400-PFPE7000) and (PFPE7000-PEG600-PFPE7000) products are presented in appendix.

Figure IV.2 shows seven major bands at 1705, 1775, 2883, 2934, 2920, 3031 and 3550 cm<sup>-1</sup>. The band at 1775 cm<sup>-1</sup> is a stretching mode of the (C=O) bond of the PFPE-carboxylic acid which is strongly blue shifted (by 50 cm<sup>-1</sup>) due to the electronegative fluor atoms in alpha position to the carboxylic group.<sup>[154]</sup> The same blue shift of the carboxylic (C=O) band was observed previously in the studies measuring the IR spectrum of the trifluoroacetic acid.<sup>[155,156]</sup> The bands which appear in the PFPE spectrum in the region 2800-3600 cm<sup>-1</sup> are assigned to the different (OH) vibrational modes. Namely, to the asymmetric stretching  $\nu_{\rm as}$ (OH), to symmetric stretching  $\nu_{\rm s}$ (OH) and to subbands complex, corresponded to  $\nu$ (C-O) +  $\nu_{\rm b}$ (OH) at 3550, 3031 and 2934 cm<sup>-1</sup>, respectively.<sup>[157]</sup> The bands at 1705, 2883 and 2920 cm<sup>-1</sup> are assigned to the PFPE-PEG-PFPE product. The bands at 2883 and 2920 cm<sup>-1</sup> are attributed to symmetric and asymmetric stretching modes of the PEG (C-H) groups, respectively.<sup>[154]</sup> The band at 1705 cm<sup>-1</sup> attributed to the ester (C=O) stretching mode. No bands of PFPE-carboxylic acid were observed in the product spectrum.



Figure IV.2.: Representative FT-IR spectra of a triblock-copolymer surfactant and the PFPE. Spectrum of PFPE2500-carboxylic acid (20 mM in FC-40, red) as a reactant and the PFPE2500-PEG600-PFPE2500 triblock product (20 mM in FC-40, black). Adapted with permission from Ref.<sup>[4]</sup>

## 1.2. Perfluorpolypropylene–Polyethylene glycol monoethyleter (PFPE7000-PEG-OMe)

The surfactant was synthesized by Gerri Kannenberg. Detailed synthesis data is published in Janiesch et al.<sup>[158]</sup> This surfactant was used in the experiments where retention of flourophores and biomolecules was tested (see Section IV.3.4) as well as in droplet deformation studies (see Section IV.2.2):

#### 1.3. Gold-Linked Surfactants

Gold nanoparticle-linked surfactants were synthesized in a one step process<sup>[4]</sup> as shown in Figure IV.3. PFPE7000-carboxylic acid or PFPE2500-carboxylic acid (2.1 mg, 0.3 µmol, molecular weight 7000 g/mol, DuPont, Netherlands; 750 mg, 0.3 µmol, molecular weight 2500 g/mol, DuPont, Netherlands) and KOH (5N, 10 µL, Sigma Aldrich, Germany) were mixed with (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles (2 % w/w solution in water, 5 ml, Sigma Aldrich, Germany) and stirred until complete flocculation of the PFPE-PEG-Gold product. To remove the KOH from the product the crude product was washed three times with ultra pure water. The water was then removed by freeze-drying (Piatowski Forschungsgeräte, Germany) the product for 24 hours. Afterwards the product was dissolved in 1 ml of fluorinated oil FC-40 (Acros Organics, Germany), 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 and stored at 4°C. The Gold-PEG-PFPE surfactant was obtained (9.9 mg, 86 %). The final product was chemically analyzed by FT-IR. Cryo-SEM was used to investigate the presence and density of the gold nanoparticles on the inner droplet periphery (Section IV.4.1).

#### 1.3.1. FT-IR Analysis of Gold-PEG-PFPE(7000) Surfactant

Figure IV.4 shows the representative IR spectra of the PFPE7000-carboxylic acid reactant and the Gold-PEG-PFPE(7000) surfactant. The spectra in Figure IV.4 show similar seven major bands at 1695, 1775, 2850, 2934, 2950, 3031 and 3500 cm<sup>-1</sup> as discussed in Section IV.1.1.1. These bands are attributed to the same vibrational modes, as previously described. The lower intensity of the PFPE-PEG-Gold product bands is attributed to much lower concentration of the gold-linked surfactants  $3 \,\mu$ M vs. 20 mM (Triblock).



Figure IV.3.: Synthesis schematics of PFPE7000-PEG-Gold diblock-copolymer surfactants Adapted with permission from Ref.<sup>[4]</sup>



Figure IV.4.: Perpresentative FT-IR-spectra of PFPE7000-carboxylic acid and PFPE7000-PEG-Gold diblock Comparison between PFPE7000-carboxylic acid as a reactant (20 mM) and the PFPE7000-PEG-Gold diblock product (30 µM). Adapted with permission from Ref.<sup>[4]</sup>

#### 1.4. Rhodamine B-PEG-Gold-PEG-PFPE

Rhodamine B-linked (RhB-linked) surfactants were synthesized in a one step process as shown in Figure IV.5.<sup>[4]</sup> The synthesis 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 IV.1.1) and dry THF (10 ml) were cooled to -78 °C. N-butyl lithium (30 µL of a 1.6 mM solution in hexane) were added to the Gold-PEG-PFPE solution and stirred for 30 min at -78 °C. The solution was slowly heated to room temperature and stirred for additional 30 min. THF solution of RhB (15 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.

To characterize the success of the esterification between the PEG hydroxyl group and the RhB carboxylic acid group (see IR measurements in next Section) a simple reaction between the PEG(150) (molecular weight 150 g/mol, Sigma Aldrich, Germany) and RhB was made under the same conditions to compare the shifts in the IR.



Figure IV.5.: Synthesis schematics of PFPE-PEG-Gold-RhB surfactants

#### 1.4.1. FT-IR analysis of Rhodamine B-PEG-Gold-PEG-PFPE(2500)

To verify the success of the RhB linked surfactants synthesis we used the information obtained by attenuated total reflectance technique used in conjunction with IR spectroscopy (ATR-IR) (Smart Orbit accessory, Thermo Electron GmbH, Germany). Figure IV.6 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<sup>-1</sup>. The bands at 1571, 1587 and 1646 cm<sup>-1</sup> are attributed to a stretching modes of the RhB aromatic conjugated (C=C) bonds.<sup>[154,159,160]</sup> The band at 1692 cm<sup>-1</sup> is attributed to the stretching mode of the RhB carboxylic acid (C=O) bond.<sup>[154,161]</sup> The bands at 1558, 1611 and 1640 cm<sup>-1</sup> are attributed to a stretching modes of the PEG-Rhodamine aromatic conjugated (C=C) bonds.<sup>[157,159]</sup> The band at 1751 cm<sup>-1</sup> is attributed to the ester (C=O) stretching mode<sup>[23]</sup> and the bands at 2887 and 2929 cm<sup>-1</sup> are attributed to symmetric and asymmetric stretching modes of the PEG (C-H) groups, respectively.<sup>[154]</sup>



Figure IV.6.: Representative FT-IR spectra of RhB and PEG-RhB. FT-IR spectra of the pure RhB powder and of the PEG-RhB product powder. Adapted with permission from Ref.<sup>[4]</sup>

#### 1.5. 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 IV.7 was carried out under argon atmosphere in dry THF solvent in a heated Schlenk flask.<sup>[4]</sup> 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 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 additional 30 min stirring. Pentadecafluorooctanoyl chloride (13.23 g, 30.6 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. At the end of the reaction the THF solvent with unreacted PEG was removed by separatory funnel. The crude product was washed with 10 ml  $H_2O$  to remove the lithium chloride. Following the washing procedures the crude product was dried at 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 (Rf = 0.28) as a bright vellow color product was collected and used as emulsion destabilizer and a colorless oil which is the triblock (Rf = 0.72) was stored for other experiments. The desired 2-(2-(2-Hydroxyethoxy)ethoxy)ethyl 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8pentadecafluorooctanoat diblock-copolymer was obtained (7.43 g, 83%) and analyzed 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 (CDClsubscript3, 22°C;  $\delta = 37.26$  for <sup>1</sup>H,  $\delta = 77,16$  for 13C) <sup>1</sup>H NMR  $\delta = 4.48$  (tr, 2 H, CH<sub>2</sub>, C9); 3.74 (tr, 2 H, CHsubscript2, C10); 3.66 (tr, 2 H, CHsubscript2, C13/14); 3.61 (s, 4 H, CHsubscript2, C11, 12); 3.54 (tr, 2 H, CHsubscript2, C13/14); 1.37 (s, 1H, OH); <sup>13</sup>C NMR  $\delta = 158.3$  (tr, COOR, C8); 117.0 (trq, CFsubscript2, C1); 114-105 (m, CFsubscript2, 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 IV.7.: Synthesis schematics of PFO-PEG diblock-copolymer surfactants

#### 1.6. PFPE(7000)-PEG-Bead Surfactant

To link polymer beads to droplets external periphery (see Section VI.3) a new type of bead-linked surfactants was synthesized. Two reaction steps were necessary to accomplish the synthesis of bead linked surfactants as shown in Figure IV.8 PFPE(7000)-PEG-bead surfactant. First, the polystyrene beads (Polyscience Inc, USA) were functionalized with Pluronic<sup>®</sup> F-127 (Sigma-Aldrich Chemie GmbH, Germany) according to the protocols described by Kim et al.<sup>[153]</sup> and the second is the coupling of the pluronic functionalized beads with PFPE(7000) carboxylic acid.

Polystyrene beads (d=2.077 µm, SD 0.045 µm, 2.61% solids) were mixed with 2 ml of 2% (w/v) PEG-PPG-PEG (Pluronic F-127) solution and stirred for 5 hours. 50 µL of toluene were added and the solution was stirred for another 2 hours before the solution was heated to 98°C to remove the toluene. The resulting milky solution was centrifuged for 12 min at 13000 rpm (Biofuge fresco, Kendro Laboratory Products, Germany) the supernatant was removed and the beads were resuspended 5 times in 2ml of ultra pure water and centrifuged again to remove unbound Pluronic F-127. Following the last centrifuging step the beads were dried in vacuum (1 x  $10^{-2}$  mbar) for 12 h.

In the second step the functionalized beads were coupled to PFPE7000-carboxylic acid via an ester bond. Towards this end, the beads were mixed with 1.5 ml of ultra pure water and  $10 \,\mu$ L of 5N KOH. PFPE7000-carboxylic acid (20 mg, 0.003 mol) was added to the beads and the solution was stirred for 30 min. The functionalized beads precipitated at the bottom and on the walls of the reaction vial. The water was removed and the

product was dried for 24 hours in vacuum (1 x  $10^{-2}$  mbar). The product was solved in 2 ml of FC-40. FT-IR was used to analyze the success of the synthesis.



Figure IV.8.: Schematics of different experimental steps for bead surfactant synthesis. The aliphatic methylene group of the polypropylene glycol (PPG) was incorporated in the swollen beads. By removing of the toluene the beads shrink and the Pluronic F-127 is incorporated solidly in the beads.

#### 1.6.1. FT-IR Analysis of PFPE(7000)-PEG-Bead Surfactants

The successful synthesis of PFPE(7000)-PEG-Bead Surfactants was confirmed by FT-IR analysis. As shown in Figure IV.9. The black line represents the transmission of PFPE carboxylic acid and the red line shows the synthesized PFPE-PEG-bead product. The results show the same shifts of the band at 1695 cm<sup>-1</sup> towards 1776 cm<sup>-1</sup> in the product, as observed in synthesis in the previous section.



Figure IV.9.: Representative FT-IR analyses to confirm successful bead surfactant synthesis.

## 2. Interfacial Tension Dynamic Analysis

#### 2.1. Pendant Drop Analysis

The pendant drop method was used to evaluate the interfacial tension at the water and oil (FC-40) interface and the influence of various surfactants. Three independent dilutions of surfactant containing oil phases were prepared for each experiment. Dilution series covered concentrations ranging from 10 n M to 10 m M. Data analysis was carried out as described in Section III.1.2.

The results in table IV.1 show a significant difference of CMC values of the diblock surfactant PFPE(7000)-PEG-OMe(750) and the two triblock surfactants PFPE(7000)-PEG(600)-PFPE(7000) and PFPE(7000)-PEG(1400)-PFPE(7000). This implements a better stabilization with triblock surfactants due to the lower concentration needed to create micelles.

	procoadi				
Surfactant	CMC	$\gamma_{CMC}$	$\Pi_{CMC}$	Γ	a
	[µM]	[mN/m]	[mN/m]	$[\mu { m mol}/{ m m}^2]$	$[\mathring{A}^2]$
PFO(500)-PEG(150)-PFO(500)	3101.0	7.9	43.0	6.5	26.7
PFO(500)-PEG(150)	1763.0	6.0	46.0	6.9	24.1
$\mathrm{PFPE}\text{-}\mathrm{COO}\text{-}(2500)~\mathrm{NH_4}\text{+}$	202.6	5.1	48.2	7.4	22.4
PFPE(7000)-PEG-OMe(750)	148.6	21.5	29.1	3.9	43.1
PFPE(2500)-diethylamin	114.5	28.1	23.5	3.5	48.3
PFPE(7000)-PEG(600)-PFPE(7000)	48.7	23.5	28.1	3.4	48.8
PFPE(7000)-PEG(1400)-PFPE(7000)	45.1	19.5	32.5	4.0	41.1
PFPE(2500)-PEG(600)-PFPE(7000	22.7	3.1	50.0	8.1	21.4
PFPE(2500)-PEG(600)	17.2	18.1	36.8	5.4	31.6
PFPE(7000)-PEG-Gold	7.8	34.6	17.2	2.8	58.9

Table IV.1.: Properties of surfactants as extracted from the interfacial tension experiments and evaluated following the procedure described in Section III.1.2:

CMC and  $\gamma_{CMC}$  represent the critical micelle concentration and the corresponding final surface tension.  $\Pi_{CMC}$  shows the minimum of surface tension of each surfactant.  $\Gamma$  and a represent the surface coverage and the available area of each surfactant.

The analysis of the PFO-PEG surfactant confirm the weak stabilization with this surfactant that can not protect droplets from fusion. Therefore this surfactant was used as a destabilizing surfactant to break up droplets and analyze the content afterwards.

#### 2.2. Deformation Measurements

A droplet production and deformation device (Section III.2.3.1) was used to analyze the deformation of the droplets in order to provide an insight on the dynamic accretion of diblock and triblock surfactants on the oil-water interface and to evaluate the dynamic interfacial tension.<sup>[139]</sup> Moreover this device was used to evaluate the influence of surfactant mixtures and protein interaction with the droplets interfaces. Both types of surfactants were used with a concentration of 2.5 m M and in all experiments ultra pure water was used as the aqueous phase. The surfactant mixtures composed of PFPE(7000)-PEG(1400)-PFPE(7000) triblock and PFPE(7000)-PEG(750)-OMe diblock were mixed at different ratios: 4:1, 3:2, 2:3 and 1:4. Analysis procedure is described in Section III.1.3. Figure IV.10 shows the deformation results as analyzed in the first and last deformation chamber of the deformation device.

The time it took droplets to float through the device from the first to the last chamber was computed from high speed video recordings resulting in a flow time of one second. The number of analyzed droplets in these videos varied from 38 to 96. An increase in deformation between the first and last deformation chamber can be seen. Moreover, it can be observed that the deformation is stronger in case of mixtures in comparison to pure surfactants. This result can be explained by faster accretion and a denser organization of the mixed surfactants on the droplet periphery. These results are in good agreement with the data from the pendant drop measurements which already indicated the difference in CMC values as well as the surface coverage of triblock and diblock surfactants. Through the different structure of these two surfactants the mixture overcomes the disadvantages of each surfactant and is offering a denser layer of surfactants on the periphery.



Figure IV.10.: Surfactant influence on droplet deformation in a deformation device. Droplet deformation using different surfactant mixtures of Tri-7000-PEG-1400 (Tri) and Di-7500-O-Me-750 (Di) recorded in the first and the last flow cell chamber (time difference: 10 ms and 1.1 sec after droplet production). The y-axes shows the deformability calculated with a ferret's diameter. The number of droplets analyzed in each data point is shown as n in grey. Deformation increases with droplet age. No differences with regard to surfactant mixtures are visible.

**Dynamic of Deformation** In a second experiment the droplet deformation was measured for every 10<sup>th</sup> chamber. The total number of chambers was 100. 2.5 m M of PFPE7000-PEG1400-PFPE7000 in FC-40 was used as oil phase and water as aqueous phase. Here, 8000 frames were recorded for each chamber. The time between the droplet production and the droplet reaching the last recorded chamber was one second. The results are shown in Figure IV.11. A constant value is reached after about 50 to 60 chambers indicating that surfactants need 500 milliseconds to create a dense layer to stabilize droplets and to shield them from fusion in case of this surfactant.



Figure IV.11.: Deformation of droplets as a function of the time following the droplet production. Droplet deformation of water droplets in FC40 with 2.5 m M of Tri-7000-PEG-1400 recorded in chambers 1 to 100. Number of droplets recorded is shown as n in grey on top. Deformation linearly increases with droplet age up to chamber 50.

## 3. Key Factors for Droplet Stability and Fluorophore Retention

#### 3.1. Droplet Stability

To optimize the conditions for stable mono-disperse droplet creation the range of the surfactant concentrations for the TRI7000 and TRI7000 surfactants was investigated.<sup>[158]</sup> For better visualization a fluorescence dye Alexa 488 was disolved in TRIS-buffer to a final concentration of 5 µM and used as an aqueous phase. Videos of freshly produced droplets,

created in a microfluidic device, were recorded immediately after the cross junction with a Phantom V7.3 camera at 10000 fps.

As can be observed in Figure IV.12 surfactant concentrations below 1mM lead to droplet coalescence as soon as droplets were in contact. At higher surfactant concentrations (above 1mM) droplets were stable and no coalescence was observed. Figure IV.12 shows five representative images at different concentrations for both tested surfactants.



Figure IV.12.: Droplet stability as a function of surfactant concentration and its molecular weight. Representative brightfield images of water-in oil droplets immediately after production. At low surfactant concentration (C < 1mM) coalescence of droplets is observed (left panel). At high surfactant concentration creation of small droplets occurs (right panel). Monodisperse and stable droplets are produced in a stable regime as it can be seen in the middle panel. Adapted with permission from Ref.<sup>[144]</sup>

In case of TRI2500 stable monodispers droplets creation was observed at a concentration range of 2,5 mM < c < 10 mM. In case of the bigger surfactant TRI7000 the concentration range was larger, ranging from 1 mM up to 20 mM surfactant concentration. Above these surfactant concentrations small droplets were created during droplet production due to jet-dripping and by friction of the droplets flown in the channels. When droplets were stabilized (less than a second after production) with a dense surfactant layer no appearance of more small droplet or a decrease in size of produced droplets was observed any more.

