Microfluidic formation of hierarchical micro and nano emulsions for biomedical applications

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Doctor of Philosophy

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"πάντα ρεῖ"

Heraclitus of Ephesus
To my family
Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation contains fewer than 65,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

Zenon Toprakcioglu
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Abstract

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The self-assembly of proteins and peptides into complex supramolecular structures provides an important avenue in the development of materials for biomedical applications. In particular, the design and fabrication of protein and peptide-based hydrogels has emerged as an attractive route toward novel materials with tunable three-dimensional chemical and physical structure, biodegradability, biocompatibility, as well as drug loading and release properties. These unique characteristics offer great potential for the utilisation of self-assembled hydrogels as drug delivery vehicles, biomedical materials, and tissue engineering scaffolds. A class of materials highly suitable for such applications due to their biodegradability and lack of cellular toxicity is silk-derived proteins. Regenerated silk fibroin (RSF) in particular, retains most of the properties of native silk but is readily available through scalable processes. This natural block copolymer has the propensity to self-assemble into a fibrillar network that is $\beta$-sheet rich, and its biocompatibility coupled with its remarkable mechanical properties make this protein an excellent candidate as a material for biomedical applications.

A facile and reproducible route towards the fabrication of novel materials, featuring organised and multicompartmental structure with high particle monodispersity can be achieved through microfluidics, and in particular, droplet-based approaches. In this thesis, microfluidic techniques are employed to generate microcapsules comprised of a protein-fibrillar network. Capsules exhibiting complex internal structure were fabricated, while droplets ranging from hundreds of nanometers up to hundreds of microns, were generated. Furthermore, different approaches as to how such microcapsules/microgels can be used for biomedical applications are presented through the generation of hierarchical emulsions, but also through the formation of Janus-like microgels, exhibiting enhanced release kinetic profiles.

Furthermore, by integrating silver nanoparticles with silk-based microcapsules, I was able to systematically form organic/inorganic microgels, and investigate their properties both in vitro and in vivo. Not only did these hybrid microgels display potent antimicrobial
properties, but in contrast to conventional treatments involving silver, the organic/inorganic microgels showed minimal cytotoxicity towards mammalian cells, making them ideal for wound healing purposes. To this effect, the antibacterial and wound healing properties of the hybrid microgels were investigated through the use of a murine model. It was determined that the efficacy of this system is comparable to results obtained when using a conventional antibiotic such as ampicillin, which clearly demonstrates the potential of the hybrid microgels for treatment of surgical site infections.

The successful delivery of cargo molecules for cell related applications mostly depends on particle size and its distribution. Although traditional methods of generating nanoparticles have resulted in significant advances, some of which are currently used for pharmaceutical treatments, systematic control over size and monodispersity remains challenging. In order to address this issue, I developed a microfluidic/nanofluidic device capable of generating nanosized water-in-oil emulsions, with sizes ranging from 2500 down to 50 nm. By adding monomeric protein to the aqueous phase, these nanoemulsions acted as templates to form nanogels, which had the ability to permeate through mammalian cancer cell membranes and deliver intracellular cargo.

Finally, in order to gain a better understanding of the processes involved in protein self-assembly, novel label-free approaches which utilise the fluorescence of the intrinsic amino acid, tryptophan, were investigated. It was found that during protein self-assembly, or more generally during protein phase transitions which involve hydrophobic burial, an increase in the tryptophan fluorescence signal was observed. This allowed for a systematic study of protein phase transitions, such as fibril, spherulite and crystal formation, without resorting to extrinsic fluorophores, paving the way for a label-free method to monitoring self-assembly events. In order to fully explore the potential of observing such events in a massively parallel way, I developed a microfluidic device capable of trapping thousands of individual droplets under zero-flow conditions. This device was used to spatially confine and temporally monitor biomolecular interactions within thousands of droplets simultaneously, and the statistical character of self-assembly could thus be highlighted.
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9. Zenon Toprakcioglu, Tuomas PJ Knowles, Shear-mediated sol-gel transition of re-generated silk allows the formation of Janus-like microgels, Scientific Reports, 2021 11 (1) 1-10

10. Zenon Toprakcioglu, Pavan-Kumar Challa, Catherine Xu, Tuomas PJ Knowles, Label-free analysis of protein aggregation and phase behavior, ACS nano, 2019, 13 (12), 13940-13948

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15. Zenon Toprakcioglu, Aviad Levin, Tuomas PJ Knowles, Hierarchical biomolecular emulsions using 3-D microfluidics with uniform surface chemistry, Biomacromolecules, 2017, 18 (11), 3642-3651


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Chapter 1

Introduction

1.1 Proteins

A group of macromolecules which play a central role in the processes of life and perform a vast array of biological functions are proteins. They consist of a long sequence of amino acid residues that are linked together through peptide bonds, while their roles range from protecting against infections (antibodies), to molecular transport through cells (membrane proteins), and as reaction catalysts (enzymes). Proteins have the ability to fold into 3-dimensional structures, which are unique to each protein.[1]

Fig. 1.1 Schematic showing the different structures that a protein can undertake. The quaternary structure in this schematic is hemoglobin. Figure adapted from [1].
Protein folding involves a series of steps; initially, a primary amino acid structure can fold into a secondary structure, which is stabilised by intermolecular hydrogen bonds. Some examples of typical secondary structures include $\alpha$-helix and $\beta$-sheet conformations. These secondary structures can then further come together to form larger, tertiary structures. In some proteins, such tertiary subunits can further assemble or co-assemble into quaternary structures. [1] This is schematically depicted in figure 1.1.

1.1.1 Protein self-assembly

The self-assembly of monomeric protein units into supra-molecular complexes plays an important role in numerous biological contexts [2, 3] and is a fundamental characteristic of life. Depending on solution conditions, these structures can range from nanometers to micrometers in size. Many living organisms utilise the propensity of proteins to fold into complex structures for the formation of functional materials. These materials consist of a fibrillar network of densely packed $\beta$-sheet structures which, for historical reasons, have been referred to as amyloid fibrils. The solubility of proteins and their phase behaviour has been of considerable interest due to the fundamental role proteins play in biological systems and the fact that their biological activity is modulated by their assembly state. Moreover, it is now recognised that different solid protein phases such as gels[4–6], aggregates[7], or crystals can co-exist with liquid condensed phases.[8–10] While most proteins self-assemble in order to fulfil their functions such as forming viral capsids, [11] molecular motors[12] or cytoskeletal filaments[13], protein self-assembly can also be connected with the formation of aberrant structures and biological malfunction. [14–16]

In particular, amyloid fibrils, which are rich in $\beta$-sheet structure, are associated with the development of neurodegenerative diseases such as Parkinson’s or Alzheimer’s. [17, 18] Such fibrils are the result of protein misfolding, where exposed hydrophobic surfaces render the structures "sticky" resulting in the formation of toxic oligomer and aggregate species. Not only do such aggregates lack normal function, but play a pivotal role in disease as they accumulate in cells and grow over time, leading to amyloidosis. Even though elaborate pathways for recycling and removing such misfolded species exist, in many cases complete removal of the toxic species is impossible leading to the neurodegenerative disorders mentioned above. A schematic of the state of a native protein and how it may misfold to form aggregates is shown in figure 1.2.
1.1 Proteins

Fig. 1.2 Schematic showing the states of a protein and how it may misfold to form different species such as toxic oligomers or aggregates. Figure adapted from [1].

1.1.2 Monitoring protein self-assembly

Understanding the mechanisms involved in protein misfolding is of fundamental importance in formulating approaches that may tackle these neurodegenerative diseases. A key aspect that is crucial in evaluating the aggregation kinetics of biomolecular species is the ability to monitor the nanofibrillar growth as a function of time. To that effect, turbidity assays [19, 20] as well as fluorescent techniques have been developed so as to observe how protein aggregates are formed [21]. A widely used fluorophore used to detect protein nanofibrils within a solution is Thioflavin T (ThT). This fluorophore has the characteristic of exhibiting an increase in its quantum yield once bound to a $\beta$-sheet rich structure, and therefore an increase in fluorescent intensity correlates with an increase of $\beta$-sheet rich structures. This technique offers a multitude of advantages such as ease of application, sensitivity and largely
Introduction

non-invasive nature [21]. However, the use of an extrinsic fluorophore raises the question whether the molecule itself affects, either by promoting or by inhibiting, the kinetics of protein aggregation. For this reason, there has been an interest in utilising intrinsic fluorescence of proteins to study them in their native state with the benefit of having minimal or no structural modifications to the polypeptide chain. Proteins containing aromatic amino acids such as phenylalanine, tyrosine and tryptophan absorb and fluoresce in the ultraviolet range (250-400 nm) [21], and because of this label-free phenomenon, a wealth of physical properties such as hydrodynamic radius [22], electrophoretic mobility [23], conformational changes [24–33] or protein binding interactions [34–37] can be probed.

1.1.3 Silk fibroin

Protein nanofibrils represent a highly ordered form of supramolecular protein structure and result from the conversion of soluble peptides or proteins into β-sheet rich supra-molecular polymers [38]. Such fibrils can be formed by a wide variety of polypeptide chains with a range of physico-chemical properties. Many living organisms, ranging from bacterial cells to mammalian systems [38–41], have harnessed this inherent ability of proteins to form functional materials in areas as diverse as forming viral capsids, acting as molecular motors or cytoskeletal filaments and are even thought to play a protective role in various fungi. A key feature of this class of structure is the formation of an extended intermolecular β-sheet network by the monomeric subunits [42, 43]. This structural arrangement makes nanofibrils stiff and resistant to physical forces, [44–46] but also relatively inert when subject to harsh external conditions such as increased temperature and high/low pH [47]. Furthermore, the self-assembly of protein fibrils can often be achieved under mild aqueous environment conditions [48–50]. Thus, these thermodynamically stable fibrillar assemblies are emerging as potential building blocks for material based applications which include drug delivery [47, 51], release of cargo molecules/particles such as nanoparticles, peptides or antibodies, but also as scaffolds for cell growth, proliferation and differentiation [52–55].

A protein that possesses remarkable properties and is of particular interest for fundamental studies but also for industrial and material based applications, is silk. The silk produced in the glands of arthropods such as spiders not only exhibits remarkable mechanical properties, but in combination with its biocompatibility, biodegradability and lack of cellular toxicity, make this protein an ideal candidate for biomedical applications. [56] Silk fibres consist of densely packed β-sheets and β-strands that are parallel to the main fibril axis. This is in contrast to amyloid fibrils, where the β-strands are arranged so that they are perpendicular to the constituent proto-filament axis while the β-sheets are parallel [57]. A schematic of this can be seen in figure 1.3.
However, obtaining large quantities of silk protein from spiders involves extraction of the protein from the glands of the spider while the animal is still alive, thus making the harvesting process quite challenging. An alternative approach is to extract the protein from the cocoons of the Bombyx mori silkworm. [58, 59] As the silk protein from these cocoons exhibits mechanical properties that are similar to those of spider dragline silk [60, 61], such use of this alternative material is preferable. Bombyx mori silk is comprised of native silk fibroin coated with the protein sericin. The silk fibroin, which is easier to manipulate as compared to sericin, consists of heavy ($M_w \sim 390kDa$) and light chains ($M_w \sim 26kDa$) that are linked together through disulfide bonds. [62] Silk fibroin is a hydrophobic block copolymer that self-assembles to forms β-sheet structures resulting in an extremely resilient and tough material, which due to its bioavailability is excellent for pharmaceutical and biomedical applications. [63] The purification process involving the removal of sericin results in a new type of fibroin, which is known as reconstituted silk fibroin (RSF), and it displays different properties to native silk fibroin.

RSF has been used as a fundamental building block for the formation and generation of a wide range of functional bio-materials such as hydrogels, bioadhesives, implantable
scaffolds, nano/microspheres and biofilms [63, 64]. Additionally, RSF exhibits high cellular compatability as well as low immunogenic potential [65–67], making it an ideal candidate for biomedical applications. Moreover, RSF biodegradation rates can be specifically tuned through proteolysis pathways [68, 69, 59, 70, 71], and numerous studies have revealed that encapsulation efficiencies of small molecules within the gel matrix of silk hydrogels are quite high [71, 72, 70]. Furthermore, RSF-based materials has gained increasing attention in tissue engineering [59, 73–75], as implantable devices [76–78] and even in drug delivery studies [79, 80, 70, 81, 71] for the reasons mentioned above.

Fig. 1.4 Schematic of the potential uses of processes silk from the cocoons of the silkworm *Bombyx mori*. Figure adapted from [73].

More recently, RSF-based materials such as hydrogels and biofilms have been utilised as platforms for the delivery of bioactive molecules, [79, 82, 83, 80, 71] and drugs [84–87, 70]. In this context, degradable and biocompatible microspheres represent a promising class of materials since they can be regulated so that release kinetics can be specifically tailored for increased therapeutic results [88–90]. It has been reported that silk microspheres not only have prolonged release rates but also slow degradability [71, 72, 70], which can be a desirable property when using such capsules for release applications. Silk microcapsules have been
generated through a variety of methods, such as ultrasonic emulsification [91], through phase separation [71, 92], or even by spray-drying [93]. Even though these techniques have shown promise, controlling and modulating the capsule morphology remains a key challenge in utilising silk microspheres for drug delivery applications. For such applications, it is essential to have a highly monodisperse sample in order to ensure that both capsule biodegradability, but more importantly, release kinetics are specifically controlled [94].

1.2 Microfluidics

Microfluidics involves studying the flow of small volumes of liquid, which are typically sub-nanolitre, through micron-sized channels. Using such small length-scales offers advantages such as having a low Reynolds number, which is the ratio of the inertial to the viscous forces within a liquid that is subject to a motion. Therefore in such microfluidic regimes, laminar rather than turbulent flow is established, which allows for various physical observations to be made. Moreover, due to the minute volumes involved, such microfluidic systems have proven invaluable in tackling challenging problems such as the detection of single-molecule species.

1.2.1 Microfluidic techniques

The systematic study of chemical and biochemical processes on such small scales is thus of increasing interest, and microfluidics offers an ideal platform for such experiments due to its high throughput. Of particular interest in this context is droplet-based microfluidics. By utilising microdroplets, produced using conventional Polydimethylsiloxane (PDMS) based microfluidic devices, each picolitre droplet effectively acts as an individual microreactor. This principle has in turn allowed for the probing of PCR reactions [95], protein nanofibrillar aggregation studies [96–98], cell based assays [99, 100] and also DNA binding assays [101] at high throughput in picolitre volumes.

Typically, water-in-oil droplets are produced by using flow-focusing droplet generating devices, where two immiscible phases intersect resulting in the formation of micron-sized droplets [102]. Parameters such as droplet size can be precisely controlled by changing the microfluidic channel dimensions or by varying the ratio of the continuous oil phase flow rate to that of the aqueous one. The medium in which the droplets are suspended is referred to as the continuous phase, while the disperse phase refers to the droplet phase. The physics behind droplet formation has been systematically studied and two main regimes have been identified; a dripping and a jetting regime. Droplet break-off occurs when the dispersed phase has an instability and the continuous phase shears part of the fluid off. A dimensionless
parameter that governs whether droplet formation will occur and under which regime this will take place, is the Capillary number (Ca). This is used to describe the relationship between the viscous and interfacial tension forces of the fluid.

\[ Ca = \frac{\mu u}{\gamma}, \]  

(1.1)

where \( \mu \) is the viscosity of the continuous phase, \( u \) is the mean speed of the continuous phase fluid and \( \gamma \) is the interfacial tension between the dispersed and continuous phases.

If this dimensionless parameter is greater than \( 10^{-2} \), then droplet break off predominantly occurs at the jetting regime. Conversely, if it is below \( 10^{-2} \), then the dripping regime is predominant.

**Fig. 1.5** Schematic of the parameters governing droplet formation with a microfluidic channel. Figure adapted from [103].

### 1.2.2 Monitoring processes using microfluidics

Processes within the droplet can be monitored by collecting and storing them off-chip in glass or quartz capillaries or on chip in an array of traps by continuously applying flow from the continuous phase. [104–111] However, this mode of operation usually results in droplet shrinkage that is proportional to the continuous phase flow rate, a process which is due to a finite concentration of water being partitioned into the oil phase when the continuous phase is constantly being renewed. [105] Alternative ways of trapping droplets can be achieved by incorporating holes [112] or wells [113] into the device design. Additionally,
1.2 Microfluidics

it has been shown that electrical fields can both manipulate and confine droplets. [114] Another approach is to trap droplets without the need of a continuous flow. Such devices have been developed in order to observe the nucleation rate of lysozyme crystal growth [115], to monitor enzyme levels in single cells using fluorogenic assays [116], to study molecular transport through membrane pores of giant unilamellar vesicles (GUVs) [117] or even to investigate conformational membrane changes due to an applied shear force. [118] More recently, multi-layered lithography [119] has been used to produce a 3-Dimensional microfluidic devices capable of trapping cells without the need for a continuous flow [120].

One key application of microdroplet arrays is the monitoring of self-assembly processes in the context of biological and synthetic systems. Array-based technologies thus have a broad variety of applications due to their high throughput and high density, and therefore offer an ideal platform for parallel processing and monitoring of microlitre volume samples, ranging from nanocrystal nucleation events to studying single cell assays. [99, 105]

1.2.3 Microfluidics as templates for material fabrication

An alternative use of droplet-based microfluidics is for the generation of novel materials such as hydrogels/microcapsules [121, 122] or micro-gels [97, 119, 123, 124], as shown in figure 1.6 left panels. By encapsulating polymers and/or proteins within the aqueous phase, the propensity of such systems to self-assemble into supra-molecular structures can be utilised, resulting in the formation of the microgels. These materials may even display different and/or beneficial properties when compared to bulk studies.[125–129]. Microemulsions generated via polydimethylsiloxane (PDMS) based microfluidics thus represent an attractive platform for fundamental and biomedical applications because of the high level of monodispersity within the system. Since protein microgels are biodegradable, biocompatible, non-toxic and immunogenic, they represent ideal candidates for the storage and delivery of cargo molecules.[63] Recently there has been an increased interest in the generation of protein based micro-gels as versatile biomaterials [97, 119, 123] for various applications including the storage and delivery of cargo molecules. Moreover, micrococoon and microscale capsules have been fabricated using PDMS-microfluidics from native silk protein (NSF) for the storage of sensitive biological materials [130]. Additionally, using non-planar and glass capillary microfluidics, micron and sub-micron droplets from the more abundant RSF have been produced [119, 131, 132]. Due to the high level of monodispersity within the sample, release kinetics for drug delivery applications are not only reproducible, but more importantly can be specifically tailored for each individual system. Additionally, the use of protein-based materials as opposed to synthetically prepared ones, offers advantages such as biocompatibility, lack of toxicity, immunogenicity [63] and natural self-assembly.
1.2.4 Microfluidics for protein-nanoparticle generation

Addressing the challenge of cellular permeability for controlled delivery applications requires the systematic formation of suitable nanoparticles. Due to their small size such particles are ideal, indeed essential, for targeted drug delivery applications [133, 134, 70] and furthermore have been used in pharmaceutical, cosmetic, food and material-based industries [135–138]. Currently, a variety of methods for generating nano-scale particles are available, such as ultrasonic emulsification techniques, spray drying, phase separation/coacervation methods, solvent extraction or even bulk emulsion and polymerization techniques [139]. However, control over size and monodispersity, which are essential in regulating molecular release, remains problematic. A technique that can reproducibly generate highly monodisperse particles on larger scales is microfluidics. Droplet microfluidics in particular, has been used, through compartmentalisation and parallelisation [102, 140, 141], for carrying out PCR reactions [95], cell based [100] and DNA binding assays [101], as well as for protein nanofibrillar aggregation studies [97, 98]. Conventional polydimethylsiloxane (PDMS) based microfluidics has been used to generate monodisperse water-in-oil microemulsions with extremely high controllability. However, for applications involving drug/gene delivery, these micron-sized emulsions are far too large, as effective particle transfer through the cell membrane typically occurs if the drug-carrier is less than 1 \( \mu \text{m} \). Currently, it remains a challenge to generate monodisperse nanoemulsions using conventional microfluidics. [142].

More recently, however, RSF has been used to produce micro-gels as it self-assembles to form nanofibrillar structures resulting in a protein capsule. Capillary microfluidics has been used to form RSF micro and nano-gels where the dispersed phase comprised of RSF and the continuous phase was poly(vinyl alcohol) (PVA). [143] In fact, by changing the concentration of fibroin and adjusting the ratio of the flow rate of the dispersed to the continuous phase nano-gels as small as 200 nm could be formed, see figure 1.6 right panel. Additionally, the release kinetics of small molecules encapsulated within the formed micro/nano-gels was monitored, and it was found that 40% of the encapsulated molecules are released within the first 24 hours. [143] However, a systematic and reproducible way of generating protein-based capsules is clearly needed.

1.2.5 Higher order droplet formation

Microfluidic devices offer a highly suitable platform for producing water-in-oil (w/o) or oil-in-water (o/w) emulsions. Control of the dispersed phase droplet sizes is well established and can readily be achieved by varying the channel widths and the ratio of flow between the dispersed and continuous phases. [145] Controlling the surface wettability, however, is
1.2 Microfluidics

Fig. 1.6 Microfluidically generated polymer and protein micro and nano-gels. Figure adapted from [144] and Mitropoulos et al. [132].

crucial for droplet formation. [146] In order to produce droplets, the continuous phase must wet the channel surface, thus preventing the dispersed phase from coming into contact with the surface, i.e. to form a w/o emulsion, the surface of the device must be hydrophobic and vice versa when forming an o/w emulsion. For example, polydimethylsiloxane (PDMS), commonly used in microfluidic device fabrication, is intrinsically hydrophobic so that o/w emulsions are challenging to form without surface modification and even w/o droplets cannot easily be produced. Surface modification techniques such as oxygen plasma, or silane treatments [147, 148] can render the microfluidic device hydrophilic or hydrophobic, depending on their intended use.

Surface chemistry plays an even greater role when generating double emulsions. This is because the first and second junction of a microfluidic device need to display different surface properties. [149] For instance to produce w/o/w droplets, the first junction must be hydrophobic and the second one hydrophilic. Yet, such changes in surface chemistry on the micrometer scale can be challenging to achieve. [149, 150] A variety of methods for producing double emulsions exist, such as making two devices; one that is rendered hydrophilic while the other is hydrophobic. The w/o emulsion is formed in the former and then transferred directly to the latter device, where the process of producing w/o/w double emulsions is finalised. [151, 152] While such methods have been shown to allow the
successful formation of double emulsion droplets, not only do they require surface treatment of the devices [153, 154], which is often temporary, [155] but may also present additional difficulties, as both devices must be optimised to allow the sequential production of the droplets, adding another level of complexity to the process and to the potential towards scaling-up. Thus, there has been an increased interest in producing non-planar (3-D) devices, [146, 156, 157] where the geometry of the microfluidic device allows for the continuous phase, or the outer phase in the case of double emulsions, to completely engulf and surround the dispersed phase. By applying such an approach, the need for surface modifications is eliminated, and instead, by utilising the properties of the inner, middle and outer phase solutions one can make w/o/w or o/w/o double emulsions using a single microfluidic device design.

Double or higher order emulsions can potentially be beneficial to a variety of applications, such as in the biomedical and pharmaceutical fields, cosmetics or even in food products [158–160]. Therefore, allowing the scale-up of such platforms using reliable microfluidic devices that have high efficiencies is of increasing appeal. The ability to encapsulate drugs [161, 162], enzymes [163], hormones [164] or even living cells [165, 166] in the core of the double emulsion (i.e. the inner phase) and then control their release kinetics by altering the middle phase shell thickness is promising [167–169]. Additionally, double emulsions may have a significant role to play in the removal of toxic waste from non-potable water, as active matter can migrate from the outer to the inner phase of the emulsion. [170] Furthermore, biomimetic materials such as liposomes can be produced in this manner. [153] It has been reported that by using oleic acid, asolectin and cholesterol as the middle phase, w/o/w emulsions can be transformed into liposomes by solvent extraction [153].
Chapter 2

Materials and Methods

2.1 Materials and Methods

2.1.1 Device fabrication

In order to generate water-in-oil droplets, a soft photolithographic process was employed to fabricate the microfluidic devices used. In brief, a 50 µm thick photoresist layer (SU-8 3050, MicroChem) was spin-coated onto a silicon wafer and soft-baked for 15 min at 95 °C. The photo-mask was placed onto the wafer, which in turn was exposed to UV light. This was postbaked for 5 min at 95 °C. In order to remove excess photoresist, the master was developed in propylene glycol methyl ether acetate (PGMEA, Sigma-Aldrich). For the double emulsion experiments, a two-step photolithographic process was used.[119]

In order to fabricate microfluidic devices, a 10:1 elastomer PDMS to curing agent (SYLGARD 184, Dow Corning, Midland, MI) mixture was used. This was cured for 3 hours at 65 °C. The hardened PDMS was cut, peeled off the master and holes of 0.75 mm were punched into the PDMS. This was then bonded onto a glass slide by treating with a plasma bonder (Diener Electronic, Ebhausen, Germany).

2.1.2 Device fabrication for double emulsion devices

Two-level photolithography was used in the production of these particular masters. The two-layered master was fabricated by firstly spin coating a 25 µm thick negative photo-resist (SU-8 3025, MicroChem) onto a silicon wafer, and then soft baking for 15 minutes at 95 °C. The mask in figure 2.1a was placed on to the wafer, exposed under UV light and post baked for 5 minutes at 95 °C. [282] A second 25 µm thick layer was spin coated onto the wafer and the second mask was correctly aligned (figure 2.1b) with the pattern formed from the first
Materials and Methods

mask and UV exposed. Finally, the master was developed in Propylene glycol methyl ether acetate (PGMEA) (Sigma Aldrich) to remove any excess photo-resist. To produce the second master, the design shown in figure 2.1b was used and the same experimental procedure was employed using a 25 \( \mu \text{m} \) thick layer of photo-resist.

