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Microfluidics for protein biophysics

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Abstract

Microfluidics has the potential to transform experimental approaches across the life sciences. In this review, we discuss recent advances enabled by the development and application of microfluidic approaches to protein biophysics. We focus on areas where key fundamental features of microfluidics open up new possibilities and present advantages beyond low volumes and short time-scale analysis, conventionally provided by microfluidics. We discuss the two most commonly used forms of microfluidics technology, single phase laminar flow and multiphase microfluidics. The understanding and control of the characteristic physical features of the microfluidic regime, the integration of microfluidics with orthogonal systems as well as the generation of well-defined microenvironments can be used to develop novel devices and methods in protein biophysics for sample manipulation, functional and structural studies, detection as well as materials processing.

Keywords: Protein biophysics, Microfluidics

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1. Introduction

Due to the central role of proteins in all biological systems, the determination of the structures, functions and interactions of these biomolecules has become a crucial element in many areas of modern biomolecular sciences. Indeed, areas as diverse as the discovery of biomarkers in diagnostics [1, 2, 3], the use and development of building blocks for biomaterials [4, 5, 6] and the development of therapeutic molecules [7, 8, 9] critically rely on a molecular level understanding of protein behaviour. Recent advances in micro and nanotechnologies have supported developments in protein biophysics for the study, detection and processing of proteins. In this review, we discuss how technologies based on the control of fluids at the microscale are promoting and enabling key developments and how these advances can contribute to the future of quantitative protein science. A number of recent developments in microfluidics have become possible through advances in a diversity of fields, reflecting the interdisciplinary nature of microfluidics. For example, the fabrication of microfluidic devices, initially inspired by the micro and nano-fabrication technologies used by the semiconductor industry in clean-room environments, [10, 11] is now commonly performed by soft lithography [12, 13, 14], which enables the rapid fabrication of devices from polymeric materials.

While the focus of many early microfluidic devices was commonly in the miniaturisation of existing macroscale laboratory experiments, enabling shorter analysis times with lower sample volumes and the possibility to multiplex measurements [10, 11, 15, 16, 17], recent advances have enabled new types of measurement approaches that have the potential to enable fundamental advances in many aspects of protein biophysics. Microfluidics offers a route to simplify, outperform or expand the possibilities offered by conventional equipment or methods used for 1) sample manipulation, 2) detection, 3) structural and functional studies as well as 4) materials processing (discussed in Sections 3.1, 3.2, 3.3 and 3.4 respectively). Such advances rely on : i) a better understanding of the distinct physical behaviour of fluids at the microscale, ii) the integration of

2

complementary systems or methods within microfluidic devices to expand their functions and iii) the possibility to create well controlled micro-environments. These three fundamental features of microfluidics are discussed in Sections 2.1, 2.2 and 2.3 respectively. Figure 1 summarises these salient features and the resulting opportunities in common biophysical operations.

Enabling features

2.1 flow properties   2.2 integration   2.3 microenvironment

Applications for protein biophysics

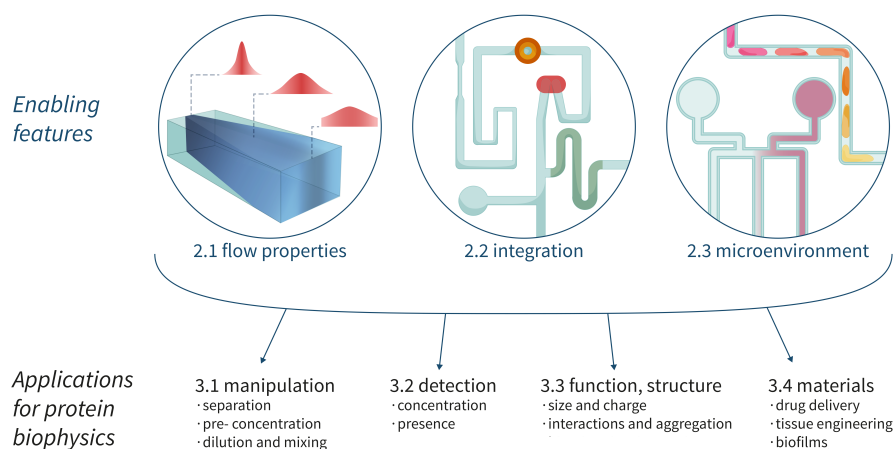| 3.1 manipulation	3.2 detection	3.3 function, structure	3.4 materials
· separation	· concentration	· size and charge	· drug delivery
· pre- concentration	· presence	· interactions and aggregation	· tissue engineering
· dilution and mixing			· biofilms

Figure 1: Schematic depicting how the basic features of microfluidics can be used and combined to develop devices and methods for protein biophysics. Each enabling feature and the resulting applications are discussed in the text, in the corresponding sections indicated.

In this review, we focus on a selected set of examples representative of the possibilities afforded by micron scale experiments in quantitative protein science. Moreover, although the review focuses on microfluidic approaches for protein biophysics, many techniques discussed here can be applied to other biomolecules.