To provide a detailed analysis of the droplet stability and their size distribution droplets were collected and stored in an analysis chamber (see Section III.2.3). The results are presented in Figure IV.13. Size distribution was measured for large droplets (size range:  $5\,\mu\text{m} < \text{R} < 40\,\mu\text{m}$ ) and small droplets (size:  $\text{R} < 5\,\mu\text{m}$ ). The measured size distribution at a given surfactant concentration was normalized with the corresponding area of the large and the small droplets, respectively. The height of the bars in Figure IV.13 represent the percentage of occupied area.



Figure IV.13.: Droplet size distribution as a function of surfactant concentration. Left panel: small droplets and micelles formed at high surfactant concentrations. Right panel: Size distribution of "mother-droplets".

Surfactant concentrations below 1mM result in a broad droplet size distribution of larger droplets, which can be attributed to coalescence. Some droplets were larger than 50  $\mu$ m radius and are not shown in the Figure but were included in the calculated percentage of occupied area by droplets. These findings are in good agreement with Baret et al.<sup>[32]</sup> On

the other hand high surfactant concentrations lead to creation of small droplet, visible with brightfield microscopy. This effect was observed at a surfactant concentration of 5mM for TRI2500 and at 20 mM for TRI7000. For high concentrations even shrinking of the "mother-droplets" is visible (for TRI2500) by the decrease of the average droplet radius. In most microfluidic droplet applications this effect is undesirable since a constant volume of all droplets is essential for quantitative analysis. Therefore in all further investigations the surfactant concentration was always kept between 2.5 and 10 mM to ensure production of stable droplets with low size distribution.

## 3.2. Effect of Droplet Size and their Formation Rate on Fluorophore Retention

To preform long term experiments with microfluidic droplets, stable encapsulation of particles, molecules and cells is necessary. Therefore, molecular retention in the droplets was analyzed according to droplet size and the rate of droplets formation.<sup>[158]</sup>

First the effect of droplet formation speeds was investigated to determine fluorophore retention. Figure IV.14 shows representative images of the fluorophore ATTO 590 SE moderate retention as a function of formation speeds (0.5, 3, and 30 kHz) (defined as described in Section III 4.4).



Figure IV.14.: Effect of droplet production rates on fluorophore retention Moderate retention of ATTO 590 SE fluorophores in the droplets does not depend on the droplet creation frequencies. ATTO 590 SE fluorophores were dissolved in PIPES-buffer and used as an aqueous phase for droplets creation. The oil phase contained 2.5 mM of TRI7000 surfactants. Adapted with permission from Ref.<sup>[144]</sup>.

As can be seen in Figure IV.14 the retention was not affected due to different droplet creation frequencies. All flurophores mentioned in Section III.4.1 were tested and showed the same result.

Next the effect of droplet size on fluorophore retention was investigated. The fluorophors were encapsulated in droplets having different sizes (Diameter = 25, 40 and 150  $\mu$ m, 3 KHz production speed, 2.5 mM of TRI7000 surfactant). As shown in Figure IV.15 the droplet diameter had no effect on the fluorophore encapsulation stability. All investigated fluorophore retentions were not affected by the size of the droplets.



Figure IV.15.: Effect of droplet sizes on the retention of fluorophores within the droplets. Retention of fluorophores in the droplets is not affected as a function of droplet size. ATTO 532 SE, ATTO 655 SE, ATTO 590 SE and ATTO 647N SE fluorophores were dissolved in TRIS buffer and used as an aqueous phase for droplet creation. The oil phase contained 2.5 mM of TRI7000 surfactant. Adapted with permission from Ref.<sup>[144]</sup>

Stable retention Moderate retention No retention

## 3.3. Effect of Buffer Type and and Cell-Culture Medium on Fluorophore Retention in Droplets

To investigate the influence of buffers and media on dissolved fluorophore retention the most common four buffers (TRIS, HEPES, PIPES and PBS) and to the cell culture media (RPMI and DMEM) were used as an aqueous phase.<sup>[158]</sup> The data concerning buffers, media and fluorphores used in this study are described in Section III.4.1. The hydrophilicity of the tested fluorophores was analyzed using the calculated distribution coefficient LogD (see Section III.4.1). Positive LogD values correspond to hydrophobic fluorophores, negative values to hydrophilic ones.

The retention summary of 12 commonly used fluorophores is presented in Figure IV.17. Fluorophores were encapsulated in droplets, produced with FC-40 oil containing 10 mM TRI2500 and  $3 \mu M$  Gold-PFPE surfactant. Droplet production and analysis were preformed according to Section III.4.1. The retention of fluorophores was categorized according to:

$$\begin{array}{l} I_0 \approx I_{t0} \approx I_{t24} \Rightarrow & \mbox{Stable flurophore retention} \\ I_0 \approx I_{t0} > I_{t24} \Rightarrow & \mbox{Moderate fluorophore retention} \\ I_0 >> I_{t0} & \Rightarrow & \mbox{No fluorophore retention} \end{array}$$

Figure IV.16.: Characterization criteria for fluorophore retention in droplets

Stable retention is marked green, moderate retention is marked yellow and no retention is marked red. Stable retention (marked green) was observed for all highly-hydrophilic fluorophores (LogD < 0). Detailed definition of these three categories is presented in Section III.4.4.

In Figure IV.17 highly hydrophilic (LogD < -7) fluorophores as Alexa 488, Alexa 647, and ATTO 488 show stable retention independently on buffers and media type. However, a buffer/medium-dependent retention was observed for less hydrophilic fluorophores with LogD values around -4. For Alexa 532 and ATTO 532 fluorophores the protonation state of secondary amines was reduced by buffers and media with higher pH values and consequently led to a moderate retention. All flourphores with LogD values > 0 showed

Fluorophore [LogD] Buffer/ Medium [pH]	Alexa 488 M [-9.47]	Alexa 532 M [-4.43]	Alexa 647 M [-7.75]	ATTO 488 SE [-7.60]	ATTO 495 SE [-0.81]	ATTO 520 SE [+0.59]	ATTO 532 SE [4.49]	ATTO 565 SE [-0.18]	ATTO 590 SE[+1.56]	ATTO 633 SE [n.a.]	ATTO 647N SE[+4.47]	ATTO 655SE [-0.61]
TRIS [7.7]												
HEPES [7.4]												
PIPES [6.8]												
PBS [7.4]												
RPMI [7.9]												
DMEM[8.2]												

no or in case of RPMI media moderate retention

Figure IV.17.: Fluorophores retention in the droplets Fluorophores retention in the droplets (diameter = 40 µm) having different buffer/medium conditions. TRI2500 (10 mM) surfactants were used to generate droplets for this experiment. Adapted with permission from Ref.<sup>[144]</sup>

Surprisingly, no retention was observed for slightly hydrophilic dyes as ATTO 495 (LogD = -0.81), ATTO 655 (LogD= -0.61) and ATTO 565 (LogD= -0.18). In case of ATTO 495 dye this behavior can be explained by the relatively small molecular weight as well as the planar structure which leads to a smaller energetic barrier for the transport through the surfactant layer.<sup>[162]</sup> For ATTO 565 and 655 dyes only half of the tested buffers and media showed moderate retention.

## 3.4. Retention as a Function of Surfactant Length, Structure and Concentration

In addition to buffer and fluorophore properties the effect of the physical properties of surfactants (i.e., geometry, concentration and molecular weight) on the fluorophore retention in the droplets were investigated.<sup>[158]</sup> Three different surfactants (TRI2500, TRI7000, and DI7000) with 10 mM concentration each were used (see Section IV.3.1). The retention summary of the 12 fluorophores encapsulated with the three surfactants is presented in Figure IV.18. As can be observed from Figure IV.18 droplets produced with the TRI7000 surfactants exhibited the best retention of encapsulated fluorophores. However,

the droplets produced with this surfactant were highly polydisperse in size due to low droplet stability. Improved retention in the droplets produced with diblock surfactants can be attributed to the denser surfactant layer on droplets periphery due to reduced steric effect on comparison to triblock surfactant geometry. However, for droplet stability it is preferable to have a dense PFPE layer on the outside droplet interface. The most efficient layer against coalescence can be provided by triblock surfactant geometry having two PFPE tails.<sup>[15]</sup> Due to the inherent requirement for stable monodisperse droplets, further experiments were performed with TRI2500 and TRI7000 surfactants. As shown in Figure IV.18, improved retention was also achieved for some of the fluorophores in droplets produced with longer surfactants (higher molecular weight). This observation is straightforward since dyes have to pass a higher energetic barrier due to a thicker surfactant layer.<sup>[162]</sup> Further optimization of fluorophore retention within the droplets was analyzed depending on the surfactant concentration.

	Fluorophore [LogD] Surfactant	Alexa 488 M [-9.47]	Alexa <mark>532 M</mark> [-4.43]	Alexa 647 M [-7.75]	ATTO 488SE [-7.60]	ATTO 495SE [-0.81]	ATTO 520SE [+0.59]	ATTO 532SE [- 4.49]	ATTO 565 SE [-0.18]	ATTO 590 SE [+1.56]	ATTO 633 SE [n.a.]	ATTO 647N SE [+4.47]	ATTO 655 SE [-0.61]
	TRI 2500												
T.7	TRI 7000												
	DI 7000												
S	TRI 2500												
1PE 6.8]	TRI 7000												
	DI 7000												
PMI 7.9]	TRI 2500												
	TRI 7000												
H _	DI 7000												

Figure IV.18.: Fluorophores retention in the droplets is a function of buffer/medium selection and surfactant physical properties. Fluorophores retention in the droplets  $(D = 40 \,\mu\text{m})$  as a function of buffer/medium selection and surfactant physical properties. The droplets were generated using three different surfactants (TRI2500, TRI7000, and DI7000 (10 mM)). Adapted with permission from Ref.<sup>[144]</sup>

The range of surfactant concentrations was chosen according to the results in Section IV.3.1. Based on these results and considering that molecular retention might be dependend on surfactant concentration, further fluorophore retention studies were preformed in droplets produced with 2.5 and 10 mM surfactant concentration. The retention summary of the 12 fluorophores encapsulated in droplets made of TRI7000 and TRI2500 surfactants at two different concentrations is presented in Figure IV.19. In both cases, improved fluorophore retention was observed with the lower surfactant concentration. However, this effect is more apparent in TRIS buffer and RPMI medium. The decrease of free surfactants in the oil phase reduces the formation of reverse micelles and small droplets, which can act as carriers for the solubilized fluorophores. These results are in agreement with previous observations where the decrease of the fluorescence intensity of fluorescein inside droplets was observed due to increased surfactant concentration.<sup>[163]</sup>

These results emphasize that with a proper selection of the physical surfactant properties such as concentration, geometry, and molecular weight the retention of most of the fluorophores can be controlled efficiently.

Buffer		т	RIS			PH	PES		RPMI			
Surfactant	TTI	RI 2500 TRI 7000			TPI	2500		7000	TRI 2500 TRI 7000			7000
Surfactant Surf.Conc. [mM]	2.5	10	2.5	10	2.5	10	2.5	10	2.5	10	2.5	10
Alexa 488												
Alexa 532												
Alexa 647												
ATTO 488												
ATTO 495												
ATTO 520												
ATTO 532												
ATTO 565												
ATTO 590												
ATTO 633												
ATTO 647N												
ATTO 655												

Figure IV.19.: Fluorophores retention in the droplets as a function of surfactant length and concentration Fluorophores retention in the droplets  $(D = 40 \,\mu\text{m})$  as a function of surfactant length and concentration. Fluorophores were dissolved in TRIS, PIPES buffers, and RPMI medium. The oil phase contained 2.5 or 10 mM of triblock surfactants. Adapted with permission from Ref.<sup>[144]</sup>

#### 3.5. Retention of Biomolecules

In most biorelevant applications with microfluidic droplets, fluorescence measurements are applied to reveal the content of biomolecules and to asses the success and efficiency of the approach. For example, fluorescently labeled proteins were used to characterize their localization within the droplets for amyloidosis investigations<sup>[164]</sup>, protein crystalization<sup>[165]</sup> and immunological applications.<sup>[4]</sup> Therefore the influence of fluorophores and surfactants on the retention of labeled biomolecules within droplets was investigated.



Figure IV.20.: Retention of ATTO 633- labeled microtubules in the droplets Retention of ATTO 633-labeled microtubules in the droplets was not observed over time. This time-lapse shows that polymerized ATTO 633-labeled microtubules diffused within minutes into the oil phase. The oil phase contained 10 mM TRI2500 surfactants. Note: Similar results were observed with and without addition of gold-linked surfactants.

The retention of highly hydrophilic biomolecules such as antibodies and streptavidin was compared with less hydrophilic biomolecules such as tubulin. Towards this end labeled streptavidin proteins and antibodies (see Section III.4.3) were dissolved in TRIS buffer to a final concentration of  $0.2 \,\mu$ M. For the labeled tubulin, PIPES buffer (the most common buffer used for tubulin experiments) was used for microtubule creation (1  $\mu$ M) in droplets. The main outcome from these experiments was that the retention is strongly dependent on the physical properties of the surfactant as well as on the hydrophilicity of the selected dye and the hydrophilic/hydrophobic properties of the biomolecule. As shown in Figure IV.21 stable retention of ATTO 633-labeled microtubules in the droplets created with 2.5 mM TRI7000 surfactants was achieved. Whereas, droplets produced with 2.5 mM TRI2500 surfactant showed no retention of ATTO 633-labeled tubulin and even pre-polymerized microtubules diffused out of the droplets immediately after encapsulation. A representative time-lapse is presented in Figure IV.20. Additionally, stable retention was observed for microtubules labeled with highly hydrophilic ATTO 488 dye (Figure IV.21).

In contrast to the labeled microtubules experiments, stable retention for more than 14 days was observed for all labeled antibodies and streptavidin proteins tested in this study. These results were independent of the hydrophilicity of the dye and surfactant selection. In case of antibodies and streptavidin proteins their high hydrophilicity and their big molecular weights are the major reasons for their stable retention within the droplets independent of the dye properties. Even the most hydrophobic ATTO 647N dye (LogD = +4.47), which showed no retention in the droplets in a pure state, did not influence the the stable retention of sheep anti-mouse antibodies or streptavidin.



Figure IV.21.: Factors influencing the retention of microtubules in micro droplets Retention of labeled microtubules in the droplets strongly depends on the degree of hydrophilicity of the dye and surfactant selection. ATTO 633- and ATTO 488-labeled microtubules were polymerized in PIPES buffer and used as an aqueous phase for droplets creation. The oil phase contained 10 mM TRI7000 and TRI2500 surfactants. Note: Similar results were observed with and without addition of gold-linked surfactants. Adapted with permission from Ref.<sup>[144]</sup>

Compared to antibodies, tubulin is less hydrophilic and has even been considered as an "amphi potential" protein, i.e., a protein that has the ability to exist in both aqueous and lipid phases (like membrane proteins). Therefore, the dyes for labeling have to be selected very carefully if less hydrophilic proteins like tubulin or other amphiphilic molecules are encapsulated in droplets.

In conclusion this section shows that stable droplets are produced using surfactant concentrations in the range of 2,5-10 mM. Retention of molecules in droplets is widely independent of the droplet production speed as well as the droplet size. Stable fluorophore retention is observed for highly hydrophilic molecules. For less hydrophilic or amphiphilic molecules and biomolecules the fluorophore selection, buffer selection (pH and salt concentrations), surfactant length and surfactant concentration are crucial for a stable encapsulation.

# 4. Gold-Nanostructured Droplets, their Biofunctionalization and Analysis

Goldnanoparticles (GNp) are commonly used as anchoring points for biomolecules in cell experiments due to their biocompatibility and flexible biofunctionalisation via thiol-gold chemistry.<sup>[166]</sup> To provide the droplets with bioactive surfaces the gold-linked surfactants were synthesized (see Section IV.1.3) and mixed with surfactants containing no GNp.<sup>[4]</sup>

#### 4.1. Cryo-SEM Analysis

To get a quantitative estimation concerning the GNp density the nanostructured droplets were characterized using high resolution cryo-SEM. The analysis was carried out as described in Section III.3. Representative top-view cryo-SEM (scanning electron microscope) micrographs of freeze fractured nanostructured droplets created with gold-linked surfactant (B)  $30 \mu$ M; (D)  $3 \mu$ M; (F)  $0 \mu$ M are presented in Figure IV.22. In all experiments here a 10 mM TRI2500 oil solution was used to create stabilized droplets. Gold nanoparticles were observed on the inner periphery of the droplets. Their density in the periphery correlates with the Gold-PEG-PFPE concentration used for the droplet creation. As shown in Figure IV.22 a higher density and more homogeneous distribution of gold NP's on the inner droplet periphery was obtained using a higher concentration of gold-linked surfactant (30 µM) (Figure IV.22 B) compared to a lower concentration (3 µM) (Figure IV.22 D).<sup>[4]</sup> To prove that the bright dots were gold NPs and not artifacts due to the cryo-SEM measurements or freeze-fracture preparation, droplets without gold-linked surfactants were created, freeze-fractured, and observed by cryo-SEM (Figure IV.22 F).



Figure IV.22.: Cryo-SEM micrographs of freeze-fractured nanostructured droplets Representative cryo-SEM micrographs of freeze-fractured nanostructured droplets obtained with different magnifications. The droplets were created using a PFPE-PEG-PFPE triblock (TRI2500) copolymer surfactant concentration of 20 mM and PFPE-PEG-Gold-naonoparticle surfactant concentrations of (A, B) 30 μM, (C, D) 3 μM and (E, F) 0 μM. Adapted with permission from Ref.<sup>[4]</sup>

#### 4.2. Biofunctionalization of Nanostructured Droplets

In this study GNp were used as anchoring points for various biomolecules. Towards this end, two biofunctionalization approaches were developed. The first approach was based on functionalization of created droplets with Ni<sup>2+</sup> mediated interaction between the (His6tag) proteins and nitrilotriacetic acid (NTA)-thiol linker. In the second approach the biomolecules were coupled covalently via ester or thiol bond to the gold-linked surfactants before droplet creation.

