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Microfluidic approaches for probing amyloid assembly and behaviour†

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The self-assembly of proteins into supra-molecular structures and machinery underpins biological activity in living systems. Misassembled, misfolded and aggregated protein structures can, by contrast, have deleterious activity and such species are at the origin of a number of disease states ranging from cancer to neurodegenerative disorders. In particular, the formation of highly ordered protein aggregates, amyloid fibrils, from normally soluble peptides and proteins, is the common pathological hallmark of a range a group of over fifty protein misfolding disorders. Because of the critical role of the process in the aetiology of such disorders, as well as the quest to understand the basic principles of protein folding and misfolding, the amyloid phenomenon has become a central area of modern biomedical research. Advances in our knowledge of the physical properties of amyloid systems have, however, also highlighted the potential of amyloid structures in the context of materials science. In this review, we explore how microfluidic approaches can be used to study aspects of amyloid assembly and behaviour that are challenging to probe under bulk solution conditions. We discuss the use of volume confinement to probe very early events in the amyloid formation process. In addition, the well-defined fluid flow properties within channels with dimensions on the micron scale can be exploited to measure the physical properties of protein aggregates, such as their sizes and charges, to shed light on the physical and chemical parameters defining amyloid species. Moreover, the molecular species formed during aggregation reactions have physical dimensions spanning at least three orders of magnitude, and microfluidic techniques are well suited to work with analytes of such disparate dimensions. Furthermore, the flexibility of the design of microfluidic devices lends itself to adaptable experimental setups, including the study of protein self-assembly within living cells. Finally, we highlight the salient features of microfluidic experiments that facilitate probing complex biological systems, and discuss their use in the exploration of amyloid as a class of functional material.

Introduction

The self-assembly of proteins to form amyloid aggregates is the hallmark of a range of protein misfolding diseases including neurodegenerative disorders, non-neuropathic conditions and systemic amyloidoses.[1–6] In recent years, much interdisciplinary research has aimed at developing an understanding of the microscopic processes involved in amyloid formation. In this context, the properties of the aggregates formed by a number of specific proteins have been characterised.[7–19] Experimental and computational approaches have been employed in studies of the binding and the effect of potential aggregation inhibitors aggregation, ranging from small molecules to proteins have been performed, both to understand the fundamentals principles underlying the process of aggregation, and to devise potential therapeutic intervention strategies.[20–32]

In recent years, high-resolution techniques from structural biology have been applied to the investigation of amyloid aggregates, revealing a detailed picture of the fibrillar architecture and highlighting the generic features of amyloid aggregates formed by proteins with a wide range of different native state structures, functions, and amino acid sequences.[1,7–9,33–35] Thus, the characteristic cross-β structure of amyloid fibrils has been shown to be stabilised by non-covalent interactions between the peptide backbones of the constituent polypeptide chains, and their generic architecture does not depend on their specific amino

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acid sequences. The amyloid structure represents a free energy minimum in the energy landscapes of many proteins, and, once formed, aggregates can therefore be highly persistent.[36] The self-assembly of peptides and proteins to form amyloid aggregates results in supramolecular structures that are not amenable to the fast and reversible assembly and regulation, which is necessary for functional intracellular self-assembly processes, such as the reversible polymerisation of cytoskeletal proteins associated with the mobility of cells. The observation that under given solution conditions, proteins with diverse native structures and functions can be induced to form amyloid aggregates, even for proteins not associated with amyloid formation *in vivo*, led to the hypothesis of amyloid as a generic protein structure.[1] Moreover, there are now multiple examples of forms of amyloid structures involved in functional roles in systems as diverse as the extracellular matrix of *E. coli*, fungal prions, spider silk, melanin synthesis and hormone storage.[37–41]

Studies of the material properties of amyloid structures have highlighted their potential as biocompatible materials for a wide range of uses.[24,42–47] Several reductionist model systems have been designed for the study of amyloid formation, including the use of short peptide sequences derived from amyloidogenic proteins. In the case of the amyloid-β (Aβ) peptides, which are found as amyloid aggregates in Alzheimer's disease, this approach has eventually led to the reduction of the model peptide to just two amino acids, diphenylalanine (FF), thus pushing the boundary between protein aggregation and crystal formation.[48,49]

Microfluidic methods can advance the study of amyloid formation, both as a platform for improved assays and in the development of strategies to address questions which have hitherto been challenging to access using bulk solution methods. Specifically, the confinement of fluid flow into small channels and the resulting laminar flow profile enables precise modelling and control of the fluid flow and thereby quantitative characterisation of the analytes present.[50] This feature is a key factor in a number of strategies aiming to probe the physical properties and morphologies of amyloid aggregates formed under flow.[43,51,52] Moreover, in order to enhance the number of species that can be analysed on chip, separation modules have been combined to achieve a multi-dimensional sample analysis.[53–55] As an example, isoelectric focusing followed by capillary electrophoresis achieves narrow pre-concentrated analyte bands for downstream analysis.[55–57]

Chip-based applications include the determination of size, electrophoretic mobility, and charge of a given system, without the need for calibration against the behaviour of a reference molecule.[58–62] Changes to the size or charge of sample molecules upon complex formation can be explored to probe non-covalent protein-protein interactions and protein-ligand binding.[59,60,63,64] These microfluidic strategies can be, and have been, applied to characterise the properties of amyloid structures and their interactions with chemical compounds or other proteins.[60,62,63,65]

Microfluidic methods have been applied succesfully to the high-throughput generation of microdroplets with highly uniform volumes. Such microdroplets present another promising approach to the further study of amyloid systems. The use of microdroplets allows a large number of independent reactions to be monitored simultaneously in volumes which are several orders of magnitude smaller than has been usual in bulk assays.[66–68] In addition to parallelised screening, volume confinement can be exploited to observe stochastic and often rare events taking place as part of the amyloid formation process and obtain statistic information on their occurrence.[69] Indeed, the amyloid formation process is governed by a series of microscopic steps, such as primary nucleation, elongation, fragmentation, and aggregate induced secondary nucleation, which vary in relative importance between different systems with the more dominant steps masking rarer ones in non-compartmentalised bulk scale assays. Furthermore, inside individual droplets the spatial propagation and distribution of amyloid species can be monitored as a function of time.[69,70] The interaction between analytes and microdroplet interfaces can be explored to characterise the physical and mechanical properties of species ranging from individual proteins to entire cells.[43,47,70,71]

The molecular and supramolecular species found during the conversion from soluble precursor protein to amyloid aggregates span dimensions from the Ångström to the micrometre scale. Microfluidic strategies have found applications in research at many levels of amyloid formation and behaviour as they can easily accommodate such a wide range of analyte sizes.[52,69,72,73] The sample volumes required for experiments within channels with dimensions on the micron scale are greatly reduced compared to experiments in bulk solution, an important consideration in cases where the available sample material is limited, for instance in the case of samples derived from *in vivo* sources.[70] Furthermore, the availability of highly sensitive optical detection methods enables samples to be studied at the single-molecule level,[74] a feature, that enables analytes to be studied at low concentrations, for example allowing amyloid precursor proteins to be characterised at physiological concentrations. The ability to study samples at the single-molecule level also allows small populations within an ensemble to be probed. Here, we explore current microfluidic strategies that can provide improvements over conventional assays in bulk solution in terms of assay times and throughput.[75,76] We review approaches which allow the study of rare events in protein aggregation to generate an understanding of the underlying protein-protein interactions, while extending these methods for performing high-throughput experiments. These approaches, in turn, give rise to microfluidic means of exploring more complex phenomena, such as the manner in which proteins interact to form aggregates in cells and tissues, while allowing control of such interactions in the development of new protein-based materials for future applications.