2. Fundamental features of microfluidics

2.1. Characteristic physical features of the microfluidic regime

Unlike flow regimes at the macroscale that can be turbulent and therefore unpredictable and difficult to control, flow regimes at the microscale are typically laminar for aqueous solutions with moderate viscosities [18, 19]. The transition

3

between turbulent and laminar flow is governed by the Reynolds number, which represents the ratio of inertial to viscous forces defined as $R_e = uL/\nu$, where u is the flow velocity of the single phase fluid, L, the characteristic length scale and ν, the kinematic viscosity. In the laminar regime, characterized by a smooth and constant fluid motion, the velocity, pressure, and other flow properties remain constant in time and vary smoothly in space. The operation in the regime of low Reynolds numbers in single phase flows is therefore useful for a range of applications in analytical biosciences where precise and predictable flow control is required, including separation strategies, quantitative mixing and dilution as well as for the study of the diffusion of biomolecules.

A further feature which is significantly modified at the micro scale with respect to the bulk scale is the surface-to-volume ratio, which increases dramatically when the system volume is decreased. In this case, the boundary conditions and interfaces play an important role, and phenomena with an insignificant influence at the macroscale can become dominant at the micro-scale.

Some examples of microfluidic devices exploiting such properties in protein biophysics are shown in Fig.2.

2.2. Integration with orthogonal methods

Microfluidic devices are usually fabricated using microfabrication techniques and are therefore, in principle, compatible with a range of micro or nano scale devices. The possibility to expand the scope of functionalities available in microfluidic devices through the integration of orthogonal methods, represents the second fundamental feature. Indeed, a range of sensors and actuators have been combined with microfludics to manipulate, detect or evaluate the functions of a variety of biomolecules. The integration of optical or acoustic elements in microfluidic devices have given rise to optofluidics [22, 23] and acoustofluidics [24, 25, 26] respectively. The integration of electrodes, that gave access to electrokinetic phenomena, are also commonly used for separation and pre-concentration strategies [20, 21]. Finally, a large variety of optically transparent materials are available for the fabrication of microfluidic devices, which makes

4

such devices compatible with a range of external optical sensing and manipulation modalities.

Figure 3 shows examples of microfluidic devices that have gained additional functions through integration with orthogonal methods.

2.3. Controlled microenvironments

Another key feature of experiments at the microscale is the possibility to create highly controlled microenvironments. For example, the compartmentalisation of aqueous solution into microdroplets in a non-miscible carrier fluid and the possibility to generate thousands to millions of essentially identical droplets of given composition are attractive advantages for the development of high-throughput experiments [27, 28]. Since its origin, droplet microfluidics has received considerable interest and has been used extensively for chemical reactions, materials characterisation and biological experiments. Digital microfluidics provides another alternative to compartmentalise and manipulate low volumes of fluid [29].

Controlled microenvironment can also be created via continuous flow, using for example the laminar flow property inherent to fluids at the microscale, to generate well-defined concentration and thermal gradients. Finally, in-vivo environments have also been recreated in microfluidic devices. A striking example is the recent development of organs on a chip [30, 31, 32], where cells and tissues are grown on chip for a range of studies including proteomics analysis, trafficking and therapeutics studies.

Figure 4 shows examples of micro-environments created using droplet microfluidics.

3. Applications

In this section, we highlight some opportunities opened up by specific enabling features of microfluidics in the context of biophysical studies of proteins and their complexes. The areas covered include the manipulation and preparation of protein solutions (Section 3.1) the development of strategies for detection
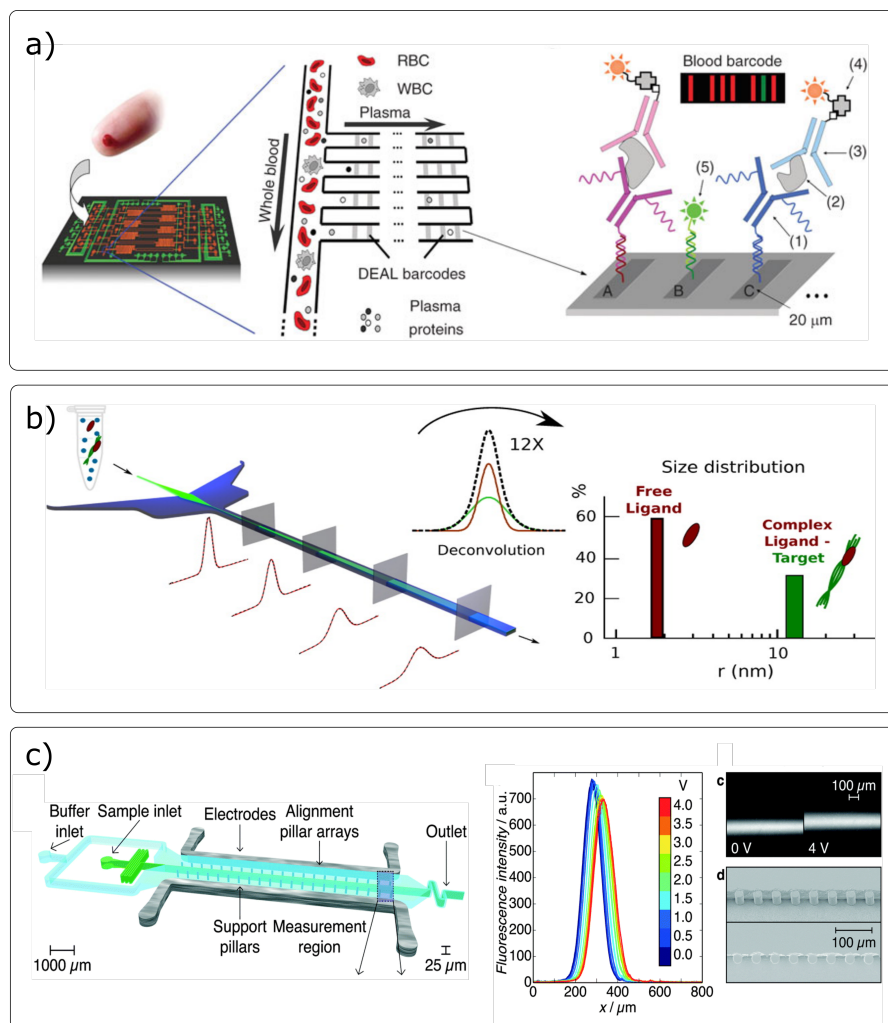
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Figure 2: A series of scenarios that take advantage of single-phase laminar flow properties at the microscale. a) Plasma proteins are separated from large particles at a branching point of capillaries in the context of an integrated blood barcode chip for the rapid measurement of plasma proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [73], copyright 2008. b) The acquisition of multiple diffusion profiles in a microfluidic channel opens attractive possibilities for the evaluation of the size distribution of heterogeneous mixtures. Reprinted with permission from [110]. Copyright 2016 American Chemical Society. c) Using free flow electrophoresis, it is possible to determine the effective charge of proteins in solution, a key parameter that modulates molecular interactions, as demonstrated here using a charge-ladder family of mutants of the calcium binding protein calbindin D_{9k}. Reproduced from [115] with permission of the PCCP Owner Societies.