The results of the first approach are presented in Figure IV.23 where GNps in the nanostructured droplets were coupled with His6-tag green fluorescence protein (His6-GFP) via a GNp-thiol linker (detailed reaction see Section III.5.1.

Images were taken (A) 1 day, (B) 4 days, and (C) 10 days after creation. The decrease in fluorescence intensity over the observed period can be explained due to oxidation of gold-sulfur bonds in the aqueous phase<sup>[167]</sup> followed by diffusion to the oil phase and by this a solvent-induced denaturation of the GFP molecules.<sup>[168]</sup> Figure IV.23 (D and E) show representative images of droplets having either no gold-linked surfactant (D) or no NTA-thiol-linker (E). As expected the fluorescence signal is equally distributed over the whole droplet.



Figure IV.23.: Representative fluorescence images of the GFP-linked goldnanostructured and nonstructural droplets (A, B, C) Gold nanostructured droplets measured (A) 1 day, (B) 4 days, and (C) 10 days after their creation. (D, E) show the negative control with images of the non-gold nanostructured droplets (D) and nanostructured droplets where the His6GFP was used without NTA-thiol linker (E), taken one day after creation, respectively. All images have the same intensity scale. Adapted with permission from Ref.<sup>[4]</sup>

#### 4.2.1. Multiple Droplet Biofunctionalization

Since in most of the biorelevant applications binary or trinary functionalization might be required, the droplets functionalization with various proteins was examined. Method 1 is to synthesize a surfactant with a covalently linked biomolecule and to produce droplets with the second NTA-thiol activated biomolecule in the aqueous phase. Method 2 uses two bioactive molecules dissolved in the aqueous phase and linked via NTA-Thiol chemistry to the GNp's on the inner droplet periphery.

Rhodamine B linked surfactants and His6-GFP NTA-Thiol were used in Method 1. Method 2 used GFP- and MHC-Alexa568-NTA-thiol linked molecules. Both methods were successful in linking the molecules to the gold NP's. Representative confocal images of encapsulated GFP- and MHC-Alexa568-NTA-thiol linked molecules taken 10 min after production are shown in Figure IV.24. The results show an even distribution of the fluorescence biomolecules on the periphery.



Figure IV.24.: Representative images of encapsulated GFP- and Alexa568-MHC-NTA-thiol linked molecules. Images were taken 10 min after production. (A) Shows GFP, (B) shows the Alexa568-MHC molecules and (C) presents the merged fluorescence from (A) and (B).

The second approach involves two steps: synthesis of PFPE-PEG-gold surfactants linked to rhodamine B (RhB) (Section IV.1.4) followed by droplet creation using a mixture of TRI2500 (20 mM) and RhB-PEG-Gold-PEG-PFPE (5  $\mu$ M) surfactants as an oil phase, while PBS was used as an aqueous phase. The droplets were stored and observed in the analysis chamber (see Section III.2.3). Figure IV.25 shows the distribution of the fluorescence signal in the RhB-linked nanostructured droplets. (A - C) show a time-lapse taken 1, 4 and 16 days after creation and (1-10) a z-stack of a droplet. All images show that the fluorescence signal is distributed equally at the droplet periphery. Due to the chemical bonding and the stable fluorescence dye the signal was stable for more than two weeks.<sup>[4]</sup>



Figure IV.25.: Representative fluorescence images of the RhB-linked gold-nanostructured droplets (A, B, C) RhB-linked nanostructured droplets measured (A) 1 day, (B) 4 days, and (C) 16 days after their creation. (1-10) Representative distribution of the fluorescence signal along the RhB-linked nanostructured droplet height, 3 days after creation. Stack 1 represents the top of the droplet, stack 10 the bottom. All images have the same intensity scale. Adapted with permission from Ref.<sup>[4]</sup>

## 5. T Cell Experiments

As described in detail in Section I.2 the effective *ex vivo* T cell activation is the crucial step for successful immune therapy. Therefore, nanostructured and biofunctionalized droplets were used as 3D APC analogues. To provide the specific interactions with the T cells, the nanostructured droplets were functionalized with cyclic arginine-glycine-aspartic acid peptide (cRGDfK) or with pMHC proteins via cysteine or Ni-NTA-thiol chemistry, respectively. Single T cell encapsulation was achieved using a microfluidic spiral device as described in Section III.2.3.1.

#### 5.1. T Cell Jurkat 6.1 Experiments

The human acute T cell leukemia cell line (Jurkat E 6.1) was used to evaluate the potential of nanostructured and biofunctionalized droplets to serve as a 3D APC analogue enabling single cell analysis in a defined compartment. Jurkat T cells express  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$ integrins and exhibit activation-dependent regulation of integrin-mediated adhesion.<sup>[169]</sup> c(RGDfK)-PEG6-cysteine which are specific for  $\alpha_5\beta_1$  were linked to PFPE-PEG-gold surfactants (Section III.5.2)<sup>[4]</sup> No significant difference between both labeling strategies was observed. However, the two step approach was preferred to minimize the amount of soluble c(RGDfK)-PEG6-cysteine peptides in the aqueous phase, to avoid blockage of active sides on the cells and therefore, hindering the cell-droplet interaction.<sup>[170]</sup>



Figure IV.26.: T cell viability and T cell-droplet-periphery interaction depending on the functionalization. (A) Representative bright-field image of Jurkat E 6.1 cells (indicated by arrows) in the cRGDfunctionalized nanostructured droplets 6 h after their creation. (B) Quantification (adherent cell %) of Jurkat E6.1 cell adhesion on cRGD-functionalized (pink, left bars) and nonfunctionalized (gray, right bars) nanostructured droplets. Data is presenting mean ± standard error of the mean (n = 5). (C) T cell proliferation time lapse. (D) T cell viability after 3 days (green= alive; orange= dead). Cells were marked with propidium iodid. Adapted with permission from Ref.<sup>[4]</sup>

Cell encapsulation was preformed with straight and spiral microfluidic devices using TRI2500 (20 mM) and RGD-gold-linked surfactants (25  $\mu$ M) as oil phase and 6 x10<sup>6</sup> cells per ml in 1604 RPMI media (composition Section III.6.1) as aqueous phase. Representative droplets with encapsulated Jurkat T cells (indicated by arrows) are presented in Figure IV.26 (A). After incubation (37°C, 5% CO<sub>2</sub>) for 1 h, 90% of T cells in cRGD functionalized droplets were observed in contact with the droplet's periphery (Figure IV.26 (A, B)) whereas cells in droplets without cRGD-linked surfactants were found randomly distributed in the droplets, similarly to previous observevations.<sup>[23]</sup> The number of cells per droplet was controlled by varying the initial concentration of cells added to the microfluidic channel as well as using the spiral device (Section III.2.3.1). Figure IV.26 (C) shows the proliferation of T cells encapsulated in cRGD functionalized droplets. Cells encapsulated in droplets with cRGD-linked surfactants showed viability for up to 3 days of incubation (tested as described in Section III.6.2). Representative brightfield and fluorescence images of cells taken after 3 days are shown in Figure IV.26 (D). This observation is in agreement with previously published results that showed the viability of Jurkat cells for up to 9 days after encapsulation in droplets (diameter 100 µm) made of triblock surfactants.<sup>[150]</sup> Considering the differences in nutrition due to the smaller droplets and by this also a smaller volume used in our experiments a shorter viability was expected.



Figure IV.27.: Cryo-SEM micrographs of encapsulated T cells within the freeze-fractured nanostructured droplets. Representative cryo-SEM micrographs of freeze-fractured biofunctionalized droplets after the Jurkat E6.1 cell adhesion experiment. Adapted with permission from Ref.<sup>[4]</sup>

For more detailed cell-droplet interaction analysis, droplets with encapsulated T cells were freeze-fractured and investigated by cryo-SEM (Figure IV.27). Representative cryo-SEM micrographs with different magnifications are presented in Figure IV.27. Cells can be observed as spherical objects with diameters of 3 to 5 µm, similarly to previous observations.<sup>[171,172]</sup> Cryo-SEM micrographs confirmed cell-droplet interactions as observed by bright-field microscopy.

#### 5.2. Primary Mouse T Cells

Primary mouse T cells (see Section III.6.1) were used to investigate the antigen specific activation by nanostructured droplets. Towards this end GNp-structured droplets were functionalised with Alexa568-labeled pMHC and Alexa568-labeled ICAM proteins via Ni-NTA-Thiol chemistry (see Section III.5). The ability of T cells to form a SMAC during immunological synapse was investigated. Results are presented in Figure IV.28.

Primary mouse T-cells in RPMI 1604 media were encapsulated in droplets which contained Alexa568-MHC and ICAM-1-NTA-thiol linked activation and adhesion molecules. The activation and adhesion mediated molecules were linked via NTA-thiol to the GNp. Images were taken with the confocal microscope as described in Section III.3. In Figure IV.28 image 1 to 3 show composed z-stacks of droplets with primary mouse T cells in contact with the MHC-Alexa568 peptide. Images were visualized using recorded z-stacks and a "volume viewer" plugin in FIJI (image J). The results show enhanced fluorescence signals (brown) at the cell droplet interaction areas.

Figure IV.28 (A, B and C) shows a fluorescence (A) and a bright filed image (B) of the cell periphery interaction. (C) shows the 3D projection of the fluorescence signal obtained in a z-stack. These results show clearly the advantage of having mobile biolinkers in the droplet periphery which can be concentrated for the cell-droplet-surface interaction as needed for an optimal cell activation which also allows the T cell to exert pushing and pulling forces. These results prove that this setup allows rearrangement and local accumulation of biolinkers for an optimal activation of T cells.



Figure IV.28.: Representative confocal images of the primary mouse T cells interactions with goldnanostructure droplets. Image 1 to 3 show composed confocal z-stacks of droplets with primary mouse T cells in contact with their periphery. In sake of clarity of presentation the periphery of the droplet and T cells were marked with yellow and brown colour, respectively. The fluorescence signal of the Alexa568-labeled MHC can be observed in image (A) and (C). A brightfiled image of the related T cell is presented in figure (B).

## 6. Bottom-Up Assembly of Minimal Actin Networks

This section summarizes the results of actomyosin cortex formation in droplet-based micro-compartments. Water-in-oil nanostructured emulsion droplets as cell-sized compartments were used to achieve the functional bottom-up assembly of minimal number of proteins required to reconstitute the actomyosin cortex. To analyze the dynamic actin properties in defined compartments all actomyosin networks were created by implementation of droplet based microfluidics. All droplet experiments in this section were preformed using 2.5 mM triblock surfactants TRI7000. In some experiments  $0.3 \,\mu\text{M}$  gold-labeled sur-

factants were included to provide a linkage between the actin filaments and the droplets periphery. If not mentioned explicitly, actin was used in a final concentration of  $10 \,\mu$ M. Detailed information for experimental conditions can be found in Section III.7.1 and Tables III.3 and III.4.

#### 6.1. Reconstitution of Actomyosin Networks in Bulk

Prior to the actin experiments in droplets the bioactivity of freshly purified actin (see Section III.7) was tested in bulk (Figure IV.29). Figure IV.29 (A) and (B) present actin networks following the experimental conditions 1 and 2 (see Table ??), respectively. In the first experiments phalloidin-FITC was used to avoid depolymerization of actin filaments and to provide the detection of actin network formation.

Phalloidin is a toxin found in the green amanita with  $LD_{50}$  of 2-3 mg/kg. In actin experiments it is used to reduce the actin depolymerization rate.<sup>[173]</sup> With a dissociation constant KD of 10<sup>-8</sup> M phalloidin binds the interaction sites between the G-actin subunits of F-actin strands, reducing the depolymerization by a factor of 30.<sup>[174]</sup>

Figure IV.29 (A) shows a typical F-actin network distribution on clean glass surfaces. Similar homogenous distribution of phalloidin-stabilized F-actin networks was observed in Fisher et al.<sup>[175]</sup>.



Figure IV.29.: Representative images of actin and actomyosin networks on glass surfaces. (A) and (B) show representative images of an actin and actomyosin networks labeled with phalloidin-FITC on the glass surfaces, respectively.

To assess the activity of the motor protein a final concentration of  $0.5 \,\mu\text{M}$  myosin II was

added to the actin solution. KCl of  $0.75 \,\mathrm{mM}$  was used to control the myosin II protein cluster length (i.e. 50-60 myosin II proteins per cluster).<sup>[135]</sup>

Figure IV.29 (B) presents the typical morphology of actomyosin network on the clean glass surfaces 20 min after solution preparation. The effect of myosin II can be clearly observed due to the conformational changes associated with the myosin contraction.

The aforementioned results show clearly the polymerization and contraction activity of actin and myosin proteins, respectively. Therefore, same actin solution (experimental condition 1) was used to reconstitute actin networks within the microfluidic droplets (see the following section).

## 6.2. Optimization and Regulation of Actin Encapsulation by Microfluidics

To encapsulate actin within water in oil microfluidic droplets two approaches were tested. In the first approach the actin was dissolved in complete G-buffer and the normal device for droplets generation (one-aqueous-inlet-channel device) was used to create actin containing droplets. The main drawback of this approach was that actin pre-polymerized already in the syringe and therefore blockage of syringe needles or microfluidic aqueous inlet channels was observed. To overcome this obstacle and to polymerize the actin just inside the droplets, a new microfluidic device was generated (Figure IV.30) with two aqueous inlet channels.



Figure IV.30.: Microfluidic droplet production device with two aqueous inlet channels. Inset (A) and (B) show representative fluorescence images of the the two phases for actin containing droplet production. Bright phases represent the actin solution containing the phallodidin-FITC. The second phase contains all molecules needed for polymerization and bundling of actin. The solutions have a contact time in the millisecond range before getting encapsulated.
In this device one aqueous inlet channel was used to introduce actin, phalloidin-FITC and anti-oxidation buffer in G-buffer containing no MgCl<sub>2</sub> (bright shining phase in inset (A) and (B) in Figure IV.30), the second aqueous channel contained ATP, MgCl<sub>2</sub> and myosin (when used) (dark half of the channels). By using this device the contact between two solutions was limited to few milliseconds before droplets creation. Herby, uncontrolled actin polymerization before encapsulation within the droplets was minimized.

Successful implementation of the two aqueous inlet channel device for encapsulation of G-actin and controlled polymerization after encapsulation was crucial for the following regulation of F-actin network distribution within the droplets. Polymerization of G-actin monomers inside the droplets (experimental condition 1) and chemical immobilization of F-actin filaments to the gold nanoparticles on the droplet periphery by means of lipoic acid PEG succinimidyl-ester (NHS-ester) were examined. Remarkably, most of the actin filaments were found to be in contact with the inner periphery of the droplets, compared to randomly distributed filaments in the case of droplets without gold-linked surfactants.

It has to be mentioned here that actin can be immobilized on the periphery of the droplets containing gold-linked surfactants also without lipoic acid linker due to the interaction between the cysteine on the actin filaments and the gold nanoparticles. Moreover, analysis of images obtained by confocal microscopy revealed no difference in F actindroplets interaction with and without linkers. Since NHS molecules interact with the primary amines on actin they can potentially block or affect the F-actin polymerization. Therefore, in all further experiments, were the interaction of actin and the droplets periphery were required direct linkage was preferential.



Figure IV.31.: Regulation of F-actin network distribution by gold-nanoparticle-linked surfactants. (A) and (B) show a schematic and real actin network distribution (labeled with phalloidin-FITC) in droplets having no gold-linked-surfactnat and with gold-linked surfactants, respectively. (A) non-goldnanostructured droplets and (B) gold-nanostructured droplets. Confocal images (A1) and (A2) show the location of actin networks in droplets without gold-nanoparticles. (B1) 3D image of actin network located on The periphery in gold nano-structured droplets. (B2) Slice of a confocal z-stack of actin network located on The periphery in gold nano-structured droplets.

Next, the effect of a lipid bilayer on the actin-droplet periphery interactions was analyzed. Towards this goal, the same experimental conditions as presented before in Figure IV.31 (B) were used. To generate a lipid bilayer within the droplets L- $\alpha$ -phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) lipids (5 µM) together with the G-actin were dissolved in G-buffer and introduced as an aqueous phase. Droplets were created using the oil phase containing triblock and gold-linked surfactants.

The results in Figure IV.32 show that following optimization of ion concentration lipids build a GUV on the inner periphery of the droplet. Furthermore, it can be observed (Figure IV.32 (A)) that the location of actin networks was only found in the center of the droplets similarly to distribution observed in Figure IV.31 (A), where no goldlinked surfactants were used. The merged 3D z-stack obtained by confocal microscopy is presented in Figure IV.32 (B) and (C).

There are two major outcomes from these experiments: 1) The actin-gold interaction can be prevented by the GUV formation within the droplet, which sealed completely the inner periphery of the droplet and; 2) the most common system to mimic cellular membranes as a GUV can be created within the polymer stabilized droplets.



Figure IV.32.: F-Actin distribution in droplets having a GUV on its inner droplet periphery (A) cross section image of a z-stack were actin (green, labeled with phalloidin-FITC) is located in the middle of the droplet with no interaction with the periphery, which is sealed by the dense lipid bilayer (red, rhodamine labeled).(B) 3D z-stack of merged confocal images with an actin network in the droplet surrounded by a lipid bilayer on the inner droplet periphery. (C) same z-stack as presented in (B), but with one side cropped to allow an insight view of the droplet showing that the actin is only located in the middle of the droplet.

#### 6.3. Actomyosin Cortex Creation

Following the ability to control the actin network distribution within the droplets, the next step was to introduce motor proteins such as myosin II in order to create an actomyosin cortex. Towards this end myosin II was added to the  $MgCl_2/ATP$  channel solution in the same concentration as in the bulk experiments (see Section IV.6.1, experimental conditions 2). As shown in Figure IV.33 (A) actomyosin networks can not interact with the periphery if no gold-linked surfactants used. However, in comparison to experiments without myosin, slight contractions were observed as represented by thicker actin bundles. Furthermore, the actomyosin network in this experiment showed a contractile and motile behavior for up to 20 minutes until all ATP was consumed. Figure IV.33 (B) shows time-lapse images of the dynamic behavior of actomyosin network within the microfluidic network.



Figure IV.33.: Representative images of actomyosin networks encapsulated in droplets without goldlinked-surfactants. Image (A) shows a low magnification fluorescence image of the actomyosin network distributed mainly in the center of the droplet. (B) represents time-lapse images of actomyosin dynamics within the droplets.

Addition of gold-linked surfactants was necessary to create an actomyosin cortex-like network close to the droplets periphery (Figure IV.34 A and B). These Figures show representative image obtained with a low and high magnification of actomyosin networks in gold-nanostructured droplets being in contact with the periphery. No dynamics and motility of the actomyosin network was observed.