Fig. 2.1 (a-d:) Design of microfluidic devices used for generating double emulsions. (a-b:) Schemes of photomask designs used in the fabrication of the 3-D device. (a) Mask 1. Middle and Inner phase inlets and their respective channels. The middle phase channel width is 100 \( \mu \text{m} \). The inner phase is 50 \( \mu \text{m} \). The first junction is completely planar, or 2-D. (b) Mask 2. Outer phase inlet with its respective channels and outlet. The outlet channel is 200 \( \mu \text{m} \), while the rest of the channels are 100 \( \mu \text{m} \). Mask 1 is aligned with mask 2 to produce a two-layer master. (c) Schematic design of a microfluidic device for the productions of double emulsions. The device consists of 3 inlets, one for each of the three phases and an outlet, where the double emulsions are collected. (d) Magnified view of the two junctions where the double emulsions are formed. At the first junction, the inner and middle phase intersect, resulting in droplet formation. At the second junction, the outer phase engulfs the other two phases-leading to the production of double emulsions.

2.1.3 Device fabrication for nanofluidic devices

A two-step photolithographic process was utilised to fabricate the master used for casting PDMS devices. A 500 nm thick negative photo-resist (SU-8 2000.5, MicroChem) was spin-coated onto a silicon wafer. This in turn, was soft-baked for 2 minutes at 95 \(^\circ\text{C}\). The chrome mask in figure 1b was then placed onto the wafer, exposed under UV light in order to induce polymerisation and then post-baked at 95 \(^\circ\text{C}\) for 3 minutes. A second 25 \( \mu \text{m} \) thick layer (SU-8 3025, MicroChem) was then spin-coated onto the wafer and soft-baked for 5 minutes 95 \(^\circ\text{C}\). The second mask (shown in figure 1c) was aligned with respect to the patterns formed from the first mask. This was in turn exposed to UV light and post-baked for 15
minutes at 95 °C. Finally, to remove uncross-linked photo-resist, the master was developed in Propylene glycol methyl ether acetate (PGMEA, Sigma-Aldrich)

2.1.4 Droplet formation

neMESYS syringe pumps (Cetoni, Korbussen, Germany) were used to control the flow rates within the microfluidic channels. The dispersed phase consisted of a 40 mg/mL protein solution, and in the case of fluorescence based experiments Thioflavin T (Siga Aldrich) was added. Moreover, G5000 particles (Thermo Scientific) were used for colloidal experiments. The continuous phase was comprised of fluorinated oil (Fluorinert FC-40, Sigma Aldrich) containing 2% w/w fluorosurfactant (RAN Biotechnologies). All bright field images were acquired using a Mikotron high speed camera.

2.1.5 Double Emulsion Droplet Formation

For the w/o/w double emulsions, deionized water was used as the inner phase, fluorinated oil (Fluorinert FC-40-Sigma-Aldrich) containing 2% w/w fluorosurfactant (RAN biotechnologies) was used as the middle phase, while an aqueous solution of 3% w/w sodium dodecyl sulfate (SDS; Sigma-Aldrich), 3% w/w Tween 20 (Fisher Scientific), and 20% w/w polyethylene glycol (PEG; Fluka BioChemika) was used as the outer phase. For w/o/w double emulsions containing protein, a solution of reconstituted silk fibroin (Mindsets (UK) Limited) with 3% w/w Tween 20 and 100 µM Thioflavin T was used as the inner phase while the middle and outer phases remained the same.

For the o/w/o double emulsions, FC-40 was used as the inner phase, an aqueous solution of 3% w/w SDS, 3% w/w Tween 20 and 20% w/w PEG was used as the middle phase, and FC-40 with 2% w/w fluorosurfactant was used as the outer phase. For o/w/o double emulsions containing protein, a solution of reconstituted silk fibroin (Mindsets (UK) Limited) with 3% w/w Tween 20 and 100 µM Thioflavin T was used as the middle phase while the inner and outer phases remained the same.

2.1.6 Contact Angle Measurements

The static contact angles for the different solutions used were measured by taking images and then analyzing them using ImageJ. A 50 µL drop was placed on the PDMS surface and the angle between the surface and the tangent to the droplet was determined. It was found that deionised water has a contact angle of 105° ± 2° with PDMS while the aqueous solution
containing both PEG and surfactants (Tween 20 and SDS) has a contact angle of $60^\circ \pm 2^\circ$ with PDMS. Moreover, the contact angle of fluorinated oil with PDMS is $25^\circ \pm 2^\circ$.

2.1.7 Viscosity Measurements

Viscosity measurements were conducted on a Physica MCR 501 Anton Paar Rheometer. Cone and plate geometry was used to ensure the shear stress remained constant throughout the sample. All measurements were taken at 25 $^\circ$C under oscillatory shear stress. The viscosity of deionized water was found to be 0.8 $\pm$ 0.2 mPas, while the aqueous solution containing both PEG and surfactants has a viscosity of 34 $\pm$ 1 mPas. Finally, the viscosity of the fluorinated oil was determined to be 4 $\pm$ 0.2 mPas.

2.1.8 Silk fibroin preparation and purification

*Bombyx mori* silk cocoons (Mindsets (UK) Limited) were used to extract the silk fibroin protein by a well-established protocol. [63] Initially, the cocoons were cut into pieces and placed in a beaker containing a solution of 0.02 M sodium carbonate. This was then boiled for 30 minutes, ensuring that the sericin that is present within the silk fibres, dissolved, while the insoluble fibroin remained in the beaker. The fibroin was then removed from the beaker, rinsed with cold water three times and left overnight to dry out.

A 9.3 M lithium bromide solution was prepared and added to the dried silk fibroin in a 1:4 ratio of silk fibroin to lithium bromide. The mixture was heated to 60 $^\circ$C and left for 4 hours, resulting in the silk fibroin dissolving in the lithium bromide. LiBr was removed from the solution by placing the mixture in a 3 kDa dialysis tube. This in turn was placed in a beaker containing ultrapure water, while the use of a large magnetic stir bar with a magnetic stir-plate was employed to ensure mixing. The water was changed a total of 6 times in 48 hours.

Finally, the silk fibroin solution was removed from the dialysis tube and placed in Eppendorf tubes. These were then centrifuged at 9000 r.p.m. at 4 $^\circ$C for 20 minutes in order to remove small impurities. The process was repeated twice and the final solution was stored at 4 $^\circ$C. All experiments were conducted within 2 weeks of extracting and purifying the silk fibroin to ensure no gelation had occurred.

2.1.9 Microgel de-emulsification

Droplets were de-emulsified and separated from the continuous oil phase by applying the following protocol. A 20% 1H, 1H, 2H, 2H-perfluoro-octanol (PFO, Alfa Aesar) in FC-40
2.1 Materials and Methods

Oil solution was prepared and added to the emulsion. An equal volume of deionised water was then added to the emulsion. The samples were centrifuged for 2 minutes at 1000 rpm resulting in separation of the phases, with the supernatant containing the micro-gels. The supernatant was collected, and the whole washing process was repeated an additional two times.

2.1.10 Formation of silk hydrogels in bulk

Silk fibroin solutions ranging from 20 to 80 mg/mL were mixed in a 1:1 volume ratio with varying ethanol concentrations (0–100 (v/v%)). The ethanol containing silk mixtures were incubated in 96-well plates at room temperature (23 °C) for 24 h to induce fibril formation.

2.1.11 Fluorescence microscopy and image analysis

An inverted Zeiss microscope was employed to detect the fluorescence signal of the fluorophore thioflavin T. Appropriate filters that had an excitation wavelength of 440 nm and emission wavelength of 480 nm were used. All image analysis including measurements of radius of curvature and intensity were performed using ImageJ software.

2.1.12 Thioflavin T (ThT) assay

The structural change of protein monomers to fibrils was monitored and studied by looking at the change in intensity of ThT dye at 480 nm. A ThT concentration of 50 µM was used for all experiments. The fluorescent changes in intensity were measured by fluorescent plate reader Fluorostar (BMG Labtech) using an excitation wavelength of 440 nm and emission wavelength of 480 nm. For each set of conditions, a total of three repetitions were conducted (n = 3).

Furthermore, droplet aggregation kinetics were monitored microscopically as well by using ThT filters. Capillaries with an inner diameter height of 50 µm were placed in the outlet and 20 µm sized droplets were collected. The capillaries were then placed onto a heating stage which was set at 37 °C and left to incubate for the appropriate amount of time.

2.1.13 Encapsulation efficiency and release kinetics of small molecules

A solution of 500 µM rhodamine 6G or fluorescein was dissolved in the ethanol phase, and droplets were generated as described previously. The dye-loaded droplets were incubated overnight in FC-40 oil at room temperature to ensure protein self-assembly and consequently
microgel formation. The droplets were then de-emulsified as described above, with the exception that PBS buffer rather than MilliQ water was used. To calculate the percentage encapsulation, the concentration of dye in the washed solution had to be determined. This was done using absorbance measurements on a CLARIOstar plate reader (BMG labtech). To investigate release kinetics, 250 $\mu$L of both microgel solution and PBS buffer were pipetted into a dialysis tube (3500 MWCO). This in turn was placed in a 20 mL beaker along with a magnetic stirrer, and finally 5 mL PBS buffer solution was added. Gentle stirring (at room temperature) was ensured so that the dye could diffuse uniformly. The release medium was collected and changed at indicated time points. The released fractions were analysed using fluorescence measurements on a CLARIOstar plate reader. For release studies, experiments were repeated three times.

2.1.14 Scanning electron microscopy (SEM)

The sample was mounted onto a coverslip glass slide which was then placed onto a multi-pin specimen mount. The micro-gels were dried under low vacuum conditions with pressure $1 \times 10^{-3}$ mbar. A 10 nm gold layer was then sputter coated onto the sample (Denton Vacuum Desk IV). Images were obtained using a JEOL JSM-840 SEM at 15 kV.

2.1.15 Scanning electron microscopy using critical point drying

In order to prepare the sample for cpd, water had to be replaced by the exchange solvent ethanol. This was done sequentially in steps of 20% (i.e. a solution of 20% ethanol and 80% water was added to the sample and left for a day. This was then removed and a solution containing 40% ethanol and 60% water was added to the sample and again this was left for a day). This sequential addition of ethanol was used to ensure that the composition and morphology of the sample remained unchanged. Once dehydration was complete and the sample was in an environment containing 100% ethanol, the critical point drying process using liquid carbon dioxide is initiated.

Finally, the sample was mounted onto a silicon wafer which was then placed onto a multi-pin specimen mount. A 5 nm platinum layer was then sputter coated onto the sample and images were obtained using a FEI Verios 460 SEM at 1 kV.

2.1.16 Transmission electron microscopy (TEM)

The sample was mounted onto holey carbon-coated copper grid for 1 minute and then submerged (for 1 minute) into a uranyl acetate aqueous solution of so that it was stained.
Images were obtained using an Electron Microscope (JEOL JEM-2100) operating at 200 kV. TEM involves firing a beam of electrons at the sample. The transmitted electrons interact with the specimen as they pass through it, forming an image. This image is then detected using a CCD camera. TEM has much higher magnifying power than optical microscopy because the wavelength (\(\lambda\)) of electrons is much smaller than that of visible light (400-700 nm). Therefore, since the maximum resolution, \(\delta\), is related to \(\lambda\) according to the following equation, \(\delta=\lambda/2\text{NA}\), where NA is the numerical aperture, as \(\lambda\) decreases so does \(\delta\). This means that extremely small structures, on the order of nanometers can be detected using TEM.

2.1.17 Cryo-scanning electron microscopy (cryo-SEM)

For cryo-sem, the sample was first mounted onto a mutli-pin specimen mount. This was then placed in liquid nitrogen to rapidly freeze, thus ensuring that the micro-gels remained intact. The samples were fractured using a knife and 4 nm of platinum was sputter coated onto the sample. Images were obtained using a Zeiss EVO HD15 SEM operating at 6 kV to minimise beam damage.

2.1.18 Fourier Transform Infrared Spectroscopy (FTIR)

The conformational changes of silk fibroin (both bulk and micro-gels) were performed by using an FTIR equinox 55 spectrometer (Bruker). For the bulk measurements, the samples were loaded onto the FTIR sample holder and were analysed by subtracting a water reference. Conversely, for the micro-gel measurements, an emulsion of water droplets in FC-40 was used as a reference. In all measurements conducted, a carbon dioxide atmospheric compensation was made by subtracting this from the FTIR spectra. Each FTIR measurement was repeated five times for every sample.

Second derivatives of the spectra were calculated to deconvolute the major structural contributions in the Amide band I. To calculate the structural contributions, we followed the vibrational changes in the amide I band, which correlates to the secondary structures of proteins.

2.1.19 Atomic force microscopy (AFM)

AFM images were taken with Park NX10 (Park Systems) using non-contact mode.
2.1.20 **Deep UV Detection**

To detect intrinsic tryptophan fluorescence a 280 nm LED (Thorlabs M280L3) based epifluorescence microfluidic station was used.[22] The excitation filter (Semrock FF01-280/20-25) was centered at 280 nm, while the emission filter (Semrock FF01-357/44-25) has a central peak at 357 nm. Additionally, the use of a Zeiss microscope was employed to detect the fluorescent signal of the extrinsic fluorophore thioflavin T. Appropriate filters that had an excitation wavelength of 440 nm and emission wavelength of 480 nm were used.

2.1.21 **Analysis of Kinetic Data**

The kinetic data obtained experimentally were analysed using a previously established software, Amylofit.[263] All data were normalised with the minimum and maximum fluorescence intensity. Each experiment was repeated three times and then fitted by assuming a secondary nucleation-dominated model. A global fit was used for all concentrations tested, so that a single rate law could be applied to all kinetic traces simultaneously. This yields strong mechanistic constraints and allows a better evaluation of the kinetic data.

2.1.22 **Confocal microscopy**

A confocal microscope (Leica TCS SP5 X) was used for imaging the samples. A diode 405 and an argon laser were used for violet and green excitation, respectively. The 3D images were reconstructed using ImageJ software.

2.1.23 **Silver nanoparticle formation within microgels**

A 40 mg/mL solution of regenerated silk fibroin was mixed with a 125 µg/mL solution of silver nitrate (Sigma Aldrich) and illuminated with a tungsten lamp for a total of 48 hours. This allowed for the reduction of silver nitrate by tyrosine residues within the protein amino acid chain, to silver nanoparticles. This solution was consequently injected into the first inlet of the microfluidic device as shown in Figure 1a-c.

2.1.24 **Kinetic growth inhibition and minimum inhibitory concentration (MIC) analysis**

*E. coli* bacteria (ATCC 25922) were grown to an OD$_{600}$ of 0.01 or 0.25 in M9 minimal media. Silk and silk-silver microgel samples in serial two-fold dilutions were added to the bacterial samples in 96-well plates and kinetic growth inhibition was determined by optical density
measurements (600 nm) using a Tecan Spark 10M microplate reader (Tecan Trading AG, Switzerland). The MIC was determined using the microdilution assay and evaluation of the reduction in colony forming units was obtained by plating and counting bacterial samples before and after overnight treatment. The MIC was considered the lowest peptide concentration that showed no increase in optical density and no CFU growth overnight. Presented kinetic analysis results are representative of three independent experiments conducted in triplets.

2.1.25 Bacterial viability analysis

Following kinetic analysis, samples were washed thrice with saline, incubated for 15 min in a solution containing Syto9 and Propidium Iodide (L13152 LIVE/DEAD® BacLight™ Bacterial Viability Kit, Molecular Probes, OR, USA) and washed with saline again. Images were captured using a Nikon Eclipse Ti fluorescent microscope fitted with a Zyla scMOS camera using a Nikon Intensilight C-HGFI fluorescent lamp (Nikon Instruments Inc). The presented results are representative of three independent experiments.

2.1.26 Bacterial adherence analysis

E. coli bacteria (ATCC 25922) were grown to an OD600 of 0.01 in M9 minimal media and incubated with the silk and silk-silver microgels for five hours. Samples were taken each hour, washed thrice with PBS and subjected to confocal microscopy utilizing a LSM510 confocal microscope (Zeiss). The presented results are representative of three independent experiments.

2.1.27 Mammalian cell cytotoxicity experiments

3T3 fibroblasts and HeLa cells grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) were subcultured (2 × 10^5 cells/mL) (100 µL) in 96-well tissue microplates and allowed to adhere overnight at 37 °C in a humidified atmosphere containing 5% CO2. Medium was then discarded and silk-silver microgels samples and controls, diluted in DMEM without FBS (100 µL) were added to each well in quadruplet. Following an 18 hour incubation at 37 °C, cell viability was evaluated using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 10 µL of 5 mg/mL MTT dissolved in PBS was added to each well. After a 4-hour incubation at 37 °C, 100 µL extraction buffer [20% SDS dissolved in a solution of 50% dimethylformamide and 50% DDW (pH 4.7)] was added to each well, and the plates were incubated again at 37 °C for 30 minutes. Finally, color intensity was
measured using an ELISA reader at 570 nm. The presented results are the mean of three independent experiments.

2.1.28 Mammalian cell viability analysis

3T3 fibroblasts and HeLa cells grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) were sub-cultured \(2 \times 10^5\) cells/mL (100 µL) in 96-well tissue microplates and allowed to adhere overnight at 37 °C in a humidified atmosphere containing 5% CO2. Medium was then discarded and silk-silver microgels samples and controls, diluted in DMEM without FBS (100 µL) were added to each well in quadruplet. Following an 18 hour incubation at 37 °C. A fluorescent Live/Dead staining assay (Sigma Aldrich) containing fluorescein diacetate (6.6 µg/mL) and propidium iodide (5 µg/mL) was then used to visualise the proportion of viable versus non-viable cells in each sample. The labelled cells were immediately viewed using a Nikon Eclipse Ti fluorescent microscope and images were captured by a Zyla scMOS camera using a Nikon Intensilight C-HGFI fluorescent lamp (Nikon Instruments Inc). The presented results are representative of three independent experiments.

2.1.29 In vivo antibacterial activity evaluation in a mice model of surgical site infections. Preparation of infected sutures

3/0 silk braided sutures (Atlas Medical, Jordan) were cut into 1 cm sections and suspended in \(E.\ coli\) samples (ATCC 25922) at \(2 \times 10^9\) CFU/ml for 30 minutes at 37 °C. Sutures were then dried on sterile petri dishes for 15 minutes. This preparation resulted in the absorbance of \(3 \times 10^4\) bacterial cells per 1 cm suture as verified via the following protocol described for the tissue samples

2.1.30 Mouse model of surgical site infections (SSIs)

All animal experiments were approved and conducted in accordance with the Guidelines of the Institutional Animal Care and Use Committee (license no. 04-18-054). Every effort was made to relief animal stress and to minimise animal usage. Male mice of strain BALB/c OlaHsd (20-25 g) were purchased from Envigo, Israel, and kept in a 12 hours light-dark cycle with free access to water and pellets. Surgery was performed under 100 mg/kg Ketamine (Fort Dodge, USA) and 20 mg/kg Xylazine (Merck, Germany) anesthesia. 4 mg/kg Carprofen (Vericore, UK) was given preoperatively by intraperitoneal injection for post-surgical pain relief. Briefly, the back of the mouse was shaved, washed with 70% ethanol and a 1 cm
full-thickness incision wound was created on the back of the mouse with a sterile scalpel. An infected suture was placed into the wound and secured by a single nylon suture over the middle of the incision. Fifty microliters of controls or silk-silver microgels, all mixed at a 1:1 ratio with carboxymethyl cellulose were then applied to the wound with a micropipette. Two hours post-treatment the mice were euthanised by 100 mg/kg Pentobarbital (CTS Chemical Industries, Israel) and an area of $2 \times 1$ cm around the wound was excised and homogenised with a rotor stator homogeniser in 2 mL ice cold BHI. The homogenate suspension was diluted in serial 10-fold dilution steps by transferring 20 $\mu$L to 180 $\mu$L BHI in a 96 well plate. These dilutions were then plated on BHI agar plates and incubated at 37 °C over night. The colonies on the plates containing were then counted and the number of CFU/wound was determined. Statistical analysis was carried out by unpaired one-tailed t test, using GraphPad Prism 5 (GraphPad Software) and $p<0.05$ was considered statistically significant. The data are presented as the percent of the control group ± standard deviation of the mean.
Chapter 3

Silk fibroin characterisation and microgel formation

This chapter is based on the following publications:

1. Xizhou Liu*, Zenon Toprakcioglu*, Alexander J Dear, Aviad Levin, Francesco Simone Ruggeri, Christopher G Taylor, Mengsha Hu, Janet R Kumita, Maria Andreasen, Christopher M Dobson, Ulyana Shimanovich, Tuomas PJ Knowles, Fabrication and characterization of reconstituted silk microgels for the storage and release of small molecules, Macromolecular Rapid Communications, 2019, 40 (8), 1800898

2. Zenon Toprakcioglu, Tuomas PJ Knowles, Shear-mediated sol-gel transition of regenerated silk allows the formation of Janus-like microgels, accepted-Scientific Reports

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In this chapter, I conducted all the experiments involving self-assembly kinetic runs, microgel formation and characterisation, as well as generation of asymmetric microgels. I would like to acknowledge the help of Francesco Simone Ruggeri for the second derivative analysis of the FTIR spectra and Xizhou Liu for all his help regarding the ethanol-based silk microgels. Moreover I would like to acknowledge the help of Alex Dear for fitting the release kinetic data.

3.1 Introduction: silk fibroin and microgels

In nature, proteins are utilised by a plethora of organisms for the formation of biomaterials necessary for a wide range of functions. Silk fibroin is a natural protein obtained from the
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_Bombyx mori_ silkworm. In addition to being the key structural component in silkworm cocoons, it also has the propensity to self-assemble _in vitro_ into hierarchical structures with desirable properties such as high levels of mechanical strength and robustness. Furthermore, it is an appealing biopolymer due to its biocompatibility, low immunogenicity and lack of toxicity, making it a prime candidate for biomedical material applications.

Due to its tendency to self-assemble, silk-based microcapsules have increasing gained popularity. Water-in-oil microemulsions generated through capillary microfluidics allow for the formation of monodisperse capsules capable of biomedically related applications. However, the generation of microcapsules/microgels from RSF using PDMS droplet-microfluidics had not yet been investigated. In this chapter, RSF aggregation kinetics from monomer to fibrils are first investigated. Microgels are then formed and characterised using the microfluidic approach mentioned above and their potential use as depots for molecular delivery applications is further investigated.

In this chapter, silk fibroin is firstly characterised using a variety of techniques and then the protein solution is used for the formation of microgels capable of storing and protecting molecules for delivery related applications.

### 3.2 Characterisation of silk fibroin morphologies

#### 3.2.1 Silk fibroin aggregation kinetics

The relationship between pH and aggregation time of RSF was investigated in bulk. Thioflavin T (ThT) was chosen as the fluorescent dye \( \lambda_{\text{ex}} = 440 \text{ nm}, \lambda_{\text{em}} = 480 \text{ nm} \), as this molecule only fluoresces when it binds to aggregated structures, so an increase in the emission intensity correlates directly with the formation of silk aggregates. \[171\] It was determined that the more acidic or alkaline the solution, the faster the aggregation, as can be seen in figure 3.1a. A range of different pH (pH 3 - pH 11) were investigated, and the fastest aggregation was found to occur at pH 11. RSF is a particularly interesting protein as it exhibits both primary and secondary nucleation. The initial increase in ThT fluorescence signal is characteristic of primary nucleation, whereas the plateau observed before the large increase in signal is referred to as the lag time and is typical of secondary nucleation. \[172\] Additionally, in figure 3.1b, where aggregation time is plotted as a function of pH, it can be seen that at pH 7, which is close to physiological pH, the aggregation time is around 75 hrs. As previously mentioned, in order to use silk micro-gels for applications such as small molecule encapsulation and controlled release, our system must work well at physiological conditions. To that end,
minimising the aggregation time of RSF at pH 7, so that the micro-gels can be readily used was a crucial step.

Fig. 3.1 (a) Normalised reaction kinetic curves of RSF conducted at T = 37 °C. The rate of aggregation depends on pH and is fastest with more acidic or alkaline conditions. RSF seems to aggregate via primary and secondary nucleation. (b) Aggregation time of RSF as a function of pH. (c) Normalised reaction kinetic curves of RSF in EtOH and MeOH conducted at T = 37 °C. Under these conditions, RSF aggregation follows a primary nucleation mechanism. (d) Normalised reaction kinetic curves for different solutions conducted at T = 37 °C. The aggregation mechanistic pathway changes according to whether EtOH is present and whether one is looking at droplets or bulk. (e) Concentration dependence of RSF aggregation time. A linear-log plot of the data indicates that the aggregation time appears to follow roughly a parabolic curve.

It was found that just by making silk droplets, aggregation time can be reduced from 75 hrs to 45 hrs (figure 3.1d), which is probably due to the micro-gels having a high surface to volume ratio, thus promoting aggregation. However, it appears that forming droplets results in an additional effect on the aggregation kinetics of the system. The sigmoidal aggregation curve does not exhibit any primary nucleation; instead, it mostly displays secondary nucleation. This behaviour is not well understood and further studies must be conducted, however, a possible explanation is that the shear in the flow focusing device induces aggregation and these aggregates break off and form the platform for secondary nucleation. It has been reported by Breslauer et al. [131], that by using ethanol and/or
methanol, silk fibroin aggregation can be promoted, and, the aggregates still retain their structure and fibrillar nature. The bulk aggregation kinetics of silk fibroin containing ethanol (EtOH) and methanol (MeOH) are shown in figure 3.1c. There is a clear difference in the sigmoidal curves in this system with the aggregation kinetics shown in figure 3.1a. It seems that when MeOH or EtOH are added to RSF, the aggregation follows primary nucleation rather than secondary. As there is minimal difference in aggregation time between the solution with methanol and that with ethanol, the latter was chosen as the aggregation promoting agent, due to its biocompatibility as opposed to the high toxicity of methanol. Figure 3.1d compares the kinetics of the different solutions used. It is clear that there is a massive difference in aggregation time between the green curve, which contains silk, ThT and 17% ethanol, and the black curve which does not contain any ethanol. Additionally, droplets containing silk fibroin, ethanol and ThT were made microfluidically, and the aggregation time was monitored using a fluorimeter. It is evident that by making droplets of silk fibroin containing ethanol, the aggregation time can be decreased from 75 hrs down to 6 hrs. Moreover, even more interestingly, the system now exhibits primary nucleation as the dominant aggregation mechanism, whereas previously for micro-gels containing only silk fibroin, the aggregation was secondary in nature.