and diagnosis (Section 3.2), the study of their functions and structures (Section 3.3) as well as their shaping into novel micro and nanomaterials (Section 3.4).

3.1. Manipulation

The readily predictable and controllable nature of laminar flows opens up possibilities to separate, sort, concentrate, merge and mix solutions of proteins and their complexes in a quantitative way. In addition, the integration of external methods and devices, in particular electrodes, are particularly useful for continuous flow separation and pre-concentration.

3.1.1. Separation

Separation strategies can be broadly divided into batch and continuous flow operations. Batch separation techniques commonly exploit the different interactions between the individual components of a mixture and a stationary phase. After a pulse injection, the individual species elute at different retention times according to the different interactions with the stationary phase, which can be modulated by changes in the mobile phase. Typical interactions involve electrostatic, hydrophobic, affinity or size exclusion effects.

For many systems, it is crucial to reduce the volume of the stationary phase to decrease the amount of valuable material required as well as the dilution of the sample during the analysis. In this perspective, microfluidic techniques offer novel opportunities for the miniaturizaton of both packed and open-tubular columns [33, 34], as well as of multiple column chromatography [35].

Moreover, in addition to reducing the scale of conventional methods, micro and nanofluidics opens the possibility to exploit novel types of interactions. A relevant example is entropic trapping, a phenomenon that occurs when biomolecules exhibit a hydrodynamic diameter that is larger than the section of the channel in which they are flowing [36]. In this case, the molecules have to adopt a specific conformation, typically as elongated chains, to pass through the channel, thus reducing their entropy. This energy cost leads to a lower transport rate for larger molecules. A further advantage of microfluidic technology

is the improvement of the control of the properties of the stationary phase. For example, active polymer films with nanometre scale components that can be thermally switched between hydrophilic and hydrophobic state have been integrated into a microfluidic device to respectively adsorb proteins from a solution and release them on demand [37].

In continuous flow separation, by contrast, the sample flows continuously under steady-state conditions and the main strategies for analyte separation consist in applying force fields parallel or perpendicular to the flow to separate the individual proteins. Microfluidic based continuous flow separation approaches present advantages relative to their bulk analogues including the elimination of band broadening from turbulence due to the laminar flow inherent to such devices. Free-Flow Electrophoresis (FFE) and Free-Flow Isoelectric Focusing (FF-IEF) based separation are common continuous flow separation techniques for protein analysis based on microfluidic technology. The large surface to volume ratios of microfluidic devices favour thermal dissipation, a feature that allows the possibility to apply large electric fields for electrophoretic separation without excessive Joule heating, thereby improving the efficiency of electrophoretic separation [20, 21, 38, 39] compared to conventional electrophoretic methods. Another type of continuous flow separation is diffusion based separation that relies on the difference in hydrodynamic resistance of biomolecules [40]. This type of separation has also been recently employed in the context of latent analysis of unmodified biomolecules [41], as described in details in Section 3.2 (Strategies for detection and diagnosis).

3.1.2. Pre-concentration

Pre-concentration techniques, also called enrichment techniques, are used to increase locally the concentration of a protein to enable an easier detection. Such techniques often rely on the fact that the concentration of mobile ions depends on the surface charge, which can be tuned through appropriate surface treatment and/or the application of an electrostatic field [42]. The exploitation of this electrokinetic phenomenon can be used for protein concentration. For

8

example, porous silica membranes, that allow buffer ions through but exclude larger migrating molecules, have achieve pre-concentration of about 600 fold in microfluidic devices [43].