Figure IV.34.: Representative images of actomyosin networks encapsulated in droplets witht gold-linkedsurfactants. Images (A) and (B) represent actomyosin networks with gold-nanoparticles on the periphery.

Very low dynamics of the actomyosin networks can be attributed to the non-physiological actin network conditions due to the artificial filament stabilization provided by phalloidin. It is known from literature that phalloidin affects the rigidity of the actin networks<sup>[175]</sup> and perturbs the interaction of myosin with actin filaments.<sup>[176]</sup> Therefore, in the following

experiments to detect the actomyosin network formation, phalloidin-FITC was replaced by more physiological approach such as addition of directly labeled actin. Furthermore, fascin was included to provide bundling of actin filaments.

## 6.4. Optimization of Actin Network Reconstitution in Better Physiological Conditions

In order to mimic better the physiological conditions of the actin network reconstitution and to overcome the afore mentioned drawbacks, phalloidin-FITC was replaced by direct labeled G-actin. Towards this end G-actin was mixed with 1-2 % of alexa (488/568/647)labeled G-actin (life technologies, Germany) to provide the detection of actin network formation. It is worth to mention here that these fluorescence dyes were chosen based on the retention studies presented in Section IV.3.3. Furthermore, we explored the effect of the fascin as a passive crosslinker on dynamic filament organization. Fascin is the major actin crosslinking protein found in a wide range of sub-cellular complexes such as filopodia and the cortex. This protein has been shown to work in concert with other crosslinkers such as  $\alpha$ -actin, although fascin itself is sufficient to form filopodia- and cortex-like bundles in a reconstitution system.<sup>[177]</sup>

To reconstitute actin networks  $0.1 \,\mu\text{M}$  Alexa488-labeled G-actin (experimental conditions 3, see Tables III.3 and III.4) was mixed with  $9.9 \,\mu\text{M}$  non-labeled G-actin and introduced in one of the two aqueous inlet channels (see Figure IV.30). Fascin ( $0.5 \,\mu\text{M}$ ) was mixed together with the MgCl<sub>2</sub>/ATP solution within the G-buffer and introduced into the second aqueous inlet channel. The concentration of fascin was adapted from the previously reported protocols for actomyosin cortex formation on the glass surfaces.<sup>[135]</sup>

Creation of actin networks with the new experimental conditions was analyzed. Figure IV.35 A and B present the distribution of the actin filaments within the droplets produced without and with gold-linked surfactants, respectively. In both cases, it can be observed that the networks formed a highly dense bundle structure. In the case of droplets containing gold-linked surfactants (Figure IV.35 B) the bundles were found in contact with the droplets periphery. This can be attributed to fast cross-linkage by fascin during the actin filaments creation. Additionally, a partial mixture of the fascin phase and the actin phase during droplet formation can create a local polymerization leading to high dense bundle formation.



Figure IV.35.: Fascin bundled actin networks encapsulated in droplets created without and with goldnanoparticels. (A) shows F-actin networks in droplets created without the gold-linked surfactants. Images (B) represent the same experimental conditions but with gold-linked surfactant. (1% of G-actin was labeled directly with Aexa488)

In an attempt to overcome the drawback of the partial mixture, a mixing channel (Figure I.2) was implemented into the microfluidic device immediately after droplets generation. Hereby, the content in the droplet is completely mixed within a few milliseconds.<sup>[178]</sup> Figure IV.36 A shows the effect of the mixing device on the actin filament distribution. Unfortunately, only small improvement can be observed. However, keeping the same concentration of the actin as in the previous experiment, and observing higher volume fraction of the actin bundles within the droplets, one can conclude that the density of the actin network bundles is lower. Therefore, in addition to better mixture of the solutions, more conditions were adjusted to achieve better distribution of the actin networks in the droplets.

To optimize the G-actin polymerization rate the pH of the G-buffer was increased from 7.4 to pH 8. At elevated pH values Crevenna et. al.<sup>[179]</sup> observed decrease in the actin

polymerization rates and increase the actin filament length.

The results for droplets containing actin networks, which polymerized at pH 8 are presented in Figure IV.36 (B). As can be observed F-actin interacts with the whole droplet periphery and no thick bundle structures are observed.



Figure IV.36.: Disstribution of F-actin networks created at different pH-values. (A) and (B) show the representative fluorescence images of actin networks created in the gold-nanostructured droplets at pH 7.4 and 8, respectively.

To mimic better the biophysical condition within the droplets in terms of diffusion coefficient and ion concentration, 2.5 % w/w of PEG (MW 10000 g/mol) was added and the concentration of MgCl<sub>2</sub> was reduced to 15 mM in the G-buffer solution. These modifications were necessary to mimic better the ion concentration<sup>[180]</sup> and diffusion co-efficient<sup>[181]</sup> as they are measured in the cellular cytosol. A representative image of actin

networks produced with this condition is shown in Figure IV.37. It can be observed that actin networks interact with the droplets periphery creating cortex-like structures and no thick bundles were detected. All further experiments creating actomyosin cortexes (see next sections) were reconstituted using the biophysical conditions according to the results achieved in this section.



Figure IV.37.: Actin networks in droplets with the optimized biohysical and biochemical conditions. The environment in the droplets allowed to mimic the cytosol diffusion conditions by addition of 2.5 % w/w of PEG (MW 10000 g/mol). By this adaptation the MgCl<sub>2</sub> could be reduced 15 mM.

## 6.5. Reconstitution of Actomyosin Cortex with Optimized Biophysical and Biochemical Conditions

Following the successful optimization of biophysical and biochemical conditions, as described in the previous Section, the next step towards the reconstitution of actomyosin cortex was to introduce myosin II in the  $MgCl_2/ATP$  channel (Experimental conditions 6, see tables III.3 and III.4). To detect the position of the myosin within the actomyosin networks Alexa 568-labeled myosin II (ratio 1:5 to non-labeled myosin II, kind gift of Anne Bernheim, Ben-Gurion University, Israel) was used. Additionally, an ATP recovery buffer (see Section III.7) was introduced in both aqueous inlets to extend the activity time of myosin II clusters. Figure IV.38 shows the representative combined images of actomyosin networks reconstituted within the droplets without (Figure IV.38 A) and with (Figure IV.38 B) gold-linked surfactants. It can be observed that in the case of droplets produced without gold-linked surfactants the actomyosin network distributed equally within the droplet, no interaction with the droplet periphery was observed. Moreover, myosin II colocalized (indicated by the arrows) within the actin fibers as it is expected. In the droplets produced with gold-linked surfactants a strong interaction with the periphery of the actomyosin networks is observed. However, myosin II was not detected on the periphery of the droplets but was colocalized within the actin filament network. This indicates that actin mainly interacts during the polymerization with the gold-nanoparticles, which make sense, since the ratio between the actin monomers and the myosin II clusters is 1000:1.

The actomyosin dynamics will be discussed and described in the following section. It is worth mentioning here that the interaction with the periphery of the droplets was essential to induce self-propelling of the actomyosin system within the droplet.



Figure IV.38.: Representative images of actomyosin networks encapsulated in droplets without and with gold-linked-surfactants, respectively. (A) and (B) show representative flourescence images of reconstitute actomyosin networks in droplets produced with and without gold-linked surfactants, respectively.

### 6.6. Analysis of Actomyosin Network Dynamics

Time-lapse images were recorded to analyze the dynamics of actomyosin networks (see Section III.7.2) in the droplets. Figure IV.39 (A) shows representative time-lapse images and schemes of the actomyosin contraction within a droplet containing no gold-linked surfactants.



Figure IV.39.: Representative fluorescence time-lapse images of actomyosin network motion in droplets and their schematics. (A) shows a schematic and real contraction of actomyosin network in a droplet without gold-linked surfactant. (B) presents a schematic and real rotation of the actomyosin network in droplets containing gold-linked surfactants. It can be observed that the contraction occures immediately after the actomyosin networks formation and lasts for about 8 min. The dynamics of actomyosin networks contraction is in a good agreement with the previously reported works, in which the actomyosin dynamics was investigated on 2D surfaces<sup>[131,135]</sup> or within the 3D compartments<sup>[137,182]</sup>.

More interesting, actomyosin dynamics was observed in the droplets containing goldlinked surfactants (Figure 8 IV.39 B). In this case, no significant network contraction was detected, as in the case were no gold linked surfactants were used, but a rotation (self-propelling) of the actomyosin cortex was observed. It is worth mentioning here that rotation was observed in different directions (i.e. clock-wise/counter clock-wise). This observations are similar to pervious works were polymer stabilized water-in-oil droplets were used to reconstitute actomyosin cortex from *Xenopus* laevis egg cytoplasmic extracts.<sup>[137]</sup> In this study the linkage to the droplets periphery was preformed via actin nucleation complexes. However, in both cases, due to the spherical structure of the droplets it is not clear if the whole droplet is rotating or only the actomyosin network.

To break the symmetry of the micro compartment droplets a new type of bead-linked surfactants was synthesized (described in Section IV.1.6).



Figure IV.40.: Representative brightfield and fluorescence images as well as cryo-SEM micrographs of the bead-linked-surfactants in the contact with the droplet outside periphery. (A) shows representative brightfield and fluorescence images of the droplet having three beads on its periphery (indicated by arrows). (B) presents high resolution cryo-SEM micrographs of beads in contact with the droplet periphery.

Detailed analysis is shown in Section VI.3. These 2 µm in diameter bead-linked surfactants were introduced through the oil phase. By diffusion the beads reached the droplet outer interface where they immobilized due to the electrostatic interactions of the PFPEtails (for more details see Section VI.3). Figure IV.40 A and B show the representative images and micrographs of the droplets-beads interactions as observed by light-microscopy and cryo-SEM microscopy, respectively.

Beads linked to the outside periphery of the droplet helped to track the rotation of the droplets and to correlate it to the motility of the actomyosin cortex. Figure IV.41 shows representative time-lapse images (total 30 min) of the droplets rotation induced by the actomyosin cortex motility. By tracking the bead displacement it can be observed that the droplet rotation is not limited to one plane. Moreover, the rotation speed of the actomyosin cortex is the same as of the bead. These results confirm that the actomyosin self-propelling can only be achieved by the interaction with the droplet interface. In the absence of the droplet-actin networks interaction only contraction of the actomyosin network can be detected.



Figure IV.41.: representative time-lapse images (total 30 min) of the droplets rotation induced by the actomyosin cortex motility. (A, B and C) show the representative brightfield, fluorescence and schematic images of the droplet rotation induced by the actomyosin cortex motility.

To provide more detailed analysis concerning the motility distribution within the droplets a radial velocity profile was tracked and plotted (see Figure IV.42, analyzed by Marco Linke from Ulrich Schwartz group, physical department Heidelbreg university).<sup>[183,184]</sup> Except at the edge of the droplet the profile is almost linear. This velocity profile hints on the scenario that the actomyosin network is the driving force for movement and the droplet rotation is induced by the frictional forces created by the surfactant layer.



Figure IV.42.: Radial velocity profile of rotating droplet.

To test if the myosin-induced contractility is the driving force for the droplets rotation, a myosin inhibitor, blebbistatin  $(23 \,\mu\text{M})^{[185,186]}$  was used (experimental conditions 7, details Tables III.3 and III.4). No rotation and actomyosin motility was observed in the droplets containing blebbistatin. To deactivate blebbistatin a 458 nm laser (1mW, applied 5 times for 100 ms) was used.<sup>[185,186]</sup> Rotation of the droplets was observed following the deactivation of blebbistatin. The main outcome is that the myosin-driving forces are essential for the droplets rotation.

A proposed mechanism for the rotation of actomyosin networks in defined compartments is a spontaneous break down of discrete symmetry due myosin induced contractility.<sup>[187]</sup> In the absence of tread-milling effects the dynamics arises solely from the contractile motion of myosin motors as also described by Tjhung et. al.<sup>[187]</sup> In more details, myosin activity induces a molecular scale torque, (Beusang 2008) which leads to the generation of active torque density at larger scales, causing an isolated part of cortex to contract and rotate. The non-symmetric immobilization of the actin cortex to the droplet periphery leads to the generation of a force- and a torque-momentum, which in a superposition provides a direction to the droplet rotation.<sup>[188]</sup> It is worth mentioning here, that no actomyosin rotation was observed when using higher actin concentration (20  $\mu$ M) for reconstitution of actomyosin cortexes. The lack of the droplets rotation at higher actin concentration can be explained by symmetric distribution of the actomyosin filaments within the droplets.

Wheel gymnastics (Figure IV.43) can serve as an analogy for the aforementioned mechanism. Due to the stable anchoring points between the person and the wheel the radial velocity profile within the wheel is linear. In the case of actomyosin cortex in the droplets, the anchoring points between the actin network (i.e. the person) and the droplet perephery (i.e. the wheel) are the gold-linked surfactants, which have certain freedom to move. Therefore, the anchoring points between the actomyosin and the droplets have a more frictional character and are hereby less stable. This is also a reason to slight deviation from the linear radial velocity profile within the droplets as shown in Figure IV.42.

Furthermore, in contrast to the wheel gymnastics, the rotation of the droplets do not generate movement to a particular direction. This can be attributed to the lack of friction between the outside periphery of the droplet and its external environment, like in the case of the friction of wheel and the floor. To provide friction between the outside periphery of the droplet and the glass surface bead-linked surfactants can be used.



Figure IV.43.: Wheel gymnastics as an analogy to actomyosin driven self propelling in droplets.

Figure IV.44 shows a hopping dynamic of the self-propelling bead-containing actomyosin droplets. Three major forces, including the gravity force ( $F_g$ , indicated by red arrow), force generated by actomyosin contraction ( $F_c$ , indicated by black arrow) and the frictional force ( $F_f$ , indicated by blue arrow) are responsible for the hopping behavior. Gravity force is generated due to the high differences in the density between the FC-40 oil ( $\rho$ =1855 kg/m<sup>3</sup>) and the water phase ( $\rho$ =998 kg/m<sup>3</sup>). This force pushes and keeps the droplets in the proximity to the cover glass of the analysis chamber (see section III.2.3). Force generated by the actomyosin contraction generates the rotation of the droplets as discussed above. The friction force is generated between the bead and the glass surface when the bead is reaching the glass due to the droplets rotation. This frictional force works against the rotation of the droplets, herby against the actomyosin contraction and it is sufficient to create a failure of the powerstroke mechanism, resulting in the rupture of the actin-myosin interactions. Hopping of the droplets, kind of spring release effect, is generated as a result of the instant rupture of the actin-myosin interactions.



Figure IV.44.: A representative time-lapse of hopping dynamics of the self-propelling bead containing actomyosin droplets. The upper row shows the time-lapse images of the rotating and hopping droplet. The lower row presents the schematics of the mechanism responsible for the self-propelling and hopping behaviour of the droplets.  $F_g$  (red arrows),  $F_f$  (blue arrows) and  $F_c$  (black arrows) represent the gravity, friction and contraction forces, respectively. The black dashed arrow represent the direction of the droplet movement.

# V. Summary and Outlook

Microfluidics is a technology that profits from minimal sample consumption, minimal exposure to hazardous materials, increased analysis speed and data precision. Due to these numerous advantages, the major aim of this thesis was to explore the capacities of microfluidics, particularly droplet-based microfluidic technology in the following synthetic biology topics: 1) Mimicry of the immune system cellular environment, with the ultimate goal of programing T cells for adoptive T cell therapy; 2) bottom-up assembly of minimal synthetic cells.

## Microfluidic Droplets as Soft Nano-Structured Biomimetic Systems for Immunological Applications.

Despite the beneficial impact of modified T cells, the road towards broadly applicable adoptive T cell therapy is still long and winding. The major challenge is the guidance and exquisite regulation of immune processes *ex vivo*. In part, this is due to the difficulties of simulating *ex vivo* the intimate cellular interactions that occur between T cells and antigen-presenting cells (APCs). The fate of T cells is not solely regulated by the presence of certain molecules on the surface of APCs but also by their density and spatial distribution. Moreover, mechanical properties of APCs and force-dependent generation of, so called, supramolecular activation clusters (SMACs) during the formation of an immunological synapse (IS), play a crucial role in T cell fate regulation.

In this thesis a novel approach to the formation of nano-structured and specificallybiofunctionalized water-in-oil emulsion droplets with the potential to serve as 3D APC surrogates was developed (references 5-8 in publication list section VI.1). Towards this end, a new class of gold-linked surfactants was synthesized and stable cell-size microfluidic droplets with various gold-nanoparticle densities were created. Two chemical approaches were used, both to test the efficiency of the GNps inside the droplets to serve as anchoring points and to provide the key chemical and biological functions of APCs. Remarkably, more than 90% of encapsulated T cells were found to be in contact with the inner periphery of the droplets, compared to less than 10% in the case of non-functionalized droplets. Moreover, T cells in the functionalized droplets exhibited increased proliferation and remained viable for up to 5 days of incubation without any external nutrition. Furthermore, the dynamic properties of the droplets surfactant layer allowed formation of SMACs due to the T cell-droplet interaction, which is a hallmark for the efficient IS.

This research highlights the advanced properties of polymer-stabilized droplets for use in T cell stimulation. The combination of flexible biofunctialization and pliable physical droplet properties work in tandem, providing a flexible and modular system that closely models *in situ* APC-T cell interactions. Moreover, the ability to create a well-defined picoliter environment for T cell stimulation is preeminent for long-term monitoring of individual T cells over the course of their activation and differentiation. Therefore, in sum, this systems bears a strong potential for clarifying individual T cell response mechanisms triggered by collective APC-T cell molecular interactions. However, it should be noted that many aspects were not explored in this research and warrant further investigation.

In this thesis only adhesion and stimulation ligands were used, but the generation of productive T cell responses, such as proliferation and differentiation, requires the engagement of co-stimulatory signals and adhesion complexes in addition to stimulation signals. Therefore, combination of these signals within the droplets together with the biophysical properties will be a necessary requirement for better understanding the force-dependent conformational changes during IS formation. Moreover, the developed system offers the possibility to achieve control over identity and quantity of bio-active ligands on the droplet periphery. This advantage is crucial and opens new possibilities to understand better the molecular mechanisms of the assembly of TCR activation clusters, and consequently to address the following very important open questions:

i) is the T-cell activation simply dose-dependent in regards to the number of stimulation microclusters; ii) what is the threshold number of stimulation microclusters required for the T-cell activation; and iii) what is the optimal ratio between the stimulation, co-stimulation signals and adhesive molecules required to create the best stimulation conditions.