Finally, aggregation time dependence on concentration was investigated. The values of concentration employed ranged across three orders of magnitude. A linear-log plot of the data is shown in figure 3.1e and the aggregation time appears to follow roughly a parabolic curve. This behaviour is highly unusual, as it appears that increasing the silk fibroin concentration results in an increase in aggregation time, which is contradictory to how most proteins behave. Typically, as protein concentration increases, monomers are closer to one another, and so can come together faster, resulting in faster aggregation. This behaviour observed in silk fibroin could be due to fibrils entangling [173] with each other, as seen in polymers, thus inhibiting aggregation. Alternatively, it is possible that the monomer may diffuse more slowly as the gel forms, thus inhibiting further aggregation.

### 3.2.2 Silk fibroin-ethanol aggregation kinetics

A systematic study of how the aggregation time varies as a function of silk and ethanol concentrations was then investigated. It has been previously shown that RSF hydrogel formation could be promoted through the use of organic solvents [174–176], and in particular, adding ethanol has been shown to have profound results on the augmentation of converting soluble, random coil silk fibroin monomers to β-sheet-rich fibrillar aggregates [175]. This was achieved by systematically varying the amount of ethanol present in the solution but also by changing the concentration of protein used. The samples were places in a multi-well
plate and left overnight at room temperature to induce the aggregation process. The resulting solutions were imaged (Figure 3.2a). It is clear that mixtures containing less than 20% ethanol appeared as transparent solutions, while mixtures containing 20% or more ethanol formed opaque gels. When varying the fibroin concentration, it was observed that the formation of opaque gels only occurs in the presence of ethanol, while increasing protein concentration led to further opaqueness within the gel. Thus, it is clear that the rate of gelation in bulk is predominantly augmented by increasing ethanol content [177, 176].

![Image](image_url)

Fig. 3.2 Effect of silk fibroin concentration and ethanol content on hydrogel formation in bulk. (a) Image of multi-well plate containing samples of silk fibroin which ranged from 10-40 mg/mL, mixed with varying amounts of ethanol. The samples were incubated at room temperature for 24 h. (b) Reaction kinetic curves of a 20 mg/mL RSF solution with varying ethanol amounts, conducted at $T = 37^\circ$C. The rate of aggregation clearly depends on ethanol content.

To further elucidate the self-assembly mechanism, the aggregation kinetics of the bulk-systems were investigated through a Thioflavin T (ThT) assay. As previously mentioned, the quantum yield of ThT is highly dependent on the concentration of intermolecular β-sheets present. [43]. Detection of ThT fluorescence has been used to monitor the assembly of monomeric silk fibroin into fibrillar gel networks [178, 174, 175]. Figure 3.2b demonstrates that in the presence of low ethanol content, there is no increase in ThT fluorescence signal, indicating that the aggregation process has not started.

However, in mixtures containing 20% or more ethanol, there is a clear increase in fluorescence intensity, which follows a typical sigmoidal curve [178, 175]. Moreover, the rate of aggregation is augmented as the ethanol concentration increases as can be seen in Figure 3.2b, where the half-time, which is defined as half the value of the maximum
fluorescence intensity, of the yellow curve (50% ethanol) is substantially less than that of the purple curve (20% ethanol). This demonstrates the role of ethanol in silk-gelation, as the time taken for aggregation is reduced by around 5 times when the total ethanol concentration in the mixture is doubled from 20% to 40%.

A microfluidic-based strategy to generate nanofibrillar microgels using the same silk-ethanol system was next explored. A flow-focusing microfluidic device [179, 180] (figure 3.3a) was used to form water-in-oil microemulsions, where both silk and ethanol were dissolved in the aqueous phase. The device design consists of two junctions. RSF solution was injected into the central channel and mixed with ethanol at the first junction, as shown in Figure 3.3a. The aqueous phase was then encapsulated by the immiscible oil phase to form a microemulsion at the second junction. These microdroplet compartments serve as templates, in which RSF self-assembled into nanofibrillar structures which then become microgels [176, 179]. The droplets were de-emulsified by washing with perfluorooctanol (see Methods) and then re-immersed in an aqueous phase. Uniform microgels could be systematically and reproducibly generated with diameters ranging from 70 µm to 110 µm as shown in figure 3.3b.

The aggregation kinetics of the silk-ethanol solution in droplets were then monitored. The formation of nanofibrils, rich in β-sheets, within the microgels was confirmed through ThT fluorescence (figure 3.3c). Droplets were confined within capillaries and then imaged over time. Moreover, the ethanol content present in the solution was systematically changed from 0-50% and the droplets were incubated overnight at room temperature in order to allow for the gelation process to occur. The fluorescent microscopy images reveal a gradual increase in ThT fluorescence intensity during the initial incubation period followed by a rapid intensity increase.

To further compare the aggregation kinetics between bulk and microfluidically formed samples, droplets were placed in a multi-well plate. Again, ThT was used to monitor the progression of the self-assembly process. Fluorescent emission profiles were normalised and are shown in figure 3.3D. While the profiles of the curves look similar, the droplet-based microgels aggregate much faster, and consequently have shorter lag phases, than their counterpart bulk assays. The high molecular weights of fibroin make it prone to self-assemble into fibers or gels upon exposure to shear forces [71], and therefore this behaviour is attributed to the shear induced aggregation occurring within the microfluidic device. This topic was further studied, the results of which are shown in the second part of this chapter. Therefore, as the rate of RSF microgel self-assemble are high tunable, with half-times ranging across orders of magnitude, such microcapsules allow for scale-up fabrication, with potential applications in the pharmaceutical or biomedical fields.
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Fig. 3.3 (a) Schematic representation of the double flow focusing device used to generate droplets. At the first junction, the solution containing silk fibroin intersects with the ethanol-based solution. At the second junction, the continuous oil phase engulfs the aqueous phases resulting the formation of water-in-oil droplets. The microemulsions are incubated at room temperature for 24 h before washing. (b) Effect of flow rate on silk microgel diameter. (Inset) Bright field microscopy images of different sized silk microgels obtained from the corresponding flow rate ratios. Scale bar represents 50 µm for all images. (c) Bright field micrograph of droplets containing RSF and ThT dye (left panel). Fluorescent micrograph sequence of the corresponding droplets demonstrate the conversion of monomer to its fibrillar, aggregated state. (d) Normalised reaction kinetic curves of RSF conducted at T = 37 °C for both bulk solutions (full sphere) and microdroplets (empty sphere). The protein concentration used was 20 mg/mL.

3.2.3 Silk fibroin micro-gel formation and characterisation

Initially, samples of silk fibroin in the absence of ethanol were incubated at 37°C in order to promote protein aggregation. Transmission Electron Microscopy (TEM) was conducted in order to determine whether bulk RSF solutions contained supramolecular fibrillar structures. The fibrils, which exhibit branching, are shown in figures 3.4a-b. Scanning Electron Microscopy (SEM) was used to probe the micro-gels. Initially, regular SEM was used, however, the high vacuum in the sample holder resulted in most of the droplets collapsing. When increasing the RSF concentration to 40 mg/mL, the problem was partially solved as some of
the micro-gels remain intact, however it is clear from figures 3.4c-d that it is impossible to determine the surface structure in detail.

Cryo-SEM was employed in order to image the micro-gels without affecting their morphology. As this technique involves rapid freezing of the sample in liquid nitrogen, the micro-gels remain intact and their spherical structure can clearly be seen in figures 3.4e-f. Unfortunately, it was still challenging to image the surface structure, as the micro-gels were enveloped in a layer of oil. However, by washing and de-emulsifying the samples (technique described in methods and materials) the surface structure of the micro-gels could be observed in great detail. It is evident that the micro-gels exhibit fibrillar structures on their surface, seen in figures 3.5a-b, as was predicted from the bulk measurements (figures 3.4a-b). Additionally, the fibrils appear to be pointing outwards, behaving in a similar manner to polymer brushes (figures 3.5c-d). This type of fibrillar behaviour has been previously observed by Shimanovich et al. [181] In this work, the formation of water-in-water droplets that are stabilised by lysozyme fibrils, and are thus able to overcome the ultralow surface tension of all-aqueous interfaces was reported. Using SEM, fibrils on the droplet interface were observed, and it seems as though the lysozyme fibrils are also pointing outwards, which is in agreement with these results.
Fig. 3.4 (a-b) TEM images of RSF obtained from bulk solution of 0.4 mg/ml at pH 7. The RSF solution was allowed to incubate at 37 °C for 4 days before the sample grids were prepared and images were taken. The fibrils, which exhibit branching, appear darker. The scale bars for the two images are 100 nm and 500 nm respectively (c-d) SEM images of RSF micro-gels. It is clear that the micro-gels have collapsed due to the high vacuum inside the chamber. The scale bars for the two images are 5 µm and 10 µm respectively. (e-f) cryo-SEM images of RSF micro-gels before washing. The droplets have remained intact but are covered in a layer of oil and thus surface structure cannot be determined. The scale bars for both images are 2 µm.
Fig. 3.5 Cryo-SEM images of RSF micro-gels after washing. (a-b) RSF micro-gels approximately 20 µm. The surface of the structures is composed of fibrils. The scale bars for the two images are 10 µm and 3 µm respectively. (c-d) Surface topology of micro-gels reveals that the fibrils are pointing outwards and seem to behave like polymer. Furthermore, the majority of the fibrils are sub-micron in width, which confirms the results obtained from the TEM images. The scale bars for both images are 2 µm.

Next, in order to investigate the effect of ethanol content and silk concentration, SEM was again employed. However, due to challenges associated with cryo-SEM, samples were freeze dried and then imaged using a regular SEM. The freeze-drying process retains both microgel topology but also, to an extent, structure. Figure 3.6a-b shows the presence of porous sheet like structures on the surface of the freeze-dried microgels. It appears that the microgels exhibit similar pore sizes (on average) even when the ethanol concentration is increased, which can be seen in figure 3.6a. This is contrary to previous reports of hydrogel formation, where it has been found that increasing ethanol concentration leads to a systematic reduction in pore size. However, an increase in fibroin concentration resulted in morphological changes;
pore sizes gradually decreased and microgels became more stable as can be seen in figure 3.6b. It is therefore clear that the surface morphology of the microgels can be controlled by varying the initial fibroin concentration.

Fig. 3.6 (a) SEM micrographs of the freeze-dried RSF microgels prepared from a 20 mg/mL protein solution with varying ethanol content. Scale bars are 50 µm, 10 µm and 2 µm from top to bottom. (b) SEM micrographs of the freeze-dried RSF microgels prepared from 20% ethanol mixed with varying fibroin concentrations as shown. Scale bars are 50 µm, 10 µm and 2 µm from top to bottom.

3.2.4 Secondary structure of silk fibroin monomer and hydrogels/microgels

To better determine the effect that ethanol concentration has on the secondary structure of the silk protein as it undergoes its transition from monomer to highly ordered β-sheet fibrils, Fourier transform infrared spectroscopy (FTIR) was performed on the bulk solutions (figure 3.7a). The amide I band is a major band of the protein infrared spectrum and gives valuable information regarding secondary and quaternary structure of proteins [182, 183]. In particular, this approach has been used to reveal the structural changes in RSF hydrogels [176]. The FTIR data obtained for the RSF hydrogels show that when 20% ethanol or higher is added to the system, there is a characteristic shift of the amide I peak towards a lower wavenumber value, which indicates the formation of intermolecular hydrogen bonds. The second derivative analysis of these FTIR curves reveal that soluble RSF (where only 0% or 10% ethanol was used) adopts a predominantly random coil conformation (1648 cm⁻¹), with
minor contributions from $\alpha$-helical (1655 cm$^{-1}$), $\beta$-turn (1675 cm$^{-1}$) and antiparallel $\beta$-sheet conformations (1695 cm$^{-1}$), which is in good agreement with the literature [176, 184, 185]. In contrast, RSF hydrogels (which consist of an ethanol content that is >20%) are rich in both parallel (1625 cm$^{-1}$) and antiparallel (1695 cm$^{-1}$) intermolecular $\beta$-sheet conformations, with minor $\alpha$-helical (1655 cm$^{-1}$) and $\beta$-turn (1675 cm$^{-1}$) contributions, demonstrating the induced effect ethanol has on the formation of intermolecular hydrogen bonds. Thus one can spectroscopically differentiates the aggregated state from the monomeric, soluble state by determining whether or not intermolecular hydrogen bonds are present. In figure 3.7b, the FTIR analysis of the RSF microgels can be seen. Again, a shift in the amide I band towards lower wavenumber indicates an increase in intermolecular hydrogen bonds. Moreover, the second derivative analysis further reveals that microgels are also rich in intermolecular $\beta$-sheet structures, with minor contributions from $\alpha$-helical and $\beta$-turns. Furthermore, by increasing the ethanol concentration, a slight decrease in the content of antiparallel $\beta$-sheet conformation and an increase in the parallel $\beta$-sheet content is seen. Thus, changing the ethanol content above 20% results in minimal structural variations, which is in close agreement to the morphological data obtained from SEM imaging.

Fig. 3.7 (a) FTIR spectra of RSF solutions which were incubated overnight at room temperature, with varying ethanol concentrations (0-50%). The chart on the right panel represents structural changes of RSF protein in bulk. The contribution from each component was calculated based on the difference in the amide I peak intensities of the FTIR spectra. (b) FTIR spectra of RSF microgels incubated overnight at room temperature, with varying ethanol concentrations (20-40%). The chart on the right panel indicates the structural changes of the protein within the microdroplets.
3.2.5 Silk microgels for release kinetic applications

Having generated and characterised the RSF microgels, a potential application of these microgels as vehicles for the encapsulation and release of small molecules was investigated. Two representative fluorescent dyes were selected; a hydrophilic, water-soluble dye, fluorescein sodium salt, and a more hydrophobic dye, rhodamine 6G. The dyes were added to ethanol channel (figure 3.8a) and thus dye-loaded microgels were formed. The encapsulation efficiency of rhodamine 6G exceeded 95% in all microgels analysed, indicating that the dye was efficiently incorporated within the fibrillar network of the capsule. This is shown in figure 3.8b. However, in contrast to this result, the encapsulation efficiency of fluorescein within the microgel network was around 40%. This different loading efficiency is probably due to the strong binding that rhodamine 6G exhibits with the silk fibrils via hydrophobic interactions, as opposed to the hydrophilic nature of the fluorescein that decreased its binding affinity to the silk fibrils.

![Fig. 3.8](a) Images of dye solutions (free) and dye-loaded microgels (loaded); (b) Encapsulation efficiency for the different microgel systems. The encapsulation efficiency was measured for each of microgel system. Red: standard microgel, Blue: reverse microgel, Green: high density microgel and finally Purple: ethanol-rich microgel.

In order to determine the release kinetics of the encapsulated dye molecules, RSF microgels were placed into dialysis membranes, which were in turn placed in a PBS buffer solution (pH 7.4) and stirred at room temperature. The buffer was changed at regular time points and fluorescence intensity measurements were conducted in order to measure the concentration of
the dye molecules which had been released. Moreover, four different microgel systems were studied in order to fully investigate release kinetic profiles: (1) ‘standard microgel’ (where a 20 mg/mL silk solution comes from the inner channel and a 20% ethanol solution comes from the side channels); (2) ‘reverse microgel’ (where a 20% EtOH solution is introduced from the inner channel and then a 20 mg/mL silk solution comes from side channels, as illustrated in figure 3.9 a); (3) ‘high density microgel’ (this is the same as (1), however, a 40 mg/mL silk solution rather than a 20 mg/mL solution was used); (4) ‘ethanol-rich microgel’ (same as (1), however, a 40% ethanol solution rather than a 20% solution was used). In figure 3.9b the cumulative release of the dye molecules as a function of time can be seen for the different microgel systems.

Using the semi–empirical Korsmeyer-Peppas model, the release kinetics of the microgels were then quantified [186–190],

\[
\frac{M(t)}{M(\infty)} = kt^n, \tag{3.1}
\]

where \(M(t)/M(\infty)\) is the fraction of dye which has been released from the microdroplets into the surrounding PBS buffer, and \(k\) is the release constant. The value of \(n\), which corresponds to the release exponent, can be used to determine which mechanism is predominantly responsible for the release of the dye. For spherical particles, it is accepted that the rate limiting step for a \(n\)-value of 0.43 is fickian diffusion. In such a system, the dye simply diffuses out of the microgel into the surrounding medium. Alternatively, polymer swelling has been found to be the rate limiting step for a \(n\)-value of 0.85. Here, the buffer penetrates the gel matrix and hydrates it. Any intermediate value corresponds to a combination of these two factors. This equation only holds for release fractions which are below 60%.

The \(n\)-values from this data, suggest that dye diffusion is the predominant rate-limiting step for rhodamine release (see figure 3.9b). However, polymer swelling seems to be the rate-limiting step in the fluorescein release (figure 3.9b right panel). Moreover, the fluorescein is released faster than the rhodamine, which is in agreement with the hydrophobicity of the two molecules, i.e. the hydrophobic rhodamine sticks to the fibrils and is released in a slower manner.

Morphological changes due to increased ethanol content within the system appear to increase the rate of rhodamine release, resulting in diffusion no longer being entirely rate-limiting. However, ethanol content has a minimal effect on the rate of fluorescein release, which still remains predominantly swelling-based, suggesting that a morphological change does not strongly effect the rate at which the gel hydrates. Moreover, when the protein concentration is increased, resulting in a denser gel matrix, slower release kinetics are expected. This is indeed observed for both dyes systems, however, the effect is more
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Fig. 3.9 (a) Schematic representation of the system used to investigate the release kinetics of dye molecules. Droplets are initially formed by having a double flow focusing device, where at the first junction the protein solution is mixed with a solution containing both the dye and ethanol. At the second junction the oil phase encapsulates the aqueous phases resulting in the formation of water-in-oil droplets. Following incubation at room temperature for 24 h, microgels are washed and then subsequently monitored to investigate the release kinetics. (b) Release profiles of rhodamine 6G (left panel) and fluorescein (right panel) dyes through silk-based microgels in PBS buffer. The Korsmeyer-Peppas model was used to fit the data (solid lines). The dashed gray line on the right panel indicates the 60% release which is the maximum fractional release that this model can be employed for. Red: standard microgel, Blue: reverse microgel, Green: high density microgel and finally Purple: ethanol-rich microgel.

dominant for rhodamine, which changes from diffusion to swelling-based release. As previously described, rhodamine has strong hydrophobic interactions with the polymer matrix. Therefore, rhodamine probably interacts more with the higher-density polymer matrix, causing it to be retained. A long period of swelling is likely needed to disrupt the matrix sufficiently for the dye to diffuse out at an appreciable rate.
These results demonstrate that the rate of release of cargo molecules from the microgels can be controlled by making small changes to the method of microgel assembly. In general, the rate of swelling can be slowed by increasing the protein content of the microgel. This would seem to be of particular use for hydrophilic cargoes, whose release is more rapid. Predicting and controlling the release kinetics for drug-related systems is of course key for any biomedical application. Finally, it is important to note that although hydrophilic cargo is released relatively rapidly, the rate of release can be further augmented by preparing a reverse microgel, where the cargo is closer to the microgel surface.

3.3 Formation of microgels through shear flow

3.3.1 Asymmetric tubular-like micro-gels

The effect of shear on aggregation and on microgel morphological variations was first investigated. It is known that when protein solution travels through a microfluidic channel, it experiences shear. In fact, it has been reported that shear can induce protein aggregation[191, 192], and this effect is particularly prominent with silk-based proteins[193]. Both the spider and silkworm are capable of pulling liquid protein from their glands and through the use of shear, a phase transition occurs which aggregates the protein into its well-known solid form. However, in the context of drug delivery, it would be particularly interesting to be able to utilise this and form capsules where the outer shell has aggregated and is solid, but internally there is still a liquid phase, i.e. a core-shell structure. To that end, and knowing that silk is shear sensitive, a high concentration of reconstituted silk fibroin (40 mg/mL) was passed through a microfluidic chip with a single junction, a schematic of which is shown in figure 3.10a, in order to generate asymmetric microgels.

It is known that the velocity profile within a channel has a parabolic curve[194], figure 3.10b, and that the shear stress is the derivative of the velocity multiplied by the viscosity, with respect to the direction of flow movement, figure 3.10c. This is represented by the following equation, \( \tau = \eta \frac{\partial u}{\partial y} \), where \( \eta \) is the dynamic viscosity of the flow, \( u \) is the flow velocity of the fluid and \( y \) is the height above the boundary.

Therefore, it is clear that the shear stress is higher at the walls of the channel. When a droplet containing protein monomers is pushed through such a channel, the shear force that the protein solution experiences within the droplet is highest at the walls and subsequently a reasonable assumption would be that protein aggregation would initiate there. A planar schematic representation of a droplet travelling through such a microfluidic channel is shown in figure 3.10d, where protein aggregation can be seen to initiate at the walls of the device.
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\[ \tau = \eta \left( \frac{\partial u}{\partial y} \right) \]

Fig. 3.10 (a) Schematic representation of the microfluidic device used. (b-c) Schematic representations of the velocity profile of the fluid through a microfluidic channel (b) and the corresponding shear stress distribution (c) (d) Schematic representation of a droplet (blue) travelling within a microfluidic channel, surrounded by the oil phase (yellow) while undergoing shear. The shear stress is highest at the walls of the channel, which is where protein aggregation initiates. Proteins are represented as orange spheres. The panel on the right is a 3-dimensional schematic of the microfluidic channel. The red area corresponds to the aggregated protein, while the blue area in the middle represents the non self-assembled and still liquid aqueous core.

However, in a 3-dimensional microfluidic channel, the droplet experiences a shear force from all areas it is in contact with, and therefore, protein aggregation occurs all around the aqueous droplet. This is schematically represented in the right panel of figure 3.10d, where the red area corresponds to protein aggregates, and the blue area in the middle of the core-shell structure, is the non self-assembled, still liquid, aqueous phase.
Characterisation of the asymmetric microgels

Fig. 3.11 (a-d:) Brightfield images showing the outlet of the microfluidic chip. A 40 mg/ml RSF protein was used as the dispersed phase and FC-40 with fluorosurfactant was used as the continuous phase. (a) Elongated fibre-like structure when $Q_{dis}=100 \mu$L/hr, $Q_{cont}=100 \mu$L/hr. (b) Asymmetric, tubular structures when $Q_{dis}=100 \mu$L/hr, $Q_{cont}=400 \mu$L/hr. (c) Mixture of tubular and spherical structures when $Q_{dis}=100 \mu$L/hr, $Q_{cont}=800 \mu$L/hr. (d) Spherical structures when $Q_{dis}=100 \mu$L/hr, $Q_{cont}=1500 \mu$L/hr. (e) Darkfield images of tubular structures following formation. (f) Darkfield images of tubular structures one week after formation. (g) FTIR spectra of 40 mg/ml RSF. The blue curve represents the monomeric form of the protein, before any gelation has occurred. The green curve represents the micro-gels 1 hour after formation, while the red curve corresponds to the micro-gels 24 hours after formation.

The generation of different microgel morphologies was next investigated by varying the flow rate ratio between the aqueous and oil phases. Initially, both the oil and aqueous phase flows were kept constant at a rate of 100 $\mu$L/hr, which resulted in elongated structures (figure 3.11a). When the oil to aqueous phase flow rate ratio was increased to 4:1, a mixture of tubular and spherical micro-gels was formed, figure 3.11b-c, while a further increase in this
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Fig. 3.12 (a-c) cryo-SEM micrographs of tubular/asymmetric microgels. (d) Micrograph of a fractured microgel which shows that the surface has aggregated whereas the core remains liquid. (e-f) High magnification cryo-SEM micrographs of the microgel surface. A dense fibrillar network is clearly present.

ratio to 15:1 led to the generation of just spherical structures being formed, figure 3.11d. Furthermore, it appears that these structures were not only stable just after formation, but remain so for longer than 24 hours, as can be seen in figures 3.11e-f. In order to determine
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the structural transitions undergone by the sheared silk fibroin, FTIR measurements were conducted. The silk fibroin prior to gelation (blue curve) has peaks at 1650 cm$^{-1}$ and 1545 cm$^{-1}$ which are characteristic of random coil conformation and correspond to the amide I and II bands respectively. As can be seen from the spectrum in figure 3.11g, following droplet generation, the amide I peak shifted, suggesting that partial aggregation (and gelation) had occurred (green curve). Moreover, an FTIR spectrum following overnight incubation (red curve) was obtained. The amide I and II peaks had further shifted to 1630 cm$^{-1}$ and 1520 cm$^{-1}$ respectively confirming the transition from random coil to beta sheet structure.

The surface structure of the asymmetric microgels was visualised using cryo-scanning electron microscopy (cryo-SEM). Microgels were formed and immediately de-emulsified (see Methods) before being re-immersed into an aqueous phase. The overall tubular/asymmetric morphology can be seen in figure 3.12a-c. A fracture on one of the microgels (figure 3.12d) reveals that the surface of the particle has aggregated and formed a fibrillar network, while the core of the microgel still contained protein monomers. The outline of the microgel remains and can clearly be seen. Furthermore, high magnification images on the surface of the asymmetric microgels was conducted. The micrographs reveal the presence of a dense fibrillar network with fibrils pointing outwards.