Dielectrophoresis, an electrostatic phenomenon that describes the motion of a dielectric particle resulting from a force produced by a non-uniform electric field, has also been used for preconcentration. Even though the mechanism of protein dielectrophoresis is not fully understood [44], it has been exploited to create a molecular dam by combining AC and DC electric fields at nanoconstrictions [45]. In this case, the effects of negative dielectrophoresis and electroosmosis oppose the net transport from electrophoresis to trap the proteins away from the nanoconstriction as shown in Figure 3.a. Other examples of micro/nanofluidic preconcentration chip include [46, 47, 48]

3.1.3. Dilution and mixing

Standard dilution processes of analytical chemistry typically rely on manual operation or expensive robotic automation. Microfluidic devices have enabled the development of high throughput quantitative assays for rapid and automated dilution, through the development of gradient generators [49, 50].

Such advances have found applications for instance in single-molecule studies, where the concentrations of proteins must be sufficiently low to allow only one molecule to reside in the probe volume at any given time. Under these conditions, at concentrations that are significantly lower than their dissociation constant, many biological complexes are not stable. By developing a microfluidic device capable of performing extremely rapid dilutions, it was possible to observe weakly bound complexes before they dissociate in single-molecule experiments [51]. The microfluidic device achieves dilution of 1:100,000 and is suitable for studying complexes that have an dissociation rate lower than approximately $1\,\mathrm{s}^{-1}$.

A reconfigurable microfluidic device, representing a good example of integration for automated dilution, was used to analyse the concentration of proteins from tissue extracts using bicinchoninic acid assay [52]. The device comprises

9

among others a quantitative microfluidic dilution generator capable of generating linear, logarithmic, or arbitrary dilution using a three steps process. Each diluted sample is collected in individual fixed volume reaction chambers, comprising an acoustic mixing mechanism, thus enabling high-throughput quantitative assays over a range of dilutions ranging from 1:1 to 1:100. Mixing in microfluidic devices has been discussed extensively in reviews [53, 54].

3.1.4. Multiphase flow, droplet-based and digital microfluidics

Through the compartmentalisation of thousands of identical microdroplets in a non-miscible carrier fluid, droplet-based microfluidcs offers the possibility to perform high-throughput experiments on a level which can exceed the throughput of conventional robotics based lab automation [17, 27, 28, 55, 56]. The resulting multi-phase fluids have been used for chemical reactions [57, 58], fluid characterisation [59, 60] biological experiments [17, 61, 62, 63, 64] and we discuss below some recent advances relevant to protein science, in the context of sample manipulation and specific measurements.

In order to increase the possibilities offered by droplet-based microfluidics, a range of strategies have been proposed to alter and manipulate individual droplets. A device was used to generate a sequence of droplets that defines a digital concentration gradient [56]. In the device, a nano-sized droplet of defined concentration, called the mother droplet, is trapped in a dilution chamber. When a buffer droplet is injected into the chamber, it merges with the mother droplet. The new droplet, of larger volume, blocks a side channel forcing the oil flow into the dilution chamber. The consequence of this process is the breaking up of the larger droplet that releases an output droplet. The droplet in the chamber returns to its initial volume, unblocking the side channel and restoring the oil flow until the next buffer droplet arrives in the chamber. The mixing and consequent dilution take place during and after the output-droplet generation. The process, that takes approximately 35 ms per output drop, enables a high throughput generation of a sequence of digitally diluted drops. The principle, demonstrated for a DNA binding assay, can also be used for other biomolecules

10

including proteins. In another example, a droplet sampling system was used to generate a concentration gradient in droplets, using small volumes of reagent [65]. This system enabled the development of a high-throughput quantitative binding assay in nanoliter droplets to determine the dissociation constant of protein-peptide interaction using fluorescence anisotropy measurements [66].

Hybrid microfluidic devices with integrated actuators have also been developed to manipulate droplets. For example, a device comprising a surface acoustic wave transducer integrated on the chip was designed to merge multiple droplets on-demand [67]. The acoustic radiation forces generated by the transducer are used to immobilize droplets as they flow into an expansion chamber. The droplets are held until successive drops arrive and merge. Once the merged droplet reaches a critical volume, the acoustic radiation forces are not sufficient to counteract the viscous drag exerted on the drop, causing the drop to flow out of the chamber and leaving space for another one to be trapped. Another example of hybrid microlfuidic device describes how a picoinjector is used to add controlled volume of reagent to individual droplets using electro-microfluidics [112].

Another area of droplet compartmentalisation is digital microfluidics that consists in the manipulation of discrete droplets of sample and reagents through the application of a series of electrical potentials to an array of electrodes coated with a hydrophobic insulator. Such approach is particularly well suited for automated protocols, and was used to perform a series of operations including precipitation, rinsing and resolubilisation to extract proteins from heterogeneous fluids [69] as shown in Fig.3.b.