## 2. Droplet-Based Microfluidics for Bottom-Up Assembly of Minimal Synthetic Cells

Actin cytoskeleton is a very important regulator of intracellular signaling pathways. Therefore, it affects many cellular processes, including, but not limited to, cell division, force generation, polarization, intracellular transport and motility. This broad range of actin-mediated processes also underscores their central physiological roles, as well as their involvement in a wide variety of disease states. Although attaining a fundamental characterization of how macroscopic cytoskeletal dynamics emerge from it molecular constituents is a compelling goal, little understanding has yet been accomplished, mainly due to the extensive complexity of the cytoskeleton network and difficulties inherent in studies *in vivo*.

The research within this thesis focused on dissection of complex cellular sensory machinery by engineering of biomimetic systems for controlled manipulation of subcellular units *in vitro*. Towards this end, an automated droplet-based microfluidic system was used to generate water-in-oil nanostructured droplets as cell-size compartments for bottom-up assembly of the minimal number of proteins required for a "simple synthetic cell". While the applied methodology has a potential for assembly of a wide range of subcellular functional units, the focus was on the reconstitution of the actomyosin cortex. (references 1 in publication list section VI.1)

As a first step, to optimize the retention of labeled biomolecules and fluorophores within emulsion droplets, key physical and chemical factors of fluorescent dyes, surfactants and buffer conditions were investigated in details (references 4 in publication list section VI.1) The outcomes of this investigation provide crucial information for the successful reconstitution of actomyosin cortex within the nanostructured droplets (references 1 in publication list section VI.1). Furthermore, the ability to control the filaments organization by linkage to the droplets periphery was very important and following addition of myosin II motor proteins allowed to achieve droplets migration and self-propulsion (references 1 in publication list section VI.1).

The described achievements highlight the potential of the droplet-based-microfluidics system for a bottom-up assembly of sub-cellular units. However, these results cover only a small part of what this technology can propose. For example, pico-injection technology has to be used more extensively in the bottom-up assembly approaches. The results as presented in Figure V.1 can guide the perspective for further implementation of pico-injection microfluidic unit in testing the importance of subsequent introduction of various proteins. It can be observed that premixed differently labeled actin was distributed equally in the droplets (Figure V.1 upper row). Figure V.1 upper row shows that premixed differently labeled G-actins formed actin networks within the droplets combined of all three labeled actin. In contrast, actin network created by pico-injection subsequent introduction of differently labeled G-actin showed segregation of differently labeled F-actin (Figure V.1 lower row).



Figure V.1.: Comparison of actin network formation as a result of premixed and subsequent introduction of differently labeled G-actin. Upper row shows representative fluorescence images of Alexa488, Alexa568 and Alexa647 labeled actin networks. The bottom row shows the actin networks generated by pico-injection (middle row) subsequent introduction of Alexa488, Alexa568 and Alexa647 labeled actin monomers.

Another experiment was performed to highlight the importance of implementation of pico-injection units for the bottom-up assembly of sub-cellular units. In this experiment, actin and myosin were introduced via pico-injection into droplets containing a GUV like lipid bilayer on the periphery (Figure V.2). The results show that this approach can produce large numbers of GUVs containing actomyosin networks stabilized by droplets. In this approach actin could be linked via talin (recently purified in our group) to integrines in the lipid bilayer. The possibilities have the potential to allow a bottom-up assembly of focal adhesion complexes in the near future.



Figure V.2.: Actomyosin distribution in the GUVs-containing droplets. Two experimental steps were required for stable droplets creation with F-actin and GUVs: the first is creation of droplets with GUVs (red, rhodamine labeled POPE), and the second is an injection of these droplets with Alexa488-labled G-actin solution by pico-injection.

# VI. Appendix

## 1. List of Publications

1) J. Frohnmayer; M. Weiss; J.-W. Janiesch; B Haller; I. Platzman; J. P. Spatz: GUVs within the Polymer-Stabilized Water-in-Oil Microfluidic Droplets. Book Chapter in preparation. 2015

2) J.-W. Janiesch; B Haller; A. Bernheim; M. Schwering; U. Schwarz; M. Linke; I. Platzman; J. P. Spatz: Reconstitution of Actomyosin Cortex in Droplet-Based Synthetic Cells. In preparation. 2015

3) M. Weiss; J. Frohnmayer; **J.-W. Janiesch**; B Haller; I. Platzman; J. P. Spatz: Generation on Stable GUVs within the Polymer-Stabilized Microfluidic Droplets. To be submitted. 2015

4) J.-W. Janiesch<sup>\*</sup>; M. Weiss<sup>\*</sup>; G. Kannenberg; J. Hannabuss; T. Surrey; I. Platzman; J. P. Spatz: Key factors for stable retention of fluorophores and labeled bio-molecules in droplet-based microfluidics. *Analytical Chemistry* 87 (4), pp. 2063-2067, 2015. (\*equally contributed)

5) I. Platzman; J.-W. Janiesch; G. Kannenberg; J. Matic; J. P. Spatz: PEG-Based Antigen-Presenting Cell Surrogates for Immunological Applications. In: Soft Matter *Nanotechnology : From Structure to Function*, pp. 187-215 (Eds. Chen, X.; Fuchs, H.). Wiley-VCH, Weinheim 2015

6) I. Platzman; K. Gadomska; J.-W. Janiesch; I. Louban; E. A. Cavalcanti-Adam; J.
P. Spatz: Soft/Elastic Nanopatterned Biointerfaces in the Service of Cell Biology. In:

*Micropatterning in Cell Biology*, Part A, pp. 237-260 (Eds. Piel, M.; Théry, M.). Academic Press, Amsterdam 2014

7) I. Platzman<sup>\*</sup>; J.-W. Janiesch<sup>\*</sup>; J. P. Spatz: Synthesis of nanostructured and biofunctionalized water-in-oil droplets as tools for homing T cells. *Journal of the American Chemical Society*, 135(9):3339–3342, March 2013. (\*equally contributed)

8) I. Platzman; J.-W. Janiesch; J. Matic; J. P. Spatz: Artificial Antigen-Presenting Interfaces in the Service of Immunology. *Israel Journal of Chemistry* 53 (9-10), pp. 655-669, 2013

## 2. Structure of Used Fluorophores



 $Figure \ VI.1.: \ \textbf{Overview of the chemical structures of used fluorescent dyes}$ 

### 3. Bead-Surfactant Analysis

To analyze the ability of the synthesized bead-surfactant (Section IV.1.6) to serve as a marker in the droplet periphery several experiments were preformed including confocal microscopy, diffusion and dislocation analysis. For diffusion and dislocation experiments the distribution of the median values of the bead-surfactant speeds in oil phase and in the droplet interface were compared.

For confocal analysis fluorescent beads (Fluorobright PLAIN Y G, radius:  $0.57 \,\mu\text{m}$ , excitation max. = 441 nm, emissions max. = 486 nm, 1.08  $10^{11}$  particles/ml, Polyscience Inc, Warrington, PA, USA) were synthesized according to the reaction presented in Section IV.1.6 and combined with droplets containing a lipid bilayer on the inner droplet periphery to enhance the visibility of the location the bead-surfactant. The results presented in figure VI.2 show the fluorescent signal of the beads in close contact to the lipid bilayer proving that the bead of the bead-surfactant is incorporated in the surfactant layer on the droplet periphery.

To create a lipid bilayer on the inner periphery of the droplets, lipids (DOPE-Rohdamin B (80%), DOPC (10%), DOPS (10%)) solved in chlorform were mixed with a 8:1:1 ratio, dried to remove the chloroform and resolved in ultra pure water with 20 mM MgCl<sub>2</sub>.



Figure VI.2.: Representative confocal images of bead-linked-surfactant in contact to the outer lipidcontaining droplet periphery.

In diffusion experiments preformed in an analysis chamber (Section III.2.3) a significant difference in diffusion speeds of free bead-surfactants and in the droplet periphery incorporated bead-surfactants could be shown (p-value:  $5.87 \times 10^{-10}$ ) (Figure VI.3). This difference in diffusion speeds decreased over time due to low density of the beads (bead  $\rho$ = 1.05 g cm<sup>-3</sup>, FC-40  $\rho$  = 1.85 g cm<sup>-3</sup>) resulting in a contact to the top glass of the analysis chamber. By this friction was increasesd and no free movement was observed afterwards. Therefore only results shortly after production were taken into consideration.



Figure VI.3.: Analysis of beads diffusion in the oil phase. The diagrams A and B present the diffusion of the bead-surfactant. (A) represents the diffusion of free bead-surfactant in the oil phase. Diagram B shows the diffusion of bead-surfactant in the droplet periphery. Red line represents the median for A and B. The diffusion for free bead surfactant was  $17.21 \pm 3.05 \,\mu m \, ms^{-1}$  before and and  $1.31 \pm 0.25 \,\mu m \, ms^{-1}$  rafter sedimentation. The median for bead-surfactant speed in the periphery was  $0.41 \pm 0.36 \,\mu m \, ms^{-1}$ 

In a second experiment the dislocation of beads was analyzed. In figure VI.4 the difference of free bead-surfactants and in the periphery incorporated bead-surfactants can be clearly seen. Here the p-value is 3.6x 10<sup>-11</sup>. The difference of the dislocation is much higher due to the free movement of free bead-surfactant compared to the beads in the periphery which can only move short distances due to the dense surfactant layer. The results of these experiments together with IR-analysis and cryo-SEM images of beads in the periphery of actin droplets (Section IV.1.6.1) show the success of the reaction and confirm the expected behaviour of this new synthesized bead-surfactant.



Figure VI.4.: Analysis of beads dislocation in the oil phase The diagrams A and B present the dislocation of the bead-surfactant. (A) represents the dislocation of free bead-surfactant in the oil phase. Diagram (B) shows the disslocation bead-surfactant in the droplet periphery. Red line represents the median for A and B;  $80.4 \pm 38.7 \,\mu m \, ms^{-1}$  and  $2.7 \pm 3.3 \,\mu m \, ms^{-1}$  respectively.

### 4. Proteins

#### 4.1. Actin

Actin is a globular protein found in all eukaryotic cells with a variation of less than 5% in the sequences of amino acids between specie.<sup>[189]</sup> 1 to 5% of cell mass is actin as monomers (length 55  $\mathring{A}$ ) or in filamentous form. The actin monomer has a weight of 42 kDa and consists of 375 amino acids in 4 subdomains called G-actin due to it's globular shape. F-actin has a double helical shape where each monomer has an offset of 166° and 2.75 nmwith a diameter of about 5-9 nm and up to several micrometer length.<sup>[190?]</sup> The persistence length of actin is about 17 µm max.<sup>[191,192]</sup>

Actin polymerization is mediated by the presence of ATP which is hydrolyzed during the actin polymerization.<sup>[193]</sup> In the first step of actin polymerization 2-4 ATP saturated G-actins assemble. This step is the time determing step in actin polymerization.<sup>[194]</sup> Polymerization occurs then at both ends of the actin filaments but has different kinetics due to structural asymmetrice of the film endings. The so called (+) and (-) end have different polymerization and depolymerization constants which are  $k_1 = 11.6 \pm 1.2 \,\mu M^{-1} s^{-1}$ and  $k_{-1} = 1.4 \pm 0.8 \,\mu M^{-1} s^{-1}$  for the (+) end and  $k_2 = 1.3 \pm 0.2 \,\mu M^{-1} s^{-1}$  and  $k_{-2} = 0.8 \pm 0.3 \,\mu M^{-1} s^{-1}$  for the (-) end.<sup>[195]</sup> Actin filaments together with Myosin II motor proteins represent the major components of muscle fibers. To investigate actomyosin dynamics in many experiments fluorescent labeled phalloidin is used although it can change the elasticity of actin filaments.<sup>[196]</sup> In the last years more direct label actin is used to analyze actin dynamics.

#### 4.2. Myosin II

Myosin II protein is a sub-group of the myosin superfamily, also called motor proteins, which in particular occurs in stress fibers and the sarcomeres of striated muscle. They are composed of six polypeptide chains, two 220 kDa heavy chains and four modulatory light chains, each with 20 kDa.<sup>[190,197]</sup> Clustering of the heavy chains results in an approximately 150 nm long  $\alpha$ -helical structure having on the N-termini two globular head regions which are about 17 nm long. The light chains are associated with the short neck regions between the head and tail region of the myosin molecule. Myosin II molecules can form filaments with 18 nm in diameter and up to 1- 2 µm in length, depending on the KCl concentration.<sup>[135]</sup> The head regions of these myosin II filaments can attach to parallel actin filaments. ATP hydrolysis and exchange leads to a conformational change, resulting in a 5 nm power stroke and associated muscle contraction.<sup>[198]</sup>

In stress fibers non-muscle myosin II creates a complex with 10-30 actin filaments and other actin binding proteins. Structural and functional elucidation was achieved by fluorescence labeling of  $\alpha$ -actinin and myosin. The case of discernible striations of filaments led to the development of models which have great similarity with sarcomeres coupled with high adaptability and broad contractility of stress fibers.<sup>[199]</sup>

#### 4.3. Phalloidin

Phalloidin is a toxin found in the green amanita with  $LD_{50}$  of 2-3 mg/Kg. In actin experiments it is used to reduce the actin depolymerization rate.<sup>[173]</sup> With a dissociation constant KD of 10<sup>-8</sup> M phalloidin binds the interaction sites between the G-actin subunits of F-actin strands, reducing the depolymerization by a factor of 30.<sup>[174]</sup> In actin experiments fluorescently labeled derivatives such as phalloidin TRITC/FITC are used to stabilize actin filaments and to observe them with fluorescence microscopy. A drawback in using phalloidin is the artificial stiffening combined with dramatically reduced depolymerization of F-actin which can lead to an inactivation of microfilamentous functions and is in stark contrast to biological bundling protein fascin.<sup>[200]</sup>

#### 4.4. Fascin

Fascin is an actin-bundling protein that was first isolated by Kane et.al.<sup>[201]</sup> from cytoplasmic extracts of sea urchin eggs. Fascin is a compact protein of 55 to 58 kDa. It bundles actin filament parallel with a distance of about 10 nm with spaced intervals (one fascia per 4-5 actin).<sup>[202]</sup> In vivo fascin is mostly located in the filopodia to decrease actin depolymerization by bundling. The bundling of actin filaments by fascin increases the mechanical stiffness of the filaments whereas  $\alpha$ -actinin increases the elasticity. Fascin has been used before in several investigations<sup>[203]</sup> as it promotes actin polymerization creating a nucleus from where actin filaments can elongate and bundle.

#### 4.5. Blebbistatin

Blebbistatin (BLEBB) is a small cell permeable selective myosin II inhibitor that was originally discovered as the result of a high throughput screen for inhibitors of nonmuscle myosin (NMM) II.7citeTakeshiSakamoto:2005wo BLEBB, discovered by means of a high through-put small molecule screening for inhibitors of non-muscle myosin IIA, was recently reported to be a selective in vitro inhibitor of the myosin II isoforms expressed by striated muscle and non-muscle cells (IC50= 0.5–5 mM) but with reported poor inhibition of purified turkey gizzard SMM II(IC50 80 mM).<sup>[204]</sup> Thus, BLEBB, in the concentration range of 0.5–5 mM, was found to be an ATPase inhibiting agent specific for myosin II. Original studies showed BLEB exhibited an interesting specificity for several striated muscle and non-muscle myosins, including Dictyosteliummyosin II, non-muscle myosins IIA and IIB, scallop striated muscle myosin II, porcine muscle myosin II, whereas SM and non-conventional myosins (I, V, and X) have been reported to be little influenced.<sup>[204]</sup>

#### 4.6. Deformability of Droplets with Actomyosin

Droplet deformability experiments were performed in oder to compare polymerized actomyosin cortexes with droplets containing G-actin and myosin as monomers and no ATP/MgCl<sub>2</sub>. The experiments were done using the same flow rates as in the experiments presented in Section IV.2.2. For these measurements experimental conditions 6 with and without  $ATP/MgCl_2$  (see tables III.3 and III.4) and a deformation device with two aqueous inlets was used.

As the results of non polymerized actin and myosin at t=0 (first and last chamber) were similar to the results of droplets where actin polymerized and because the deformation after 30 minutes had the same value as in the last chamber (t=0), only the deformation results of actomyosin that polymerized are represented.

The results presented in Figure VI.5 show that actomyosin encapsulation in droplets had little to no effect on the change of deformability of droplets compared to droplets with water (see Section IV.2.2), if values of the first and the last chamber (t=0 min) are compared to the results in chambers 1 and 50 to 100 in Figure IV.11. The results show that in the first second after droplet production the accretion of surfactants is the dominating effect and that the encapsulation of proteins dose not change the deformability of droplets.

After 30 minutes droplets with non-polymerized- and polymerized-actomyosin networks were reintroduced into the deformation device using same flow rates as in the first measurement at t=0, to be able to compare the data. Here, the results of polymerized-actomyosin networks of the first and last chamber show deformation values which are smaller that in the first chamber after droplet production. This indicates that actomyosin polymerization stiffed the droplet and that networks were in contact with the droplet periphery. Furthermore, the deformation in the first and last chamber have the same value indicating that actomyosin networks are stable, even if they get squeezed 100 times in the narrow channels between the deformation chambers.



Figure VI.5.: Deformability of droplets within encapsulated actomyosin networks.

## 5. Surface Tension

**Tri-7000-PEG-1400** Pendant Drop measurements characterized the influence of surfactants on the FC40 water interface. For Tri-7000-PEG-1400 two dilution series were prepared and tested. One showed signs of contamination as seen in figure VI.6. The other dilution series, shown in dark blue, showedhas an exponential decay of IFT which and yields  $\gamma_{CMC} = (16.47 \pm 8.47) \,\mathrm{mN} \,\mathrm{m}^{-1}$  after surpassing the CMC. At this point surface pressure  $\Pi_{CMC}$  was  $(27.50 \pm 8.47) \,\mathrm{mN} \,\mathrm{m}^{-1}$ . The CMC is extrapolated to  $(45.10 \pm 113.19) \,\mathrm{\mu}$  M. Surface excess  $\Gamma$  is  $(4.04 \pm 0.33) \,\mathrm{\mu} \,\mathrm{M/m^2}$  and therefore the area *a* one molecule occupies in the interface is  $(41.11 \pm 12.93) \,\mathrm{\AA^2}$ .