Effect of flow rate on shear-induced microgels and quantification of aggregates on microgel surface

The effect that flow rate, and subsequently shear rate and shear stress, have on the morphology of the formed droplets was determined and correlated to the amount of aggregates present at the surface. This was done by measuring the radius of curvature of the microgels as a function of flow rate. To correlate the flow rate with the shear stress at the walls of a rectangular channel, the following relation was used, $\tau = \eta \frac{OPA}{8A}$, where $\eta$ is the dynamic viscosity of the flow, $Q$ is the flow rate, $A$ is the cross-sectional area of the microfluidic channel, $P$ is the hydraulic diameter (or the wetted perimeter) and $\lambda$ is a shape factor defined by $\lambda = 24/[((1 - 0.351\frac{b}{a})(1 + \frac{b}{a}))^2$, where $a$ represents the long side of the rectangle and $b$ represents the short side.[194]

Therefore, by changing the flow rate, the shear stress was systematically varied and the amount of surface aggregation could be monitored. The most stable configuration of a water in oil droplet is a sphere. In fact, if the droplets only contained an aqueous phase then the interfacial tension would promote the minimisation of the surface energy resulting in the droplets adopting a spherical shape. Therefore, any asymmetric microgel must contain elements of aggregated protein. Consequently, the radius of curvature is proportionate to the
3.3 Formation of microgels through shear flow

Fig. 3.13 (a-c) Asymmetric microgel formation due to variations in the flow rate and shear stress. The dispersed phase flow rate remained constant at 100 µL/hr, while the continuous phase flow rate was 200 µL/hr, 300 µL/hr and 500 µL/hr respectively for each panel. The red circle indicates how the radius of curvature was determined for each system with different flow conditions. (d) Fluorescence micrograph of asymmetric microgels. The microgels exhibit a higher fluorescence signal at the asymmetric areas. (e) Graph of normalised intensity against position along the microgel for the two line profiles of the microgel in (d). The red curve corresponds to the line profile along the spherical part of the microgel while the green curve represents the line profile at the asymmetric part. (f) Double logarithmic plot of the radius of curvature against shear stress. The fit to the data is shown as a solid red line. Inset: Graph of radius of curvature against shear stress. (g) Graph of radius of curvature against percentage of aggregates.

amount of aggregates present. Moreover, the more the surface has aggregated, the smaller the radius of curvature. A systematic change of the shear flow was thus conducted and the
radius of curvature was measured. Examples of asymmetric microgel formation through varying flow rates are shown in figure 3.13a-c. Furthermore, a red circle around the edge of the microgel indicates how the radius of curvature at that particular area was determined for each flow rate system.

The graph of wall shear stress as a function of radius of curvature is plotted in figure 3.13f inset. It is clear that the data follow an asymptotic trend, where a high shear stress corresponds to a smaller radius of curvature, which in turn suggests a larger amount of aggregates. A double logarithmic plot of the radius of curvature against the shear stress, shown in figure 3.13f, indicates a power law with an exponent of -0.22, as obtained by the fit.

In order to further investigate how shear affects the protein solution within the droplet, a fluorescent dye was added to the aqueous solution. The fluorescent dye Thioflavin T (ThT), has the tendency to increase its quantum yield when it interacts with protein aggregates and in particular with $\beta$-sheet structures. Therefore, a higher fluorescence intensity corresponds to the presence of protein aggregates. Following droplet generation through the microfluidic chip, the microgels were collected and imaged using fluorescence microscopy. Figure 3.13d shows characteristic asymmetric silk-based microgels. In figure 3.13d it can be seen that the microgels have a higher fluorescence intensity at regions which are not spherical, indicating the presence of a solid, aggregated surface and the formation of a shell at that particular area.

Moreover, it is evident from figure 3.13d that only areas which are asymmetric exhibit a higher fluorescence signal. A comparison between the spherical and non-spherical part of the microgel was conducted. A line profile along the spherical part (red line) and along the asymmetric part (green line) was taken and is shown in figure 3.13e. From the data, which are normalised with respect to the maximum intensity, it is clear that the mean intensity value within the droplet is around 0.7. The red curve, which represents the line profile at the circular region follows a gradual intensity increase, with a maximum in the middle of the droplet, as is expected. However, the line profile along the asymmetric area (green curve) has two prominent peaks, which correspond to the aggregated part of the microgel. The relative difference between the mean intensity value within the droplet and at the surface of the asymmetric part for this particular microgel, is around 25%. Such an analysis was conducted for 10 droplets and it was found that the relative change between the asymmetric and circular areas was $27\% \pm 3\%$.

Finally, by comparing the increase in fluorescence signal of ThT as it binds to $\beta$-sheets, the amount of aggregates present at the surface were quantified for each system of microgels formed under different shear stress conditions. The graph of surface aggregates formed as a function of the radius of curvature is shown in figure 3.13g. The shear stress has thus been
correlated to the amount of surface aggregates, and therefore for a given flow rate one can predict how many surface aggregates will be formed.

**Asymmetric tubular microgels for storage and release of cargo molecules**

Finally, the potential use of the asymmetric microgels for storage and release of cargo molecules was investigated. 5 µm sized colloidal particles were added to the protein solution and following droplet generation, the colloids were encapsulated within the microgel. The tubular microgels were then collected and imaged using brightfield microscopy. A time-lapse image sequence (figure 3.14a-d and SI videos 1-3) reveal that even though the surface of the microgels has aggregated/gelled, the core remains liquid, which is clear from the diffusion exhibited by the colloidal particles. The yellow circles in figure 3.14a-d show an example of a colloidal pair, where there is clear diffusion of these two particles with respect to each other. Such a system, where the surfaced has gelled but the core remains liquid, can be used for pharmaceutically related applications such as antibody delivery. In this approach, anything encapsulated within the core of the microgel is protected, while it is still free to diffuse, making these asymmetric capsules ideal for cell related encapsulation studies.

Moreover, an alternative route to controlling molecular diffusion for delivery related applications is through the use of double emulsions.[195–197] Water-oil-water (wow) or oil-water-oil (owo) hierarchical structures have previously been shown to be quite effective at tailoring release kinetics of encapsulated molecules.[198, 199, 119] To that effect, tubular microgels were encapsulated within a wow double emulsion. This was done by using two consecutive junctions, where the second junction has a non-planar (3-D) geometry.[119] At the first junction, the protein solution is intersected by the oil phase, leading to the formation of the asymmetric microgels. In the second, non-planar junction, the external aqueous phase intersects the microgel in oil resulting in the generation of the double emulsion. By varying the flow rates, tubular microgels with different aspect ratios, but also the number of asymmetric microgels encapsulated within the double emulsion, could be varied and controlled systematically. This is shown in figure 3.14e-h, where wow double emulsions with different number of encapsulated asymmetric structures as well as varying tubular aspect ratios can be seen.

Finally, by slightly changing the device geometry so that the angle at the junction is 60° rather than 90°, janus-like microgels could be formed (figure 3.14i-k). Moreover, it can be seen that the microgels have a higher fluorescence intensity at regions which are not spherical, indicating the presence of a solid, aggregated surface and the formation of a shell at that particular area. Such structures have particular promise as "smart materials" and have significant advantages in directed motion as the two parts of the particle posses different
Fig. 3.14 (a-d) Time-lapse microscopy sequence of asymmetric core-shell microgels with colloidal particles encapsulated within them. The yellow circles show an example of a colloidal pair, where the particles move with respect to each other freely within the microgel, indicating that the core is liquid and only the external surface has aggregated/gelled. (e-h) Wow double emulsions with asymmetric tubular microgels as the core part of the hierarchical structure. For (e-g), the outer phase flow ranged from 400-600 $\mu$L/hr respectively while the oil to protein phase flow rate ratio was kept constant at 4:1. For (h), the oil to protein phase flow rate ratio was 7:1, while the outer phase flow rate was 400 $\mu$L/hr. (i-k) Fluorescence microscopy images of janus-like microgels formed using a microfluidic device with a 60$^\circ$ junction.

physical/chemical properties which can be utilised. Furthermore, as the production of protein-janus particles is challenging, this microfluidic approach to generating monodisperse silk-based janus microgels can be quite advantageous.
3.4 Conclusions

Silk fibroin is a particularly interesting material due to the combination of its remarkable mechanical properties but also because of its highly promising biomedical applications. In this chapter the aggregation kinetics of silk were first investigated and the pH dependence as well as the effect of alcohol on the self-assembly process was determined. Moreover, by using droplet microfluidics, silk-based microgels were generated and their morphologies and structures were characterised using electron microscopy and FTIR. Additionally, the potential application of these microgels as drug delivery vehicles was showcased. Both hydrophobic and hydrophilic molecules were encapsulated within the microgels and their release kinetics were monitored and fitted to a well established model. Finally, due to the propensity of silk to aggregate under shear flow, the formation of asymmetric, tubular-like microgels was achieved by modulating the flow rates within the microfluidic channels. The resulting microgels exhibited a core-shell structure, where the surface had aggregated while the internal core was still liquid. This structure was confirmed from the electron micrographs and from the experiment involving the encapsulation of colloidal particles within the microgels. Here, the colloids were free to move within the tubular capsules, indicating that while the surface had gelled, internally the microgel was still liquid. Furthermore, the morphologies of the asymmetric microgels could be controlled by varying the shear rate, which affected the degree of surface aggregation, as corroborated by fluorescence microscopy. Such microfluidically generated protein-based microgels and capsules show great promise in delivery related applications. Due to their biocompatibility and biodegradability, protein, and in particular silk, capsules offer an alternative and possibly better route for approached involving the biomedical and pharmaceutical industries.
Chapter 4

Inorganic/organic microgels for therapeutics and biomedical applications

This chapter is based on the following publications:


* Authors contributed equally

In this chapter, I conducted all microfluidic based experiments as well as electron microscopy characterisation. I would like to acknowledge the help of Tuuli Hakala for experiments related to the encapsulation of air bubbles within microgels, and Lee Schnaider who conducted all the cell culture and viability experiments as well as the murine model.

4.1 Introduction

Self-assembling peptides and proteins have the potential to serve as multi-functional building blocks for the generation of versatile materials for a wide range of biomedical applications. [200–205] Supramolecular hydrogels, comprised of self-assembled protein nanofibrils, are a particularly versatile class of biomaterial and have been used in contexts ranging from
tissue engineering to drug delivery. [206–209] Due to the rapid emergence of multidrug resistant bacteria, the development of biomaterials with intrinsic antimicrobial properties has been continuously increasing. [210, 211] Despite continuous improvements in biomedical material development; nosocomial and bacterial-associated infections, still pose major global healthcare problems. [210, 211] Silver-based antiseptics, which have shown broad-spectrum antimicrobial activity and lower propensity to induce microbial resistance as compared to classical antibiotics, represent an important class of antimicrobial agents which could potentially be utilised to overcome these pressing matters. [212, 213] Indeed, silver has been used for hundreds of years for antimicrobial applications and recent strides in the fields of nanotechnology have led to the development of silver nanoparticles with substantial biocidal activity. [212–217] Crucially, however, these nanoparticles commonly display significant cellular toxicity towards mammalian cell lines, limiting their biomedical use. [216, 218, 219] Furthermore, the antibacterial properties of the silver nanoparticles are highly dependent on their size and spatial arrangement, thus generating challenges for reproducible formulation. [219, 220] In the first part of this chapter, the production of a hybrid organic/inorganic nanofibrillar silk microgel decorated with silver nanoparticles is shown. These microgels display potent antimicrobial activity \textit{in vitro} and \textit{in vivo} and are able to adhere bacterial cells to their surfaces and subsequently eradicate them through a two-step mechanism of action. Importantly, in contrast to treatments involving conventional silver nanoparticles, these silk-silver microgels are non-hemolytic and non-cytotoxic towards mammalian cell lines. Finally, it is shown that these hybrid microgels display substantial efficacy as topical antimicrobial agents in a murine model with surgical site infections. These results demonstrate the ability of inorganic decorated silk microgels to act as powerful antimicrobial agents through a two-step mechanism combining adherence and eradication, and open up a route towards the future development of highly tunable and biocompatible microgels which can encapsulate active compounds for various biomedical applications.

The encapsulation of microbubbles has recently been demonstrated in the context of modulating the porosity of microcapsules for a wide range of biomedical related applications [221, 222] such as targeted drug delivery[223, 224], gene therapy[225], delivery of biocatalysts[226] and as tumour/thrombus-destruction materials [227]. Moreover, hollow particles have also attracted attention in the field of energy-storage[228] and in cell culture assays [221, 222]. Furthermore, bubbles form the basis of generating foamed porous materials which are essential in the cosmetics, pharmaceutical and food industries [223, 229]. Such porous materials may offer orthogonal routes for controlled release of chemicals and are thus quintessential in these industries. Controlling the surface area, pore size and pore volume of microcapsules are thus key parameters, yet remain challenging to achieve using conventional
bulk techniques. In the second part of this chapter, a microfluidics-based approach for the formation of monodisperse silica-coated micron-scale porous capsules of controllable sizes is described. This method involves the generation of gas-in water-in oil emulsions, and the subsequent rapid precipitation of silica which forms around the encapsulated gas bubbles resulting in hollow silica capsules with tunable pore sizes. By varying the gas phase pressure, control over both the diameter of the bubbles formed and the number of internal bubbles enclosed within the silica microcapsule can be achieved. Moreover, it is further demonstrated through the use of optical and electron microscopy, that these silica capsules remain stable under particle drying. Such a systematic manner of producing silica-coated microbubbles and porous microparticles thus represents an attractive class of biocompatible material for biomedical and pharmaceutical related applications.

4.2 Hybrid inorganic/organic silk-silver microgels

4.2.1 Formation and characterisation of the hybrid inorganic/organic silk-silver microgels

In order to fabricate micron-scale hybrid organic/inorganic materials a microfluidics-based platform was developed. The FDA approved protein regenerated silk fibroin (RSF) was used as the basic building block. Not only does this protein have the propensity to self-assemble into nanofibrillar structures, but it can also reduce silver nitrate which leads to the formation of silver nanoparticles (AgNPs). Micron-sized hydrogels were thus generated which exhibited antibacterial properties while simultaneously displayed little to no toxicity towards mammalian cells. Finally, a mice model was used to demonstrate the potential of this material as a local treatment against surgical site infections.

The hybrid material was produced by a two-step process. Initially, silver nitrate was added to a monomeric RSF solution. In the presence of white light, the silver nitrate was reduced by tyrosine residues, within the protein structure, resulting in the formation of AgNPs. This is done through the reduction of Ag\(^+\) to Ag\(^0\) via electron donation by the phenol group on the tyrosine amino acid, resulting in a cyclic radical as the oxidation product. [230–232] This is schematically depicted in figure 4.1a. The composite materials were subsequently synthesised using a droplet microfluidic approach. [141] Water-in-oil (w/o) microdroplets were generated on-chip as shown in figure 4.1b. The device consisted of 2 junctions; in the first junction, a solution consisting of the monomeric RSF with silver nanoparticles is mixed with a 40% v/v ethanol solution (figure 4.1c). This aqueous mixture then intersects with the oil phase in the second junction, resulting in the formation of w/o microdroplets. Following
Fig. 4.1 Formation and characterization of regenerated silk fibroin-silver (RSF) microgels. (a) Schematic depicting the synthesis of the silver nanoparticles through the reduction of silver nitrate by tyrosine. (b) Scheme of the microfluidic device used consisting of two junctions. (c) Schematic representation of water-in-oil microdroplet generation and consequent de-emulsification. In the first junction the protein solution is mixed with the ethanol solution. Droplet formation occurs at the second junction where the oil intersects the aqueous phase. (d-g) SEM images of RSF microgels with increasing magnification. (e-f) SEM micrographs of a microgel which has fractured. The surface of the microgel has the same porous morphology as the internal structure of the particle. (g) High magnification micrograph showing the highly dense fibrillar network within the microgel. (h) TEM micrographs of RSF nano-fibrils without (left panel) and with silver nanoparticles (middle panel). Scale bar is 200 nm for both panels. The size distribution of the silver nanoparticles within the RSF fibrillar network was determined from the TEM micrographs and is depicted in the right panel.

generation, the soluble monomeric protein within the microdroplets was converted into a nanofibrillar gel by utilising the propensity of RSF to self-assemble into a supra-molecular complex under appropriate conditions. The hierarchical self-assembly of RSF, which contains long-range ordered spatial arrangement through light and heavy chains linked together by disulfide bonds, has been known to play an essential role in various bio-inspired applications [63, 205, 233, 234]. This self-assembly process was promoted by the addition of ethanol in the aqueous phase and by incubating at room temperature for 48 hours (figure 4.1c).

RSF nanofibrils within the microgels were visualised by conducting scanning electron microscopy (SEM). The samples were initially freeze dried (see Methods) and then imaged, resulting in the micrographs shown in figure 4.1d-g. The overall spherical microgel morphology can be seen in figure 4.1d, while a fracture on one of the microgels reveals that the surface of the particle has the same dense fibrillar network as the interior (figure 4.1e-f). The
mechanical properties of hydrogels have been shown to play a significant role for various biomedical applications [235]. In this study, however, as the weight fraction of the silver nanoparticles present within the hybrid microgels is around 0.03% it was expected that the mechanical properties of the microgels with and without the nanoparticles would be quite similar, and thus mechanical testing for the microgels was not conducted.

Furthermore, transmission electron microscopy (TEM) was conducted on RSF fibrils without the presence of (figure 4.1h left panel) and in the presence of (figure 4.1h middle panel) silver nanoparticles. From the TEM images, it was determined that the average size of the nanoparticles was 7.6 nm (figure 4.1h right panel). Furthermore, microgel stability over time was investigated. Once formed, in the absence of external environmental factors such as bacteria or cells, the microgels should not degrade. In an aqueous environment, it was determined using SEM that after 12 months, the microgels were still stable. Moreover, following their formation and de-emulsification, the microgel mean size was found to be 103 \( \mu \text{m} \) with a sigma value of 6 \( \mu \text{m} \) (figure 4.2 a-b).

![Image](attachment:image.jpg)

Fig. 4.2 Optical micrograph and size distribution of the microgels following their formation and de-emulsification. The microgels showed a uniform size distribution with a mean value of 103 \( \mu \text{m} \) and a sigma value of 6 \( \mu \text{m} \) (n = 120).

### 4.2.2 Evaluation of the antimicrobial properties of the silk-silver microgels

Following microgel formation, the antibacterial activity of the silk-silver microgels against *E. coli* (ATCC 25922) was evaluated. This was done via a minimum inhibitory concentration analysis, as well as by kinetic growth inhibition assays. Silk-silver microgels and control samples were added to *E. coli* cultures at early log-phase and incubated overnight. **Silk-silver**
microgels at a concentration of 3% v/v were found to completely inhibit bacterial growth, causing a 7.5 log reduction of bacteria, with lower concentrations partially inhibiting growth in a dose-dependent manner (figure 4.3a). Complete bacterial growth inhibition was observed for bacteria treated with silver nitrate at a concentration of 8 µg/mL and also with silver nanoparticles with a diameter of 10 nm at 10 µg/mL, while treatment with the silk microgels alone did not affect bacterial growth (figure 4.3a and figure 4.4a). To directly assess bacterial viability, bacteria were subjected to Live/Dead viability analysis using Syto9 (indicating live bacteria) and propidium iodide (indicating dead bacteria). Following 3 hours of treatment, bacterial cell death was observed for samples treated with the silk-silver microgels at 3% v/v and silver nitrate, this persisted over 17 hours of treatment, while the control and silk microgel treated bacteria remained viable and proliferated over time. (figure 4.3b).

The ability of the composite microgels to inhibit bacterial growth at a higher bacterial load, similar to that of an active infection was next evaluated. *E. coli* cultures were grown until reaching a mid-log phase and were then treated with 3% v/v samples of the silk-silver microgels. Kinetic growth inhibition analysis coupled with Live/Dead viability assays revealed that the silk-silver microgels inhibited bacterial proliferation but did not induce lysis at this bacterial load (figure 4.3c-d).

### 4.2.3 Evaluation of the silk-silver microgel mechanism of action

In order to gain insights into the mechanism of action of the silk-silver microgels, bacterial adherence kinetics of both the silk microgels alone and the composite microgels were evaluated. The *E. coli* strain utilised in these analyses is a free-living non-biofilm forming strain and yet the bacteria readily adhere to the silk microgel surface, which could in itself be utilised by the bacteria as a nutrient source. Bacterial adherence to the periphery of the microgels comprised of silk only was observed via confocal light microscopy following one hour of co-incubation (figure 4.5a). Over the course of 5 hours, bacterial adherence increased and spread to the central regions of the silk microgels, completely covering the surface of the particles in the process (figure 4.5a). A schematic representation of bacterial adherence on silk microgels with and without the presence of silver is depicted in figure 4.5b. Bacterial viability was assessed via the live/dead assay previously described, which demonstrated that the cells remained viable when adhered to the surface of the silk microgels. In the case of composite microgels, bacterial adherence to the periphery was also observed following one hour of incubation but to a significantly lower extent than that of the silk microgels (figure 4.5c). Bacterial adherence decreased over time in this case and propidium iodide staining revealed that the adhered cells were not viable (figure 4.5c).
4.2 Hybrid inorganic/organic silk-silver microgels

Fig. 4.3 Characterisation of the antibacterial capabilities of the silk-silver microgels. (a) Bacterial growth inhibition kinetics evaluated by turbidity analysis via absorbance readings at 600 nm. (b) Bacterial viability evaluation following overnight growth inhibition kinetic analysis using the Live/Dead backlight bacterial viability kit. (c-d) Addition of the silk-silver microgels to mid-log phase bacteria causes bacterial growth inhibition. (c) Bacterial growth inhibition kinetics following the addition of the silk-silver microgels to mid-log phase bacteria, evaluated by turbidity analysis via absorbance readings at 600 nm (d) Bacterial viability evaluation taken at the end of the kinetic experiment following 4.5 hour incubation of mid-log phase bacteria with silk-silver microgels. The magnification used for all microscopy images was 40X.
Inorganic/organic microgels for therapeutics and biomedical applications

4.2.4 Biocompatibility of the silk-silver microgels

The biocompatibility of the silk-silver microgels with mammalian cells was next evaluated via an MTT-based cell viability assay in which 3T3 fibroblasts and HeLa cells were grown overnight in 96-well plates in the presence of the silk-silver microgels and controls. Cell viability was not significantly altered by the presence of the silk microgels and the silk-silver microgels in the case of these cell lines (figure 4.6a-b). Cellular viability was reduced to 75-80% when treated with silver nanoparticles with a diameter of 10 nm (figure 4.4b-c) and 25% when treated with the silver nitrate solution (figure 4.6a-b). Similar results were obtained using Live/Dead staining, containing fluorescein diacetate (indicating live cells) and Propidium Iodide (indicating dead cells) of cells treated in the same manner (figure 4.6c-d). The hemolytic activity of the silk-silver microgels was also evaluated by spectroscopic measurements of the amount of hemoglobin released from red blood cells following treatment with the silk-silver microgels and controls. Following five-hour incubation with the silk-silver microgels, over 97% of the red blood cells remained intact and undisrupted (figure 4.7). These results demonstrate that the encapsulation of the silver nitrate within the microgels shields the mammalian cells from its cytotoxic effect, thus making the silk-silver microgels biocompatible, an important factor in the development of clinically relevant materials.
4.2 Hybrid inorganic/organic silk-silver microgels

Fig. 4.5 Characterisation of the bacterial adherence activity of the silk-silver microgels. (a) Kinetics of bacterial adherence with the silk and silk-silver microgels. In the top row (silk microgels alone) accumulation of bacteria over time is apparent while in the second row (silk-silver microgels) any bacteria that adhere to the surface are eradicated. (b) Schematic representation of the bacterial adherence over time (for silk microgels) and their eradication (for silk-silver microgels). (c) Viability analysis of the bacteria adhered to the two different types of microgels. For the silk microgels alone, no cell death is apparent. However, bacterial cell death is evident when silk-silver microgels are used. The magnification used for all microscopy images was 60X.

4.2.5 In vivo antibacterial activity of the silk-silver microgels

Despite many precautions and protocols that are taken in order to prevent infection, surgical site infections (SSIs) are one of the most frequently reported nosocomial infections. [236, 237] To evaluate the potential of the silk-silver microgels as a novel local treatment for SSIs, their antimicrobial effect was investigated in a murine SSI model. In this model (presented in figure 4.8a), a silk suture contaminated with *E. coli* bacteria was implanted into an incision wound on the back of mice and the infected incision site was treated with the silk-silver microgels and controls. An assessment of the infection was performed by counting viable bacteria in the tissue homogenate. In order to establish the model, it was first validated that
sutures carrying an inoculum of *E. coli* were able to cause marked infection in the wounds, which persisted during the entire period of observation. Variation between bacterial counts in individual wounds assessed was small, indicating that a contaminated suture could cause
Fig. 4.7 Hemolytic activity of the silk-silver microgels. Hemolytic activity was assessed by incubating defibrinated sheep blood with the silk-silver microgels and controls and calculations of the subsequent hemolysis from absorbance readings at 451 and 405 nm. The percentage of non-disrupted blood cells is presented.

a reproducible experimental infection (figure 4.8b). Once the model was established, the antibacterial effect of the silk-silver microgels was evaluated. These microgels were mixed at a 1:1 ratio with carboxymethyl cellulose (CMC) in order to increase the viscosity of the samples and allow for uniform coverage of the suture site. As the in vitro Live/Dead analysis
revealed that vast bacterial death occurred by three hours of treatment, viable counts in the wounds were assessed at two hours post treatment. A single treatment with the silk-silver microgels produced bacterial counts in wounds significantly lower than those obtained with the control, eliminating over 75% of the bacteria, as compared with the control of CMC alone (figure 4.8c). These results are comparable to those obtained in the case of treatment with 250 mg/mL of Ampicillin which resulted in killing of over 90% of the bacteria, thus demonstrating the potential of the silk-silver microgels as topical local drug delivery vehicles.

Fig. 4.8 Efficacy of the silk-silver microgels in a mouse model of surgical site infections (SSI). (a) Schematic representation of the SSI model. (b) Establishment of infection in a mouse SSI model. 1 cm-long, full thickness incision wound was created on the dorsal side of the mouse. Approximately 1 cm of silk suture infected with *E. coli* bacteria (∼ 3 × 10⁴ cells/cm of suture) was placed into the wound. Viable counts of bacteria per wound were analyzed in the tissue homogenate at 2 hours post infection. (c) Antimicrobial effect of the silk-silver microgels and controls. For all experiments n=12 per group; ***p<0.001.
4.3 Microporous silica microcapsules fabricated using gas-in water-in oil emulsions

In order to generate microcapsules with variable multi-scale pore sizes, gas-in water-in oil emulsions (g/w/o) were first generated. This was done using a non-planar microfluidic device, where the inner phase contained the gas, while the middle and outer phases consisted of silicic acid (with buffer) and oil respectively. Once the silicic acid comes into contact with the sodium phosphate buffer (figure 4.9), the precipitation of silica occurred rapidly [238] around the encapsulated gas bubbles resulting in the formation of microporous silica capsules. It was determined that by varying the pressure at which gas was injected through the device, while keeping the flow rates of the liquid phases constant, both bubble size but also the number of internal bubbles could be specifically controlled. The bubbles suspended in the aqueous phase, were then encapsulated by the oil phase resulting in the formation of a bubble loaded aqueous droplet in oil. This gas-water-oil (g/w/o) three-phase system was the basis for all subsequent experiments. The silica microparticles were then de-emulsified and re-injected into an aqueous medium, before being imaged using optical and electron microscopy. The latter of which revealed the cavities within the porous microparticles.