3.2. Strategies for detection and diagnosis

In contrast with applications in genomics, where the detection of low concentrations of nucleic acids can be achieved via target amplification techniques such as PCR, the detection of proteins present in low abundance remains highly challenging. This problem has prompted the development of both label-mediated and label-free detection strategies in microfluidic devices, which, in most cases,
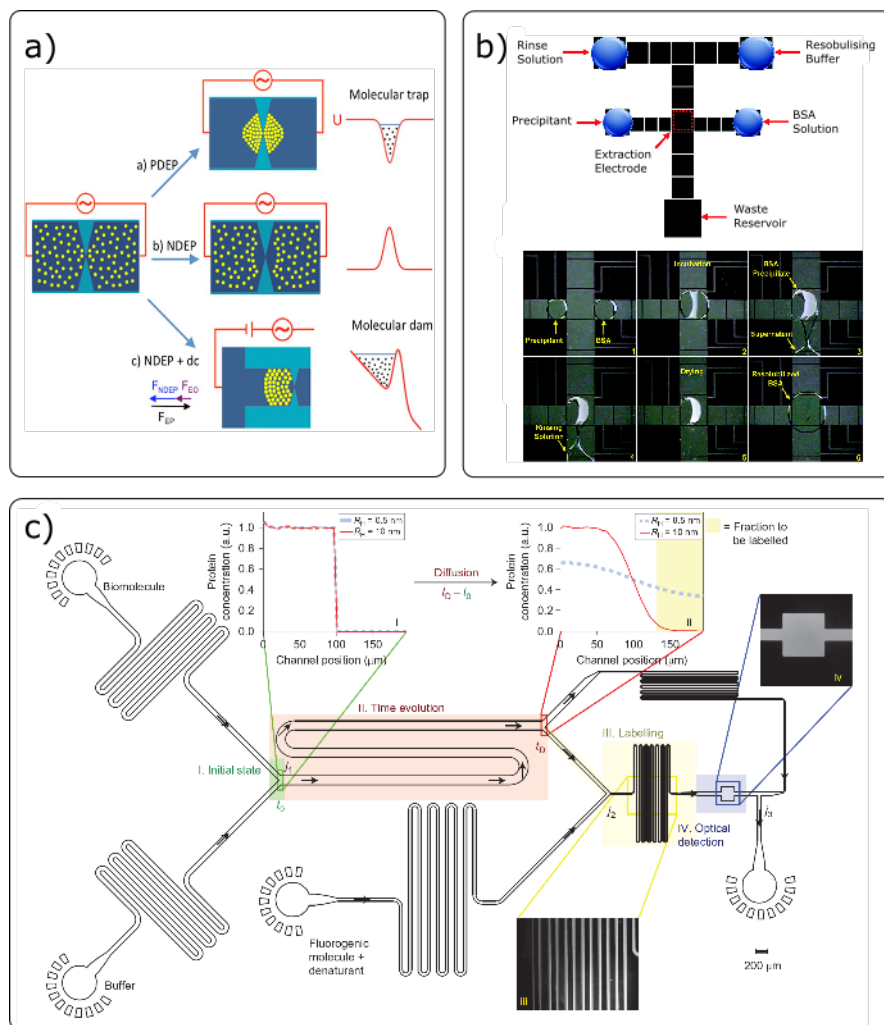
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Figure 3: The combination of microfluidics with orthogonal techniques has opened up new possibilities in protein biophysics. a) A molecular dam to enrich proteins was created by combining AC and DC electric fields at nanoconstrictions. Reprinted with permission from [45]. Copyright 2012 American Chemical Society. b) An automated protocol, comprising precipitation, rinsing and resolubilisation steps was developed on a microfluidic digital platform to extract proteins from heterogeneous fluids. Reprinted with permission from [69]. Copyright 2009 American Chemical Society. c) A platform for the latent analysis of unmodified biomolecules has enabled attomole detection sensitivity. Reprinted by permission from Macmillan Publishers Ltd: Nature Chemistry [41], copyright 2015.

take the form of hybrid sensor/microfluidic devices that provide a cost-effective platform for diagnosis [70, 71]. In addition to these integration strategies, the operation in the laminar flow regime is useful for sample pre-processing.

3.2.1. Optical label-mediated detection

The fabrication of microfluidic devices made of optically transparent materials has enabled the implementation of optical based detection methods, including fluorescence microscopy. Even though some developments exist where the optical sensing element is directly integrated in the microfluidic device itself [22, 23, 72], most measurements are made under laboratory microscopes.

For example, an integrated blood barcode chip, enabling on-chip blood separation and rapid measurement of plasma proteins is detailed in [73]. The detection relies on channels patterned with a dense, barcode-like, array of single stranded DNA oligomers and a DNA encoded antibody library with appropriate fluorescent labels. The separation technique exploits the Zweifach-Fung effect, a hydrodynamic effect that describes the attraction of particles towards high flow rate at branching points of capillaries, to separate plasma proteins from blood cells (see Fig.2.a). This method enabled the measurement of proteins over a 10^5 concentration range within 10 minutes of sample collection. In another example of optical label-mediated strategy, the detection of the membrane protein tyrosine kinase-7 (PTK7) on single cells was achieved using a highly sensitive method using aptamer and nicking enzyme assisted signal amplification in microfluidic droplets [74].

3.2.2. Label-free detection

Recent developments aiming to integrate label-free detection techniques with microfluidic devices hold promise for non-perturbative detection of proteins at low concentrations. It is increasingly accepted that, even in the case of careful choice of the labelling position, the labels can affect the conformational ensemble of the protein studied and can therefore influence the measurement [75]. A recent study addresses the issue by introducing a platform for the latent anal-

13

ysis of unmodified biomolecules [41]. The paper describes a technique where the protein of interest is first subjected to diffusion and then denatured and labeled just before optical detection (see Fig.3.c). Under these conditions, the protein evolves in a label free micro-environment and is therefore unaffected when the diffusional motion is probed. The technique, that has demonstrated attomole detection sensitivity was used to characterize a clinically relevant α-synuclein complex as well as protein-protein interactions. Another label-free optical method consists in measuring the intrinsic fluorescence of proteins [76].