**Tri-7000-PEG-600** Three dilution series were tested as shown in figure VI.7. The exponential decay with  $\alpha$ ,  $\beta$  and  $\eta$  yielded a  $\gamma_{CMC}$  of



Figure VI.6.: Summary of surface tension measurements of droplets containing TRI7000 surfactants. Influence of Tri-7000-PEG-1400 on IFT of FC40 in water, displayed as  $IFT(= y) [mNm^{-1}]$  over concentration(= x) [ $\mu$ M]. Measured IFTs for different dilution series are displayed in distinct colours, the final exponential fit with  $\alpha$ ,  $\beta$  and  $\eta$  is displayed in red. The 95% confidence interval for pure FC40 in water is shown in grey. Error bars indicate 2 standard deviations. 2 dilution series were tested.

 $(19.55 \pm 2.11) \,\mathrm{mN}\,\mathrm{m}^{-1}$  with surface pressure  $\Pi_{CMC} = (23.08 \pm 2.11) \,\mathrm{mN}\,\mathrm{m}^{-1}$ , a CMC at  $(48.75 \pm 35.52) \,\mathrm{\mu}\,\mathrm{M}$  and surface excess  $\Gamma$  of  $(3.40 \pm 0.33) \,\mathrm{\mu}\,\mathrm{M/m}^2$  with one surfactant molecule occupying  $(48.82 \pm 4.78) \,\mathrm{\AA}^2$  in the interface. Two of three dilution series show an increase in IFT at 1 and 10 m M. In analogous measurements with sodium dodecyl sulfate (SDS) this is usually a sign of impure SDS, which might contain hydrolysed SDS.

**Di-7500-O-Me-750** Three dilution series were measured as shown in figure VI.8. One of which, shown in light blue, seemed to be an outlier. The exponential decay with  $\alpha$ ,  $\beta$  and  $\eta$  yielded a  $\gamma_{CMC}$  of  $(18.50 \pm 2.98) \,\mathrm{mN} \,\mathrm{m}^{-1}$  with surface pressure  $\Pi_{CMC} =$  $(26.08 \pm 2.98) \,\mathrm{mN} \,\mathrm{m}^{-1}$ , a CMC at  $(148.65 \pm 9.68) \,\mu$  M and surface excess  $\Gamma$  of  $(3.86 \pm 0.46) \,\mu \,\mathrm{M/m^2}$ with one surfactant molecule occupying  $(43.06 \pm 9.69) \,\mathrm{\AA^2}$  on the interface. Measured data however show a plateau at about  $1 \,\mu \,\mathrm{M}$ . This might indicate the presence of a polymer. If so, the critical aggregate concentration (CAC) of this polymer would be  $(0.27 \pm 0.31) \,\mu \,\mathrm{M}$ at a surface pressure of  $\Pi_{CAC} = (7.17 \pm 5.92) \,\mathrm{mN} \,\mathrm{m}^{-1}$ .



Figure VI.7.: Summary of surface tension measurements of droplets containing TRI7000short surfactants. Influence of Tri-7000-PEG-600 on IFT of FC40 in water, displayed as  $IFT(=y) [mN m^{-1}]$  over concentration(= x) [µM]. Measured IFTs for different dilution series are displayed in distinct colours, the exponential fit with  $\alpha$ ,  $\beta$  and  $\eta$  is displayed in red. The 95% confidence interval for pure FC40 in water is shown in grey. Error bars indicate 2 standard deviations. 3 dilution series were tested.



Figure VI.8.: Summary of surface tension measurements of droplets containing DI7000 surfactants. Influence of Di-7500-O-Me-750 on IFT of FC40 in water, displayed as  $IFT(= y) [mNm^{-1}]$  over concentration(= x) [µM]. Measured IFTs for different dilution series are displayed in distinct colours, the exponential fit with  $\alpha$ ,  $\beta$  and  $\eta$  is displayed in red. The 95% confidence interval for pure FC40 in water is shown in grey. Error bars indicate 2 standard deviations. 3 dilution series were tested. A plateau at 1µM might indicate presence of a polymer.

## 6. FT-IR Analysis

Figure VI.9 (A) shows the representative FTIR spectra of the PFPE(7000)-carboxylic acid reactant and the triblock surfactant product PFPE(7000)-PEG(1400)-PFPE(7000) (TRI7000). This figure presents five major bands at 1701, 1775, 2848, 2956 and 3556 cm<sup>-1</sup>. The band at  $1701 \text{ cm}^{-1}$  is attributed to the ester (C=O) stretching mode. The band at 1775 cm<sup>-1</sup> is attributed to a stretching mode of the (C=O) bond of the PFPE-carboxylic acid which is strongly blueshifted (by  $50 \text{ cm}^{-1}$ ) due to the electronegative fluor atoms in alpha position to the carboxylic group. The same blueshift of the carboxylic (C=O) band was observed previously in the studies measuring the FTIR spectrum of the trifluoroacetic acid. The bands at 2848 and 2956 cm<sup>-1</sup> are assigned to symmetric and asymmetric stretching modes of the PEG (C-H) groups of the PFPE-PEG-PFPE product.2 The band at 3556 cm-1 is assigned to the asymmetric stretching (OH) vibrations. Figure VI.9 (B) shows representative FTIR spectra of the DI7000 and PFPE(7000)-carboxylic acid. This figure presents major bands at 1698, 1775, 2889, 2993 and 3556  $\rm cm^{-1}$ . The band at 1698  $cm^{-1}$  represents a stretching mode of the ester (C=O). The broad band at 2889 and 2993 cm<sup>-1</sup> represents the symmetric and asymmetric stretching of PEG (CH). The band at 3556 cm<sup>-1</sup> is assigned to the asymmetric stretching (OH) vibrations.



Figure VI.9.: Representative FT-IR spectra of a triblock-copolymer surfactant and the PFPE. Figure. FTIR spectra of the reactants and the products of the surfactant synthesis. FC-40 perflourinated oil was used as a background solvent to obtain the spectra. (A) Comparison between the PFPE(7000)-carboxylic acid (20 mM) as a reactant and the PFPE(7000)-PEG(1400)-PFPE(7000) triblock product (20 mM). (B) Comparison between PFPE(7000)-carboxylic acid as a reactant (20 mM) and the PFPE(7000)-PEG-OMe(750) diblock product (20 mM).

### 6. FT-IR Analysis

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## 8. R scripts

### 8.1. R plot

########### # Inhalt ## ###########

- ## 1. Dateien laden (11)
- ## 2. Geschwindigkeite
  ## 2.1 Beads in Öl (46) Geschwindigkeite der Beads (24)
- ## 2.2 Beads in Droplets (80)
  ## 2.3 Signifikanz (113)
- ## 3. Dislokation (124)

######################## # 1. Dateien Laden # 

getwd() setwd("/Users/service/Desktop/Auswertung") # !!!!!Muss angepasst werden!!!!!

# Beads in Öl Laden

Beadsoel<-read.csv("BOELLINEAR.csv", header=TRUE, sep=";") # !!!!Muss angepasst werden!!!!!</pre>

# Beads in Droplets Laden

Beadsdroplets<-read.csv("Beadsdroplet2.csv", header=TRUE, sep=";") # !!!!!Muss angepasst werden!!!!!</pre>

\*\*\*\*\* #### 2. Geschwindiakeite der Beads #### 

# Definition des Vektors vBeadoel. vBeadoel ist TracK Median Speed (Spalte 21) von Beads in Öl # vBeaddropl = TracK Median Speed (Spalte 21) von Beads im Droplet

vBeadoel<-c(Beadsoel[,21])</pre>

class(vBeadoel)

vBeaddropl<-c(Beadsdroplets[1:58,21])</pre>

class(vBeaddropl)

### 2.1 Beads in Öl ### 

# Barplot vBeadOel # #----

mp<-barplot(vBeadoel,space=2,xlim=c(0,200),ylim=c(0,25))</pre> mp<-Darplot(VBeadoel, space=2, %llm=(U0,200), yllm=(U0,25))
title(xlab="Spurnummer der Beads", mgp = c(2.5, 0, 0))
title(ylab="Geschwindigkeit der Beads [µm/ms]", mgp=c(2.5,1,0))
axis(1, at=mp, seq(from=0, 57, labels=FALSE), mgp = c(0, 0.2, 0.2), col.ticks="white")
title("Geschwindigkeiten der Beads in Öl", line = +2)</pre>

### 8. R scripts

# Median

m<-median(vBeadoel)</pre>

m

# Standart Abweichung

sd<-sd(vBeadoel)</pre>

sd

#Zeichnet horizontale Linie für Median und Standart Abweichung

abline(h=m, col="red")
abline(h=m-sd,col='blue')
abline(h=m+sd,col='blue')

# Barplot vBeaddropl #
#-----

mp2<-barplot(vBeaddropl,space=2,xlim=c(0,200),ylim=c(0,5),main="Geschwindigkeiten der PEG-Krytox\ngekoppelten Beads")
title(xlab="Spurnummer der Beads",mgp = c(2.5, 0, 0))
title(ylab="Geschwindigkeit der Beads [µm/ms]",mgp=c(2.5,1,0))
axis(1, at=mp2, seq(from=0, 57,labels=FALSE),mgp = c(0, 0.2, 0.2), col.ticks="white")</pre>

# Median

mdb<-median(vBeaddropl)</pre>

mdb

# Standart Abweichung

sdII<-sd(vBeaddropl)</pre>

sdII

#Zeichnet horizontale Linie für Median und Standart Abweichung

abline(h=mdb, col="red")
abline(h=mdb-sdII, col='blue')
abline(h=mdb+sdII, col='blue')

# Wilcoxon Rang-Summen-Test. Infos: http://www.ipd.uka.de/Tichy/uploads/folien/149/Emp11Wilcoxon.pdf

"p-value Beads in Oel zu Beads in Droplets"<-wilcox.test(x=vBeadoel, y=vBeaddropl, paired = TRUE)

### 8.2. Deformation Anlysis R

# read the script and execute it line after line (Ctrl+Enter in RStudio)

```
#### read .txt files ####
# this loop searches the working directory and the subdirectories for '.txt' files
# each '.txt' file will be uploaded as a data frame while the first row of each '.txt' file
# will specify the names of the columns
# everything is sorted in a nested list 'input'
# 1. layer = subdirectories of working directory ("" = working directory itself)
# 2. layer = '.txt' files found
# 3. layer = columns of '.txt' file
subs<-list.dirs(".",full.names=FALSE) # Subdirectories Index 'i'</pre>
subs # here's all directories found in the working directory ("" = working directory itself)
input<-list() # creates an empty list for sorting all the data</pre>
for(i in 1:length(subs)){ # loop through working directory and subdirectories
  if(length(list.files(subs[i],pattern="*.txt"))>0){ # if a file ending with '.txt' is found
       input[[i]]<-list() # create another empty list (within the list 'input')
files<-list.files(subs[i],pattern="*.txt") # list all found '.txt' files in 'files'</pre>
       for(j in 1:length(files)){ # loop through 'files' (with j)
    input[[i]][[files[j]]<- # make list entry within the empty list (within 'input')</pre>
           read.table(file=paste(subs[i],files[j],sep="/"), # read '.txt' file
sep="\t",dec=".",header=TRUE,skip=0,na.strings="1.#R",fill=TRUE) # line 1 = headder
       }
  }
}
names(input)<-subs # define first layer names of nested list = directories/ subdirectories</pre>
```

# 'input' is a nested list names(input) # its first layer elements are the subdirectories ("" is the working directory) names(input[[2]]) # the second layer are the '.txt' files found in the subdirectory names(input\$Sample\_1) # you can also call them by their name with '\$name' names(input\$Sample\_1\$video\_1.txt) # in the third layer are the actual columns of the '.txt' file input\$Sample\_1\$video\_1.txt\$Feret[1:10] # the first 10 entries for 'Feret' in 'video\_1.txt'

#### 8. R scripts

```
#### Deformation ####
# we just need deformation Delta = (Feret-MinFeret)/(Feret+MinFeret) and 'Slice' from input
# this loop creates another list 'data' which is sorted the same way as 'input'
# but it only contains 'Delta' and 'Slice' for each '.txt' file
data<-list() # create an empty list</pre>
for(i in 1:length(input)){ # go through 'input' (first layer)
  if(length(input[[i]])>0){ # if there's stuff in (1st layer) 'input' entry
    data[[i]]<-list() # create empty list within the list 'data'</pre>
    files<-names(input[[i]]) # save names of stuff in (1st layer) 'input' entry</pre>
    for(j in 1:length(files)){ # go through these names
      attach(input[[i]][[files[j]]]) # attach: now 'Slice' will call 'input[[i]][[files[j]]]$Slice'
      data[[i]][[files[j]]]<-list("Slice"=Slice,"Delta"=(Feret-MinFeret)/(Feret+MinFeret))</pre>
      detach(input[[i]][[files[j]]]) # detach afterwards
    }
 }
3
names(data)<-subs # define names of 1st layer 'data'</pre>
rm(input) # we don't need this anymore
names(data) # this list is sorted the same way as 'input'
names(data$Sample_1)
names(data$Sample_1$video_1.txt) # but it only contains 'slice' and 'Delta' (what we need)
data$Sample_1$video_1.txt$Delta[1:10] # first 10 'Delta' entries of 'video_1.txt'
#### look at data ####
# if experimental procedures were new, you need to look at the deformations first
# to see how far apart the 'Delta' maxima are
# this is needed for setting the right mask size for calculating the maxima
# if the masksize if too big, not all maxima will be detected
# if it's too small, some irrelevant local maxima will be detected
# so far a mask of 21-51 was always good (I took 31)
# the function I wrote for this is 'lokex' (description within 'function_local_extrema.r' script)
# define some nice colors for plotting
colorlist<-list("firebrick1","gray85","red3","deepskyblue2","lightskyblue2","royalblue2","slateblue1")
# Now I load a .R function from basedir '../Experiment 1' because I saved it there</pre>
# if you need to use functions more often, it makes sense to save them all in one directory somewhere
# on your PC and then always load them from that directory
source(paste(basedir,"function_local_extrema.r",sep="/")) # I call the function from basedir
str(lokex) # function 'lokex' with arguments Vektor, maske, max, rand
# now try out some values for argument 'maske'
attach(data$Sample_2$video_1.txt) # 'Slice' will now call 'data$Sample_1$video_1.txt$Slice'
masksize<- 31# put in the mask size you want to test
titel<-paste("local maxima with mask size",masksize)</pre>
plot(Slice,Delta,pch=20,cex=0.2,col=colorlist[[7]], # the actual plot
     bty="l",main=titel,xlab="frame",ylab="deformation")
# now I highlight the points which would be found as local maxima
# give function 'lokex' a vector, it will calculate local maxima based on a mask
# it gives back these maxima as 'Wert' and the index of these maxima as 'Stelle'
# so now I plot these maxima over the Slices where they appeared
points(Slice[lokex(Delta,maske=masksize,rand=FALSE)$Stelle], # Slice[Stelle]
       lokex(Delta,maske=masksize,rand=FALSE)$Wert, # Wert
       pch=20,cex=0.73,col=colorlist[[1]]) # highlighting
# look at the plot, are these maxima correct? if not choose another masksize and plot again
# masksize = 11 for example is way to small (red points somewhere near 0)
# with masksize = 111 not all maxima are detected
detach(data$Sample_2$video_1.txt) # always detach afterwards
# attach another video to check. always detach afterwards
```

```
#### Funktion: bxplt ####
#
# Pseudocode:
#
# Start Funktion
#
bxplt<-function(data,spacing,colors,title="title",ylabel="ylab",grid=TRUE,white=TRUE,</pre>
                y.lim=c(min(unlist(data)),max(unlist(data))))
{
  Gitter<-grid # boolean</pre>
  #### create pos
  pos<-numeric(length(data))</pre>
  count<-1
  for(i in 1:length(data)){
    pos[i]<-count</pre>
    count<-count+spacing[i]</pre>
  }
  if(white){ #### wenn heller Hintergrund
    par(col="gray40", # Farbe der Achsen
        cex.lab=1.2, # Größe der Achsentitel
        las=1, # Achsenbeschriftung: 0=90°gedreht an yAchse, 1=waagrecht an yAchse
        bty="l" # Ramen: l = eine yAchse links und eine xAchse unten
    )
  } else { #### wenn dunkler Hintergrung
    par(bg='black', # Plot Hintergrundfarbe
        col.axis='white', # Achsenbeschriftung
        col.lab='white', # für Labels
col.main='white', # für Titel
        col.sub='white', # für Untertitel
        fg='gray90', # Plot Vordergrundfarbe
col="gray60", # Farbe der Achsen
        cex.lab=1.2, # Größe der Achsentitel
        las=1, # Achsenbeschriftung: 0=90°gedreht an yAchse, 1=waagrecht an yAchse
        bty="l" # Ramen: l = eine yAchse links und eine xAchse unten
    )
  }
  #### 1. Boxplot zeichnen
  boxplot(data, # richtig geordnetes data frame oder list
          ylab=ylabel, # y-Achsenbeschriftung
          main=title, # Titelei
          notch=FALSE, # Vertrauensintervall für Median darstellen
          col=colors,
          yaxt="n", xaxt="n",
          at=pos,
          ylim=y.lim
  )
```