4.3.1 Device design

The silica-coated microcapsules were synthesised using a g/w/o emulsion strategy that relies on the formation of monodisperse micro-droplets. These consisted of a gas core, surrounded by an aqueous phase, comprised of silicic acid with sodium phosphate buffer, all of which is encapsulated within a continuous immiscible oil phase. In order to generate such droplets, the use of a non-planar microfluidic device design was employed. This ensured that the aqueous phase did not wet the microfluidic channel surface and allowed for successful droplet generation. The master used for all experiments was fabricated using a two-step soft lithography process. To this effect; 25 µm high structures were formed using a film mask (figure 4.9b) and then aligned with channels fabricated using a second film mask shown in figure 4.9a. This resulted in the generation of a non-planar device which is schematically depicted in figure 4.9c (see Methods for additional details on device fabrication).

Typically, droplet generation involves an aqueous phase intersecting with an immiscible oil phase resulting in the formation of water-in-oil or oil-in-water droplets. However, in order to generate g/w/o emulsions, an additional inlet was introduced. The device architecture consists of two main regions, each of which plays a fundamental role in the operation of the device. In the first region, silicic acid is mixed with sodium phosphate buffer and with
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Fig. 4.9 (a-c) Design of the microfluidic device used to generate air-in water-in oil emulsions. A two-step lithographic process was used in order to fabricate the device shown in (c). (a) Mask 1: Outer oil phase inlet with its respective channels and outlet. (b) Mask 2: middle and inner phase inlets. The aqueous phase consists of two inlets. Inlet 1: silicic acid. Inlet 2: sodium phosphate buffer with Tween 20. Once these two solutions, intersect, a long channel allows for successful mixing before they reach the gas channel. (c) Schematic representation of the device used to generate gas-in water-in oil droplets. The third junction is the non-planar (3-D) junction.

Tween 20, as shown in the top right inlet of figure 4.9c. A long serpentine channel separating this junction from the second junction allows for the two solutions to mix well before being introduced to the channel containing the gas phase. The channel length separating the first and second junction is crucial in increasing the viscosity of the aqueous phase (as the reduction of silicic acid to silica commences upon mixing with the buffer) which in turn allows for successful bubble formation and encapsulation due to increased solution surface tension. The two aqueous solutions could not be pre-mixed before being pumped through the microfluidic channels due to the speed at which the silicic acid and sodium phosphate/Tween 20 solutions precipitate and gel (see figure 4.10a), which is why they were mixed on-chip. In the second junction, the gas intersects with the silicic acid/sodium phosphate/Tween 20 solution resulting in the production of bubbles. This in turn is encapsulated by the oil phase in the third junction. This entire process of g/w/o monodisperse droplet formation is schematically represented in figure 4.9c (bottom right inlet).
4.3 Microporous silica microcapsules fabricated using gas-in water-in oil emulsions

![Image](image_url)

**Fig. 4.10** (a) Different concentrations of silicic acid and Tween 20, with 50 mM phosphate buffer. Even at extremely low concentrations, the solutions gel in less than 20 minutes, which explains why the two aqueous solutions had to be mixed on-chip. (b) g/w/o emulsion droplets generated from the microfluidic device with 6 internal gas bubbles.

### 4.3.2 Silica-coated microcapsule formation and characterisation

The generation of micron-sized g/w/o emulsions using nitrogen as a model gas was next explored. The relationship between the gas pressure and the number of internal bubbles encapsulated was further investigated. This was determined by keeping the respective aqueous phase flow rates ($Q_{aq}$) constant at 300 $\mu$L/h and the outer oil phase flow rate ($Q_{oil}$) at 800 $\mu$L/h, while the gas pressure values ranged from 110 up to 200 mbar. As previously mentioned, once the silicic acid comes into contact with the sodium phosphate buffer, the precipitation of silica initiates. If the solutions remain within the microfluidic device for too long, then silica will form in the micro-channels and block the device. To this effect, determining the residence time after which the two aqueous phases (silicic acid and buffer) intersect is crucial for proper droplet generation. It was determined that for aqueous phase flow rates of 300 $\mu$L/h, the residence time for the solutions to reach the junction where the gas bubbles are encapsulated is approximately 0.5 seconds. For the concentration of silicic acid used, it was found that if this residence time was further increased, then silica formed within the micro-channels.

Once formed, the number of encapsulated internal bubbles was determined by high speed imaging and frame by frame analysis of the data. The number of internal bubbles ($N$) was found by averaging over a total of 10 subsequent droplets. Figure 4.11a shows g/w/o emulsions which are formed at the third junction of the device. Figure 4.11a shows g/w/o emulsions with a range of number of internal bubbles, varying from $N = 1$ up to $N = 20$. 
Fig. 4.11 (a) g/w/o emulsion droplets generated from the microfluidic device for a range of gas pressures when $Q_{aq} = 300 \mu$L/h for both phases while $Q_{oil} = 800 \mu$L/h. As can be seen from the optical micrographs, the number of internal bubble droplets could be specifically controlled based on the gas pressure, and ranged from $N = 1$ to $N = 20$. The scale bar for all images is 50 $\mu$m. (b) Dependence of the number of internal gas droplets ($N$) on the pressure exerted within the microchannel.

The precise and systematic control of this microfluidic setup is demonstrated in each of the optical micrographs, with 1, 2, 3, 8 and 20 internal gas bubbles being encapsulated with increasing pressure. Moreover, an optical image showing $N = 6$ internal bubbles is shown in figure 4.101b. As expected, the higher the gas pressure, the more internal bubbles that can
be encapsulated. Interestingly, a plot of N against gas pressure, which is shown in figure 4.11b, indicates an almost linear relation, with a gradient close to 0.2. Following formation, droplets were then collected and placed on a cover slide in order to investigate whether the microcapsules remained stable over time. As can be seen in figure 4.12a-d, not only are the particles stable but during silica precipitation, the gas remains trapped within the droplet long enough for a silica shell to be formed around the bubbles. This allows for a precise way of modulating porosity, which suggests that these microcapsules can be used for various biomedical applications where pore sizes are instrumental. Additionally, the high level of monodisperse droplets and encapsulated bubbles formed using this setup can be seen in the micrographs of figure 4.12a-d.

Fig. 4.12 (a-d) Optical micrographs of g/w/o emulsions with varying numbers of internal gas bubbles placed on a cover slip. It is clear that following their generation, the gas loaded droplets remain stable while drying resulting in the formation of a silica shell around the bubbles. The scale bar for all images is 100 µm.
Scanning electron microscopy (SEM) was conducted on the silica-coated microcapsules in order to further characterise them. Following emulsion generation, droplets were incubated at room temperature for 1 hour before washing and de-emulsification. The silica particles were then re-emulsified in deionised water, placed on a glass slide and left to dry for 24 hours. The SEM images in figure 4.13a show monodisperse, spherical particles with a rough surface morphology. Moreover, the microcapsules remain mostly stable and do not seem to collapse upon drying. They do, however, shrink by approximately a factor of 2, which is due to the water diffusing out of the microbeads.

The pores that can be seen on the surface of some silica particles is probably the result of a gas bubble coming too close to the droplet interface during silica formation. However, this can be resolved by increasing the viscosity within the aqueous solution during microfluidic droplet generation in order to restrict gas movement within the emulsion. This can either be done by increasing the silicic acid concentration, or by increasing the serpentine length between the first and second junction. Furthermore, cross-sectional micrographs of the particles were taken by cutting the silica beads in half. The images in figure 4.13b-g reveal the cavities within the capsules where the bubbles were and give an insight into the internal structure of the particle. It is clear that by regulating the number of encapsulated bubbles one can tailor the porosity of microcapsules to the point where molecular release/uptake through the silica network and into the environment can be specifically controlled.

The pore size of the capsules is essential in controlling the release kinetics for drug delivery applications. In order to investigate what the pore size of the silica shell is, two solutions of equal dye concentration were used. A solution consisting of 500 μM bovine serum albumin (BSA) labelled with fluorescein (FITC) was encapsulated within the silica capsule and the release kinetics were investigated by measuring the fluorescence intensity as a function of time. Conversely, a 500 μM solution of purely FITC molecules was encapsulated within silica capsules and the release kinetics of that system were also investigated. The relative intensity of the two systems was then compared in order to determine the relative amount of molecules that escape from the two different systems. From figure 4.14, it is clear that even though the concentration of dye molecules is the same for both cases, the BSA-FITC system has 10 time less relative intensity than the FITC-alone system. The initial increase in fluorescence from the BSA-FITC system can be attributed to the protein-dye molecules present on the surface of the capsule.

Therefore, it can be concluded that the pore size of the system must lie between the sizes of the two molecules, FITC and BSA-FITC, i.e. between 1 - 6.8 nm.
Fig. 4.13 (a-g) Scanning electron micrographs of silica-coated microcapsules. The microparticles remain stable and do not collapse upon drying. (b-g) Cross-sectional micrographs reveal the areas (cavities) within the capsules where the bubbles were. The scale bars depict 10 µm.
Fig. 4.14 Release kinetic profiles for FITC (orange circles) and BSA-FITC conjugate (purple circles). The concentration of the solutions was 500 µM in both cases.

4.4 Conclusions

The production of inorganic/organic microcapsules for their potential use in biomedically related application has gained increasing interest. In particular, the emergence of multidrug resistant bacteria has led to the continuing development of biomaterials capable of combating such bacteria. In this chapter, a microfluidics-based method for the production of hybrid organic/inorganic silk-based microgels which display strong antibacterial properties is showcase. The results presented here demonstrate the encapsulation and reduction of silver nitrate to silver nanoparticles within these silk microgels. Importantly, as compared to previously developed silver-containing antibacterial systems utilising silk fibroin as colloids, films and composites, which lack the uniform morphology and size that the microgels developed here display, these microgels have increased surface to volume ratio which facilitates enhanced antibacterial activity. It is demonstrated that this hybrid material exhibits potent antibacterial
4.4 Conclusions

activity which involves a unique two-step mechanism; bacterial adherence and consequent eradication. These results further show that the hybrid microgels are both non-hemolytic and non-cytotoxic towards mammalian cell lines, which is not only crucial for any potential biomedical application, but is in contrast to the majority of conventional treatments involving silver nanoparticles. Moreover, cellular viability of silver nitrate treated cells was significantly reduced whereas silk-silver microgels had viabilities greater than 90% for both cell lines, suggesting that the encapsulation of the silver nitrate within the silk microgels shields the mammalian cells from its cytotoxic effect. Finally, the use of a murine model with surgical site infections was employed to demonstrate microgel ability as a topical antimicrobial agent. This capability, coupled with the biocompatibility of the silk microgels, and the versatility of this platform allow for the utilisation of this approach for numerous biotechnological and biomedical applications.

Encapsulating microbubbles within microcapsules for modulating the porosity of the latter, has gained increase interest in the context of biomedical related applications [221, 222] which include gene therapy[225], drug delivery[223, 224], delivery of biocatalysts[226] and as tumour/thrombus-destruction materials [227]. Controllable generation of monodisperse micro-sized gas-in water-in oil (g/w/o) droplets in a reproducible manner is desirable for next-generation delivery strategies, yet remains challenging. In this chapter it is shown that by utilising non-planar microfluidics, a scalable platform for generating silica-coated microcapsules can be developed, mimicking the formation of diatoms in nature. G/w/o droplets were generated on chip, and by mixing silicic acid with sodium phosphate buffer, multiple microbubbles stabilised by a silica shell within the same microcompartment could be formed. Using this approach, control over bubble size and number of encapsulated bubbles within individual capsules can be precisely achieved by modulating the pressure at which the gas-phase is introduced on-chip. Crucially, these important parameters can easily be changed within seconds, allowing for the formation of core-shell silica capsules with varying dimensions. In addition, following droplet generation, optical microscopy reveals that these emulsions are stable and that the gas remains trapped within the microparticles long enough for the precipitation of silica to form around the bubbles. Moreover, scanning electron micrographs further corroborates that these particles are stable when dried and that cavities formed due to the presence of gas bubbles during droplet generation contribute towards the silica capsule microporous morphology. Such silica-based microcapsules represent a class of biocompatible and non-toxic material, and in conjunction with the high level of control over their formation, these multi-scale microporous capsules have favourable characteristics enabling them to serve as a platform to explore various delivery and related biomedical applications.
Chapter 5

Formation of hierarchical and nano-emulsions for controlled release and intracellular delivery

This chapter is based on the following publications:

1. Zenon Toprakcioglu, Pavan-Kumar Challa, David B Morse, Tuomas PJ Knowles, Attoliter protein nanogels from droplet nanofluidics for intracellular delivery, Science Advances, 2020, 6 (6), eaay7952

2. Zenon Toprakcioglu, Aviad Levin, Tuomas PJ Knowles, Hierarchical biomolecular emulsions using 3-D microfluidics with uniform surface chemistry, Biomacromolecules, 2017, 18 (11), 3642-3651

In this chapter, all experiments were conducted by myself, with the exception of the confocal microscopy and cell culture, which was arranged by David Morse.

5.1 Introduction

The production of single, double and higher order emulsions, through the use of droplet microfluidics where sizes can be precisely controlled and modulated have potential for the storage and study of biomolecules, including peptides and proteins. In this chapter, an approach for producing both water-oil-water (w/o/w) and oil-water-oil (o/w/o) double emulsions without any need for surface modification, an enabling feature for biomolecular encapsulation is demonstrated. Using this strategy, control over the number of monodisperse encapsulated internal droplets is systematically and reproducibly shown and modulated
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by suitable adjustment of the relevant flow rates. Moreover, by adding silk solution to the aqueous phase, microgels surrounded by an external oil shell, or core-shell structures with encapsulated oil droplets can be formed. More importantly, by changing the shell thickness, control over the release kinetics of encapsulated molecules may be achieved. This microfluidic strategy allows for multiple emulsions to be generated drop by drop for biomolecular solutions with potential applications in the biomedical and pharmaceutical fields.

Micro-scale hydrogels consisting of macromolecular polymer networks in an aqueous continuous phase are the focus of increasing attention due to their potential use in tissue engineering, cell encapsulation and for the storage and release of cargo molecules. However, for applications targeting intracellular delivery, their micron-scale size is not suitable for effective cellular uptake. Nano-scale analogues of such materials are thus required for this key area. A micro and nanofluidics-based strategy for generating monodisperse nano-sized water-in-oil emulsions with controllable sizes ranging from 2500 ± 110 nm down to 51 ± 6 nm is demonstrated. These nanoemulsions can act as templates to form protein nanogels stabilised by supra-molecular fibrils from three different proteins. It is further shown that these nanoparticles have the ability to penetrate the cell membrane of mammalian cells and deliver intracellular cargo. Nanoparticles based on natural proteins thus emerge as useful materials for the storage and delivery of important cargo molecules such as future drugs, and due to their biocompatibility and lack of toxicity, possess advantageous characteristics in the context of pharmaceutical and biomedical applications.

In this chapter, improvements over conventional microgel production are showcased. By making hierarchical emulsions, one can tailor the release of the cargo molecules. Moreover, by scaling down the sizes of the microgels to nanogels, drug delivery applications and intracellular uptake may be achieved.

5.2 Generation of hierarchical emulsions using non-planar microfluidic devices

Both double emulsions of w/o/w and o/w/o were formed using the same 3-D microfluidic device presented here. The ratio between the flow rates of the inner ($Q_{in}$), middle ($Q_{mid}$) and outer ($Q_{out}$) phases was optimised in order to control droplet size and/or number of encapsulated droplets. A top view image of the microfluidic device used is shown in figure 5.1a. The arrows represent the flow of the three phases. The first part of the microfluidic device, up to the second junction, is planar (2-D), while the second junction is non-planar
5.2 Generation of hierarchical emulsions using non-planar microfluidic devices

(3-D). A schematic representation of the device is shown in figure 5.1b. At the first junction, inner phase droplets are formed in a medium of middle phase (effectively forming the first emulsion); while at the second junction, the outer phase engulfs the middle and inner phases, resulting in the double emulsion.

![Schematic representation of the device](image)

Fig. 5.1 (a) Schematic representation of the 3-D microfluidic channels. In the first junction droplets of inner phase surrounded by the middle phase are formed, while in the second junction, the outer phase encapsulates the other two phases forming a double emulsion. In the schematic shown above, there are three internal droplets within the external droplet. (b) Image showing a perfectly aligned non-planar microfluidic device where the second junction is 3-D. The arrows represent the flows of the three different phases.

### 5.2.1 Water/Oil/Water double emulsions

The time evolution of droplet formation was monitored using a high speed-camera (figure 5.2). For $Q_{out}=500 \, \mu L/hr$, $Q_{mid}=200 \, \mu L/hr$ and $Q_{in}=50 \, \mu L/hr$, the external droplets were formed after 250 ms. The capillary number, $Ca=\mu u/\gamma$ ($\mu$ is the viscosity of the outer phase, $u$ is the mean speed of the outer phase fluid and $\gamma$ is the interfacial tension between the middle and outer phases), was found to be $<10^{-2}$. Upon initial droplet formation (figure 5.2-left panel) the contact angle and its time evolution was studied. The dynamic contact angle was found to remain practically constant at $>90^\circ$ during formation and detachment of the droplets (figure 5.2-left panel). As has been reported [146], droplet formation can only occur when the contact angle is $>90^\circ$. The argument presented refers to both planar and non-planar devices, but not to double emulsions. These results demonstrate that this condition clearly still holds for double emulsions formed in 3-D microfluidic devices.

The relationship between $Q_{out}$ and the number of internal droplets encapsulated was further investigated. This was determined by keeping $Q_{in}$ and $Q_{mid}$ constant, at 50 $\mu L/hr$ and 500 $\mu L/hr$ respectively. The $Q_{out}$ values ranged from 200 $\mu L/hr$ up to 5000 $\mu L/hr$. Monodisperse encapsulated internal droplets were formed, and these were counted by observing the
images taken and analysing them frame by frame. For each case, the number of internal droplets (N) was taken to be the average of 10 different images. Figure 5.3a shows double emulsions with 8 internal droplets, while in figure 5.3b, N is 4. As expected, decreasing $Q_{\text{out}}$ leads to increasing N. This can be seen in figure 5.3d. These data further suggest that very large numbers of internal droplets can be encapsulated, since N increases asymptotically when $Q_{\text{out}}$ decreases. As the outer phase flow rate is decreased, the external droplet size increases, and so more droplets can be encapsulated. In practice, however, $Q_{\text{out}}$ cannot, be made arbitrarily small if w/o/w double emulsions are to be produced. If one were to continue decreasing $Q_{\text{out}}$, a phase separation between the middle and outer phases would occur (figure 5.5d), which explains why N increases asymptotically. Effectively, one would obtain inner phase water droplets surrounded by an oil phase medium, forming water droplets dispersed in an oil "droplet" of effectively infinite size. Therefore, for a given device geometry, there is a practical upper limit to N. If a smaller inner phase channel and a larger outer phase channel was used, then N could theoretically be increased. A double logarithmic plot of N against $Q_{\text{out}}$, shown in figure 5.3e, indicates a power law with an exponent close to -1. Up to 20 internal droplets were encapsulated by increasing $Q_{\text{mid}}$ with respect to $Q_{\text{in}}$ and decreasing $Q_{\text{out}}$ to as low as 200 $\mu$L/hr, (figure 5.3c).

Droplets were collected, placed on a glass slide and imaged, as shown in figures 5.4a-c. In figures 5.4a and 5.4b all of the collected droplets either contained 2 or 3 internal droplets respectively, while for figure 5.4c, all but one of the double emulsion droplets does not contain 4 internal droplets. Encapsulation of multiple internal droplets, but without effective control over N, has been reported. [239] Here, it has been shown that within the geometric limitations of this microfluidic device, N can be predicted and reproducibly manipulated by adjusting the flow rates and the geometry of the devices.
5.2 Generation of hierarchical emulsions using non-planar microfluidic devices

Fig. 5.3 (a) w/o/w double emulsion droplets when \( Q_{\text{out}} = 700 \, \mu\text{L/hr} \), \( Q_{\text{mid}} = 200 \, \mu\text{L/hr} \) and \( Q_{\text{in}} = 50 \, \mu\text{L/hr} \) with \( N = 7 \). (b) w/o/w double emulsion droplets when \( Q_{\text{out}} = 1500 \, \mu\text{L/hr} \), \( Q_{\text{mid}} = 200 \, \mu\text{L/hr} \) and \( Q_{\text{in}} = 40 \, \mu\text{L/hr} \) with \( N = 4 \). (c) w/o/w double emulsion droplets when \( Q_{\text{out}} = 200 \, \mu\text{L/hr} \), \( Q_{\text{mid}} = 300 \, \mu\text{L/hr} \) and \( Q_{\text{in}} = 40 \, \mu\text{L/hr} \) with 20 encapsulated internal droplets. The scale bar for all three images is 200 \( \mu\text{m} \). (d) Graph of \( N \) against outer phase flow rate. \( N \) increases asymptotically as \( Q_{\text{out}} \) decreases. (e) Log-log plot of \( N \) as a function of \( Q_{\text{out}} \). The gradient is approximately -1.

Fig. 5.4 (a) Collected droplets consisting of 2 internal droplets, (b) 3 internal droplets and (c) 4 internal droplets. The scale bar for these images is 100 \( \mu\text{m} \).

The variation of internal droplet size as a function of flow rate was further studied. Internal droplets of 50 \( \mu\text{m} \) are shown in figure 5.5a while droplets as small as 35 \( \mu\text{m} \)
Formation of hierarchical and nano-emulsions for controlled release and intracellular delivery are shown in figure 5.5b. The internal droplet size was changed by varying the oil phase, $Q_{\text{mid}}$, from 200-1200 $\mu$L/hr, while keeping $Q_{\text{in}}$ and $Q_{\text{out}}$ constant at 20 $\mu$L/hr and 1000 $\mu$L/hr respectively. As $Q_{\text{mid}}$ is increased, and the ratio $Q_{\text{mid}}/Q_{\text{in}}$ increases, the droplet size decreases as can be seen in figure 5.5c. The relationship between flow rate and droplet size is asymptotic, which suggests that as the middle phase is decreased with respect to the inner phase, droplets will not form and phase separation will occur. Moreover, as previously mentioned, if $Q_{\text{mid}}$ exceeds a certain value (in this case 1200 $\mu$L/hr when $Q_{\text{out}}$ is 1000 $\mu$L/hr), droplet formation cannot occur at the second junction as seen in figure 5.5d.

Fig. 5.5 (a) w/o/w double emulsions where the internal droplet size is 50 $\mu$m. $Q_{\text{out}}$=1000 $\mu$L/hr, $Q_{\text{mid}}$=200 $\mu$L/hr, $Q_{\text{in}}$=20 $\mu$L/hr (b) w/o/w double emulsions where the internal droplet size is around 35 $\mu$m. $Q_{\text{out}}$=1000 $\mu$L/hr, $Q_{\text{mid}}$=1000 $\mu$L/hr, $Q_{\text{in}}$=20 $\mu$L/hr (c) Graph showing the size dependence of the internal droplet as a function of flow rate, $Q_{\text{mid}}$. Internal droplet diameters were varied by more than 30%. (d-f) w/o/w double emulsions. (d) $Q_{\text{out}}$=1000 $\mu$L/hr, $Q_{\text{mid}}$=1200 $\mu$L/hr, $Q_{\text{in}}$=20 $\mu$L/hr. The middle flow rate is too high and does not allow for the outer phase to form the double emulsion. (e) Single internal droplet encapsulation by droplet formation in the first junction where $Q_{\text{out}}$=1000 $\mu$L/hr, $Q_{\text{mid}}$=150 $\mu$L/hr, $Q_{\text{in}}$=100 $\mu$L/hr (f) Co-flowing of the inner and middle phases resulting in a single internal droplet of around 150 $\mu$m, where $Q_{\text{out}}$=1000 $\mu$L/hr, $Q_{\text{mid}}$=200 $\mu$L/hr, $Q_{\text{in}}$=400 $\mu$L/hr. Scale bar for all images is 200 $\mu$m.
5.2 Generation of hierarchical emulsions using non-planar microfluidic devices

Fig. 5.6 (a) Graph showing the external droplet size dependence on outer phase flow rate, when $Q_{in}=50 \mu L/hr$ and $Q_{mid}=500 \mu L/hr$. There is an asymptotic relation between the droplet size and $Q_{out}$. Inset: Schematic representation of elongated droplets formed in a microfluidic channel and ellipsoid with its three main axes a, b, and c. (b) Log-log plot of the external droplet size as a function of $Q_{mid}/Q_{out}$. The gradient is 0.25 and most of the data points are in the regime where $Q_{mid}/Q_{out} < 1$.

Furthermore, by decreasing the ratio of $Q_{mid}/Q_{in}$, extremely large internal water droplets could be formed. This could be achieved using two different techniques; either by making water-in-oil droplets at the first junction followed by double emulsion formation in the second junction, or by carefully tuning $Q_{in}$ and $Q_{mid}$, so that one can co-flow the inner and middle phases in the first junction, allowing the outer phase to encapsulate both phases at the second junction. In the former situation, $Q_{in}$ and $Q_{mid}$ were 100 $\mu L/hr$ and 150 $\mu L/hr$ respectively while $Q_{out}$ was kept at 1000 $\mu L/hr$. Figure 5.5e shows that large internal droplets, of around 85 $\mu m$ can be produced using this technique. In the latter case, internal droplets of up to 150 $\mu m$ can be produced, as seen in figure 5.5f, where $Q_{in}$ is 400 $\mu L/hr$, $Q_{mid}$ is 200 $\mu L/hr$ and $Q_{out}$ is again 1000 $\mu L/hr$.