The physical properties of amyloid aggregates

The majority of the measurement techniques currently in use for the characterisation of individual proteins and the species found on the amyloid formation pathway are bulky and operate over long timescales. Furthermore, such characterisation measurements are typically performed either in the gas phase (e.g. mass spectrometry) or on chromatography columns which employ support matrices in order to limit the diffusion of analyte molecules

and allow their controlled movement. This is in contrast to the well-defined non-turbulent fluid flow profiles characteristic of small length scales, such as micron scale channels, which can keep analyte molecules localised even without the involvement of any external support medium. As such, not only can microscale channels be used to characterise the chemical and physical properties of aggregated species using only very small sample and reagent volumes, the analysis can be carried out on orders of magnitude shorter timescales than with conventional approaches where the involvement of the support matrix restricts fast movement of individual molecules. These features have enabled both the size and electrophoretic properties of amyloid species to be determined under steady state flow conditions, Fig. 1. Notably, the laminar flow enables the sample diffusion and migration in an electric field to be analysed in a quantitative manner, yielding absolute values for physical parameters, which can be compared between solution conditions.[60,64,65]

In addition, the ability to perform analysis directly in aqueous environments lends itself effectively to probing the physical properties of the amyloid aggregates, in particular as the species formed on aggregation pathways are transient and commonly held together by reversible non-covalent interactions. For instance, the species formed during the aggregation of α-synuclein, a protein whose aggregation is associated with the aetiology of Parkinson's disease, have been characterised using thermophoretic and electrophoretic approaches, Fig. 1b.[65] By determining the electrophoretic and thermophoretic mobilities of the monomeric, oligomeric and fibrillar forms of α-synuclein, it was found that these parameters could be employed to differentiate between the species of the aggregation reaction. This approach could in the future enable separation of the reaction mixture into the individual components to then further characterise the physical properties of the different species either directly on chip or via an off-chip analysis approaches, such as atomic force microscopy or mass spectrometry.[77,78]

The process of amyloid formation in confined volumes

The presence of an air-water interface can be eliminated by observing the amyloid formation process in microdroplets, enabling surface effects to be studied in a controlled manner.[70] To probe the effect of different liquid-liquid interfaces, a plug-based microfluidic approach modified with teflon tubing was used for the study of Aβ40 aggregation, Fig. 2a.[70] Once Aβ40 is encapsulated inside a plug, it is protected from the surfaces of microchannels by a layer of fluorocarbon oil, and it is the surface chemistry of the aqueous/fluorous interface that starts affecting the aggregation process. In plugs with fluorocarbon/water interfaces, an increase in the fluorescence signal of the labelled Aβ40 peptide at the plug edges was observed, indicating adsorption of the peptide to the interface. In contrast, in plugs with an amphiphilic fluorinated surfactant/water interface, the fluorescence signal of the labelled peptide remained evenly distributed, indicating that the amphiphilic surfactant prevents Aβ40 adsorption to the interface. These findings further uncovered a general driving force, originating from confinement, which, together with biological quality control mechanisms, helps proteins remain soluble and therefore functional in nature.[70] The importance of interfaces and lipid membranes in particular has been illustrated in bulk solution studies, microfluidics provide an ideal platform for further studies of the effects of interfaces on amyloid formation.[79]

During the amyloid formation process, the products of relatively rare primary nucleation events are amplified through elongation and secondary events, which include fragmentation and surface-induced nucleation.[80] Thus, once initial nucleation events have occurred, the kinetics of the formation of amyloid species in a sample are often dominated by such processes.[81] Primary nucleation events are therefore challenging to detect and quantify in bulk solution assays. The compartmentalisation of a sample solution into microdroplets enables the propagation of the aggregation reaction to be monitored from single nucleation events, and permits the tracking of both the spatial and temporal evolution of such events, Fig. 2b.[69] Through this approach, the specific secondary processes dominating amyloid formation by insulin could further be identified. The study highlighted the dependence of the aggregation lag time on the system size.[69,82,83] The picolitre volumes of microdroplets represent a closer approximation of cellular dimensions than the tens of microlitres used in plate reader assays, and can help to explain the relative stability of proteins in confined cellular compartments.[84]

The process of amyloid formation under flow

The well-defined flow properties within microfluidic channels can be used to address additional questions relative to measurements in bulk solution. For example, performing experiments under flow enables the amyloid formation process to be probed under a different set of mass transport constraints compared to bulk measurements or microtiter plate assays. Specifically, the effect of mass transport on the rate of the amyloid formation reaction has been investigated for insulin fibril formation, Fig. 3a.[51,85] The results showed that for channels coated with insulin fibril seeds, the amyloid formation process was substantially accelerated by an increase in the flow of protein monomer in the channels. This finding highlights the importance of performing measurements on amyloid formation under well-controlled mass transport constraints for the purpose of reliable comparison.[51,85]

The introduction of flow has also enables the effect of fluid shear stress on aggregate morphology to be probed. For example, it has been shown that the morphology of insulin aggregates can be affected when the amyloid formation process takes place in a flow field in a confined microfluidic channel, rather than in bulk solution. Specifically, structures with fibre-like morphology have shown to dominate when the fibrils are formed in channels where the flow rates are high, while low flow rates lead preferentially to the formation of spherical aggregates or spherulites.[52] Such an ability to exert control over the morphology of the amyloid structures by a simple combination of volume confinement and movement of fluid could be especially valuable for the applications that employ amyloid fibrils for functional purposes.

Investigating reactions under steady state flow conditions allows multiple processes to be spatially decoupled from one an-

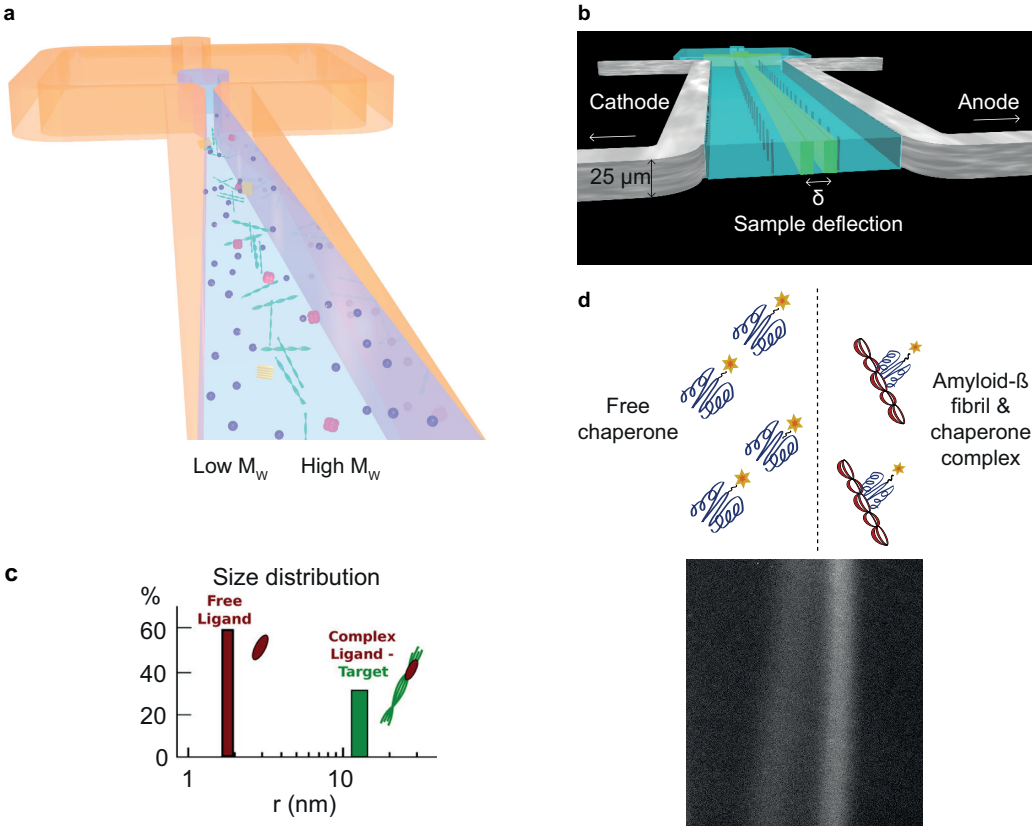

Fig. 1 The precise control over fluid flow at micron scale enables quantitative characterisation of biomolecules and their complexes on chip. **(a)** Schematic rendering of a microfluidic device with the different scales of species involved in amyloid formation: monomers; oligomers [16] and fibrils. [34] Microfluidic methods can be used to explore the interactions of these species with small molecules, with each other, and with macromolecules such as chaperones, as well as with fibril networks and gels. [45] The laminar flow within microfluidic channels enable analytes to be separated and characterised based on their diffusion coefficients. [60] **(b)** Integrating electric fields with microfluidic channels has enabled physical characterisation of individual proteins, protein oligomers and amyloid fibrils. Here, solid metal wall electrodes were automatically aligned with the fluidic channels by micropillar arrays. An electric field was generated perpendicularly to the direction of flow and the electrophoretic mobility of the sample was quantified. [58,64] **(c)** Microfluidic diffusional sizing has been used to determine the hydrodynamic radii of individual biomolecules in solution. A stream of protein molecules was flanked by buffer streams, and the diffusion of the protein molecules into the adjacent fluid flows was observed. The acquired data can be used to extract the concentrations of individual molecules and their complexes. [60] **(d)** Integrating high electric fields with microscale channels has enabled rapid fractionation of proteins and their complexes. This approach allows real-time probing of the interactions between the transient species formed on the aggregation pathway and external molecules, such as potential therapeutic agents interfering with these species. [62]