### 8. R scripts

```
#### 1. Boxplot zeichnen
 boxplot(data, # richtig geordnetes data frame oder list
         ylab=ylabel, # y-Achsenbeschriftung
         main=title, # Titelei
         notch=FALSE, # Vertrauensintervall für Median darstellen
         col=colors,
         yaxt="n", xaxt="n",
         at=pos,
         ylim=y.lim
 )
                #### wenn Gitter
 if(Gitter){
   if(white){
     grid(nx=NA, ny=NULL,col="gray60") #grid over boxplot
   } else {
     grid(nx=NA, ny=NULL,col="gray20") #grid over boxplot
   }
   bxplt.data <- boxplot(data, #### 2. Boxplot zeichnen</pre>
                          ylab=ylabel, # y-Achsenbeschriftung
                          main=title, # Titelei
                          notch=FALSE, # Vertrauensintervall für Median darstellen
                          col=colors,
                          at=pos,xaxt="n",
                          add=TRUE,
                         ylim=y.lim
   )
 }else{ #### wenn kein Gitter
   bxplt.data <- boxplot(data, #### 2. Boxplot zeichnen</pre>
                         ylab=ylabel, # y-Achsenbeschriftung
                         main=title, # Titelei
                         notch=FALSE, # Vertrauensintervall für Median darstellen
                         col=colors,
                         at=pos,xaxt="n",
                         add=TRUE,
                         ylim=y.lim
   )
 }
# par(paralt.fun) # nicht aufrufen, damit plotting einstellung bleiben (zB xaxt)
 return(list("xticks"=pos,"bxplt"=bxplt.data))
}
    #### Ende Funktion
```

```
#### Funktion: lokex ####
# Pseudocode:
#
# Start Funktion
# vektor = numerisches array mit den zu analysierenden Werten
# maske = ungerader numerischer Wert, welcher als Fenster bei Extremwertsuche benutzt wird
# max = boolean: TRUE => Maxima suchen, FALSE => Minima suchen
# rand = boolean: TRUE => Randmaxima suchen
#
# n = Länge(vektor)
# Stelle = leer (numerisches Array: Kontainer für Stelle der gefundenen Extremwerte)
# Wert = leer (numerisches Array: Kontainer f
ür gefundene Extremwerte)
# count = 0 (numerischer Wert: Zähler für die Kontainer Arrays 'Stelle' und 'Wert')
#
# if 'max' == TRUE
     if 'rand' == TRUE
#
#
          for Randbereich am Anfang von 'vektor' (bestimmt durch Fenstergröße 'maske')
#
              if Maximum gefunden dann
                  count++; 'Stelle' und 'Wert' eintragen
#
#
    for Bereich von 'vektor' in welchem Fenster 'maske' nicht über 'vektor'-Grenzen ragt
#
          if Maximum gefunden dann
#
              count++; 'Stelle' und 'Wert' eintragen
     if 'rand' == TRUE
#
          for Randbereich am Ende von 'vektor' (bestimmt durch Fenstergröße 'maske')
#
#
              if Maximum gefunden dann
#
                 count++; 'Stelle' und 'Wert' eintragen
# else
#
     das ganze nur mit Minimum anstatt Maximum
#
# return Liste aus 'Stelle' und 'Wert'
#
# Ende
lokex<-function(vektor,maske=3,max=TRUE,rand=TRUE)</pre>
{
 vektor<-as.numeric(vektor)</pre>
 maske<-as.numeric(maske)</pre>
  if(maske%%2==0)
  {
    print("Maske muss ungerade sein!")
    print(paste("Maske wird von",maske,"auf",maske+1,"geändert!"))
   maske<-maske+1
  }
  if(maske<=1)</pre>
  {
    print("Maske von <3 ist sinnlos.")</pre>
   print(paste("Maske wird von",maske,"auf 3 geändert!"))
    maske<-3
  }
```

```
n<-length(vektor)</pre>
Stelle<-numeric(0)</pre>
Wert<-numeric(0)</pre>
count<-0
if(max==TRUE)
{
  if(rand==TRUE){ #### Anfang Anfangsrand
  for(i in 1:((maske-1)/2))
  {
    if(vektor[i] == max(vektor[1:(i+(maske-1)/2)]))
    {
      count<-count+1
      Stelle[count]<-i</pre>
      Wert[count]<-vektor[i]</pre>
    }
  }
  }
       #### Ende Anfangsrand
  for(i in (((maske-1)/2+1):(n-(maske-1)/2)))
  {
    if(vektor[i] == max(vektor[(i-(maske-1)/2):(i+(maske-1)/2)]))
    {
      count<-count+1
      Stelle[count]<-i</pre>
      Wert[count]<-vektor[i]</pre>
    }
  }
  if(rand==TRUE){ #### Anfang Endrand
    for(i in (n-(maske-1)/2+1):n)
    {
      if(vektor[i] == max(vektor[(i-(maske-1)/2):n]))
      {
        count < -count + 1
        Stelle[count]<-i</pre>
        Wert[count]<-vektor[i]</pre>
      }
    }
  }
       #### Ende Endrand
} else {
            #### Ende Maxima
  if(rand==TRUE){ #### Anfang Anfangsrand
```

Chapter VI. Appendix

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# Eidesstattliche Versicherung gemäß § 8 der Promotionsordnung der Naturwissenschaftlich-Mathematischen Gesamtfakultät der Universität Heidelberg

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These drawbacks are mainly due to complications related to controlled permeability of the traditional polymersomes<sup>[96]</sup> and due to the lack of technological means that allow precise and efficient injection of different biological components into polymersomes. To overcome these obstacles, a new highly innovative and creative approach in bottom-up synthetic biology had to be created, as described in the following section.

#### 3.4. Droplets

Microfluidic block copolymers-stabilized water-in-oil droplets can also be classified as polymersomes, but due to the continuous oil phase they are considered in the scientific community as emulsion droplets. The advantages of water-in-oil emulsion droplets for biological applications have been described in details in Section I.1.1. In the context of bottom-up assembly of artificial cells droplet-based compartment systems represent the advantages of the polymersomes, but can also be easily adapted to the droplet-based microfluidic technologies for precise delivery of bio-relevant ingredients. The continuous oil phase can play an important role in preserving the droplet material content (see Section IV.3). Moreover, a continuous oil phase is required to control an injection (details see Section I.1.1) of precise amounts of high-value biomaterials exclusively into the droplets Figure I.9.



Figure I.9.: A droplet based pico-injection unit. (A) Shows a junction to control the spacing between the droplets through addition of oil via the second oil channel. Figure (B) shows a injection channel and the electrodes of an alternating electric field with 30kHz and 600V used to reduce the stability of the surfactants in the droplet periphery for an aqueous injection of other cellular components (injection volume can range between 2 to 100 pL dependend on the applied pressure) from a pico-injection channel.

Due to the variety of required cellular components in the droplets the microfluidic device with small and compact electric systems was integrated to apply electric fields in microthe membrane, and by addition of frog egg extract to the membrane actin was able to reconstitute long filopodia-like actin structures. Other studies added G-actin or F-actin to ponticulin-containing SLBs and created cortex-like actin structures with the average actin layer thickness of 15 nm as revealed by AFM measurements.<sup>[122]</sup>

Together, these studies point to a powerful role of SLBs in modulation of spatial dynamic organization of actin filaments. However, unlike in biological membranes, the planar bilayers do not allow pulling of the proteins and instead are rigid in the vertical dimensions due to the trapping at the glass surface. Therefore, the ability of actomyosin cortex to exert forces in all three directions is restricted in studies on supported rigid surfaces. Moreover, to emulate cortical dynamics it is essential not only to incorporate actin turnover dynamics to soft interfaces, but also reproduce a cell-like geometry.

Lipid vesicles or droplets can overcome the aforementioned limitations due to their 3D cell-like geometry and soft interfaces. For example, Liu et al.<sup>[110]</sup> used PIP2 containing GUVs to show that elastic interactions between the synthetic lipid vesicle membrane and reconstituted actin cytoskeleton can cooperate without accessory proteins to induce the formation of actin filament protrusions (Figure I.10).



Figure I.10.: Role of membrane in formation of thin actin filament protrusions. A model of membraneinduced formation of actin filaments. Left: Small, local deformations of the membrane induced by actin filament polymerization against the membrane (black arrows).

Merging attractions to create a larger deformation (curly brackets). Middle: Deformations that fail to create a larger deformation (grey arrows). Right: After bundling of filaments to overcome the resistance of the membrane. Actin filaments bundle and can elongate without further physical constraint. Adapted with permission from Ref.<sup>[110]</sup> Copyright 2008, Nature Physics.

Model of membrane-induced formation of a thin actin filament protrusion. Left: Smallamplitude local deformations of the membrane arise as actin filaments polymerize against A 24 x 24 mm glass (#1) was glued on top. The chamber was filled completely with FC-40 oil containing the same surfactant concentration as used for droplet creation. To avoid evaporation the sides of the chamber were sealed by two-component glue (Twinsil, Picodent GmbH, Germany). Droplets stored in these chambers remained stable for up to several month.<sup>[4,144]</sup>



Figure III.7.: Droplet production and storage for analysis. (A) Droplet-based microfluidic device with red and blue colourings indicating oil and aqueous inlets, respectively. (B) Insight of a flow-focusing junction where oil (red) and aqueous (blue) phases meet at a 20 µm wide nozzle and droplets are generated. (C) Illustration of an analysis chamber to store droplets for analysis and characterization. (D) Phase contrast image of 40 µm diameter droplets in an analysis chamber.

#### 2.3.1. Different Microfluidic Operation Units

For various research purposes different microfluidic units were designed by using a QCADprogram. Most of the designs were adapted from literature and optimized for our needs. In the following paragraphs three main designs are described.

**Deformation Measurements** A droplet deformation device (figure III.8.A) was designed<sup>[20]</sup> and implemented for dynamic physical analysis of surfactants and proteins accretion and actin polymerization in microfludic droplets. The results achieved by this device are presented in Section IV.2.2. In general, two aqueous inlets (blue) and two



Figure III.8.: Representative schematics and images of different droplet-based microfluidic units. (A) Representation of droplet deformation devices and a magnified area of the deformation chambers. (B) Schematic representation of spiral device for single cell encapsulation and a magnified area of dropspot chamber. (C) Picoinjection device for controlled introduction of different bioactive components into droplets. The inset represents an example for the injection where colored liquids were used.

oil inlets (red) for droplet production and droplet spacing, respectively, were used to adjust the flow rates for high throught droplet deformation analysis, allowing high-speed recording. Installation and recording was done as described in subsection 2.3 (*Droplet production and Storage*) and section 3 (*High-speed microscopy*). Flow rates were set constant:  $50 \,\mu l \,h^{-1}$  aqueous phase (blue),  $170 \,\mu l \,h^{-1}$  oil phase (red) and  $220 \,\mu l \,h^{-1}$  spacing oil (red) (see figure III.8A).

Single cell encapsulation Single cell encapsulation and analysis experiments were performed with spiral devices as shown in figure III.8 B. This device allows Dean flow-based (De) separation of cells or particles by an inertial lift and viscous drag force and can be calculated with the presented formula where  $\rho$  is the density of the fluid, V the axial velocity scale,  $\mu$  is the dynamic viscosity, d is the channel diameter for non circular channels



Figure III.9.: Representative cryo-SEM micrograph of freeze fractured microdroplets (A) Cryo-SEM micrograph with low magnification of freeze fractured microdroplets. The droplets were fractured by the cooled knife in the preparation device chamber in the 10<sup>-6</sup> - 10<sup>-7</sup> mbar vacuum chamber at -160 °C. (B) Representative cryo-SEM micrograph of the fractured droplets obtained with higher magnification. (C) Following the water sublimation in the preparation chamber a Cryo-SEM micrograph with a single fractured droplet is presented.

were recorded in bright-field with 60  $\mu s$  exposure time and 16 000 pps with a resolution of  $256 \times 512.$ 

# 4. Retention Analysis of Fluorophores and Biomolecules in Droplets

### 4.1. Fluorophores

ATTO 488 N-Hydroxysuccinimidyl ester (SE), ATTO 495 SE, ATTO 520 SE, ATTO 532 SE, ATTO 565 SE, ATTO 590 SE, ATTO 647N SE and ATTO 655 SE were purchased from ATTO-TEC GmbH (Siegen, Germany). Alexa Fluor<sup>®</sup> 488 C5 Maleimide (M), Alexa Fluor<sup>®</sup> 532 C5 M and Alexa Fluor<sup>®</sup> 647 SE were purchased from Thermo Fisher Scientific (Schwerte, Germany).

The pure fluorophores were dissolved in anhydrous DMSO at a concentration of 1 mM and stored at -20°C. In retention experiments the fluorophores were further diluted with each buffer and both cell culture media (see next Section 4.2) to a final concentration of  $5 \,\mu\text{M}$  and used as an aqueous phase for droplets creation.

The hydrophilicity of the fluorophores was analyzed by calculating the distribution coefficient, LogD, which is a measure of the expected ratio of the sum of concentrations of all forms of the fluorophore (ionized plus un-ionized) in water and in a non-polar solvent (octanol). Negative LogD values correspond to hydrophilic fluorophores, positive values orophore retention was categorized in three different groups according to Figure 4.4:



Figure III.10.: Characterization criteria for fluorophore retention in droplets

Figure III.11 shows the fluorescence intensities  $I_{t0}$  of droplets produced with three different buffers and different fluorophores and representing the three different categories of fluophore retention.<sup>[144]</sup>



Figure III.11.: Flurophore retention characterization (A, B and C) show droplets produced with 10 mM TRI7000 surfactant, containing ATTO 633 in TRIS (A), ATTO 655 in RPMI (B) and Alexa 488 in TRIS (C). The droplets were analyzed immediately after production (Intensity I<sub>t0</sub>). Upper panel: Selected fluorescence images demonstrating different fluorescence retention in droplets. Middle panel: Intensity profiles of cross-sections marked in upper panel. Lower panel: Red, yellow and green blocks illustrate the color marking used in all tables.

#### 6. Cell experiments

and the supernatant with unbound cRGD peptides were removed. The precipitant was freeze-dried for at least 24 h to remove remained water completely. Afterwards, the dried product was dissolved in 1 ml of fluorinated oil FC-40 (Acros Organics, Germany) and filtered with a hydrophobic filter (PTFE  $0.2 \,\mu\text{m}$ ) to remove unreacted, hydrophilic (11-Mercaptoundecyl)tetra(ethylene glycol) functionalized gold nanoparticles and cRGD traces. The obtained surfactant was used in different concentrations for cell adhesion experiments.<sup>[4]</sup>



Figure III.12.: Chemical structure of the c(RGDfK) peptide 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 the 3 cysteine-linkers.

### 5.3. Rhodamine functionalization (Two step Approach)

Gold nanoparticle functionalization with rhodamine involved two steps. First the synthesis of PFPE-PEG-Gold surfactants linked to rhodamine B (RhB) (see Section IV.1.4) followed by droplet creation using a mixture of TRI2500 (20 mM, PFPE2500-PEG600-PFPE2500, synthesis described in Section IV.) and RhB-PEG-Gold-PEG-PFPE (5  $\mu$ M) surfactants as an oil phase, while PBS was used as an aqueous phase.<sup>[4]</sup>

### 6. Cell experiments

Mercaptoundecyl-tetra(ethylene glycol) functionalized gold nanoparticles and cRGD were coupled in advance. The obtained surfactant was used in different concentrations for cell adhesion experiments.<sup>[4]</sup> created in a microfluidic device, were recorded immediately after the cross junction with a Phantom V7.3 camera at 10000 fps.

As can be observed in Figure IV.12 surfactant concentrations below 1mM lead to droplet coalescence as soon as droplets were in contact. At higher surfactant concentrations (above 1mM) droplets were stable and no coalescence was observed. Figure IV.12 shows five representative images at different concentrations for both tested surfactants.



Figure IV.12.: Droplet stability as a function of surfactant concentration and its molecular weight. Representative brightfield images of water-in oil droplets immediately after production. At low surfactant concentration (C < 1mM) coalescence of droplets is observed (left panel). At high surfactant concentration creation of small droplets occurs (right panel). Monodisperse and stable droplets are produced in a stable regime as it can be seen in the middle panel. Adapted with permission from Ref.<sup>[144]</sup>

In case of TRI2500 stable monodispers droplets creation was observed at a concentration range of 2,5 mM < c < 10 mM. In case of the bigger surfactant TRI7000 the concentration range was larger, ranging from 1 mM up to 20 mM surfactant concentration. Above these surfactant concentrations small droplets were created during droplet production due to jet-dripping and by friction of the droplets flown in the channels. When droplets were stabilized (less than a second after production) with a dense surfactant layer no appearance of more small droplet or a decrease in size of produced droplets was observed any more.

To provide a detailed analysis of the droplet stability and their size distribution droplets were collected and stored in an analysis chamber (see Section III.2.3). The results are the other hand high surfactant concentrations lead to creation of small droplet, visible with brightfield microscopy. This effect was observed at a surfactant concentration of 5mM for TRI2500 and at 20 mM for TRI7000. For high concentrations even shrinking of the "mother-droplets" is visible (for TRI2500) by the decrease of the average droplet radius. In most microfluidic droplet applications this effect is undesirable since a constant volume of all droplets is essential for quantitative analysis. Therefore in all further investigations the surfactant concentration was always kept between 2.5 and 10 mM to ensure production of stable droplets with low size distribution.

## 3.2. Effect of Droplet Size and their Formation Rate on Fluorophore Retention

To preform long term experiments with microfluidic droplets, stable encapsulation of particles, molecules and cells is necessary. Therefore, molecular retention in the droplets was analyzed according to droplet size and the rate of droplets formation.<sup>[158]</sup>

First the effect of droplet formation speeds was investigated to determine fluorophore retention. Figure IV.14 shows representative images of the fluorophore ATTO 590 SE moderate retention as a function of formation speeds (0.5, 3, and 30 kHz) (defined as described in Section III 4.4).



Figure IV.14.: Effect of droplet production rates on fluorophore retention Moderate retention of ATTO 590 SE fluorophores in the droplets does not depend on the droplet creation frequencies. ATTO 590 SE fluorophores were dissolved in PIPES-buffer and used as an aqueous phase for droplets creation. The oil phase contained 2.5 mM of TRI7000 surfactants. Adapted with permission from Ref.<sup>[144]</sup>.

As can be seen in Figure IV.14 the retention was not affected due to different droplet creation frequencies. All flurophores mentioned in Section III.4.1 were tested and showed the same result.

Next the effect of droplet size on fluorophore retention was investigated. The fluorophors were encapsulated in droplets having different sizes (Diameter = 25, 40 and  $150 \,\mu\text{m}$ , 3 KHz production speed, 2.5 mM of TRI7000 surfactant). As shown in Figure IV.15 the droplet diameter had no effect on the fluorophore encapsulation stability. All investigated fluorophore retentions were not affected by the size of the droplets.



Figure IV.15.: Effect of droplet sizes on the retention of fluorophores within the droplets. Retention of fluorophores in the droplets is not affected as a function of droplet size. ATTO 532 SE, ATTO 655 SE, ATTO 590 SE and ATTO 647N SE fluorophores were dissolved in TRIS buffer and used as an aqueous phase for droplet creation. The oil phase contained 2.5 mM of TRI7000 surfactant. Adapted with permission from Ref.<sup>[144]</sup>

Fluorophore [LogD] Buffer/ Medium [pH]	Alexa 488 M [-9.47]	Alexa 532 M [-4.43]	Alexa 647 M [-7.75]	ATTO 488 SE [-7.60]	ATTO 495 SE [-0.81]	ATTO 520 SE [+0.59]	ATTO 532 SE [4.49]	ATTO 565 SE [-0.18]	ATTO 590 SE[+1.56]	ATTO 633 SE [n.a.]	ATTO 647N SE[+4.47]	ATTO 655SE [-0.61]
TRIS [7.7]												
HEPES [7.4]												
PIPES [6.8]												
PBS [7.4]												
RPMI [7.9]												
DMEM [8.2]												

no or in case of RPMI media moderate retention

Figure IV.17.: Fluorophores retention in the droplets Fluorophores retention in the droplets (diameter = 40 µm) having different buffer/medium conditions. TRI2500 (10 mM) surfactants were used to generate droplets for this experiment. Adapted with permission from Ref.<sup>[144]</sup>

Surprisingly, no retention was observed for slightly hydrophilic dyes as ATTO 495 (LogD = -0.81), ATTO 655 (LogD= -0.61) and ATTO 565 (LogD= -0.18). In case of ATTO 495 dye this behavior can be explained by the relatively small molecular weight as well as the planar structure which leads to a smaller energetic barrier for the transport through the surfactant layer.<sup>[162]</sup> For ATTO 565 and 655 dyes only half of the tested buffers and media showed moderate retention.