Moreover, the thickness of the oil shell can be controlled directly during droplet formation. This may be of particular importance when the internal droplet is loaded with cargo molecules, as the release kinetics will vary considerably based on the thickness of the oil phase engulfed within the emulsion. In the co-flowing regime, shell thickness was found to be $11 \pm 0.5 \mu m$ (figure 5.5f), while for the droplet regime (figure 5.5e), where $Q_{mid}/Q_{in}$ is low, shell thickness was $22.2 \pm 0.5 \mu m$, with largest shell thickness determined at $38.6 \pm 0.5 \mu m$ (figure 5.5b). Shell thickness could be further decreased down to $10.1 \pm 0.5 \mu m$ by adjusting the flow
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rates. Therefore, shell thicknesses of only a few micrometers could potentially be obtained by manipulating the ratio of the three flow rates.

Finally, the external droplet size dependence on the ratio of the flow rates $Q_{\text{mid}}/Q_{\text{out}}$ was investigated. When $Q_{\text{mid}}/Q_{\text{out}}$ is small, droplets form an elongated morphology (figure 5.6a inset). In order to work out the volume of these structures, ellipsoidal geometry to model their shape was assumed. The three axes a, b, and c for each ellipsoid (schematic shown in figure 5.6a) were thus determined. Since the droplets conserve their volume but eventually assume a spherical shape upon exiting the channel, the final diameter of the spheres from the volume of the corresponding ellipsoid was calculated. To explore this dependence, the value of $Q_{\text{in}}$ and $Q_{\text{mid}}$ were kept constant at 50 µL/hr and 500 µL/hr respectively, while $Q_{\text{out}}$ ranged from 200-5000 µL/hr. The same asymptotic behaviour is displayed on the graph of external droplet size against $Q_{\text{out}}$ (figure 5.6a) as has previously been seen for the internal droplet size and N (figures 5.5c and 5.3d) respectively. It has been shown [145] that droplet size as a function of the ratio between the dispersed phase ($Q_{\text{dis}}$) to the continuous phase ($Q_{\text{con}}$) flow rate displays two regimes. In the first regime, where $Q_{\text{dis}}/Q_{\text{con}} < 1$, droplet size stays approximately constant while in the second regime, where $Q_{\text{dis}}/Q_{\text{con}} > 1$, the droplet size increases linearly with increasing dispersed phase flow rate. [145] Assuming that $Q_{\text{in}}$ plays no role on this relationship, in this system the dispersed phase flow rate is $Q_{\text{mid}}$ while the continuous phase flow rate is $Q_{\text{out}}$. A double logarithmic plot of external droplet size against $Q_{\text{mid}}/Q_{\text{out}}$, figure 5.6b, shows that these data display similar behaviour as that seen by Garstecki et al. for $Q_{\text{mid}}/Q_{\text{out}} < 1$. [145] In this regime, the data presented here indicate a weak external droplet size dependency on this ratio, the gradient and hence the apparent exponent of the corresponding power law being approximately 0.25. High $Q_{\text{mid}}/Q_{\text{out}}$ values could not be reached, however, because droplets are not successfully formed if the outer phase flow rate is not high enough (figure 5.5d). Thus, for the regime $Q_{\text{mid}}/Q_{\text{out}} > 1$ it could not be determined whether 3-D double emulsion microfluidics exhibit the same linear behaviour (i.e. a scaling exponent of 1) as in the simple planar case.

Water/Oil/Water double emulsions with encapsulated silk fibroin

Forming an emulsion where proteins are involved is particularly challenging due to the propensity of proteins to adhere to the surfaces of microfluidic devices. Thus, when attempting to form w/o emulsions, it is essential to render the surface of the channels more hydrophobic in order to avoid protein surface wetting. However, in double emulsion formation, controlling the surface properties of the two junctions can be challenging in the case of protein solutions. Therefore, using a device that does not need surface modifications and yet allows double emulsion formation with protein as the inner phase is important if one is to
5.2 Generation of hierarchical emulsions using non-planar microfluidic devices

Fig. 5.7 (a-f) w/o/w double emulsion where the inner phase contains reconstituted silk fibroin. A concentration of 0.1 mg/mL was used. (a) $Q_{\text{out}}=500 \mu \text{L/hr}$, $Q_{\text{mid}}=200 \mu \text{L/hr}$, $Q_{\text{in}}=20 \mu \text{L/hr}$. 4 internal droplets were encapsulated within the external droplet. (b) $Q_{\text{out}}=150 \mu \text{L/hr}$, $Q_{\text{mid}}=200 \mu \text{L/hr}$, $Q_{\text{in}}=20 \mu \text{L/hr}$ with 12 encapsulated internal droplets. It is clear that the protein does not wet the surface of the channels in the first junction. (c) $Q_{\text{out}}=400 \mu \text{L/hr}$, $Q_{\text{mid}}=100 \mu \text{L/hr}$, $Q_{\text{in}}=100 \mu \text{L/hr}$. Double emulsions were generated via co-flowing the inner and middle phases in the first junction followed by encapsulation in the second junction. The concentration of protein used was 10 mg/mL. (d) $Q_{\text{out}}=350 \mu \text{L/hr}$, $Q_{\text{mid}}=250 \mu \text{L/hr}$, $Q_{\text{in}}=20 \mu \text{L/hr}$. Double emulsion formation where the internal droplets are 15 $\mu \text{m}$ in diameter. The ratio $Q_{\text{mid}}/Q_{\text{in}}$ is kept $>10$ so that the aqueous phase does not wet the surface. The concentration of protein used was 10 mg/mL. (e-h: left panels) Bright field images of double emulsions with two and three internal droplets respectively. (e-h: right panels) Fluorescent microscopy images of the same droplets as shown in the left panels.

make double emulsions in a reproducible and facile manner. By correctly tuning the ratio $Q_{\text{mid}}/Q_{\text{in}}$, double emulsions which contained reconstituted silk fibroin as the inner phase were successfully formed. The protein-containing droplets exhibit the same behaviour as water droplets, and parameters such as number of internal droplets or external droplet size can be clearly controlled as can be seen in figure 5.7a and 5.7b respectively. The values of protein concentration employed ranged across three orders of magnitude, from 0.01 mg/mL
Formation of hierarchical and nano-emulsions for controlled release and intracellular delivery up to 10 mg/mL. It was found that for protein concentrations greater than 1 mg/mL, double emulsions formation was possible by co-flowing the inner and middle phases in the first junction followed by encapsulation in the second junction, which can be seen in figure 5.7c. Using a protein concentration of 10 mg/mL, double emulsions with internal droplets as small as 15 µm were made, shown in figure 5.7d. This was achieved by employing a large value of $Q_{\text{mid}}/Q_{\text{in}}$ so as to ensure that the protein would not wet the surface of the channels.

Double emulsions containing a solution of reconstituted silk fibroin with ThT as the inner phase were produced. The droplets were incubated at 37 °C for 2 days in order to promote aggregation and as a result internal droplets gelate and silk fibroin micro-gels are formed. As ThT is a fluorescent dye that mostly binds to β-sheet structures, protein-containing droplets that have undergone a structural transition can be detected. ThT-stained internal droplets were thus studied using fluorescent microscopy. To avoid droplet generation in the co-flowing regime, a concentration of 0.1 mg/mL silk fibroin was used. w/o/w emulsions with 2 and 3 internal droplets were imaged both in bright field and by using ThT filters. In the bright field images (left panels on figure 5.7e-h) both internal and external droplets are clearly visible. However, in the images where ThT filters were used (right panels on figure 5.7e-h) only droplets containing aggregated silk fibroin can be detected.

**Oil/Water/Oil double emulsions**

Given the intrinsic hydrophobicity of PDMS, producing o/w/o double emulsions without surface treatment is particularly challenging. However, when the first junction is fabricated as a 3-D structure, the geometry of the microfluidic device favours the formation of such emulsions, as has been previously speculated. [146] Nevertheless, using the geometry of this device (i.e. the second junction being 3-D), o/w/o emulsions were generated by tuning the ratio between $Q_{\text{in}}$, $Q_{\text{mid}}$ and $Q_{\text{out}}$ appropriately. Unlike w/o/w double emulsions, in o/w/o emulsions, there is less control over internal droplet formation. This is because after the first junction, the inner oil phase tends to wet the hydrophobic surface of the channel, making it challenging to produce o/w droplets. Co-flowing the oil and water phases in the first junction, followed by encapsulation in the second junction seems to be the best approach of preparing double emulsions. However, by regulating $Q_{\text{mid}}/Q_{\text{in}}$, multiple small internal droplets were successfully encapsulated within the external droplet, where instead of co-flowing the inner and middle phases, oil-in-water droplets were first formed at the first junction. Moreover, it appears that the role played by the surfactant in the middle phase is extremely vital for co-flowing to occur. It appears likely that the hydrophobic part of the amphiphilic surfactant adsorbs onto the surfaces of the hydrophobic PDMS channel, while the hydrophilic part remains in contact with the aqueous solution. Thus, the aqueous middle phase wets the
surfactant-bearing PDMS channels and prevents the oil from doing so. This idea is supported by experiments conducted using lower amounts of surfactant. It was found that for surfactant concentrations of 1% SDS and 1% Tween 20 the oil was able to wet the surface despite following the same experimental procedure, i.e. flowing the middle phase before the inner one and having a high $Q_{\text{mid}}/Q_{\text{in}}$ ratio. The same result was obtained when both surfactant concentrations were doubled. In fact, double emulsion formation was only evident when 3% SDS and 3% Tween 20 were used.

Double emulsions consisting of multiple internal droplets were observed when $Q_{\text{mid}}/Q_{\text{in}}$ was 10. By keeping $Q_{\text{out}}$ constant at 1000 µL/hr and ranging $Q_{\text{mid}}/Q_{\text{in}}$ from 10 to 1, the size of the internal droplets increased to the point where only one droplet was encapsulated within the external droplet (when $Q_{\text{mid}}/Q_{\text{in}}$ was equal to 2). Further increase of $Q_{\text{in}}$ resulted in augmentation of the internal droplet. The effect of flow rate on size and number of internal droplets is summarised in figure 5.8a-c. The largest droplets formed had a diameter of 114 ± 2 µm, while the smallest appear to be ~10 µm. Furthermore, internal droplet diameters of 5 - 10 µm were generated by applying a large $Q_{\text{mid}}/Q_{\text{in}}$. The first emulsion, oil-in-water, is formed in the jetting regime. This multiphase flow is then encapsulated by the outer phase as seen in figure 5.8d.

Emulsions consisting of silk fibroin solution in the middle phase were formed. Avoiding surface wetting proved to be challenging when using high concentrations of protein, as the aqueous solution adhered to the channels of the device. However, it was determined that for protein concentration of 0.1 mg/mL, o/w/o double emulsions could be produced. ThT was added to the middle phase to detect aggregated protein. Using microscopy, silk fibroin aggregation was monitored at different stages of aggregation. Measurements were taken across a period of 2 days. Initially, after 12 hours of incubation at 37 °C protein aggregates start to appear in the middle phase as small white points, where the black circles correspond to the non fluorescent internal oil droplets, as can be seen in figure 5.8e (left panel). After 24 hours of observation, silk fibroin further aggregates resulting in the formation of larger clumps, which are clearly seen in figure 5.8e (right panel), and they correspond to the brighter areas, with the darker areas again being the internal oil droplets. Droplets were unstable when incubated in bulk for more than 24 hours. In order to circumvent this problem, the use of capillaries was employed. Droplets were therefore trapped inside capillaries and imaged after 24 and 48 hours of incubation. After 24 hours few aggregate clumps were detected, which suggests that most of the silk fibroin has gelled, as can be seen in the left panel of figure 5.8f. However, after 48 hours, there are no clumps present (right panel of figure 5.8f), indicating that the aggregation is complete and that the core-shell structure has fully gelled.
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Fig. 5.8 (a-d) o/w/o double emulsions. (a) $Q_{\text{out}}=1000$ µL/hr, $Q_{\text{mid}}=100$ µL/hr, $Q_{\text{in}}=10$ µL/hr. Internal droplet size is 10 µm. (b) $Q_{\text{out}}=1000$ µL/hr, $Q_{\text{mid}}=100$ µL/hr, $Q_{\text{in}}=40$ µL/hr. Internal droplet size is 25 µm. (c) $Q_{\text{out}}=1000$ µL/hr, $Q_{\text{mid}}=100$ µL/hr, $Q_{\text{in}}=100$ µL/hr. The inner and middle phases co-flow in the first junction. At the second junction, the outer phase encapsulates both the inner and middle phases resulting in internal droplets of 114 ± 2 µm. (d) $Q_{\text{out}}=4000$ µL/hr, $Q_{\text{mid}}=3000$ µL/hr, $Q_{\text{in}}=100$ µL/hr. The first o/w emulsion was formed in the jetting regime, producing oil droplets of 5-10 µm.

(e-f) Fluorescent microscopy images of o/w/o droplets consisting of 0.1 mg/mL silk fibroin solution with 100 µM ThT as the middle phase. For all images, the incubation temperature was 37 °C. (e: left panel) Image taken after 12 hours of incubation. The white points indicate the formation of small aggregates and give an insight towards the first steps of aggregation whereas the black circles correspond to the internal oil droplets. (e: right panel) Image taken after 24 hours of incubation. Aggregation has proceeded resulting in larger clumps being visible. (f) Fluorescent microscopy images of an o/w/o droplet trapped in a capillary. (f: left panel) Image taken after 24 hours of incubation. There are few clumps present, which suggests that the protein has almost fully aggregated. (f: right panel) Image taken after 24 hours of incubation. There are no clumps present, indicating that the protein has fully aggregated and the core-shell structure is stable.

5.3 Formation of protein-based nanoparticles using nanofluidic techniques

5.3.1 Device design

The nanoparticles were synthesised using a water-in-oil emulsion strategy that relies on the formation of monodisperse nanodroplets of an aqueous phase, consisting of a protein solution, in a continuous immiscible oil phase. In order to generate nano-sized droplets, micro-channels were integrated with nano-channels using a soft lithographic process, allowing for a robust
nano-to-micro and chip-to-world interface. The master used in all experiments was fabricated using two-step lithography. 800 nm wide structures were formed from a chrome mask (figure 5.9b) and aligned with micron-sized channels fabricated from a film photomask (figure 5.9c) resulting in the device shown schematically in figure 5.9a, the junction of which can be seen in magnification in figure 5.9d (see Materials and Methods for further details on device fabrication). The integration between the micro and nano areas of the device is shown in the schematic representation in figure 5.10.

**Fig. 5.9 (a-d)** Design of the nano/microfluidic device used to generate nanoemulsions. A two-step lithographic process was used in order to fabricate the device shown in (a). (b) Mask 1: chrome mask used which enabled the fabrication of the nano-channels. (c) Mask 2: conventional film photomask is carefully aligned with mask 1 resulting in a two-layered master. (d) Schematic design of the T-junction used to generate nanodroplets.

**Fig. 5.10** Schematic representation of water-in-oil nanodroplet generation and subsequent de-emulsification resulting in the formation of nanogels.

Droplet generation occurs when the phase to be dispersed intersects with an immiscible continuous phase, resulting in a break off of the former into the bulk fluid stream [143]. This is schematically represented in figure 5.10, which depicts how monodisperse nanodroplets were formed. Typically, droplet size depends on volumetric flow rates of both the continuous and dispersed phases, the viscosity of the fluids and on the interfacial tension. However, a crucial factor in determining droplet size is channel dimensions at the junction (i.e. where the two liquids intersect) and in particular the cross sectional area of the rectangular channel.
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As such, both the width and the height of the junction need to be precisely known. To that effect, the dimensions of the nanosized channels were characterised. Scanning electron microscopy (SEM) was used to determine the width of the channels, which was found to be approximately 800 nm (figure 5.11a-b) while atomic force microscopy (AFM) was employed to measure the height of the junction. The latter was found to be (400 ± 30) nm as seen in the micrograph in figure 5.11c and the corresponding height profile, acquired by atomic force microscopy, is shown in figure 5.11d. This is in agreement with the theoretical height that should have been achieved (∼ 500 nm) using the spin-coating process described in the Methods section.

![Figure 5.11](image_url)

Fig. 5.11 (a-b) SEM micrographs of the nanodroplet generating device. (b) Magnified SEM image of the junction. (c) AFM micrograph of the nanochannels shown in (b). (d) Corresponding height profile of the junction shown in (c)
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5.3.2 Nanodroplet and nanoparticle formation

Next, the generation of micron and sub-micron scale water-in-oil droplets using the nanofluidic device was explored. The flow rate of the dispersed phase $Q_{dis}$ was kept constant at 10 $\mu$L/hr while the continuous phase flow rate $Q_{cont}$ was varied from (10 - 60) $\mu$L/hr. Optical micrographs of droplet generation at the junction can be seen in figure 5.12. Having established the reproducible generation of nanoemulsions on chip, their ability to act as templates for the formation of protein nanogels was then explored. To this effect, reconstituted silk fibroin was added to the aqueous phase and a 4 mg/mL solution was prepared. This was then used as the dispersed phase. Following formation, nanodroplets were collected from the outlet, as shown in figure 5.13d and placed on a glass cover slip to image (figure 5.13a-c). Conversion of soluble monomer protein molecules into nanofibrillar aggregates was initiated through incubating the nanoemulsion at 37 $^\circ$C for 24 hrs. The droplets were then de-emulsified (see Methods) and re-emulsified into an aqueous phase.

Fig. 5.12 (a-d) Optical micrographs of droplet generation in the integrated nano to-micro-channels. Different sized droplets were obtained by varying the ratio of two flow rates, $Q_{cont}/Q_{dis}$

Figure 5.15a-e shows transmission electron microscope (TEM) and scanning electron microscope (SEM) micrographs of silk fibroin nanospheres obtained using this approach. The samples were obtained using a silk fibroin concentration of 4 mg/mL, while different
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Fig. 5.13 (a-c) Optical micrographs of nanodroplets on a cover slip. (d) Optical micrograph of the nanodroplets at the outlet of the device following their generation at the T-junction.

Fig. 5.14 (a-c) SEM micrographs of different sized nanoparticles.

sized nanodroplets were generated by varying the ratio of the flow rates $Q_{\text{cont}}/Q_{\text{dis}}$. The SEM images show monodisperse, spherical particles with a smooth surface morphology. $Q_{\text{cont}}/Q_{\text{dis}}$ values of 1, 1.5 and 2 were used to obtain particle sizes of 1500 nm, 900 nm and 500 nm, SEM micrographs of which are shown in figure 5.14a-c respectively. Furthermore, when the
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Fig. 5.15 (a) TEM micrograph of silk nanoparticles that have a diameter of \( \sim 190 \) nm. (b-e) SEM micrographs of silk nanoparticles. Particle size is strongly dependent on the flow rates used within the microfluidic channels. For all images the dispersed phase was kept constant at 10 \( \mu \text{L/hr} \) while the continuous phase varied from 10-60 \( \mu \text{L/hr} \). 200 nm sized nanoparticles were obtained when \( Q_{\text{cont}} = 40 \ \mu \text{L/hr} \) (c), 100 nm sized nanoparticles were obtained when \( Q_{\text{cont}} = 50 \ \mu \text{L/hr} \) (d), and finally, 50 nm sized nanoparticles could be formed when \( Q_{\text{cont}} = 60 \ \mu \text{L/hr} \) (e). The concentration of silk used for all the nanodroplet generation was 4 mg/mL. (f-g) Size distribution histograms of the silk nanoparticles corresponding to (d) and (e) respectively.
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ratio $Q_{cont}/Q_{dis}$ was 4, nanodroplets as small as 200 nm were formed. Transmission electron microscopy (TEM) was used to characterise these nanoparticles and it was found that the diameter measured through TEM agrees with dynamic light scattering (DLS) measurements (figure 5.16a), implying that there is minimal shrinkage of the nanoparticles upon drying, which has been shown to be the case by other synthesis methods [143, 131]. Further evidence of this can be seen from the micrographs in figure 5.13b-c, where no shrinkage is observed following droplet formation.

In order to synthesise silk nanoparticles that are sufficiently small to penetrate through cell membranes (typically < 200 nm), the continuous phase flow rate was further increased while the dispersed phase flow rate was kept constant at 10 μL/hr. Figure 5.15c-e shows SEM micrographs of the resulting silk nanogels as a function of the different continuous phase flow rates used. Particles ranging from 204 ± 19 nm up to 51 ± 6 nm could be synthesised by varying the continuous phase flow rate from (40 - 60) μL/hr. Particle size could be decreased by a factor of 2 by slightly increasing the continuous phase flow rate (from 50 μL/hr to 60 μL/hr) as can be seen in the SEM micrographs (figure 5.15d-e) and the corresponding histogram plots (figure 5.15f-g). This suggests that control over emulsion size can be precisely achieved even at the sub-micron scale. The histograms shown in figure 5.15f-g were obtained by measuring particle diameters of the corresponding SEM images and were fitted to a Gaussian distribution. The mean diameters ($\mu$) and standard deviation ($\sigma$) of the samples shown in figure 5.15d and 5.15e respectively were 101 ± 8 and 51 ± 6. The coefficient of variation (which is the ratio of $\sigma/\mu$ and gives an indication of the dispersion of the distribution) for these samples was calculated as 8% and 12% respectively, demonstrating a fairly narrow distribution for both cases.

The high versatility of this approach was further exhibited by generating sub-micron droplets using either β-lactoglobulin or lysozyme solutions. In both cases, droplet formation was successful and consequently, nanodroplets were incubated at 65 °C for 2 days in order to promote protein aggregation. Following the same procedure as previously mentioned for silk nanogels, β-lactoglobulin and lysozyme droplets were de-emulsified and placed in an aqueous phase before imaging. Figure 5.17a-c shows (SEM) micrographs of β-lactoglobulin nanospheres, while lysozyme nanoparticles are shown in figure 5.16b-d. The samples were obtained using a concentration of 50 mg/mL for both β-lactoglobulin and lysozyme, and again, nanodroplet size could be specifically controlled by varying the ratio of the flow rates $Q_{cont}/Q_{dis}$. The SEM images in figure 5.17a-c show high monodispersity, while the spherical β-lactoglobulin particles exhibit smooth surface morphology just like their silk counterpart. Furthermore, nanogel stability over time was investigated. Once formed, in the absence of external environmental factors such as bacteria or cells, the nanogels should not degrade. In
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Fig. 5.16 (a) DLS data for silk nanoparticles. (b-d) SEM micrographs of lysozyme nanoparticles.

In fact, it was found that the nanoparticles were stable in the oil phase after three months at room temperature, and it would appear that in the oil phase, the nanogels should in theory be stable even longer. In an aqueous environment, it was determined using SEM that after one month, the nanogels were still stable. However, due to bacterial contamination, nanogel stability will degrade over time as the bacteria can digest the proteins and break the nanoparticles apart.

The relationship between $Q_{\text{cont}}$ and the size of the droplets formed was further investigated for both protein solutions. This was determined by keeping $Q_{\text{dis}}$ constant at 10 $\mu$L/hr, while $Q_{\text{cont}}$ values ranged from 10 up to 60 $\mu$L/hr. Monodisperse nanodroplets were formed and their sizes were measured by analysing the corresponding SEM micrographs. As expected, a decrease of $Q_{\text{cont}}$ leads to increasing droplet size, which can be seen in figure 5.17d. This data further suggest that not only do all three protein solutions exhibit the same asymptotic relation between particle diameter and the flow rate ratio ($Q_{\text{cont}}/Q_{\text{dis}}$), but also that particle diameters can vary by more than one order of magnitude (which is the case for silk nano-
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Fig. 5.17 (a-c) SEM micrographs of β-lactoglobulin nanoparticles obtained by varying the continuous oil phase flow rate while keeping the aqueous dispersed phase constant at 10 μL/hr. $Q_{\text{cont}}$ was 40 μL/hr and 30 μL/hr for (a) and (b-c) respectively. (d) Graph showing the dependence of droplet diameter as a function of the ratio of the two flow rates, $Q_{\text{cont}}/Q_{\text{dis}}$ for different proteins. For all three cases, the droplet size increases asymptotically as $Q_{\text{cont}}/Q_{\text{dis}}$ decreases.

spheres). Hence, in view of this asymptotic behaviour, for a given device geometry there is a practical lower limit to droplet size. However, if smaller nano-channels were fabricated, then theoretically particle size could be further decreased.

The viscosity of both the continuous and dispersed phase solutions affects the break-off of droplets and play a role on defining droplet size. This is evident in figure 5.17d, where for the same flow rates and continuous phase, different sized droplets were formed when the disperse phase was changed. Both lysozyme and β-lactoglobulin exhibited similar behaviour, while smaller sized droplets were formed from the solution containing silk fibroin. In fact, it
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has been shown that the higher the viscosity of the dispersed phase, the smaller the resulting droplet diameter [143]. This result is in agreement with the observations made in this thesis, where for the same $Q_{cont}/Q_{dis}$, the higher viscosity silk fibroin solution was used to form 51 nm ± 6 sized nanogels while droplet diameters of 350 nm ± 21 and 550 nm ± 36 were generated from the lower viscosity β-lactoglobulin and lyszyme solutions respectively.

5.3.3 Nanogels for intracellular delivery

Finally, in order to determine whether the nanoparticles produced using this droplet-based approach can be used for intracellular applications, cell studies were conducted. Increasing evidence in the literature suggests that cellular uptake of nanoscale particles occurs predominantly via a phagocytosis mechanism and it has been previously reported that nanoparticles can be endocytosed via such a pathway [240–242]. Ovarian cancer cells (PEO1) were stained with CellTracker™ Violet BMQC Dye ($\lambda_{ex} = 415$ nm and $\lambda_{em} = 516$ nm) and cultured. Nanodroplets comprised of 4 mg/mL silk fibroin and 0.1 mg/mL green fluorescent protein (which was used as a fluorescent marker) were generated and incubated off-chip in order to promote protein aggregation. Two different size distributions were produced: 200 nm and 1000 nm. The droplets were then de-emulsified (see Methods) and re-immersed into deionised water before being added to the cell culture and left to incubate for 4 hours. Finally, the cells were washed in order to remove any excess nanoparticles and then imaged using confocal microscopy. Cells are depicted in red throughout figure 5.18, while the nanoparticles are shown in green.