Various affinity-based sensors, that rely on capturing analytes, including proteins, using immobilized ligand molecules, have been combined with microfluidics for label-free biosensing. Such examples include the integration of electrochemical [77, 78, 79, 80, 81], acoustic [82, 83, 84, 85] or surface plasmon resonance sensors [86, 87]. In such cases, microfluidics can be used to optimise the capture of the analytes. Ultimately, the number of molecules binding to an affinity-based biosensor depends on the transport of the target molecule to the interface and the kinetics and thermodynamics of the binding reaction to the ligand [88].

Innovative approaches, developed to interface microfluidic with gravimetric sensors based on mechanical resonators, that report on mass changes through a resonance frequency shift, are described here. This class of sensors that can detect the presence of molecules, such as a proteins, bound to their surface via tethered capture molecules, normally suffer significant viscous losses when operating in a viscous medium such as liquids, the native environment of biomolecules. The issue was addressed by integrating a microfluidic channel inside a micro-cantilever excited into resonance, in a so-called suspended micro-channel resonator [82]. This type of sensor architecture has enabled ultra-high resolution measurements. Sub-attogram resolution was recently achieved using a vacuum packaged suspended nano-channel resonator [89]. Another approach where the microfluidic device, comprising a spray nozzle, is used to deposit the solution of interest onto a Quartz Crystal Microbalance [90] and micro-cantilevers [91] was also proposed recently. The small droplets generated evaporate quickly on the

14

surface of the sensor leaving the dry mass of the protein of interest on the sensor. Even though this novel approach holds interesting promises, the integration

₃₁₅ of upstream separation techniques will be necessary to reveal its full potential. Similar techniques were used to interface microfluidic devices with mass spectrometry and have enabled the detection of low concentration of proteins against a complex biological background [16, 55, 92, 93].

3.2.3. Nanotechnology enabled sensing

₃₂₀ Nanopore sensing measures changes in the ion current induced by molecules passing through a pore separating two large electrolyte reservoirs and represents a miniaturisation of Coulter counting approaches to the nanoscale. This technique with high statistical throughput, which allows the translocation of tens of thousands of single molecular species to be probed, has been combined

₃₂₅ with microfluidic to enable the measurement of a range of monomeric proteins [94] and amyloid fibrils [95, 96] and can reveal important information about heterogeneous populations [97, 98]. In the context of protein studies, solid state nanopores were used to record the translocation time of lysozyme at different incubation times during the aggregation process [95]. By analysing the results

₃₃₀ obtained from thousands of events, the authors were able to show that protein fibril translocation time scales linearly with the length, giving information on the sizes of supra-molecular protein structures. Another nanopore enabled sensing approach reported in [99] made use of digitally encoded DNA nanostructures for the simultaneous detection of four different antibodies of the same isotype

₃₃₅ at nanomolar concentration levels. It is also noted that proteins have been used as nanopores [100].

Nanofluidic devices have also been used for the creation of smaller probe volumes allowing enhanced spatial resolution for various sensing techniques including fluorescent detection [101] for single molecule studies. The applica-

₃₄₀ tion and fabrication of nanofluidic devices are detailed in the following reviews [102, 103, 104].
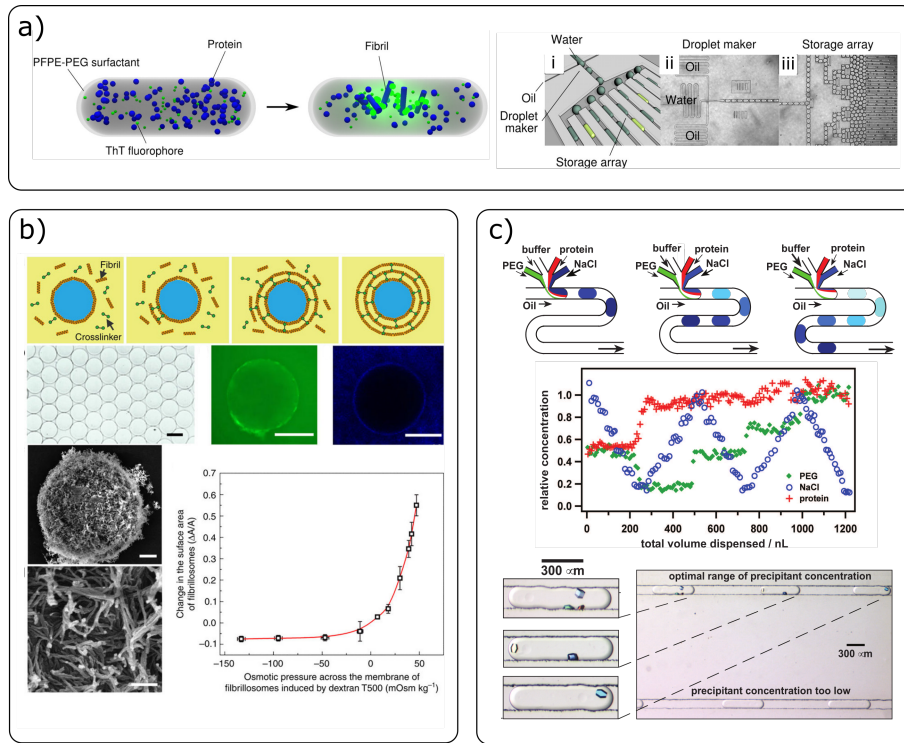
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Figure 4: Figure showing examples of the creation and control of fluidic microenvironments using microdroplets. a) Microdroplets assay enables the detection of single primary nucleation events and the monitoring of their temporal and spatial evolution. Reprinted from [116]. b) Fibrillosomes, composed of protein nanofibrils, were used to create stable aqueous emulsion in an aqueous phase. Reprinted from [140]. c) A microdroplet platform was used to screen for the best conditions for protein crystalisation by dynamically varying the ratio of additives inside the droplet Reprinted and adapted with permission from [119]. Copyright 2003 American Chemical Society.