### 3.4. Retention as a Function of Surfactant Length, Structure and Concentration

In addition to buffer and fluorophore properties the effect of the physical properties of surfactants (i.e., geometry, concentration and molecular weight) on the fluorophore retention in the droplets were investigated.<sup>[158]</sup> Three different surfactants (TRI2500, TRI7000, and DI7000) with 10 mM concentration each were used (see Section IV.3.1). The retention summary of the 12 fluorophores encapsulated with the three surfactants is presented in Figure IV.18. As can be observed from Figure IV.18 droplets produced with the TRI7000 surfactants exhibited the best retention of encapsulated fluorophores. However,

the droplets produced with this surfactant were highly polydisperse in size due to low droplet stability. Improved retention in the droplets produced with diblock surfactants can be attributed to the denser surfactant layer on droplets periphery due to reduced steric effect on comparison to triblock surfactant geometry. However, for droplet stability it is preferable to have a dense PFPE layer on the outside droplet interface. The most efficient layer against coalescence can be provided by triblock surfactant geometry having two PFPE tails.<sup>[15]</sup> Due to the inherent requirement for stable monodisperse droplets, further experiments were performed with TRI2500 and TRI7000 surfactants. As shown in Figure IV.18, improved retention was also achieved for some of the fluorophores in droplets produced with longer surfactants (higher molecular weight). This observation is straightforward since dyes have to pass a higher energetic barrier due to a thicker surfactant layer.<sup>[162]</sup> Further optimization of fluorophore retention within the droplets was analyzed depending on the surfactant concentration.

	Fluorophore [LogD] Surfactant	Alexa 488 M [-9.47]	Alexa 532 M [-4.43]	Alexa 647 M [-7.75]	ATTO 488SE [-7.60]	ATTO 495SE [-0.81]	ATTO 520SE [+0.59]	ATTO 532SE [- 4.49]	ATTO 565 SE [-0.18]	ATTO 590 SE [+1.56]	ATTO 633 SE [n.a.]	ATTO 647N SE [+4.47]	ATTO 655 SE [-0.61]
	TRI 2500												
T.7	TRI 7000												
	DI 7000												
S	TRI 2500												
IPE 6.8]	TRI 7000												
	DI 7000												
н_	TRI 2500												
PM 7.9	TRI 7000												
H _	DI 7000												

Figure IV.18.: Fluorophores retention in the droplets is a function of buffer/medium selection and surfactant physical properties. Fluorophores retention in the droplets  $(D = 40 \,\mu\text{m})$  as a function of buffer/medium selection and surfactant physical properties. The droplets were generated using three different surfactants (TRI2500, TRI7000, and DI7000 (10 mM)). Adapted with permission from Ref.<sup>[144]</sup>

The range of surfactant concentrations was chosen according to the results in Section IV.3.1. Based on these results and considering that molecular retention might be dependend on surfactant concentration, further fluorophore retention studies were preformed in droplets produced with 2.5 and 10 mM surfactant concentration. The retention summary of the 12 fluorophores encapsulated in droplets made of TRI7000 and TRI2500 surfactants at two different concentrations is presented in Figure IV.19. In both cases, improved fluorophore retention was observed with the lower surfactant concentration. However, this effect is more apparent in TRIS buffer and RPMI medium. The decrease of free surfactants in the oil phase reduces the formation of reverse micelles and small droplets, which can act as carriers for the solubilized fluorophores. These results are in agreement with previous observations where the decrease of the fluorescence intensity of fluorescein inside droplets was observed due to increased surfactant concentration.<sup>[163]</sup>

These results emphasize that with a proper selection of the physical surfactant properties such as concentration, geometry, and molecular weight the retention of most of the fluorophores can be controlled efficiently.

Buffer	TRIS			PH	PES		RPMI					
Surfactant	TRI	2500	TRI	7000	TRI	2500	TRI	7000	TRI	2500	TRI	7000
Surf.Conc. Filiporopilore	2.5	10	2.5	10	2.5	10	2.5	10	2.5	10	2.5	10
Alexa 488												
Alexa 532												
Alexa 647												
ATTO 488												
ATTO 495												
ATTO 520												
ATTO 532												
ATTO 565												
ATTO 590												
ATTO 633												
ATTO 647N												
ATTO 655												

Figure IV.19.: Fluorophores retention in the droplets as a function of surfactant length and concentration Fluorophores retention in the droplets  $(D = 40 \,\mu\text{m})$  as a function of surfactant length and concentration. Fluorophores were dissolved in TRIS, PIPES buffers, and RPMI medium. The oil phase contained 2.5 or 10 mM of triblock surfactants. Adapted with permission from Ref.<sup>[144]</sup>

As shown in Figure IV.21 stable retention of ATTO 633-labeled microtubules in the droplets created with 2.5 mM TRI7000 surfactants was achieved. Whereas, droplets produced with 2.5 mM TRI2500 surfactant showed no retention of ATTO 633-labeled tubulin and even pre-polymerized microtubules diffused out of the droplets immediately after encapsulation. A representative time-lapse is presented in Figure IV.20. Additionally, stable retention was observed for microtubules labeled with highly hydrophilic ATTO 488 dye (Figure IV.21).

In contrast to the labeled microtubules experiments, stable retention for more than 14 days was observed for all labeled antibodies and streptavidin proteins tested in this study. These results were independent of the hydrophilicity of the dye and surfactant selection. In case of antibodies and streptavidin proteins their high hydrophilicity and their big molecular weights are the major reasons for their stable retention within the droplets independent of the dye properties. Even the most hydrophobic ATTO 647N dye (LogD = +4.47), which showed no retention in the droplets in a pure state, did not influence the the stable retention of sheep anti-mouse antibodies or streptavidin.



Figure IV.21.: Factors influencing the retention of microtubules in micro droplets Retention of labeled microtubules in the droplets strongly depends on the degree of hydrophilicity of the dye and surfactant selection. ATTO 633- and ATTO 488-labeled microtubules were polymerized in PIPES buffer and used as an aqueous phase for droplets creation. The oil phase contained 10 mM TRI7000 and TRI2500 surfactants. Note: Similar results were observed with and without addition of gold-linked surfactants. Adapted with permission from Ref.<sup>[144]</sup>

gold-linked surfactant (30 µM) (Figure IV.22 B) compared to a lower concentration (3 µM) (Figure IV.22 D).<sup>[4]</sup> To prove that the bright dots were gold NPs and not artifacts due to the cryo-SEM measurements or freeze-fracture preparation, droplets without gold-linked surfactants were created, freeze-fractured, and observed by cryo-SEM (Figure IV.22 F).



Figure IV.22.: Cryo-SEM micrographs of freeze-fractured nanostructured droplets Representative cryo-SEM micrographs of freeze-fractured nanostructured droplets obtained with different magnifications. The droplets were created using a PFPE-PEG-PFPE triblock (TRI2500) copolymer surfactant concentration of 20 mM and PFPE-PEG-Gold-naonoparticle surfactant concentrations of (A, B) 30 μM, (C, D) 3 μM and (E, F) 0 μM. Adapted with permission from Ref.<sup>[4]</sup>

### 4.2. Biofunctionalization of Nanostructured Droplets

In this study GNp were used as anchoring points for various biomolecules. Towards this end, two biofunctionalization approaches were developed. The first approach was based on functionalization of created droplets with Ni<sup>2+</sup> mediated interaction between the (His6-tag) proteins and nitrilotriacetic acid (NTA)-thiol linker. In the second approach the biomolecules were coupled covalently via ester or thiol bond to the gold-linked surfactants before droplet creation.

The results of the first approach are presented in Figure IV.23 where GNps in the nanostructured droplets were coupled with His6-tag green fluorescence protein (His6-GFP) via a GNp-thiol linker (detailed reaction see Section III.5.1.

Images were taken (A) 1 day, (B) 4 days, and (C) 10 days after creation. The decrease in fluorescence intensity over the observed period can be explained due to oxidation of



Figure IV.25.: Representative fluorescence images of the RhB-linked gold-nanostructured droplets (A, B, C) RhB-linked nanostructured droplets measured (A) 1 day, (B) 4 days, and (C) 16 days after their creation. (1-10) Representative distribution of the fluorescence signal along the RhB-linked nanostructured droplet height, 3 days after creation. Stack 1 represents the top of the droplet, stack 10 the bottom. All images have the same intensity scale. Adapted with permission from Ref.<sup>[4]</sup>

## 5. T Cell Experiments

As described in detail in Section I.2 the effective *ex vivo* T cell activation is the crucial step for successful immune therapy. Therefore, nanostructured and biofunctionalized droplets were used as 3D APC analogues. To provide the specific interactions with the T cells, the nanostructured droplets were functionalized with cyclic arginine-glycine-aspartic acid peptide (cRGDfK) or with pMHC proteins via cysteine or Ni-NTA-thiol chemistry, respectively. Single T cell encapsulation was achieved using a microfluidic spiral device as described in Section III.2.3.1.

#### 5.1. T Cell Jurkat 6.1 Experiments

The human acute T cell leukemia cell line (Jurkat E 6.1) was used to evaluate the potential of nanostructured and biofunctionalized droplets to serve as a 3D APC analogue enabling single cell analysis in a defined compartment. Jurkat T cells express  $\alpha_4\beta_1$  and  $\alpha_5\beta_1$ integrins and exhibit activation-dependent regulation of integrin-mediated adhesion.<sup>[169]</sup> c(RGDfK)-PEG6-cysteine which are specific for  $\alpha_5\beta_1$  were linked to PFPE-PEG-gold surfactants (Section III.5.2)<sup>[4]</sup> No significant difference between both labeling strategies was observed. However, the two step approach was preferred to minimize the amount of soluble c(RGDfK)-PEG6-cysteine peptides in the aqueous phase, to avoid blockage of active sides on the cells and therefore, hindering the cell-droplet interaction.<sup>[170]</sup>



Figure IV.26.: T cell viability and T cell-droplet-periphery interaction depending on the functionalization. (A) Representative bright-field image of Jurkat E 6.1 cells (indicated by arrows) in the cRGDfunctionalized nanostructured droplets 6 h after their creation. (B) Quantification (adherent cell %) of Jurkat E6.1 cell adhesion on cRGD-functionalized (pink, left bars) and nonfunctionalized (gray, right bars) nanostructured droplets. Data is presenting mean ± standard error of the mean (n = 5). (C) T cell proliferation time lapse. (D) T cell viability after 3 days (green= alive; orange= dead). Cells were marked with propidium iodid. Adapted with permission from Ref.<sup>[4]</sup>



Figure IV.28.: Representative confocal images of the primary mouse T cells interactions with goldnanostructure droplets. Image 1 to 3 show composed confocal z-stacks of droplets with primary mouse T cells in contact with their periphery. In sake of clarity of presentation the periphery of the droplet and T cells were marked with yellow and brown colour, respectively. The fluorescence signal of the Alexa568-labeled MHC can be observed in image (A) and (C). A brightfiled image of the related T cell is presented in figure (B).

## 6. Bottom-Up Assembly of Minimal Actin Networks

This section summarizes the results of actomyosin cortex formation in droplet-based micro-compartments. Water-in-oil nanostructured emulsion droplets as cell-sized compartments were used to achieve the functional bottom-up assembly of minimal number of proteins required to reconstitute the actomyosin cortex. To analyze the dynamic actin properties in defined compartments all actomyosin networks were created by implementation of droplet based microfluidics. All droplet experiments in this section were preformed using 2.5 mM triblock surfactants TRI7000. In some experiments  $0.3 \,\mu\text{M}$  gold-labeled sur-

In diffusion experiments preformed in an analysis chamber (Section III.2.3) a significant difference in diffusion speeds of free bead-surfactants and in the droplet periphery incorporated bead-surfactants could be shown (p-value:  $5.87 \times 10^{-10}$ ) (Figure VI.3). This difference in diffusion speeds decreased over time due to low density of the beads (bead  $\rho$ = 1.05 g cm<sup>-3</sup>, FC-40  $\rho$  = 1.85 g cm<sup>-3</sup>) resulting in a contact to the top glass of the analysis chamber. By this friction was increasesd and no free movement was observed afterwards. Therefore only results shortly after production were taken into consideration.



Figure VI.3.: Analysis of beads diffusion in the oil phase. The diagrams A and B present the diffusion of the bead-surfactant. (A) represents the diffusion of free bead-surfactant in the oil phase. Diagram B shows the diffusion of bead-surfactant in the droplet periphery. Red line represents the median for A and B. The diffusion for free bead surfactant was  $17.21 \pm 3.05 \,\mu m \, ms^{-1}$  before and and  $1.31 \pm 0.25 \,\mu m \, ms^{-1}$  rafter sedimentation. The median for bead-surfactant speed in the periphery was  $0.41 \pm 0.36 \,\mu m \, ms^{-1}$ 

In a second experiment the dislocation of beads was analyzed. In figure VI.4 the difference of free bead-surfactants and in the periphery incorporated bead-surfactants can be clearly seen. Here the p-value is 3.6x 10<sup>-11</sup>. The difference of the dislocation is much higher due to the free movement of free bead-surfactant compared to the beads in the periphery which can only move short distances due to the dense surfactant layer. The results of these experiments together with IR-analysis and cryo-SEM images of beads in the periphery of actin droplets (Section IV.1.6.1) show the success of the reaction and confirm the expected behaviour of this new synthesized bead-surfactant.



Figure VI.4.: Analysis of beads dislocation in the oil phase The diagrams A and B present the dislocation of the bead-surfactant. (A) represents the dislocation of free bead-surfactant in the oil phase. Diagram (B) shows the disslocation bead-surfactant in the droplet periphery. Red line represents the median for A and B;  $80.4 \pm 38.7 \,\mu m \, ms^{-1}$  and  $2.7 \pm 3.3 \,\mu m \, ms^{-1}$  respectively.

### 4. Proteins

#### 4.1. Actin

Actin is a globular protein found in all eukaryotic cells with a variation of less than 5% in the sequences of amino acids between specie.<sup>[189]</sup> 1 to 5% of cell mass is actin as monomers (length 55  $\mathring{A}$ ) or in filamentous form. The actin monomer has a weight of 42 kDa and consists of 375 amino acids in 4 subdomains called G-actin due to it's globular shape. F-actin has a double helical shape where each monomer has an offset of 166° and 2.75 nmwith a diameter of about 5-9 nm and up to several micrometer length.<sup>[190?]</sup> The persistence length of actin is about 17 µm max.<sup>[191,192]</sup>

Actin polymerization is mediated by the presence of ATP which is hydrolyzed during the actin polymerization.<sup>[193]</sup> In the first step of actin polymerization 2-4 ATP saturated G-actins assemble. This step is the time determing step in actin polymerization.<sup>[194]</sup> Polymerization occurs then at both ends of the actin filaments but has different kinetics due to structural asymmetrice of the film endings. The so called (+) and (-) end have different polymerization and depolymerization constants which are  $k_1 = 11.6 \pm 1.2 \,\mu M^{-1} s^{-1}$ and  $k_{-1} = 1.4 \pm 0.8 \,\mu M^{-1} s^{-1}$  for the (+) end and  $k_2 = 1.3 \pm 0.2 \,\mu M^{-1} s^{-1}$  and  $k_{-2} = 0.8 \pm 0.3 \,\mu M^{-1} s^{-1}$  for the (-) end.<sup>[195]</sup> Actin filaments together with Myosin II motor proteins

## 6. FT-IR Analysis

Figure VI.9 (A) shows the representative FTIR spectra of the PFPE(7000)-carboxylic acid reactant and the triblock surfactant product PFPE(7000)-PEG(1400)-PFPE(7000) (TRI7000). This figure presents five major bands at 1701, 1775, 2848, 2956 and 3556 cm<sup>-1</sup>. The band at  $1701 \text{ cm}^{-1}$  is attributed to the ester (C=O) stretching mode. The band at 1775 cm<sup>-1</sup> is attributed to a stretching mode of the (C=O) bond of the PFPE-carboxylic acid which is strongly blueshifted (by  $50 \text{ cm}^{-1}$ ) due to the electronegative fluor atoms in alpha position to the carboxylic group. The same blueshift of the carboxylic (C=O) band was observed previously in the studies measuring the FTIR spectrum of the trifluoroacetic acid. The bands at 2848 and 2956 cm<sup>-1</sup> are assigned to symmetric and asymmetric stretching modes of the PEG (C-H) groups of the PFPE-PEG-PFPE product.2 The band at 3556 cm-1 is assigned to the asymmetric stretching (OH) vibrations. Figure VI.9 (B) shows representative FTIR spectra of the DI7000 and PFPE(7000)-carboxylic acid. This figure presents major bands at 1698, 1775, 2889, 2993 and 3556  $\rm cm^{-1}$ . The band at 1698  $cm^{-1}$  represents a stretching mode of the ester (C=O). The broad band at 2889 and 2993 cm<sup>-1</sup> represents the symmetric and asymmetric stretching of PEG (CH). The band at  $3556 \text{ cm}^{-1}$  is assigned to the asymmetric stretching (OH) vibrations.



Figure VI.9.: Representative FT-IR spectra of a triblock-copolymer surfactant and the PFPE. Figure. FTIR spectra of the reactants and the products of the surfactant synthesis. FC-40 perflourinated oil was used as a background solvent to obtain the spectra. (A) Comparison between the PFPE(7000)-carboxylic acid (20 mM) as a reactant and the PFPE(7000)-PEG(1400)-PFPE(7000) triblock product (20 mM). (B) Comparison between PFPE(7000)-carboxylic acid as a reactant (20 mM) and the PFPE(7000)-PEG-OMe(750) diblock product (20 mM).

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