Cell controls containing no nanoparticles show very low fluorescence signal at an emission wavelength of 510 nm, which is a characteristic peak for GFP (figure 5.18a). However, with the addition of the 200 nm sized nanoparticles, green areas that clearly overlap with cells can be seen in figure 5.18b-c. The 1000 nm sized nanoparticles were also tested, however, the particle diameter was too large and the cell was not able to uptake these micron-sized particles (figure 5.19a-b). Finally, a three-dimensional reconstruction of single cells was performed in order to determine whether the nanoparticles just adhere to the cell membrane or whether they do in fact penetrate into the cell. The same cell is shown at three different angles (rotated with respect to the z-axis) in figure 5.18d in order to better exhibit that the protein nanoparticles are indeed within the cell, while in figure 5.18e the same conclusion can be reached for a different cell.
Formation of hierarchical emulsions where control over parameters such as droplet sizes, number of internal droplets and shell thickness can be systematically varied, is investigated. By adding silk fibroin solution to the aqueous phase, microgels surrounded by an oil droplet, or a core-shell structure with encapsulated oil droplets can be reproducibly formed. In such an approach, by modulating the shell thickness, the release profile of any encapsulated molecules may be altered and tailored to suit one’s needs. The ability of this device to reproducibly form both w/o/w and o/w/o emulsions, combined with its simplicity and versatility, render it particularly promising for potential applications in the pharmaceutical, cosmetic and food industries due to its ease of production and scale-up potential.

Fig. 5.18 (a-e) Confocal microscopy images of ovarian cancer cells (PEO1), shown in red, with and without protein based nanoparticles, shown in green. (a) Control experiment: fluorescent microscopy image of the ovarian cancer cells without any protein based nanoparticles. (b-c) Confocal microscopy images for the same cancer cells in the presence of the nanoparticles. (d-e) 3-D reconstruction of single cancer cells imaged at different angles with respect to the z-axis in order to better show that the nanoparticles have in fact penetrated the membrane and are within the cell.

### 5.4 Conclusions

In this chapter, formation of hierarchical emulsions, where control over parameters such as droplet sizes, number of internal droplets and shell thickness can be systematically varied, is investigated. By adding silk fibroin solution to the aqueous phase, microgels surrounded by an oil droplet, or a core-shell structure with encapsulated oil droplets can be reproducibly formed. In such an approach, by modulating the shell thickness, the release profile of any encapsulated molecules may be altered and tailored to suit one’s needs. The ability of this device to reproducibly form both w/o/w and o/w/o emulsions, combined with its simplicity and versatility, render it particularly promising for potential applications in the pharmaceutical, cosmetic and food industries due to its ease of production and scale-up potential.
5.4 Conclusions

The issue of forming monodisperse nanoparticles in the context of cellular permeability for delivery applications remains challenging. The need for uniformly sized particles lies in the ability to regulate molecular release and specifically tailor the rate of cellular permeation for different nanoparticle diameters. [133, 134, 70] There are currently numerous methods for generating nano-scale particles such as spray drying or solvent extraction [139], however, systematic control over monodispersity and size is difficult to achieve. In this respect, droplet microfluidics has gained popularity due to the ease with which highly monodisperse water-in-oil microemulsions can be formed. [102] However, owing to their size, these micro-particles are far too large to permeate the cellular membrane. Controllable formation of monodisperse nano-sized water-in-oil droplets in a reproducible manner is thus desirable for next-generation delivery strategies, yet remains a challenge. In this chapter, it is shown that by integrating micro and nanofluidics using soft lithography, a scalable platform for nanoemulsion-templated material synthesis can be developed. Using this approach, control over nanodroplet diameter can be achieved by varying the ratio of the continuous to the dispersed phase flow rate. When protein monomer is added to the dispersed phase, protein self-assembly can be induced by incubating the nanodroplets at high temperatures resulting in the formation of protein nanogels. Nanoparticles as small as 50 nm with narrow size distributions can be generated from a range of different proteins. Finally, assays with living cells revealed that the protein-based nanoparticles have the ability to penetrate through the membrane into cells and deliver small-molecule cargo. On the other hand, micron-sized

Fig. 5.19 (a-b) Confocal microscopy image of ovarian cells (PEO1) with the addition of 1000 nm sized nanoparticles. The particles are far too large to penetrate through the cellular membrane.
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particles were far too large and did not go through the cellular membrane. Such protein nanogels represent a class of biocompatible and non-toxic materials, and in conjunction with the high level of control over their formation, they exhibit favourable characteristics as a platform to explore drug-delivery and related biomedical applications.

By designing novel microfluidic devices capable of producing hierarchical or nanoemulsions, it is shown within this chapter that improvements over conventional methods of producing microgels can be beneficial for delivery strategies.
Chapter 6

Microfluidic techniques for monitoring self-assembly events

This chapter is based on the following publications:

1. Zenon Toprakcioglu, Pavan-Kumar Challa, Aviad Levin, Tuomas PJ Knowles, Observation of molecular self-assembly events in massively parallel microdroplet arrays, Lab on a Chip, 2018, 18 (21), 3303-3309

2. Zenon Toprakcioglu, Pavan-Kumar Challa, Catherine Xu, Tuomas PJ Knowles, Label-free analysis of protein aggregation and phase behavior, ACS Nano, 2019, 13 (12), 13940-13948

In this chapter, all experiments were conducted by myself. Cell lysate was kindly provided by Catherine Xu.

6.1 Introduction

Array-based technologies have a broad variety of applications due to their high throughput and high density, and thus offer an ideal platform for parallel processing and monitoring of microlitre volume samples, ranging from nanocrystal nucleation events to studying single cell assays. [99, 105] The systematic study of chemical and biochemical processes on such small scales is thus of increasing interest, and microfluidics offers an ideal platform for such experiments due to its high throughput. By using microdroplets, produced using conventional Polydimethylsiloxane (PDMS) based microfluidics, each picolitre droplet effectively acts as an individual microreactor. This principle has in turn allowed for the probing of PCR reactions [95], protein nanofibrillar aggregation studies [96–98], cell based assays [99, 100]
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and also DNA binding assays [101] at high throughput in picolitre volumes. Processes within the droplet can be monitored by collecting and storing them off-chip in glass or quartz capillaries or on chip in an array of traps by continuously applying flow from the continuous phase. [104–111] One key application of microdroplet arrays is the monitoring of self-assembly processes in the context of biological and synthetic systems. Self-assembly is a fundamental process found in nature that can result in the formation of various types of nano-structures. [243, 244] These structures are the result of non-covalent interactions between the building blocks, which ultimately leads to the formation of structural species that offer a range of functionalities. [245] Understanding self-assembly of protein and polypeptide molecules into ordered structures is of key importance, as there is a clear correlation between the formation of distinct structural species and human neurodegenerative disorders such as Parkinson’s or Alzheimer’s. [246]

A key aspect that is crucial in evaluating the aggregation kinetics of biomolecular species is the ability to monitor the nanofibrillar growth as a function of time. To that effect, turbidity assays [19, 20] as well as fluorescent techniques have been developed so as to observe how protein aggregates are formed [21]. A widely used fluorophore used to detect protein nanofibrils within a solution is Thioflavin T (ThT). This fluorophore has the characteristic of exhibiting an increase in its quantum yield once bound to a β-sheet rich structure, and therefore an increase in fluorescent intensity correlates with an increase of β-sheet rich structures. This technique offers a multitude of advantages such as ease of application, sensitivity and largely non-invasive nature [21]. However, the use of an extrinsic fluorophore raises the question whether the molecule itself affects, either by promoting or by inhibiting, protein aggregation. For this reason, there has been an interest in utilising intrinsic fluorescence of proteins to study them in their native state with the benefit of having minimal or no structural modifications to the polypeptide chain. Proteins containing aromatic amino acids such as phenylalanine (F), tyrosine (Y) and tryptophan (W) absorb and fluoresce in the ultraviolet (UV) range (250-400 nm) [21], and because of this label-free phenomenon, a wealth of physical properties such as hydrodynamic radius [22], electrophoretic mobility [23], conformational changes [24–33] or protein binding interactions [34–37] can be probed.

In this chapter, through the combination of microfluidic techniques for trapping droplets containing protein molecules and by using amino acid intrinsic fluorescence, protein phase transitions are monitored and observed.
6.2 Microdroplet trapping arrays for the observation of molecular self-assembly events

Monitoring the molecular phenomena involved in self-assembly is essential in elucidating the processes behind protein folding or misfolding. Moreover, understanding the mechanism of self-assembly can help us for the formation of next generation materials, where one can potentially modulate the exact properties required. In this chapter, a microfluidic device capable of trapping thousands of water-in-oil droplets simultaneously, and under no-flow conditions, is portrayed. Additionally, the aggregation of the dipeptide diphenylalanine (FF), the core recognition motif of the $\beta$-42 peptide associated with Alzheimer’s disease is monitored. The ability to reproducibly generate and confine monodisperse water-in-oil droplets with an extremely high trapping efficiency while maintaining entrapment under zero-flow conditions, on timescales compatible with observing molecular self-assembly events, thus renders this approach promising for numerous potential further applications in the biological and biophysical fields.

6.2.1 Device Operation

In order to successfully trap droplets and maintain their confinement under zero-flow, a device consisting of two compartments that are separated by two thin structures, which are referred to as "necks", was designed. These necks are slightly different in diameter (the wider one being 25 $\mu$m while the thinner neck is 15 $\mu$m), which can be seen in figure 6.1d. The narrow neck acts as an exhaust for the oil phase and is crucial in the operation of the trapping device. There are two alternative 'pathways' available to the fluid as it enters the inlet. It can follow the full length of the serpentine channel, all the way to the outlet, or it can flow through the traps, in a perpendicular direction to the serpentine channel. Even though the traps have a narrow exhaust, their large number ensures that they present a much larger total cross-sectional area for flow than the serpentine, while also providing a shortcut to the outlet. Hence, flow occurs predominantly through the traps, and this is where the droplets are driven. Once a droplet is formed, the flow transfers it through the primary trap (funnel region), as shown in figures 6.2 and 6.4. Once in the funnel, the droplet is squeezed through the first neck and forced into the second trapping area (trapping chamber) which is represented experimentally in figure 6.2a-c and computationally in figure 6.2d-f. There, the droplet stops at the secondary neck and thus cuts the flow through this exhaust. Now, the flow will follow the path of least resistance and will go through the adjacent trap, which is unoccupied. The next droplet will now go and occupy the second trap, thus blocking the
Fig. 6.1 (a) Design of microfluidic device used that can store up to 10,000 droplets. (b-d) Progressively higher magnification images of the trapping array. (d) Schematic representation of the drop-trap device consisting of 4 parts: the primary trap, the first neck, the secondary trap or trapping chamber and the second neck. (e) Schematic representation of the flow focusing junction where water-in-oil droplets are formed. (f-g) Images showing an overlay of bright field and fluorescence. (f) An array of uniformly trapped droplets, constructed by merging microscopy images from different areas on the device. (h) High magnification bright field image of trapped aqueous droplets.
corresponding exhaust and so on as is verified by the computer simulations in figure 6.1f. As more droplets are formed, more traps and exhausts are occupied and blocked respectively, resulting in the entrapment of thousands of droplets.

Fig. 6.2 (a-c) Bright field time lapse microscopy of droplet trapping. (a) Droplet 1 is confined within the first trap (T1), while droplet 2 is entering the second trap (T2) by deforming through the first neck. Droplet 3 on the other hand, circumvents the first two traps as they are already occupied and begins to enter the adjacent trap (T3). (b-c) T1-T3 are occupied so droplet 4 bypasses these and moves on to an adjacent trap. (d-f) COMSOL simulations representing the flow profile of the continuous oil phase with the presence of droplets. White arrows indicate motion of the continuous oil phase while the velocity magnitude (ms$^{-1}$) is represented by the colour gradient ranging from blue (lowest) to red (highest). (d) Initially all traps are unoccupied and the continuous oil phase pushes a droplet towards the first trap. (e) The droplet enters the first trap and blocks off the oil flow through the narrow exhaust, resulting in the following droplet to pass outside this trap and move towards the adjacent trap. (f) The oil flow guides the second droplet into the second trap.
Water-in-oil droplets were generated on-chip using a flow focusing junction as shown in figure 6.1e. Droplets were then directly flowed into the array of traps and subsequently confined, shown schematically in figure 6.1a-d and experimentally in figure 6.1f-h. By applying a continuous phase flow rate ($Q_{\text{con}}$) of 800 - 1300 $\mu$L/hr and a disperse phase flow rate ($Q_{\text{dis}}$) of 100 $\mu$L/hr, systematic control over droplet diameter could be achieved (figure 6.3) while still maintaining a trapping efficiency of up to 99%, as seen from the microscopy images in figure 6.1f-h. It was found that the smallest size of droplets that could be trapped with a 99% trapping efficiency was 60 $\mu$m.

Once droplets are trapped, they remain confined within their respective array positions. Importantly, due to droplets being isolated from one another by the PDMS chamber, coalescence is inhibited and thus, it is possible to form water-in-oil droplets without the need for surfactants, making this device ideal for studying surface phenomena between aqueous and oil phases without the added complexity of a third component.

![Graph showing the size dependence of the trapped droplet diameter as a function of the continuous phase flow rate, $Q_{\text{con}}$.](image)
The time evolution of droplet trapping and recovery was investigated using high speed imaging. It was found that the balance between surface tension and the continuous phase flow rate governs whether droplets will be trapped or not. Effectively, in order to trap a droplet, the pressure exerted on it must be sufficient to deform the droplet through the first neck but not the second. As has been previously discussed, droplets are initially funnelled towards the first part of the trapping area (figure 6.4a and 6.4b). The oil flow exerts a pressure on the droplet, which results in its deformation across the first constriction, allowing the droplet to be confined within the trapping chamber. This is schematically represented from the simulation results in the middle panel of figure 6.4a and shown experimentally in 6.4c. Due to the pressure exerted by the oil flow, the droplet continues to move forward towards the second neck. Therefore, for a short time interval it deforms on both sides of the trapping chamber, i.e. it is simultaneously squeezing through both the primary and secondary neck (figure 6.4d). The droplet then further pushes its way into the secondary neck (figure 6.4e). However, the pressure is not sufficient to sustain further deformation through the narrower secondary neck and thus it bounces back and remains confined to the trapping chamber (figure 6.4f and fourth panel in figure 6.4a). The small deformation seen in the narrow neck (figure 6.4f) is the result of the oil flow which exerts continuous pressure on the droplet. Finally, when the oil flow is greatly increased, the pressure requirement to overcome the secondary neck and allow the droplet to deform through this constriction is met and droplets are thus recovered (figure 6.4g and right panel of figure 6.4a).

As mentioned above, droplet trapping is strongly dependent on channel geometry. It was found that the length ratio of the primary to the secondary neck must be $> 1.25$. If a 1:1 ratio is used, droplets simply squeeze through both necks and are not confined to the trapping chamber. Additionally, not only is the length ratio of the necks important in successfully trapping droplets, but also the ratio of the two neck widths. If a 1:1 primary to secondary neck width ratio is used droplets are confined to the funnel region and cannot squeeze through the primary neck constriction. Thus, the width of the primary neck ($w_p$) must be greater than the width of the secondary neck ($w_s$). For efficient trapping, it was determined that $w_p$ must be less than one third of the size of the trapping chamber ($w_t$), however, in order to increase the trapping yield, $w_p$ is set to be one quarter of the trapping chamber diameter. Another important parameter that must be controlled is droplet surface tension. If this is too high, droplets will not sufficiently deform through the constrictions; if it is too low, droplets will not remain confined within the traps. [247] A typical value for the interfacial tension between an aqueous phase and fluorinated oil-surfactant system is around 5 mN/m [247]. Table 6.1 summarises the geometrical and physical parameters mentioned above.
Fig. 6.4 (a) COMSOL simulation results of droplet manipulation through each part of the trapping device. White arrows indicate motion of the continuous oil phase while the velocity magnitude is represented by the colour gradient ranging from blue (lowest) to red (highest). Following their confinement in the secondary trap, droplets can squeeze through the narrow neck and can thus be recovered by applying extremely high continuous phase flow rates ($Q_{con} > 5000 \, \mu\text{L/hr}$). (b-g) Time evolution bright field image sequence of droplet trapping and recovery. (b) The droplet is pushed into the primary trap by the continuous phase. (c) The droplet is then deformed as it is pushed through the first neck into the secondary trap. (d) Droplet deformation across both necks. (e) Droplet deformation along the narrow neck. (f) Droplet confinement within the secondary trap. The small deformation seen in the narrow neck is due to the continuous oil flow which exerts pressure on the droplet. (g) Droplet recovery by changing the oil phase flow rate from 1000 $\mu\text{L/hr}$ to 5000 $\mu\text{L/hr}$.
6.2 Microdroplet trapping arrays for the observation of molecular self-assembly events

Table 6.1 Geometrical and physical parameters for successfully trapping droplets

<table>
<thead>
<tr>
<th>Geometrical and physical parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>First neck diameter ($w_p$)</td>
<td>25 $\mu$m</td>
</tr>
<tr>
<td>Second neck diameter ($w_s$)</td>
<td>15 $\mu$m</td>
</tr>
<tr>
<td>Length ratio of first to second neck (&gt;1.25)</td>
<td></td>
</tr>
<tr>
<td>Width of secondary trap ($w_t$)</td>
<td>120 $\mu$m</td>
</tr>
<tr>
<td>$w_p/w_t$</td>
<td>0.25</td>
</tr>
<tr>
<td>Interfacial tension between aqueous and fluorinated oil-surfactant system</td>
<td>5mN/m</td>
</tr>
</tbody>
</table>

As the trapping chambers in this device are in parallel and not in series (that is to say that confinement of a droplet within each trap is independent from its neighbour), the pressure required to deform the droplet through the neck is consistent for each trap. In order for a droplet with a radius of 50 $\mu$m to squeeze through a constriction having a width of 25 $\mu$m, the curvature of the droplet must increase to achieve a local radius of $r = 12.5 \, \mu m$. If an assumption is then made, that the surfactant is uniformly distributed across the interface, the Laplace pressure implied by this deformation is:

$$\Delta P \approx \frac{\gamma}{r} = 400 \, Pa$$

(6.1)

where the interfacial tension between the aqueous and the fluorinated surfactant-oil phases has been taken as 5 mN/m [247]. This is the minimum pressure required to force a droplet into the neck, while pressures greater than this produce droplet flow into the trapping chamber.

In order to test whether this predicted pressure value corresponds to experimentally observed flow rates needed to fill the traps, the Hagen-Poiseuille relation for a channel with a rectangular cross section [248, 249] was considered:

$$Q = \frac{\Delta P h^3 w}{12 \eta L} - \frac{16 \Delta P h^4}{L \eta \pi^2} \sum_{n=1,3,5,...}^{\infty} \frac{1}{n^2} \tanh\left(\frac{n \pi w}{2h}\right)$$

(6.2)

where $w$ is the width, $h$ is the height, $L$ is the length of the channel and $Q$ is the flow rate for a given pressure gradient $\Delta P/L$. It suffices for this calculation to retain only the first three terms of the series, as convergence is rapid and neglecting higher-order terms introduces an error of less than 1%. Thus the following relation is obtained:

$$Q \approx 8.8 \times 10^{-11} \, m^3 \, s^{-1} \approx 320 \, \mu L \, hr^{-1}$$

(6.3)

where the width of the neck is 25 $\mu$m, the height of the channels is 50 $\mu$m, the length of the trap is 60 $\mu$m and the viscosity of the fluorinated oil is 3.5 mPas. This estimate
seems reasonable in comparison to the flow rates employed in these experiments (typically 800-1300 µL/hr). The minimum resistance of the first neck can also be calculated using:

\[ R = \frac{\Delta P}{Q} = 4.55 \times 10^{12} \text{ Pa s m}^{-3} \]  

(6.4)

Fig. 6.5 (a) Finite elements simulation results showing the effect of a gradual increase of the primary constriction size ranging from 35 µm (left panel) to 130 µm (right panel). The aqueous droplet (blue) is set at the center of the trapping chamber, surrounded by the continuous oil phase (red). (b) Graph of droplet displacement as a function of constriction size based on simulation results. The velocity used for all simulations was 0.3 ms\(^{-1}\).
In order to probe droplet release, COMSOL simulations were conducted. Six different constriction sizes were considered: 25, 35, 50, 75, 105 and 130 µm. For each simulation conducted, the velocity of the continuous (oil) phase was set at 0.3 ms\(^{-1}\), while each simulation was run until completion, i.e. until an equilibrium was reached and the aqueous droplet stopped moving through the microfluidic channels. The constriction sizes were systematically varied (figure 6.5a), and the droplet displacement as a function of constriction size is plotted in figure 6.5b. The distance that the droplet travelled was determined by measuring the interval from the center of the microdroplet to the final "resting" point reached. It is clear from figure 6.5b that initially, droplet movement does not significantly vary for smaller constriction sizes. However, at 75 µm, the flow rate is high enough to allow droplet deformation through the primary constriction thus allowing for a successful escape event, i.e. there is a threshold beyond which droplets can be released. This behaviour increases sharply for larger constriction sizes until there is essentially no constriction (right panel in figure 6.5a).

6.2.2 Diphenylalanine self-assembly

The power of the parallel drop-trap device to investigate single nucleation events in molecular nanoscale self-assembly was next explored. The assembly of diphenylalanine (FF) into nano and micro fibrils was investigated. [250–254] FF containing microdroplets were produced on-chip as shown in figure 6.1e and then confined using the drop-trap device. A stock solution of 300 mg/mL diphenylalanine (FF) dissolved in acetic acid was prepared. This was then diluted in equal part with water to yield a final peptide concentration of 150 mg/mL, which was used as the dispersed phase. It has been shown that FF self-assembles into tubular nanostructures as a result of partial evaporation of the aqueous/acetic acid phase [243]. Therefore, following trapping, FF containing droplets were left for 3 minutes before imaging in order to allow for droplet shrinkage. This happens in a two step process. Initially, the aqueous phase can partition into the oil phase. This is then followed by the diffusion of the aqueous phase through the highly porous PDMS, which leads to droplet shrinkage, and consequently results in the self-assembly of FF. This process is illustrated in figures 6.6 and 6.7. Initially no tubular structures are present (figure 6.6a). However, as time progresses, more of the aqueous/acetic acid phase diffuses into the oil, and FF self-assembly begins with tubular structures starting to appear in the middle droplet of the top row (figure 6.6b). Self-assembly propagation continues throughout this droplet leading to further tube assembly. Furthermore, the stochastic initiation of self-assembly in adjacent droplets (figure 6.6c) can now be observed. The tubes finally un-buckle causing them to break through the droplet interface which can be seen in figures 6.6d-e. Finally, after 180 seconds practically all the
monomer in the droplets has been consumed and only tubular structures are present within the microdroplet.

Fig. 6.6 (a-f) Bright field time-lapse microscopy of FF tube self-assembly and un-buckling due to droplet shrinkage. (a) Initially no tubular structures are present. (b) As droplets start shrinking, one can observe the initiation of self-assembly in the middle droplet of the top row. (c) Tube self-assembly occurs in adjacent droplets, namely the top two and bottom one on the right in a stochastic manner. (d-e) The tubes un-buckle and break through the droplet interface as a result of the released kinetic energy. (f) Final stage of FF self-assembly.

Furthermore, due to the device being able to efficiently trap a large number of droplets, it is ideal for the parallel observation of self-assembly events. FF based droplets were thus produced and continuously imaged throughout the experimental time course. The images in figure 6.7a (n = 144) were taken immediately following droplet generation while those in figure 6.7b and 6.7c were taken 20 and 40 minutes later respectively. This ensured that water/acetic acid had sufficiently diffused into the oil phase, thus increasing the local
peptide concentration allowing for aggregation initiation. It can be seen that only some droplets display self-assembled FF tube formation after 40 minutes of incubation. These results highlight the statistical character of self-assembly in this system. It seems likely that nanotubular aggregates are formed when a nucleation threshold is exceeded and that the nucleation process is intrinsically stochastic in nature rather than dependent on a deterministic mechanism. Numerous investigations supporting the stochasticity of such aggregation phenomena have been reported [255–259] and the observations in this chapter lend support to these studies.

The rate of loss of the aqueous phase as a function of time was then investigated. The rate of volume loss of a given droplet can be written as:

\[-\frac{dV}{dt} = \frac{dV}{dr} \frac{dr}{dt}\]  \hspace{1cm} (6.5)

where for a sphere of volume (V) and radius (r):

\[V = \frac{4}{3} \pi r^3\]  \hspace{1cm} (6.6)

and thus the rate of volume loss can now be written as:

\[-\frac{dV}{dt} = (4\pi r^2) \frac{dr}{dt}\]  \hspace{1cm} (6.7)

However, if it is assumed that the aqueous phase can only partition into the oil phase from the surface of the sphere, then the rate of volume loss must be proportional to the surface area of the sphere,

\[-\frac{dV}{dt} = k(4\pi r^2)\]  \hspace{1cm} (6.8)

where k is a constant that corresponds to how easily the aqueous phase can diffuse into the oil phase, i.e. it is proportional to the diffusion coefficient of the system.

Combining equation 6 with equation 7, it is finally obtained that:

\[\frac{dr}{dt} = k\]  \hspace{1cm} (6.9)

Therefore, in a plot of radius (r) against time (t), one expects to observe a linear behaviour, where the gradient corresponds to the value of k. As can be seen in figure 6.7d, this linear relationship is indeed verified for two different systems. In the first case (black points) there is no perturbation to the device and once the aqueous phase partitions into the oil it can diffuse through the PDMS and finally evaporate into the atmosphere. Under these conditions the value of k was found to be \(\sim 1.0 \times 10^{-8}\) ms\(^{-1}\). In the second case (blue points), a glass
slide was placed on top of the device, so as to inhibit the aqueous phase from diffusing through the PDMS and consequently into the atmosphere. It is clear that this method reduces the rate of droplet volume loss even with the presence of volatile solvents, since the gradient obtained from this data set was $\sim 1.4 \times 10^{-9} \text{ ms}^{-1}$. Since partial loss of the aqueous phase enhances aggregation, regulating the rate of droplet shrinkage allows control over the rate of FF self-assemble into the observed tubular structures.