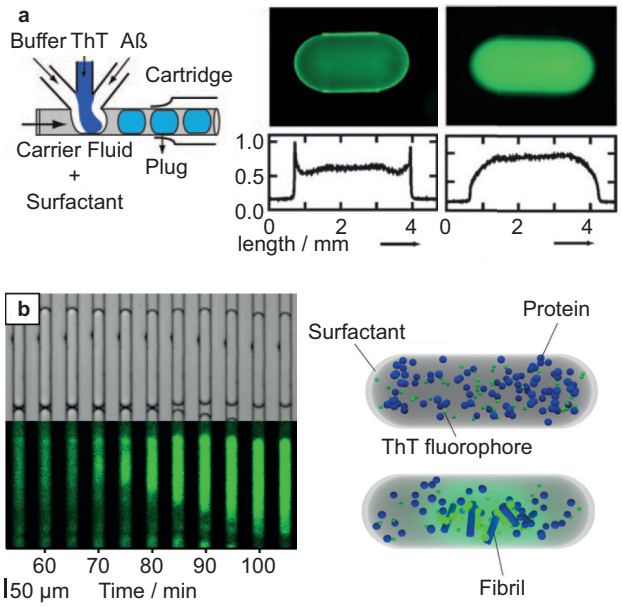

Fig. 2 Exploring the process of amyloid formation under volume confinement. **(a)** Encapsulation and aggregation of Aβ40 in plugs with controlled interfaces compared with plugs without interfacial control and with well plates. Left - schematic of the microfluidic droplet generating device. Fluorescence images of HiLyte-488-labelled Aβ40 in plugs with fluorocarbon/water (middle), showing how the protein is enriched at the interface, and Rf-OEG3/water interface (right), displaying an even protein distribution.[70] **(b)** Microdroplets incubated within microfluidic storage array channels enabled single nucleation events and proliferation of the aggregation reaction to be observed.[69]

other while keeping the whole process integrated on a single chip. Specifically in the context of amyloid formation, the key processes involved, and to be maintained as isolated events, are the protein aggregation step and the aggregate labelling step employed for probing the process through optical means. Indeed, many of the frequently used probes for monitoring the process of amyloid formation, such as Congo Red, Thioflavin S and Thioflavin T, have been shown in some situations to influence self-assembly processes, and as such, may preclude the reliable extraction of quantitative information regarding the concentration of aggregated material present.[86–88]

When protein self-assembly is studied in the presence of small molecules with high affinity for the aggregates, a further risk of interference between the amyloid forming species, the inhibitor molecules and the probe molecules is introduced. To illustrate this consideration, some of the very effective molecules observed to inhibit protein aggregation have structures that are modelled on amyloidophilic dyes.[22,89,90] The possibility of interference by probe molecules is effectively reduced by using a flow-based microfluidic platform where the aggregation process takes place in a label-free environment, whilst the detection downstream still benefits from the high sensitivity afforded by label-based detection, Fig. 3b.[73] This feature is in contrast to conventional approaches for monitoring fibril formation, where the aggregation process normally occurs in the presence of labels. In this study the control over fluid flow at small length scales enabled the mixing between the aggregating protein and the probe molecules to occur in a fast and non-disruptive manner. Because of the small scale of the channels, low molecular weight probe molecules can diffuse into the protein stream within a few seconds, allowing rapid detection of the extent of aggregation immediately following their introduction, without inducing any potentially disruptive shear stresses that would be required for rapid mixing on larger scales.[73]

Finally, the flow through microfluidic channels can be used to accelerate the incidence rate of sample molecules passing through a detection area, thus overcoming the limits that are otherwise set by sample diffusion. This feature has been put to elegant use in the context of performing measurements on the amyloid formation process at the single molecule level, Fig. 3c.[74,91] By using microfluidic channels and performing a series of sample dilutions, single molecule data on protein aggregation could be acquired over 100 times faster than in conventional confocal measurements, that do not take place under flow, increasing the achievable time resolution from hours to minutes. This enhanced resolution opens up the possibility of probing aggregation processes on time scales that are significantly more relevant to understanding in detail the kinetics of the microscopic processes of protein self-assembly.

Interactions between amyloid structures and other molecules

In addition to the characterisation of the physical properties of the species formed during a protein self-assembly process, there is great interest in developing methods that would enable studies to be made of the chemical properties of the species formed during protein self-assembly, in particular, their interactions with one another, with potential binding partners, and with aggregation inhibitors. It has recently been illustrated, for example, that the ability to model precisely the molecular diffusion of individual molecules in microscale channels can be used to determine accurately and rapidly the size of particles, such as proteins in free solution, Fig. 1c.[60] The diffusion of sample molecules into co-flowing carrier medium was monitored as a function of residence time in the analysis channel. Analysis of the spatial sample distribution thus enabled the diffusion coefficient of the analyte molecule to be extracted. By deconvoluting average signals into those originating from the individual species, this method was shown to facilitate the sizing of both individual biomolecules and mixtures of biomolecules. The approach was used to determine the binding constant between α-synuclein and its single domain antibody fragment directly in a cell lysate. As part of this study, it was observed that the aggregation-suppressing antibody could bind to both the monomeric and the aggregated forms of the protein - a finding that is in agreement with the available structural data, as the antibody binding epitope is known to be located at the C-terminus of α-synuclein, in a part of the sequence that is not incorporated into the core of the aggregated species.[60]

The highly reproducible prototyping of microfluidic chips via soft-lithography approaches, and the tight control that can be exerted over fluid flow in micron scale channels, can yield highly quantitative data on the physical parameters of individual

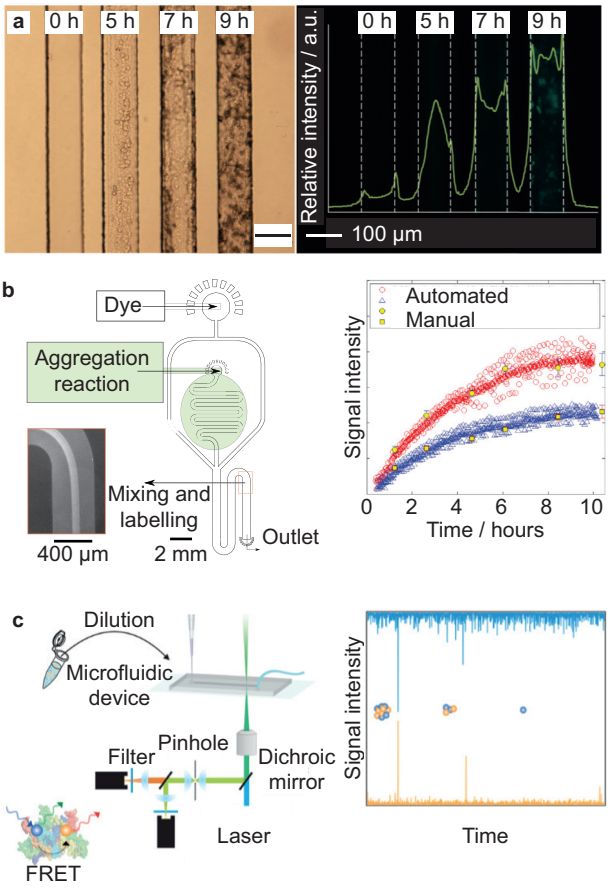

Fig. 3 The use of microfluidics to monitor the process of amyloid formation. **(a)** The growth of surface-tethered insulin fibrils within microfluidic channels. Bright field image of the microchannels (left). Fluorescence image of the microchannels and fluorescence intensity profiles (right).[85] **(b)** Microfluidic approaches have been employed to automate the measurements of protein aggregation while enabling fibril formation to occur in the absence of external molecules, such as dyes used to probe the process (left). Eliminating manual fluid handling steps allows the aggregation to be studied at a higher throughput than conventional manual measurements (right).[73] **(c)** A flow-based single-molecule detection microfluidic system which in contrast to conventional single molecule confocal measurements on protein aggregation can achieve measurement times of minutes rather than hours.[74]

molecules. For example, tracking the electrophoretic mobility of a complex between creatine kinase and calmodulin was achieved by titrating the kinase into a calmodulin solution of a fixed concentration.[64] These data allowed the construction of a binding curve for the interaction between the two proteins and ultimately, determination of the equilibrium parameters for the interaction. Importantly, the latter approach indicates the strength of the interaction directly in free solution, free of the biases, that can be introduced by conventional surface-based techniques into the measurements on binding interactions. In the future, such a platform could be applied with the aim of determining the equilibrium parameters for the species that are formed on the aggregation pathway and the affinity for their binding partners.