16

3.3. Structure and function

Recently, the exploitation of the characteristic physical features of the microfluidic regime, the integration with orthogonal methods and the generation of micro-environments have been used to quantify and understand a range of biophysical phenomena [105, 106, 107]. In this section we concentrate on examples in the domain of quantitative biophysics for the determination of protein structure and function.

3.3.1. Size and charge

A particularly convenient approach to size objects with dimensions in the nanometer range consists in measuring their diffusion coefficient, which is a direct reporter of their hydrodynamic radius. This type of measurement is made possible by controlling carefully the diffusion transport under laminar flow in microfluidic channels and was demonstrated for single molecules [108] and for the study of molecular interactions [109]. The evaluation of the size distribution of heterogeneous mixtures, which still represents a great challenge for conventional biophysical approaches such as light scattering or nuclear magnetic resonance (NMR) techniques, was also recently demonstrated through the acquisition of multiple diffusion profiles in both space and time in a microfluidic channel [110] as shown in Fig.2.b.

Microfluidic approaches have also been used to evaluate the net charge of proteins, another fundamental property associated with their structure and function. The separation of heterogeneous mixtures of biomolecules according to their electrophoretic mobilities has been demonstrated using free flow electrophoresis on the microscale [20, 111, 112]. However, in most cases the electrodes are confined behind the fluidic channel walls, which prevents the use of low dielectric constant solutions. A technique recently developed enables the integration of electrode directly in contact with the fluid in a microfluidic channels [113]. The technique, inspired by microsolidic approaches [114], consists in flowing a molten low eutectic point alloy in a side channel separated by micropillars from the main fluidic channels. The capillary forces confine the alloy

17

behind the pillars and prevent its flow into the fluidic channel. The alloy is then solidified before use by cooling at room temperature. This technique has enabled the quantification of the electrophoretic mobility of proteins without the need for reference molecules as mobility standards. Using the same technique and independently measuring the diffusion of proteins (in addition to their electrophoretic motility), it was possible to determine the effective charges of a charge-ladder family of mutants of the calcium binding protein calbindin D_9k [115] as shown in Fig.2.c. The same platform was used to detect the chelation of a single calcium ion to a calcium binding protein.

3.3.2. Interactions, aggregation and phase transitions

Many of the techniques developed for the sizing of biomolecules find relevant applications also for the measurement of intermolecular interactions. Indeed, the evaluation of diffusion coefficients allows the measurement of binding events and protein-protein interactions by monitoring changes in size upon the formation of complexes [110].

Interactions between biomolecules in solution lead often to phase separation or to the formation of aggregates which can span very different length scales and vary from oligomers of few monomeric units to large insoluble clumps. In many applications, it is of crucial importance to understand the microscopic mechanisms underlying the aggregation process. In addition, to increase throughput and decrease material consumption, microfluidics offers novel opportunities to investigate events which cannot be observed on a bulk scale. For instance, confinement of protein solution in microdroplets assays can be used to detect single primary nucleation events and monitor their temporal and spatial evolution [116]. In this paper, insights into the formation and propagation of the single primary nuclei were obtained by varying the size and shape of the droplets. The use of non-spherical droplets showed that the reaction front driving the amyloid conversion propagated as a wave along the long axis of the droplet and provided evidence about the importance of secondary nucleation events in amyloid formation (see Fig.4.a). The reduction of the droplets below a critical size markedly

increased the lag time showing how confinement induces a decrease in primary nucleation, thus offering insights which would not be achievable on a bulk scale.

Microfluidic techniques also offer possibilities to elucidate the connection be-
tween the aggregation of biomolecules and a series of functional and pathological cellular behaviors [117]. For instance, a microfluidic device was used to measure the force generated by the growth of amyloid fibrils [118]. Individual spherulites injected into the microfluidic device were trapped between polydimethyl siloxane (PDMS) cantilevers, and the forces generated by the growth of the spherulites were inferred by measuring the deflection of the cantilever. The results of this study showed that amyloid growth can generate a force comparable to other functional protein self-assembled structures such as actin and tubulin.