![Fig. 6.7](image_url)

Fig. 6.7 (a-c) Bright field images of an array of trapped FF containing microdroplets ($n = 144$) and their aggregation over time. The images have been stitched together from different areas on the device. (a) Images taken immediately following droplet generation and trapping. (b) Images taken following 20 minutes of incubation. (c) Images taken following 40 minutes of incubation. FF aggregation in droplets is a stochastic nucleation process which explains why all the droplets do not contain tubular structures. (d) Graph showing the linear relationship between droplet radii as a function of time when the aqueous phase partitions into the oil phase.

### 6.3 Monitoring self-assembly and protein phase behaviour through a label-free manner

Phase transitions of protein molecules are central to biological function and malfunction. One such transition commonly encountered in nature is the conversion of soluble monomeric states into solid phases, which include crystals and amyloid fibrils, the latter of which are associated with the onset and development of neurodegenerative diseases. Monitoring aggregate formation and protein phase behaviour is essential in gaining mechanistic insights into
these fundamental processes. Fluorescence techniques have proven invaluable in observing biological molecules; yet, most such approaches rely on the use of an extrinsic fluorophore that binds to the molecule of interest, the installation of which can perturb the molecular systems under study. However, most proteins also possess aromatic amino acids within their peptide sequence, and therefore exhibit intrinsic fluorescence. In this section, it is shown that by measuring in space and time tryptophan auto-fluorescence for three proteins, reconstituted silk fibroin, $\beta$-lactoglobulin and lysozyme, fibrillar self-assembly can be monitored accurately and without the need for extrinsic dyes. When fibrillar protein self-assembly takes place, hydrophobic burial occurs, resulting in the minimisation of exposed tryptophan residues to the solvent and consequently leading to an increase in protein auto-fluorescence. Moreover, by employing a droplet-microfluidic approach to confine protein self-assembly in space, intrinsic fluorescence can be used to image protein nano-fibrils in a label-free manner and that the micro-structural analysis obtained from intrinsic fluorescence microscopy correlates well with that from samples treated with extrinsic dyes. Finally, these results show that protein auto-fluorescence is not limited to the observation of $\beta$-sheet rich structures, but can also be used to distinguish between different types of solid phases including spherulites and crystals, making this approach suitable for overall characterisation of protein phase transition phenomena.

### 6.3.1 Detecting protein aggregation through monitoring intrinsic tryptophan fluorescence emission

In order to probe how protein intrinsic fluorescence changes both spatially and temporally during fibrillar assembly, protein solutions of varying concentrations were prepared and confined in micro-capillaries or in micro-droplets and incubated in order to promote phase transitions. Different conditions were employed for three representative proteins; reconstituted silk fibroin (RSF), $\beta$-lactoglobulin and lysozyme, and it was found that for all cases, a change in fluorescence intensity could be detected as a function of time which correlated with the environment in which the tryptophan amino acid residue was exposed to. The change in intrinsic fluorescence intensity was measured as a function of time and compared with the time evolution of Thioflavin T (ThT) fluorescence at visible wavelengths for the same sample. It is accepted that an increase in a ThT fluorescence signal correlates with an increase in $\beta$-sheet content, and can consequently be used to monitor protein aggregation. RSF, which is rich in tryptophan (W) amino acids (as it contains 13 tryptophans) [260, 261], was first used to monitor protein self-assembly. RSF was mixed with 20% Ethanol, which has been reported to act as an aggregation promoter [262, 119, 124], and separated into
two aliquots. In one of the aliquots, 20 µM of ThT was added to the solution, while the other aliquot remained unchanged. Quartz capillaries were filled from the two corresponding aliquot solutions and subsequently monitored at room temperature as a function of time. The capillary containing ThT was imaged using filters that have an excitation wavelength (λ_{ex}) of 440 nm and emission wavelength (λ_{em}) of 480 nm, while UV filters (λ_{ex} = 280 nm, λ_{em} = 357 nm) were used to detect the sample within the other capillary. The results from the two different methods of observation were directly compared and it was found that not only is an intensity change evident in the label-free case, but furthermore both intensity increases take place in a highly correlated manner. This fact can be seen in figure 6.8a-b and in figure 6.9, where intrinsic fluorescence from protein monomer exhibits a low signal (figure 6.9a) while following self-assembly there is a clear increase in the overall intensity (figure 6.9b). This parallels directly with the corresponding images of monomer and aggregate taken using ThT extrinsic fluorescence, figures 6.9c and 6.9d respectively.

Furthermore, the kinetics of RSF aggregation and fibrillisation for different protein concentrations are presented in figures 6.8 and 6.11a-b. The concentration ranged from 36 mg/mL (360 µM assuming a molecular weight of 100 kDa [63]) down to 1 mg/mL (10 µM). Three replicates were conducted for each set of conditions and the kinetic data were then fitted (solid lines) by using a global fit and assuming a secondary nucleation dominated model [263] (see Materials and Methods for more details). The combined microscopic rate constants were evaluated as global fitting parameters and were found to be \(k_+k_n = 0.47 \, M^{-2}s^{-2}, k_+k_2 = 6.96 \times 10^9 \, M^{-3}s^{-2}\) for the ThT assay and \(k_+k_n = 0.51 \, M^{-2}s^{-2}, k_+k_2 = 7.24 \times 10^9 \, M^{-3}s^{-2}\) for the tryptophan-based assay, where \(k_n, k_+, k_2\) are the primary nucleation rate, the elongation rate and the secondary nucleation rate constants respectively and \(n_e = n_2 = 2\). It is thus clear from the data that this kinetic process is strongly secondary nucleation driven and more importantly that the ThT assay and tryptophan assays yield very similar rate constants. Such a large difference between \(k_+k_n\) and \(k_+k_2\) is to be expected since \(k_2\) dominates and agrees well with other protein models where the process was also secondary nucleation dominated. [264, 265] Moreover, misfits depicting primary and elongation nucleation were also tested in order to showcase how a global fit of secondary nucleation works best for this system (see figure 6.10).

It should be mentioned that the detection limit is determined by the number of tryptophans within the amino acid sequence of a given protein. It has been previously reported that the detection limit using this setup for a solution of monomeric protein of BSA, which contains 3 tryptophan amino acid units, was 500 nM. [22] As the aggregated form of the protein has a higher fluorescence signal than its monomeric counterpart, the detection limit depends only on the initial monomer concentration. The data show that both intrinsic fluorescence and
6.3 Monitoring self-assembly and protein phase behaviour through a label-free manner

Fig. 6.8 (a) Aggregation kinetics of different silk fibroin concentrations detected by fluorescence emission of tryptophan and (b) ThT. For each condition, three replicates are shown and the kinetic data were then fitted (solid lines) by using a global fit and assuming a secondary nucleation dominated model (see Methods for more details). The rate constants are (a) \( k_+k_n = 0.51 \text{ M}^{-2}\text{s}^{-2} \), \( k_+k_2 = 7.24 \times 10^6 \text{ M}^{-3}\text{s}^{-2} \) with \( n_c = n_2 = 2 \); (b) \( k_+k_n = 0.47 \text{ M}^{-2}\text{s}^{-2} \), \( k_+k_2 = 6.96 \times 10^6 \text{ M}^{-3}\text{s}^{-2} \) with \( n_c = n_2 = 2 \). (c-d) Schematic representation of protein aggregation from random coil to \( \beta \)-sheet conformation. (c) Initially the protein is in its soluble monomeric state and exhibits low intrinsic fluorescent intensity due to tryptophan exposure to collisional quenchers. However, once the protein self-assembles into a \( \beta \)-sheet structure, hydrophobic amino acids (represented as blue spheres) including tryptophan amino acids (red spheres) can get buried within the protein and are less available to quenching molecules, resulting in an increase in the intrinsic fluorescent signal. Hydrophilic amino acids are shown as gray spheres. (d) Sigmoidal curve representing the increase in fluorescent intensity as a function of time, due to protein self-assembly from a random coil to a \( \beta \)-sheet rich structure.

ThT labelled extrinsic fluorescence exhibit very similar kinetic behaviour even at low protein concentrations (figure 6.8 and 6.11a-b). The typical sigmoidal curve observed from these kinetic profiles is indicative of a nucleation-dependent fibril assembly that proceeds via a three-phase pathway. [266, 263] The first phase, often referred to as the lag-phase[267, 268], is a combination of multiple reactions that happen in parallel, and is associated with one or more of the following processes; primary nucleation,[269] elongation, secondary nucleation and fragmentation. [267, 268, 266, 270, 263] This is then followed by the growth phase, in
which the rate of protein conversion into its fibrillar form is at its greatest. The final plateau phase, corresponds to the system reaching a steady state. [24, 267, 268, 270, 266, 263]

Whether a similar label-free approach could be used to monitor the self-assembly of β-lactoglobulin, which contains 2 tryptophan residues was next explored. To this effect, a
6.3 Monitoring self-assembly and protein phase behaviour through a label-free manner

Fig. 6.10 Aggregation kinetics of different silk fibroin concentrations detected by fluorescence emission of tryptophan. For each condition, three replicates are shown and the kinetic data were then globally fitted (solid lines) by assuming primary and elongation nucleation using Amylofit. These misfits clearly indicate that this model is incorrect and a secondary nucleation dominated model is more appropriate.

80 mg/mL (4.3 mM) solution with 10% acetic acid v/v% was prepared and incubated at 60°C for 72 hours. The use of high concentration was to ensure that the aggregation process happened on a relatively fast time-scale. The increase in intrinsic fluorescent emission intensity between the monomeric protein and its aggregated state is clearly visible in figure 6.12, showing that this strategy to monitor self-assembly can be utilised for different proteins.

It is known that physiologically relevant ions such as Cu\(^{2+}\), Co\(^{2+}\), Fe\(^{3+}\) and Ni\(^{2+}\), which are paramagnetic, can quench intrinsic fluorescence. [271] In order to determine the extent to which such ions may quench tryptophan fluorescence intensity and thus interfere with the assay readout, 200 mM of Cu\(^{2+}\) ion solution, a concentration in excess of that found under physiological conditions was added to RSF solutions. It was found that for both protein concentrations tested, the metal quenches the fluorescent signal by a factor of c.a. two (see figure 6.11c-d). This finding suggests that the quenching effect of paramagnetic metals does not hinder this method of detection, but rather for the systems tested, only reduces the sensitivity roughly by a factor of two.
Fig. 6.11 Probing the role of background fluorescence and quenching. (a-b) Aggregation kinetics of low concentration reconstituted silk fibroin (RSF) solutions detected using tryptophan and ThT fluorescence respectively. For each condition, three replicates are shown and the kinetic data were then fitted (solid lines) by using a global fit and assuming a secondary nucleation dominated model (see Methods for more details). The rate constants are (a) $k_+k_n = 0.51 \text{M}^{-2}s^{-2}$, $k_+k_2 = 7.24 \times 10^6 \text{M}^{-3}s^{-2}$ with $n_c = n_2 = 2$; (b) $k_+k_n = 0.47 \text{M}^{-2}s^{-2}$, $k_+k_2 = 6.96 \times 10^6 \text{M}^{-3}s^{-2}$ with $n_c = n_2 = 2$. (c-d) Aggregation kinetic curves of RSF solutions with and without 200 mM CuCl$_2$. For both protein concentrations tested, the metal quenches the fluorescent signal by a factor of around 2.

6.3.2 Mechanism of tryptophan fluorescence emission increases due to protein aggregation

The fluorescence emission of tryptophan is known to be highly dependent on its local physico-chemical environment [34]. It has been shown that the aromatic amino acid is susceptible to fluorescence quenching when solvent exposed. [272–276] Collisional quenching of proteins has previously been used to determine to what extent tryptophan units are exposed to the aqueous phase in the context of protein folding [277, 35, 278, 279]. As collisional quenching is a contact phenomenon, the fluorophore and the quencher must be in direct proximity for quenching to occur. Denatured proteins often have tryptophan residues exposed and are therefore accessible to quenching molecules. However, if the protein folds, these residues can get buried within the protein structure resulting in an increase of the fluorescent signal due to the inaccessibility of water-soluble quenchers [275].
When a protein aggregates, a similar type of hydrophobic burial can occur as the hydrophobic tryptophan is subjected to a driving force favouring removal from solvent exposed positions, leading to an increase in the fluorescence emission. As more protein monomers self-assemble to form aggregates, fewer tryptophan molecules are exposed to quenchers and therefore the overall fluorescent signal rises even further. This process is schematically shown in figure 6.8c. Initially, the protein is in its soluble monomeric state and tryptophan amino acids (represented as red spheres) are exposed to the environment, allowing for collisional quenching to occur. However, during self-assembly, hydrophobic amino acids (blue spheres) including tryptophan molecules are buried within the protein structure resulting in an increased intrinsic fluorescence signal, with the hydrophilic amino acids (gray spheres) on the outside (as shown in figure 6.8c). This mechanism is also represented in the sigmoidal curve in figure 6.8d, where the fluorescent emission increases as a function of time as the protein folds. Hence it is possible to monitor protein self-assembly processes by detecting an increase in the tryptophan fluorescent emission, which is indeed observed throughout the experiments detailed in this study.
Microfluidic techniques for monitoring self-assembly events

6.3.3 Monitoring fibrillar morphology and microstructure through microfluidic confinement

Having established that protein aggregation can be monitored through temporal measurements of intrinsic fluorescence, further insights into the morphology of the formed structures through label-free deep UV fluorescence microscopy was gained by exploiting the increased quantum yield in the assembled state. To this effect, in order to localise the self-assembly process in space, the aggregation reaction was carried out within micro-droplets. It has been shown that the protein lysozyme (which contains 6 tryptophans), is prone to form micron sized fibrillar structures under certain conditions. [280, 281] Lysozyme containing droplets were thus generated using a droplet-making device as shown in figure 6.13a and collected in a capillary and incubated at 60 °C for 60 hours. Fluorescence microscopy images were taken at regular time intervals to determine whether protein aggregates within the droplets could be detected using the setup shown in figure 6.13. Initially, all the droplets have uniform intensity, figure 6.14a. However, as time progresses, small aggregates that have a higher fluorescence intensity than the background of the droplet start to appear (figure 6.14b-c). After 40 hours of incubation (figure 6.14d), these structures become more prominent due to protein self-assembly taking place, until finally it is clear that a fibrillar network is present within the droplets as can be seen in figure 6.14e-f. Therefore it is clear that by using intrinsic fluorescence alone, one can not only detect the presence of protein monomer, but also follow the self-assembly process that leads to the formation of a fibrillar network.

Intrinsic fluorescent microscopy was employed to acquire the images shown in figure 6.14, whereas those shown in figure 6.14g were obtained using ThT fluorescence. The similarity in fibril structure between droplets imaged via intrinsic fluorescence and those imaged via extrinsic fluorescence suggests two things; the use of ThT does not affect the fibrillar network in this particular system, and more importantly protein auto-fluorescence...
6.3 Monitoring self-assembly and protein phase behaviour through a label-free manner

Fig. 6.14 (a-f) Fluorescent microscopy image sequence of lysozyme containing droplets detected using tryptophan fluorescence ($\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 357 \text{ nm}$). Droplets were incubated at 60 °C for 60 hours. (a) Initially the intensity within the droplet is uniform, indicating the presence of monomer species only. (b-c) As time progresses, small bright clumps start to appear within the droplets. (d) The small aggregate species grow further and their intensity increases as they become more apparent inside the droplets. (e-f) Self-assembly propagation continues until a fibrillar network is established within the droplets. (g) Fluorescence microscopy images of fibrils formed within droplets, acquired using intrinsic tryptophan fluorescence emission. (h) ThT stained fluorescence microscopy images of droplets which also contain dendritic fibrils. The similarity between the two data sets suggests that protein auto-fluorescence can be used to detect the morphology of protein aggregates without the requirement of extrinsic dyes.

can be used for the monitoring of aggregation phenomena as well as to probe single aggregate structures.

Furthermore, in order to demonstrate that this label-free approach of detecting protein phase transitions within micro-droplets is applicable for most systems, RSF and $\beta$-lactoglobulin self-assemblies were also monitored. A 20 mg/mL (200 µM) reconstituted silk protein solution was mixed with 20% v/v ethanol and droplets were generated using a microfluidic device (as shown in figure 6.13a). Similarly, 80 mg/mL (4.3 mM) $\beta$-lactoglobulin was mixed with 10% acetic acid v/v% before droplet formation. The aggregation kinetics of the two proteins were observed using tryptophan fluorescence. It is again evident from these data sets (figure 6.15a-d for RSF and figure 6.15e-h for $\beta$-lactoglobulin) that the protein in its
Fig. 6.15 (a-d) Time lapse fluorescence microscopy of reconstituted silk fibroin containing droplets detected using tryptophan fluorescence ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 357$ nm). Droplets were incubated at room temperature for 7 hours. (e-h) Fluorescent microscopy image sequence of $\beta$-lactoglobulin containing droplets detected using tryptophan fluorescence. Droplets were incubated at 60 °C for 72 hours. Initially, for both proteins, the intensity within droplets is approximately uniform, indicating the presence of monomeric species only. However, as time progresses, individual droplets start to appear brighter, which is a result of stochastic self-assembly processes taking place. This continues until the intensity within all droplets increases, which indicates the final (plateau) stage of the phase transition.

The monomeric form has a lower fluorescent intensity that in its aggregated form. Moreover, due to proteins self-assembling into nanofibrills rather than micron-sized structures, individual fibrils within the micro-droplets cannot be detected, but rather an overall intensity is observed. Interestingly, the stochastic nature of such aggregation phenomena can also be observed. As all the droplets do not simultaneously appear brighter, it seems likely that a nucleation threshold within each droplet must be exceeded before aggregation can progress. [141]

6.3.4 Spatial dynamics of the formation of different protein phases

In addition to observing protein fibrillisation into $\beta$-sheet rich structures, other forms of solid protein phases can also be monitored using this intrinsic fluorescence approach. The advantage of using an intrinsic approach is that extrinsic labels may depend on specific processes occurring, such as structural changes. For example, a ThT fluorescent emission increase requires $\beta$-sheets to be present. However, if the protein in question contains...
tryptophan, any conformational change within the protein that may occur should, in principle, be detectable via intrinsic fluorescence.

Fig. 6.16 (a-c) Microdroplets containing needle shaped spherulite-like structures formed from the protein lysozyme. (d) Left panel: Design of the trapping array device. Middle and right panels: Progressively higher magnification micrographs of the trapping array. (e-g) Image sequence showing the growth of lysozyme crystals confined within droplets.

It has been shown that under specific conditions, lysozyme can form both spherulitic structures and crystals [280]. By following the same protocol [280], spherulite and crystal growth within microdroplets was monitored. Small needle shape spherulite-like structures were obtained by heating the droplets to 40 °C for a total of 2 days. These structures, that lie within the droplets, can clearly be seen in figure 6.16a-c. Furthermore, by incubating droplets at room temperature, tetragonal crystals could be formed. Again, their growth was
monitored using tryptophan intrinsic fluorescence, the image sequence of which is shown in figure 6.16e-g. In order to track the same droplets and observe crystal growth, the use of a microfluidic trapping device was employed as has been shown above (figure 6.1 and 6.16d) [141], whereby droplets were generated and consequently confined on chip without the need for a continuous flow. Finally, in order to determine whether protein phase transitions can be observed using this label-free approach within a complex mixture, a solution containing lysozyme was mixed with cell lysate (which is inherently fluorescent at this wavelength). The growth of lysozyme crystals within this complex mixture was then monitored through tryptophan intrinsic fluorescence, which shows that this method of detection even works with complex mixtures.

6.4 Conclusions

In this chapter, a device that is capable of confining 10,000 monodisperse droplets in an array of trapping chambers with a trapping efficiency of 99% is demonstrated. These droplets can then be recovered for further studies simply by increasing the flow rate of the oil phase. Additionally, the diameter of the trapped droplets can be controlled by varying $Q_{\text{con}}/Q_{\text{dis}}$. High speed imaging was used to study the deformation of a droplet as it passes through the different areas of the trap. It appears that droplet entrapment requires a minimum pressure gradient that is associated with the increased curvature of the droplet during its passage through the neck before it can enter the trapping chamber. Finally, the ability of this device as a platform for the parallel study of nanofibrillar self-assembly processes along with aggregation kinetics on a large scale is shown.

Monitoring protein aggregation is crucial to elucidating the mechanisms that underpin biological function and malfunction. These results show that by exploiting the intrinsic fluorescence of the aromatic amino acids, and in particular tryptophan, it is possible to observe self-assembly events without the need of additional extrinsic fluorophores. By combining deep UV microscopy with droplet microfluidics, in this chapter it is shown that this label-free method can be used to observe aggregation kinetics for three different proteins, reconstituted silk fibroin, $\beta$-lactoglobulin and lysozyme. Furthermore, the comparison of protein aggregation monitored via intrinsic fluorescence correlates very strongly with the results obtained when using the established amyloidogenic fluorophore Thioflavin T, suggesting that this method can be used as an alternative non-invasive label-free modality of detection. In addition it has been demonstrated that this approach is not only limited to fibrillar self-assembly, but can be extended to observing the formation of other solid phases such as crystals. Therefore, the ability of this method to study and probe protein or
peptide self-assembly processes intrinsically, rather than through the use of labels, renders it particularly suitable for unbiased observations of protein phase transitions.

By combining novel microfluidic designs capable of trapping thousands of droplets with intrinsic fluorescence-based methods of detecting protein phase transitions, it is shown in this chapter that aggregation phenomena can be observed label-free, in a massively parallel scale.
Chapter 7

Concluding remarks

Microfluidic techniques, and in particular droplet microfluidics, offer a route towards the detection of biomolecules, analysis of biophysical processes and biomedical diagnostics, in a high throughput manner. An important area that branches from this broad field is that of generating materials from microfluidic approaches. By utilising the propensity of biomolecules such as protein monomers to self-assemble into supramolecular structures, and employing emerging microfluidic technologies, advancements in new material fabrication were made possible and have proven invaluable for the formation of capsules and microgels.

In this thesis a range of approaches have been used for the generation of hierarchical capsules ranging from hundreds of micrometers down to nanometres. The FDA approved protein, silk, was mainly utilised throughout the projects as it is an ideal biomolecule which exhibits biocompatibility, non-toxicity and biodegradability. Through the use of a flow-focusing droplet maker, silk-based microgels were formed and characterised. Furthermore, the release kinetics of small molecules were investigated in order to establish that such systems can be utilised for delivery related applications. Moreover, asymmetric, Janus-like microgels comprised of silk protein were also generated and core-shell structures, where the internal part remains liquid whereas the external shell has gelled, were formed. Such capsules are ideal for protecting valuable cargo, which is retained within the internal part of the capsule.

The issue of combining capsule production with biomedical applications has been difficult to address. In fact, generation of monodisperse capsules which are readily available for pharmaceutical needs can be challenging. In chapter 3 of this thesis, silk microgels decorated with silver nanoparticles which show potent antibacterial activity but exhibit significantly reduced cytotoxicity are fabricated. A murine model is studied whereby a suture containing bacteria is treated using the antimicrobial microgels. Such microcapsules can be particu-
larly promising in the context of drug-resistant bacteria which is becoming an increasing nosocomial issue.

The main challenges in most delivery related systems involve controlling the rate of molecular diffusion from the cargo holder to the intended target. To that effect, I designed a microfluidic device capable of generating hierarchical emulsions where parameters such as the number and sizes of internal droplets, shell thickness, but also the size of the external droplets could be specifically controlled, thereby allowing one to tailor the system so that release kinetics can be precisely regulated. Moreover, by adding silk protein to the aqueous phase, microgels surrounded by an oil shell or conversely, core-shell capsules, with oil droplets encapsulated within a microgel could be formed in a reproducible and high throughput manner, showing that such techniques are invaluable for generating monodisperse emulsions with control over the parameters mentioned above.

There has been considerable interest in forming monodisperse nanosized emulsions/capsules in the context of drug delivery which can successfully penetrate cell membranes. However, to date, this has remained challenging and has mostly been focused on polymer-based materials rather than proteins, the latter of which are readily biocompatible and biodegradable. It is shown in this thesis that by integrating micro and nanofluidics, nanodroplets comprised of protein solutions as small as 50 nm can be formed and that control over their sizes is relatively easy to achieve. Again, utilising the tendency of proteins to self-assemble, protein nanogels could be generated and by adding a fluorescent-labelled protein (GFP), intracellular delivery to ovarian cancer cells was monitored and established. Such systems thus represent excellent candidates for drug-delivery and related biomedical applications.

Elucidating the mechanisms behind biological function and malfunction is crucial and can only be achieved through the observation and monitoring of protein aggregation phenomena. In order to study such events in a microfluidic system, a device geometry capable of trapping thousands of water-in-oil droplets under zero-flow conditions was developed, thereby allowing self-assembly events to be studied in detail. Furthermore, by exploiting the intrinsic fluorescence of the aromatic amino acid tryptophan, protein aggregation including crystal, amyloid and spherulite formations could be observed without the need of additional extrinsic fluorophores. Through the integration of microfluidic trapping arrays capable of confining thousands of droplets, with intrinsic methods of observing protein phase transitions, it is shown in this thesis that such approaches are powerful in determining the mechanisms of self-assembly on a massively parallel scale, without resorting to extrinsic fluorophores.

In conclusions, the work described on this thesis focused on microfluidic-based approaches for the generation of hierarchical emulsions capable of biomedical applications and for intracellular delivery. Through the use of novel microfluidic designs, a variety of
capsule sizes could be achieved, ranging across 3 orders of magnitude, and aggregation phenomena within microdroplets could be monitored via intrinsic fluorescence and studied by confinement in micro-trap arrays. In future work, further microfluidic development has to be achieved in order to truly utilise the potential of generating capsules through microfluidic-based approaches. In particular, even though monodisperse nanodroplet production has been made possible, throughput remains relatively low and scalability of these devices is needed for producing high enough volumes that are essential for \textit{in vivo} experiments. Moreover, due to the small sizes involved, interfacial effects play an enhanced role and thus investigating the physics of droplet formation at the nanoscale is of key scientific interest. Finally, generating hierarchical nanoemulsions would be an interesting route to follow as it would not only achieve control over release kinetics, but would also allow effective intracellular delivery.
References


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