Integration of strong electric fields with microfluidic channels has also enabled the development of an electrophoresis-based separation technique that can be used to identify interactions between transient species on the aggregation pathway and the molecules that bind to such species.[62] Specifically, using such a rapid fractionation platform the interaction between Aβ fibrils and the Brichos domain chaperone was detected and the free chaperone separated from the chaperone-fibril complex in a couple of seconds and directly in free solution without the involvement of a support matrix, Fig. 1d. By binding to Aβ fibrils, the Brichos molecules can potentially inhibit further secondary nucleation events from occurring on the surfaces of the fibrils.[27] Detecting such interactions between specific transient species on the aggregation pathway and potential inhibitor molecules provides key insights into the specific steps of the aggregation pathway selected inhibitor molecules intervene with, and through this approach facilitates rational therapeutic design strategies.[92]

Another on-chip study has specifically focused on detecting the presence of early events on the aggregation pathway. The analysis employed a two-dimensional separation approach involving a combination of isoelectric focusing and capillary zone electrophoresis.[93] The combined platform allowed the time-course of α-synuclein aggregation to be studied and provided a strategy for real-time detection of early and intermediate species on the aggregation pathway. As part of the study it was shown that the chip could be used to analyse the effect of small molecules on the aggregate formation process and could as such serve as a platform for screening for potential aggregation inhibitors.

Last but not least, the ability to exert control over fluid flow at small length scales and thereby analyse molecules in their label-free state has been explored. It has been demonstrated that the physical parameters of biomolecules and biomolecular complexes can be accurately probed by quantitatively labelling them after the process of interest has incurred. This order of events is achieved through the sequential arrangement of the separation, labelling, and detection elements on chip. Such post-process labelling ensured that the physical properties determined in this way describe the unlabelled rather than the labelled state of the molecule or the complex.[63] Using this strategy the interactions could be described between α-synuclein monomers and nanobodies in their label-free states. Such microfluidic devices provide effective platforms for probing biomolecular processes and interactions in a label-free manner while ensuring that the molecules

of interest can in principle be analysed at physiologically relevant concentrations.

Chemo-mechanical force transduction

In biological systems, the generation of mechanical force is central to a wide range of phenomena, including cellular movement and traction to surfaces. Movement, for example, is controlled through the self-assembly of cytoskeletal proteins that enables extension and contraction of cells.[94,95] In this context, mechanotransduction has been a topic of considerable interest, ever since early studies demonstrated a link between a mechanical force and the corresponding biological response. Mechanotransduction is a process which involves mechanical stimulation that elicits a response from the cell, which can itself alter the intrinsic mechanical properties of the cell and can change the cellular microenvironment. Recent studies using microfluidic techniques have allowed precise control to be exerted over the multiple factors that influence cellular responses.[96]

In addition to the involvement of native proteins in force production in cells, tissues and organisms, force generation by the effect of aberrant self-assembly is of much interest in the context of amyloid formation. The capacity for high power force generation in this context is not related to their function in cell motility.[95,97] The forces emerging from the self-assembly of native proteins have been studied in detail, yet the potential for force generation by aberrant protein polymerisation has only recently been explored by utilising microfluidics based approaches, Fig. 4.

The self-assembly of biologically relevant components is also an attractive route towards force generation in an active materials, since such processes can take place under ambient conditions, with no, or minimal, requirements for external energy input. In such systems, force generation corresponds to the release of free energy due to addition of monomers and is best expressed in terms of the energy density. In order for these systems to generate a significant amount of force, the molecular building blocks must be arranged in such a way that there is some directionality in the system. In particular, this aim has been achieved by harnessing microfluidic techniques to measure the forces released by the growth of amyloid fibrils formed by insulin and lysozyme. It was demonstrated that such fibrils are capable of unleashing mechanical forces on the piconewton scale for individual filaments.[43] By trapping insulin protein aggregates between microcantilevers within a microfluidic channel, under a continuous flow of precursor protein, a precise control over the growth of the fibrils was achieved, Fig. 4a. The level of force measured for amyloid growth in both insulin and lysozyme systems was found to be comparable to those observed for actin and tubulin, systems that have evolved to generate force as part of their native functions and, unlike amyloid growth, rely on the input of external energy in the form of nucleotide triphosphate hydrolysis for maximum force generation. The force generated by amyloid aggregate growth would be sufficient to deform materials with properties similar to cells. This aspect of amyloid formation could therefore be a contributing factor to the pathology of systemic amyloidoses.

Recently, a minimalist model system for amyloid formation, FF, was investigated and found to generate forces of a similar

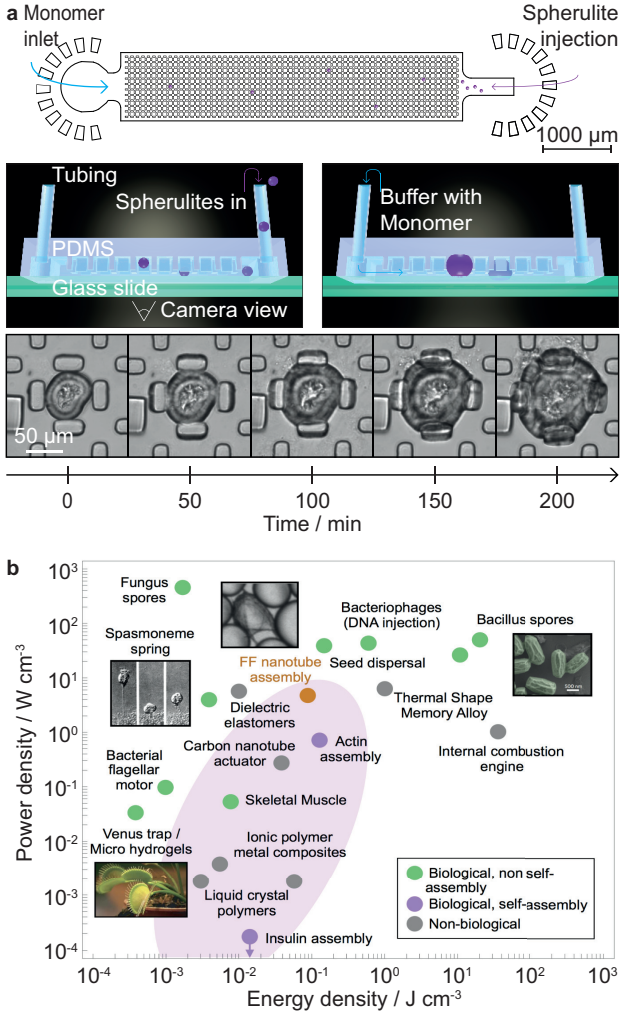

Fig. 4 (a) Design of the microfluidic device used to study forces produced through protein self-assembly. The pre-formed insulin aggregate clusters were introduced into the device, and a continuous supply of monomer was flowed through the channel (top). Schematic diagram showing the introduction of preformed spherulites into the microfluidic device, where they were trapped between microcantilevers (middle). To monitor the cantilever deflection, time course images were acquired through the glass slide at the bottom of the device (bottom) exhibiting spherulite growth within the device.[43] **(b)** Comparison of the chemo-mechanical transduction in FF nanotubes with energy (J cm^{-3}) and power (W cm^{-3}) densities by synthetic actuator systems, biological stimuli-responsive materials and biological self-assembly systems[47].

order of magnitude to those of complex biological systems and synthetic polymers.[47] The ability of the FF molecules to self-assemble into ordered tubular structures was reported over a decade ago, yet, due to the highly dynamical nature of molecular-level self-assembly phenomena, it has been challenging to achieve forces for actuation on length scales exceeding that of the building blocks themselves.[48] By employing a microfluidic platform in which super-critical solutions of FF were encapsulated within microdroplets, and could be triggered later to self-assemble by evaporation, it was shown that linear FF assembly could generate forces that were sufficient to rupture water-in-oil double layer interfaces. In this case, no large-scale actuation on sub-second timescales was permitted, since the rate of energy release was limited by the rate of FF self-assembly. However, this limitation could be overcome by tailoring the geometry of the system to accumulate elastic energy through chemo-mechanical transduction, followed by a rapid release as a result of an elastic instability.[47]

Such elastic instability could be further controlled through the rate of solvent evaporation within the droplets, thus allowing the regulation of the self-assembly kinetics and of the force generated by the tube unbuckling. Moreover, this system was employed to generate external work using an actuating self-assembly system, as the energies released in the unbuckling events were sufficient to displace physically neighbouring droplets. Through a comparison of the results for the chemo-mechanical transduction of FF tubular assemblies with values for the power generated by synthetic actuators, biological self-assembly systems, as well as biological systems that exploit mechanical instabilities to release stored energy, it has been shown that the energy and power densities calculated for the energy released from the unbuckling of a single nanotube when normalised to the volume of the nanotube, and the upper limit of actuation time provided by the FF system exceeded those resulting from force generation by conventional biological self-assembly, including actin polymerisation Fig. 4b. Thus, at a fundamental level, the exploitation of elastic instabilities has allowed the generation of a system which leverages biomimetic self-assembly to generate power-densities that are higher than those reported for biological self-assembly, and similar to systems that were specifically designed during evolution to yield high energy release.