In addition to aggregation, phase separation and crystallization are crucial processes in protein science, which find important applications in both biological and biotechnological systems. A microdroplet platform was used to screen for the best conditions for protein crystalisation by changing dynamically the ratio of additives inside the droplet [119]. In this case, each droplet has a different composition resulting in a varying number of crystals in each drop (see Fig.4.c) and another example describes how to control the number of protein crystals in each droplet by tuning the drop radius [120]. It is particularly important to have a large single crystal, rather than a number of small ones, to obtain clear X-ray diffraction patterns for crystallography studies. Other approaches were developed to create protein crystals. A vapour-diffusion method, relying on the diffusion of water vapour from a droplet containing a mixture of proteins and precipitants to a droplet containing a high salt concentration, was demon-
strated [121]. In this case, each pair of drop is separated by a water permeable carrier fluid to allow transport of the water vapour. This transport increases the concentration of the supersaturated solution and promotes the nucleation and growth of the crystal. Interestingly in this case, the droplets were interfaced with an X-ray transparent capillary to perform X-Ray diffraction measurements directly within the capillary.

Other examples of droplet microfluidics are discussed here. The first ex-

19

ample reports a ultra-high throughput screening for directed evolution [122]. This microfluidics based screening achieved a 1,000-fold increase in speed and

435 a 1-million-fold reduction in cost compared to state-of-the-art bulk automated screening systems. In another example, the difficulty of tracking individual droplets using wide-field imaging methods due to the low frame rates of high-sensitivity CCD cameras has been overcome by a stroboscopic manipulation of the excitation laser light which allowed extraction of kinetic data from droplets

440 generated at frequencies above 150Hz in an enzymatic assay [123]. This example shows an interesting case of a combination of integration of orthogonal methods with the generation of microenvironments for high throughput enzymatic assays.

Microfluidic devices have also been used to mimic in-vivo environments to

445 study proteins in a physiologically relevant yet highly controllable environment. For instance, it is well known that blood flow in the circulatory system can generate considerable shear stress [124]. Microfluidic devices, designed to generate different shear rates using a single perfusion pump, have been used to study the effect of shear stress on the Human von Wilderbrand factor, a blood plasma

450 protein that plays an important role in the blood coagulation cascade [125, 126]. Moreover, a wide range of microfluidic devices were developed to trap and culture cells on a chip [30, 31, 32, 127, 128]. For instance, these devices have offered a highly versatile platform for proteomic analysis from isolated cells [129], for imaging trafficking events [130, 131] and to evaluate the toxicity of α-synuclein

455 on cells [132].

3.4. Protein biomaterial development

Even though a range of materials have been synthesised and processed using microfluidics approaches, using for example flow templating or microdroplets [133, 134], proteins have only recently been recognized as a promising material in

460 nanotechnology. In particular, protein nanofibrils originally investigated in the context of pathologies [117], have received considerable interest in recent years as a novel class of nanomaterials. For instance, several peptides can self-assemble

20

in aqueous solutions in amyloid-like fibrillar structures which exhibit remarkable mechanical properties in terms of high Young's modulus and tensile strength. Because of their bio-compatibility and bio-degradability, such materials have found applications in cell culture scaffolds [135] and drug delivery capsules [4, 136].

The precise control of micro-environments and flow conditions offered by microfluidic devices have been used to design a variety of protein-based materials. For example, drug carrier microgels and capsules were developed by combining the inherent self-assembly process of lysozyme with droplet microfluidics, thus enabling nanoscale control and microscale structuring respectively [4]. These microgels enable local release of the encapsulated molecules and enhance microbial action in case of penicilin V and tetracyclin compared to homogeneous delivery.

In another example, the synthesis of peptides of highly uniform composition distribution was demonstrated using a combination of droplet microfluidic and biocatalytic self-assembly [137]. The formation of multiphase protein microgels composed of protein nanofibrils was demonstrated in [138]. In another study, proteins were assembled into high-resolution patterns by actively controlling nanoscale surface arrangement of a triblock copolymer monolayer. The arbitrary patterns are obtained by applying an electric potential to a set of microelectrodes in a microfluidic device [139]. Recent advances have also enabled the stabilisation of all-aqueous emulsions using protein nanofibrils [140]. The crosslinked nanostructures that are called fibrilosomes, enable the formation of stable structures even in the absence of an interface (Fig.4.b).

Using a two-level flow-templating approach, biofilms patterns with controllable dimensions were generated [141]. The properties of silk fibers were also controlled using a flow templating approach [142] The design and assembly of micro and nanofibers (not only protein-based) in the context of tissue engineering and regenerative medicine application has been discussed in a recent review [143].

21

4. Conclusions

In this paper we have discussed some of the recent advances in microfluidics in the context of applications to protein biophysics. In particular, we have explored how the use of the charactristic behaviour of fluids at the micro and nano-scale, the integration of complementary devices or methods, and the possibility to create well controlled micro-environments have enabled the development of innovative methods and devices that can simplify, outperform or expand the possibilities offered by conventional equipment and methods. We have discussed applications in sample manipulation, functional and structural studies, detection as well as materials processing.

Microfluidics is a truly multidisciplinary area that brings together physical sciences, engineering as well as biological and chemical sciences, and we expect that close collaborations between experts in each of these disciplines will enable advances in protein biophysics. This progress has the potential to advance the field of quantitative biophysics where biological phenomena will not only be observed and reported, but increasingly quantified and understood. In this context, the development of highly controlled micro-environments to study proteins and the synergies with orthogonal methods are likely to enable a deeper understanding of fundamental biomolecular processes.

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6. Bibliography

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