Through the combination of diverse microfluidic-based approaches with real-time imaging analysis and modelling, the force generation and actuation by proteins and supra-molecular polymers have been established as high power-density modes of chemo-mechanical force transduction, opening up the possibility of using elastic instabilities for future applications in other short peptide, protein, and synthetic polymer systems. Nucleation, growth and phase transitions in biological and synthetic self-assembling systems may elucidate the natural occurrence of such phenomena in nature and in amyloid related diseases.

High-throughput assays

As a result of their rapidity and low consumption of reagents for a given reaction, microfluidic devices are an attractive platform for performing high throughput screens, for example to identify inhibitors of amyloid formation. The majority of protein aggregation assays to date have been performed in multiwell plates where the required volumes are orders of magnitude larger than those of microfluidic channels or microdroplets, and this is therefore an important area for the development of microfluidic techniques.

Miniaturised channels have been used successfully as individual compartments for probing the process of aggregation and thus for exploring the inhibitory effects of drug candidates and metal ions on the fibril formation process.[51] The sample volumes of the assay chambers in these experiments were around 0.1 μL. To achieve a further reduction in reagent consumption and an increase in throughput, sub-nanolitre aqueous droplets compartmentalised by an immiscible phase, such as an oil, can be employed.[66,68] For example, such an aqueous microdroplet-based platform has been used to study the inhibitory effects of Orange G on the aggregation of AcPHF6 tau-hexapeptide[98] and to track the aggregation of Aβ in the cerebrospinal fluid (CSF) of mice.[70] With the volume of the CSF in a single mouse being only a few microlitres, the dramatically reduced volumes of microdroplets compared to microwell plates and the possibility of performing repeated analysis on the CSF from the same animal illustrate the effectiveness of microdroplet-based approaches on monitoring the aggregation process in non-readily accessible biological fluids.

Compartmentalising bulk solutions between individual microdroplets and subsequently amplifying specific species in these droplets forms the basis for precisely and accurately quantifying the presence of specific species of interest. This approach has been applied in the context of nucleic acid detection and has led to the development of widely used technologies, such as the digital polymerase chain reaction, which enables highly sensitive detection of individual DNA strands.[99,100] A similar approach has recently shown to facilitate effectively the development of a rapid and highly sensitive assay for the quantification of amyloid propagons in protein solutions, Fig. 5a.[101] The assay developed in this study reached the ultimate sensitivity limit of detecting insulin propagons down to a digital level, where a single aggregate is detected. Its performance compared favourably with existing and alternative assays in terms of its speed and sensitivity. Such assays have the potential to be used to detect small amounts of aggregates in biopharmaceutical preparations where the presence of even the smallest traces of protein aggregates can lead to immune responses.[102]

The extension of the microdroplet platform to applications that require experiments to be performed under non-identical conditions, such as the screening of molecular libraries of potential aggregation inhibitors, has so far been limited by the rate of translation from proof-of-concept to general use in a biological context. Titrating the potential candidates into microdroplets at different dilutions or testing mixtures of different candidates at different ratios could be achieved to exploit already established approaches, such as merging aqueous streams on chip before emulsification or, alternatively, performing fusion steps after the emulsification.[103] A key challenge that still has to be addressed in this context, involves establishing a strategy for uniquely labelling each of the compounds in the library to enable the identification of successful candidates after the high-throughput screening pro-

cess. Possible approaches for addressing this challenge, and add a unique identifier to act as a barcode for the different droplets, have been reviewed in contexts beyond aggregation assays [104] but are likely to be equally applicable to the protein self-assembly process. The identification labels have been proposed to include dilution series of fluorophores, quantum dots or DNA-sequence based barcoding libraries. [67,104,105]

Last but not least, in addition to the advantage of the reduced reagent consumption, high-throughput microfluidic assays also enable the work flow of screening assays to be simplified and automated. As an example, a common strategy for identifying biomarkers, including those for the onset of neurodegenerative disorders, involves enzyme-linked immunosorbent assays (ELISAs). ELISA typically requires multiple washing steps between each stage to remove unbound material from the samples and to avoid non-specific interactions, and as such consume relatively large quantities of the samples and reagents (e.g. 50-150 μL per condition). An elegant droplet-based magnetic bead immunoassay was recently demonstrated as an alternative to conventional ELISAs. By connecting wells with microchannels, analytes attached to magnetic beads were transported between the large droplets trapped in the wells, eliminating the need for the washing steps involved in conventional bead based immunoassays and minimising the quantity of the sample required and the reagents involved, Fig. 5c. This technique has been employed to target oligomeric Aβ, which has been suggested as a biomarker in the characterisation of Alzheimer's disease. [106]

Amyloid formation in cells

Microfluidic devices have increasingly been used to separate cells, and the accuracy and efficiency of recently developed sorting methods are comparable to their macroscopic counterparts. These advances have stimulated their use as novel strategies for cell separation, which is an integral step to processes involved in applications ranging from food production to environmental monitoring, and the process used by pharmaceutical industry. A variety of force fields have been implemented to generate microfluidic cell separation mechanisms, ranging from gravity to hydrodynamic, electric, acoustic, optical and magnetic forces. In particular, fluorescence and magnetic activated cell sorters use external labels of the targeted or non-targeted cells to establish specificity. [111,112]

Microfluidic compartments can also be used to encapsulate living cells in order to probe *in vivo* systems. For example, microscale cultures of primary central nervous system (CNS) cells have been integrated with micro-compartments and exposed to different types of Aβ aggregates to mimic the environment in Alzheimer's disease, Fig. 6a. In four parallel experiments, the response of the CNS cells to treatment with different drugs was examined and the compound FTY720 was suggested as a promising candidate for preventing neudrogeneration after the cells have been exposed to neurotoxic oligomeric forms of Aβ. [107] Lab-on-a-chip techniques may help to accelerate drug development by providing alternatives to traditional cell-based methods. Thus, biomimetic microsystems that reconstitute cell-to-cell communications are well suited for the development of models that mimic

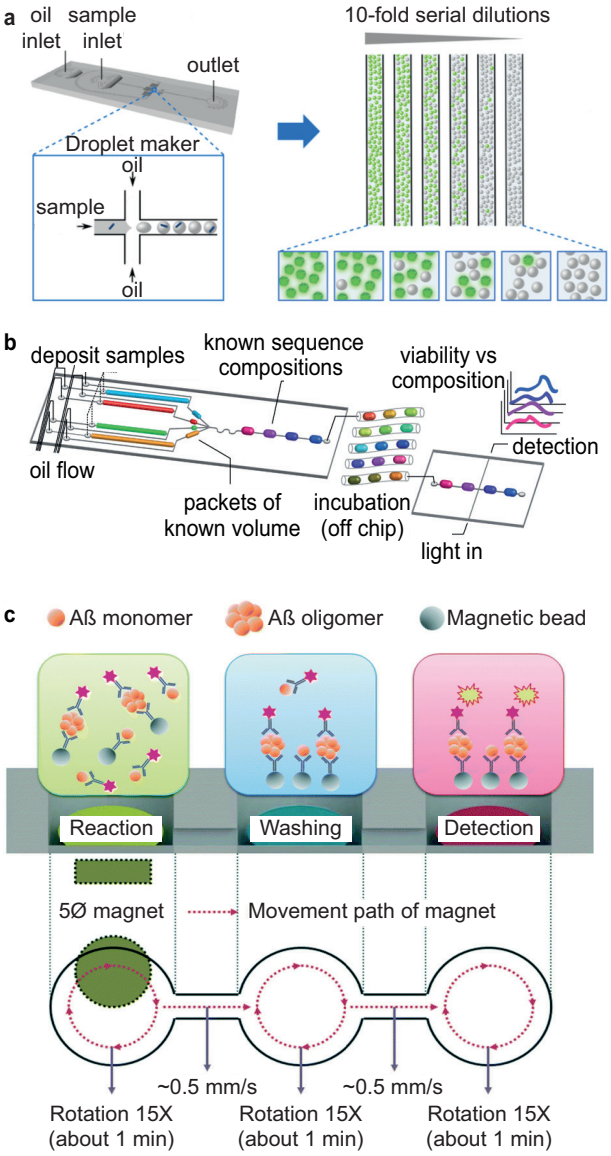

Fig. 5 High throughput microdroplet assays in studies of amyloid systems. **(a)** Distributing a protein solution between multiple microdroplets and tracking the rate at which aggregates are formed in these droplets has enabled ultrasensitive assays for determining the concentrations of individual propagons to be developed. The assay was demonstrated to reach the ultimate sensitivity limit of detecting a single propagon. [101] **(b)** The extension of the microdroplet platform to applications that require parallellised experiments to be perfomed under non-identical conditions, such as the screening of potential aggregation inhibitors, has remained limited due to the lack of appropriate barcoding strategies. One of the potential strategies for overcoming this limitation and devising a barcoding strategy for identifying effective inhibitors involves the use of multiple fluorophores in a dilution series, [105] with promising alternatives including quantum dot and DNA sequence based barcodes. [67,104] **(c)** By connecting individual microfluidic wells with microchannels analytes attached to magnetic beads can be moved between droplets trapped in individual wells, allowing multi-step analysis, such as ELISA, to be simplified and automated while limiting sample and reagent consumption. [106]

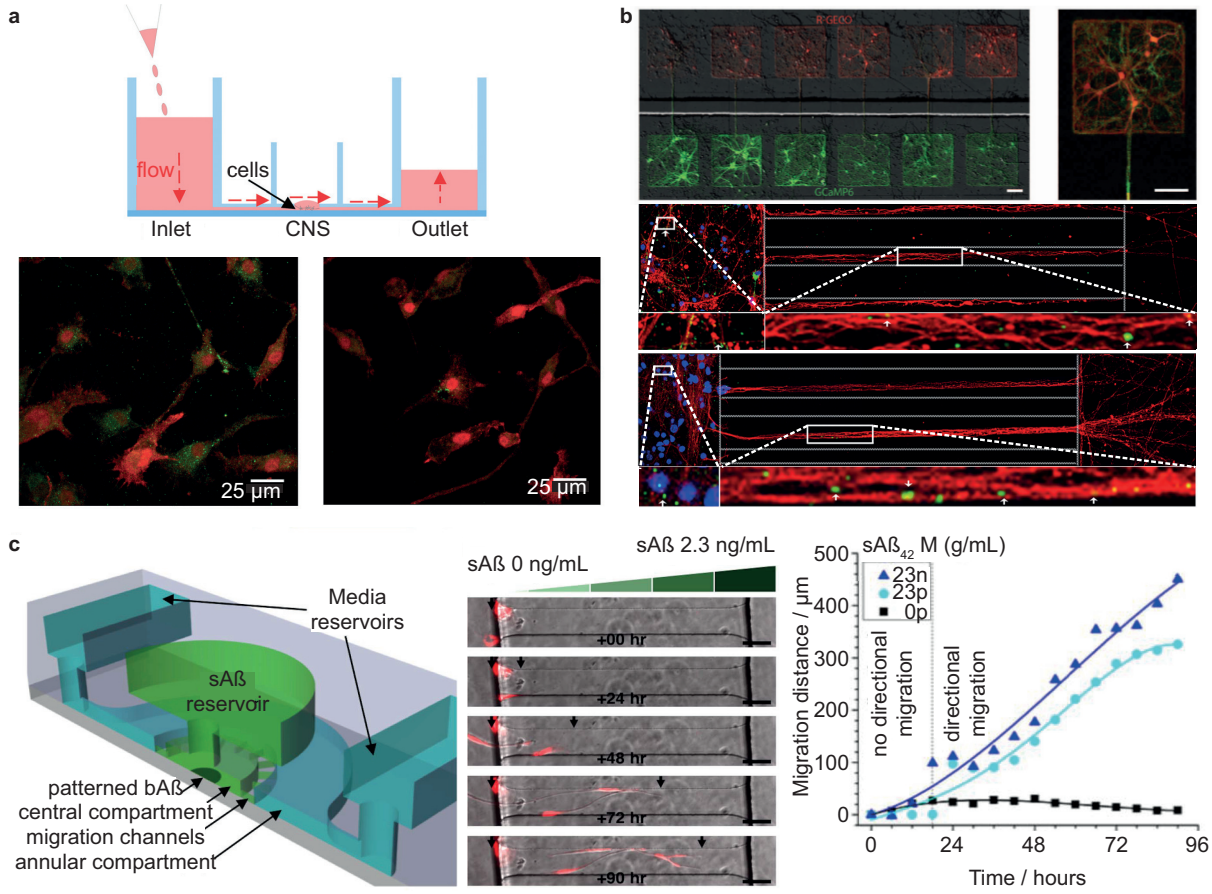

Fig. 6 Cell based assays utilising microfluidic techniques. **(a)** Illustration of the principle of operation via gravity-induced flow (top; CNS indicates the chamber where primay cells are plated). Representative images of pure microglia cultures incubated with oligomeric (bottom left) and fibrillar (bottom right) Aβ, fixed and immunostained with IBA1 (red) for microglia and 6E10 (green) for Aβ.[107] **(b)** Top- Microfluidic chambers connected through a narrow channel were used to investigate interactions between neurons exposed to glutamate.[108] A similar approach was used to study the internalisation and retrograde transport of tau aggregates in neurons[109]. Transport of short (middle) and long fibrils (bottom) is shown in green within axons (red). Magnified areas of the channel are shown at the bottom of each micrograph. **(c)** Right - a microfluidic chemotaxis platform models the pathological Aβ environments in Alzheimer's disease brains and provides surface-bound Aβ inside the central compartment, and gradients of soluble Aβ between the central and annular compartments, along a migration channel.[110] The symmetric configuration enhances the microglia counting during Aβ recruitment and the long and thin migration channels help to avoid counting errors due to spontaneous migration. Middle - induction of microglial directional migration by gradients of Aβ42. Individual microglia migrate directionally along the gradient of sAβ42 monomers formed in migration channels. Right - activation of directional motility can be discerned after 24 hours of exposure to gradients of sAβ42 monomers.[110]

more closely human tissue, with the additional advantage of using reduced amounts of cells and reagents, therefore decreasing the impact on the use of animals or human tissue. Such chips have the potential to form an easy-to-use alternative to standard cell cultures, increasing the throughput and reproducibility in assessment of toxicity and drug efficacy in living systems.

As microfluidic systems are increasingly applied in the investigation of biological phenomena and are increasingly utilised as cell culture platforms, the properties of the materials used to manufacture the devices, such as gas permeability, optical transparency, and flexibility, must be considered in a biological context.[113] By exploiting these intrinsic material properties, production of devices which combine microfabrication, microfluidics and surface micropatterning techniques can be used to create a multi compartment neuronal culture platform. This technology has potential uses in a number of neuroscience applications including studies of amyloid formation, where the ability to expose portions of neurons to local insults is desired.[114,115]

Traditional approaches of cultivating neural cells without orienting their interactions have had only a limited success in revealing neural network properties. However, by combining the concepts of micropatterned surfaces and microfluidic devices, various cell types, such as neurons and astrocytes, can be positioned in defined locations, thus allowing the study of neuron-neuron and neuron-astrocyte interactions under both healthy and pathophysiological conditions, Fig. 6b.[108] A similar approach was used in the study of cell-cell interactions in neuroinflammation in the context of neurodegenerative diseases. Different cell types were plated in separate chambers of a microfluidic network and could then be selectively primed with different stimuli. When chambers are connected by microfluidic channels, the specific contributions of the various cell types can be finely monitored by analysing their morphologies, vitality, calcium ion dynamics, and electrophysiological parameters.[116]

One set of central questions involves efforts to understand how and why microglia accumulate in the vicinity of Aβ plaques. Within microfluidic devices, microglial responses to gradients of soluble Aβ can be maintained for weeks, and patterns of surface-bound Aβ in microfluidic chemotaxis platforms have been quantified, Fig. 6c.[110] This study demonstrates that soluble monomeric and oligomeric Aβ can serve as a recruiting signal, while bound fibrillar and oligomeric surface-bound Aβ acts as a targeting signal during microglia recruitment and localisation. Soluble and insoluble Aβ have synergistic effects on microglial accumulation to sites of Aβ deposits, and could explain microglial accumulation in the vicinity of Aβ plaques in the cortex of those suffering from Alzheimer's disease cortex. Using a microfluidic chamber allowed CNS axons to be isolated in a compartment, where molecules, such as brain-derived neurotrophic factor (BDNF), could be selectively introduced. It was found in this study that Aβ reduces BDNF signalling by impairing axonal transport and this may underlie the dysfunction and loss of synapses observed in Alzheimer's disease.[117] This study has recently been further expanded to show that Aβ oligomers compromise BDNF-mediated retrograde transport by impairing the velocities of endosomal vesicles.[118] Microfluidic strategies have further been developed to perform sophisticated cell-based studies of amyloid formation. Microsystems can provide an approach for presenting cells to conditions in a controllable and reproducible fashion that cannot be easily achieved by standard tissue culture methods, including exposure to triggers, that vary in time and space, as well as to gradients of molecules and secreted proteins from neighbouring cells and physiological shear stresses from fluid flow.[119]

The contribution of cell-cell interactions can be studied by allowing the communication of different brain cells through the diffusion of soluble factors or through contact between specific cell regions. Thus, cells in chips have been exposed to differently aggregated forms of β-amyloid, mimicking an Alzheimer's disease environment, and treated with CNS drugs in order to assess the contribution of glial cells during pharmacological treatments. In this context, the neurotoxicity of diffusible moieties was studied by regulating he surrounding microenvironments and creating a biomimetic microfluidic system generating spatial gradients of diffusible oligomeric assemblies and assessing their effects on cultured neuronal cells. It was found that although the number of fibrils increased dramatically, the change in neurotoxicity with time was small, suggesting that fibrils contribute little to neurotoxicity. Moreover, exposure to Aβ was found to produce an atrophy effect, and observed neurite extension during the differentiation of neural progenitor cells increased when cells were cultured under continuous flow.[120] Such an approach may establish a prospective microfluidic platform for studying the neurotoxicity of Aβ and the potential neurotoxicity of oligomeric assemblies.

Such studies have mainly focused on the effects of soluble Aβ species on cell-to-cell communication, but these approach can be expanded further to study other amyloid-related protein systems. Microfluidic cell culture chambers have been used recently to study the uptake of different conformations of full-length human tau in primary neurons, the mechanism by which they occur, and the transport of tau aggregates within cultures of primary neurons.[109] It was shown that full-length tau readily aggregates into low molecular weight species and fibrils, yet only certain aggregates are internalised by neurons through bulk endocytosis. Additionally, aggregates from recombinantly expressed tau were found to enhance tau pathology *in vivo*. Similarly, tau oligomers were shown to be capable of entering human neurons and triggering the formation of aggregated and hyperphosphorylated pathological tau. These effects have been associated with long-term defects in neurite outgrowth, synaptic and neuronal loss, abnormal neurotransmission, and intracellular calcium ion mobilisation. These findings provide molecular insights into observations from post-mortem studies of human Alzheimer's disease brains and mouse models of propagation.[121]

Technological applications for amyloid structures

While the phenomenon of formation of aberrant protein self-assembly has historically been studied in the context of its relevance to human health, over the past decade significant efforts have focused on utilising amyloid-like protein assemblies for the development of functional biomaterials. Protein- and peptide-

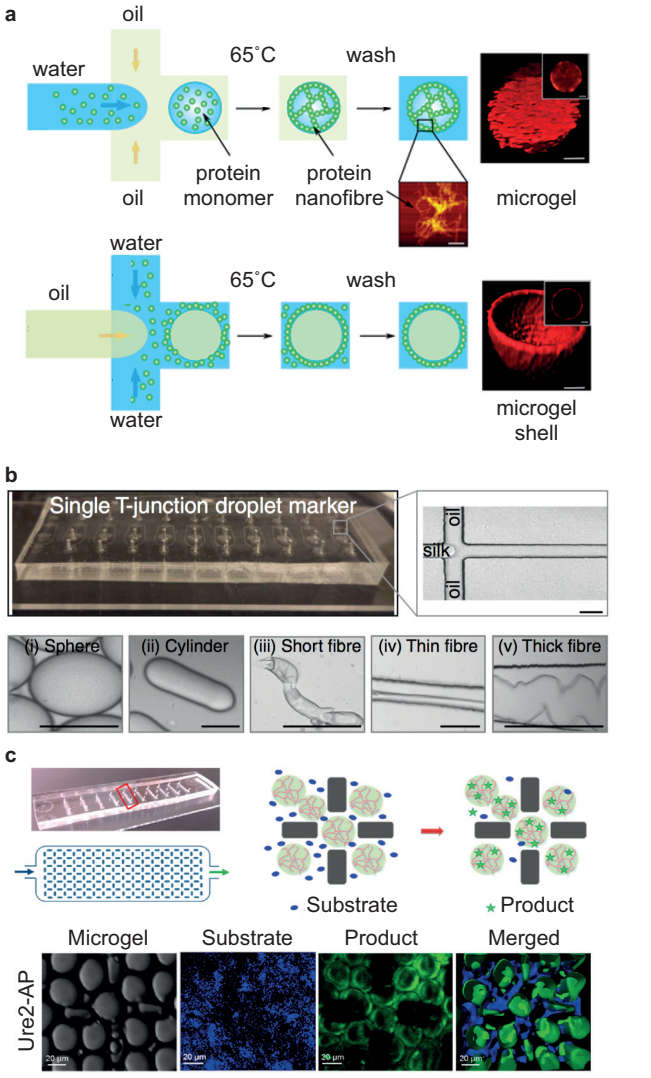

Fig. 7 Protein fibril microgel synthesis and their applications. **a)** Schematic representation of protein microgel synthesis: (top) water-in-oil microgels and oil-in-water microgel shells (bottom). The corresponding 3D confocal images of microgel and microgel shell particles stained with Nile Red are shown on the right-hand side of each scheme. **(b)** The device design and experimental setup for processing NSF into droplets (top), and examples of the various microgel geometries achieved by modulating solution flow rates (bottom).[122] **(c)** Top left panel, droplet trapping device diagram showing the structure of the droplet trapping device fabricated from PDMS with a schematic diagram of enzymatic activity of trapped Ure2-AP microgel particles in the droplet trapping device.[46] The substrate flows into the device by hydrodynamic pumping and diffuses into the trapped Ure2-AP microgel particles where it is efficiently converted into the product by the fibril-displayed AP. Lower panel, observation of trapped Ure2-AP microgel particles by bright-field DIC (far left). Fluorescence of the substrate (blue) and product (green) after pumping the substrate into the trapping device (middle panels). The three-dimensional microgel particles and the fluorescence of the substrate and product were reconstructed from serial two-dimensional scans by confocal microscopy (far right). Scale bars are 20 μm[46].

based structural biopolymers are common building blocks for biological scaffold materials in nature, either in their native forms, such as collagen, silk or fibronectin, or as related synthetic materials.[37–41,123] Such structures have inspired the production of self-assembling materials, primarily via non-covalent cross-linking to produce hydrogels as cell scaffolds by a large number of diverse patterning techniques, utilising organic and polymeric materials, including solvent-assisted micro-molding, micro-contact printing and nanolithography, which form the basis of contemporary soft lithography.[124] Yet, such materials are limited in their ability to provide an optimised environment for all cell types as the resulting scaffolds possess size scales and compositions which may or may not promote cell viability.

Fibrous networks assembled by naturally derived peptides and proteins offer unique opportunities for the production of biomimetic cell culture platforms and implantable biomaterials as they allow physiological cell-matrix interactions. While the synthetic complexity of such systems can limit their applications, amyloid fibril networks derived from common or readily available proteins such as lyzosyme with controllable topographies can readily be designed and fabricated.[125] In particular, the surface chemistry of the fibrils has been shown to promote focal adhesions of cultured cells, eliminating the need for complex protocols for fibril decoration with bioactive moieties. The self-assembly of proteins into amyloid fibrils on the nanoscale has been further utilised as a micron-scale templating approach for droplet microfluidic techniques to create a class of hierarchically ordered functional materials. By utilising the aqueous phase separation of dextran and polyethylene glycol (PEG) in a double T-junction microfluidic device, a core-shell structure was produced through the self-assembly of lysozyme into supramolecular structures that could be tuned on the basis of changes in the *in vitro* environment such as pH and temperature.[126] Furthermore, by employing the intrinsic phase separation of dextran and PEG based aqueous solutions, control over the selective partitioning of fibril-forming protein molecules within each phase could be accomplished, giving rise to the formation and stabilisation of supramolecular core-shell microgels with localised tunable properties. Such protein nanofibrils can be used to form the basis of monodisperse microgels and gel shells with the potential to act as drug carrier agents.[45] These microfluidially produced particles have also been demonstrated to enable the controllable release of four different encapsulated drug-like small molecules, as well as the component proteins themselves. Recently, hybrid organic/inorganic microcapsules containing metal-based nanoparticles and lysozyme nanofibrils as a nanocomposite were produced by a microfluidic droplet making device. In this study carboxyl-modified Fe_3O_4 nanoparticles were introduced into the lysozyme monomer or fibril solutions and could functionalise the resulting fibrils, thus allowing the production of nanofibril-inorganic nanoparticle microcapsules.[127]

Other self-assembling proteins have been explored in the context of the search for control of material properties through the application of microfluidic techniques. One recent example is the use of spun silk for the production of 'micrococoons' through microfluidic processing, Fig. 7b.[122] Silk fibroin protein has been

found to assemble into fibrils rich in β-sheet structures by a combination of small-angle and wide-angle X-ray scattering and Raman spectroscopy, revealing intrinsic similarities to amyloidogenic proteins.[128] The ability of fibroin to form ordered fibrillar structures has been employed as the basis for the production of micron-scale capsules with controllable geometry and variable levels of intermolecular β-sheet content in their protein shells. These capsules have been shown to maintain the long-term stability of soluble monomeric proteins, which would otherwise be prone to self-assembly in bulk solution. Moreover, the micrococoons demonstrated effective encapsulation of other aggregation-prone antibodies while allowing their storage and controlled release.[122]

Recently, microfluidic generation of enzymatically active microgels that were stabilised by amyloid nanofibrils have been demonstrated, Fig. 7c.[46] The scaffolds formed by the prion domain of Ure2 self-assembled under mild conditions and enabled the formation of catalytic microgels while maintaining the integrity of the encapsulated enzyme alkaline phosphatase. The enzymatically active microgel particles exhibited further robust material properties and their porous architecture allowed for the diffusion of reactants and products. This approach has been further implemented in droplet arrays, demonstrating their use as platforms for enzyme immobilisation and recycling, as well as for performing biological flow-chemistry. Moreover, along with utilising microfluidic-based techniques to maintain control over the material properties and morphology of the capsules, the spectroscopic characterisation of specific species encapsulated within microdroplets has recently been demonstrated through the acquisition of infrared spectra from single microdroplets containing aggregation-prone proteins, such as lysozyme.[78] Using this approach, structural differences in the amide bands of the spectra of monomeric and aggregated lysozyme from single microdroplets with picolitre volume could be resolved.

Outlook and conclusions

In this review we have discussed a wide range of microfluidic approaches for the study of amyloid formation, properties, and behaviour. Many of the techniques discussed have been reported as proof-of-concept studies, but not yet adopted by the wider amyloid research community.[129,130] The majority of the instruments currently used to analyse proteins and protein self-assembly complexes are bulky (e.g. mass-spectrometers) and operate over long time-scales (e.g. chromatographic or gel-based protein purification techniques combined with off line analysis techniques). These features are in marked contrast to microfluidic techniques which facilitate fast analysis on palm-sized chips. For the purpose of most types of analysis these chips are operated in combination with appropriate fluid handling and detection systems, although paper and cloth microfluidics provide an exception to this requirement.[131,132] Translation of cutting edge microfluidic methods to applications in the life sciences is in many cases achieved through collaboration between research groups in the physical and life sciences.[133] In addition, microfluidics-based technology is introduced into non-specialist laboratories through commercially available equiptment.[134,135]

Combining rapid analysis and small sample consumption with the ability to control tightly the channel dimensions during fabrication, and the flow within these channels, microfluidic chips present a still largely untapped potential to yield quantitative information about both individual proteins and the products formed during their self-assembly process, including protein oligomers and amyloid fibrils; thus for example, diffusional sizing analysis has demonstrated equal or superior performance to conventional dynamic light scattering setups.[60,63] On-chip screening of the electrophoretic mobilities of protein complexes has allowed determination of binding curves and dissociation constants without the biases that are often intrinsic to conventional surface-based methods, such as surface plasmon resonance.[64]

We have explored how the physical properties of biomolecules and supramolecular complexes can be quantified through the use of microfluidic approaches, and furthermore how these parameters can be monitored to characterise molecular interactions. Such assays show great promise in applications to improve our understanding of the nature of amyloid species and how these interact with cells and with potential inhibitors of the aggregation process. In addition, microfluidic separation techniques could provide a platform for sample purification from small volumes. Although proof-of-concept experiments on separating a small number of species on chip have been performed, microfluidic separation techniques are yet to become a routine approach in biomolecular sciences.[62,136] Notably, many of the interactions occurring on the pathway of protein self-assembly into amyloid fibrils and aggregates are not only transient, and hence accessible only to rapid analysis techniques, but also reversible and so can potentially be affected by the presence of a matrix, such as chromatography columns or acrylamide gel media, which further emphasises the value of using solution phase based techniques for their analysis, such as the aforementioned microfluidic approaches described here.

Intrinsic biomarkers have been exploited to sort cells in label-free separation methods, including separation according to size, shape, density, charge, deformability, and other properties of the aggregating systems. The sorting of live and dead cells is critical to the diagnosis of the early stages of many conditions and to test the efficacy of drugs. Micro-electromagnets are suitable for the production of variable field gradients combined with different channel designs, such as single wires and micro-coil arrays, a microfluidic device containing integrated thermo-magnetically patterned NdFeB films for cell separation using immunomagnetic beads has been produced. This droplet based microfluidic approach allowed for high-throughput analysis of proteins released from or secreted by cells, thereby overcoming one of the major limitations of traditional flow cytometry and fluorescence-activated cell sorting. This approach enables the dynamic reconfiguration of the magnetic field patterns, but has the drawback of generating Joule heating, which limits the maximum value of the usable magnetic field considerably, and thus the field gradient that can be achieved; for example Joule heating may have adverse effects on cell viability.[137] In order to overcome these limitations micro-patterning of permanent magnets within a solid substrate upon which the microfluidic channel was placed were

incorporated.[138]

The reduced volumes of microdroplets relative to conventional microtiter plate assays make droplets an attractive platform for high-throughput studies. Although the benefits of reduced reagent consumption, faster assay time scale and parallelisation have been demonstrated effectively for the analysis of a range of assays, including those used for the study of protein aggregation and neurodegenerative diseases, their extension to high-throughput screening assays has so far been limited. This has been partly due to the lack of effective barcoding techniques that would enable successful candidates to be identified after the assays have been performed. Recently, barcoding of the genetic material present in individual cells has been demonstrated through the use of DNA-based barcode technology, which has led to a proliferation of the studies in the field of single cell transcriptomics and allowed the discovery of new cell types in blood and a number of tissues.[139,140] The extension of such barcoding strategies to molecules beyond the genetic material, for example to small molecules that could serve as potential therapeutic compounds, would open up the possibility of screening millions or even billions of compounds simultaneously, whilst consuming an amount of sample that in non-droplet based methods would enable the study of only a few dozen compounds. Such high throughput screening assays have the potential to identify new therapeutic molecules, which would be especially relevant for neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases.

The presence of interfaces can influence the aggregation kinetics for a wide range of proteins and peptides, for example, in the case of Aβ aggregation, surface properties are of great importance in modulating the aggregation process, leading to self-assembly at concentrations that are many orders of magnitude below the critical aggregation concentration in bulk solution.[141] Although it has been generally recognised that the amyloid aggregation process can be assisted or accelerated by surface chemistry[142] and topography, characterisation of the specific impact of different types of surfaces and interfaces has been limited.[141] The effect of surfaces can be broadly divided into those arising from combinations of three distinct interfaces, namely, solid, liquid and air interfaces. Thus, the specific molecular surfaces found at the interface of cell membranes can enhance the effective protein or peptide concentration by specific binding or adsorption. As the effects of solid, liquid and even air-water interfaces have mainly been studied under bulk conditions, the increased availability of novel microfluidic approaches presents a potential means of studying surface interactions.[85,143–146] In particular, the use of microdroplet-based techniques, where the properties of the interfaces encapsulating solutions can be modulated easily, can allow for detailed analysis of such effects.[70]

The well-defined flow properties within microfluidic channels enable strategies to be developed to address questions, that would be inaccessible to bulk solution methods. In this review, we have discussed how the physical properties of biomolecules and supramolecular complexes, particularly in the context of amyloid formation can be defined through the use of microfluidic approaches and furthermore how these parameters can be monitored to characterise specific interactions. Such assays have shown great promise in applications to improve our understanding of the nature of amyloid species and how these interact with potential inhibitors of protein aggregation. We have discussed above some of the applications of amyloid structures as novel materials and cases where microfluidic methods have already been integrated into the production of materials. Indeed, the use of amyloid structures as scaffolds and in smart materials is an expanding field, with applications emerging in water purification, nutrient delivery, and in the production of composite materials.[130,147,148] The use of microfluidic strategies to explore the potential of amyloid-based materials is therefore set to increase substantially. Microfluidic methods have been applied to study a large number of aspects of amyloid formation and behaviour. With the continuous development and adaptation of these techniques, there is the potential for such strategies to play an even larger role in the rapidly developing field of amyloid research.

The number of individuals affected by amyloid disorders is set to increase over the coming years, resulting in a devastating impact on society and a great demand on resources. Developing a detailed understanding of the formation and behaviour of amyloid structures is therefore a pertinent objective.[6] In addition to the impetus of disease association to amyloid research, amyloid structures are showing a considerable potential as a basis for smart and active materials.While microfluidic approaches are constantly developed and optimised in the context of chip design, translation of such techniques now bridges the gap between fundamental physicochemical studies and a clearer understanding of natural processes while further implementation of these techniques are now progressing to large scale production.

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