



Microfluidics in Chemical Biology

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Abey Joseph, Akash Karthikeyan, Shamili G. Nair, V. R. Prince, P. E. Nafrin, and Baiju G. Nair

Abstract

Chemical biology is the field where two scientific disciplines, that is, chemistry and biology, join. Techniques derived from chemistry are used to study or manipulate biological or natural products. Chemical biology helps develop bioassays for the quantification of various compounds. The wisdom from chemistry is used to develop techniques to purify proteins and nucleic acids or use small molecules for multiple applications, such as sensing, drug discovery, tissue engineering, disease modelling and molecular genetics. The 1970s witnessed the development of a novel field of engineering that manipulates micro- or nano-quantities of fluids using channels and pumps for specific applications called microfluidics. The development of various microfabrication techniques and novel materials in the past few decades has led to efficient microfluidic platforms. The convergence of microfluidics, chemistry and biology gives us a platform where minute quantities of samples are orchestrated to ensemble an overall result that helps researchers in various scientific fields. This chapter briefly discusses the application of microfluidics in chemical biology in bioassays, separation and purification of proteins and nucleotides, molecular self-assembly, tissue engineering and nucleotide sequencing. These aspects include exposure to electrophoresis and chromatography in microfluidic devices, gradients in microfluidic devices, surface modification strategies, and polymerase chain reaction (PCR).

A. Joseph · A. Karthikeyan · S. G. Nair · V. R. Prince · P. E. Nafrin · B. G. Nair (✉)
School of Biotechnology, National Institute of Technology Calicut, Kozhikode, Kerala, India
e-mail: bnair@nitc.ac.in

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4.1 Introduction

Chemical biology is the convergence of chemistry and biology. It involves the study of reactions concerning biological processes. Chemical biology deals with reactions involving biomolecules such as lipids, carbohydrates and proteins and small molecules such as metals, probes and drugs which shine a light on the basic working of a living body. Chemical biology is also of paramount importance to researchers since it forms the basis for various laboratory techniques. It is impossible to obtain outcomes for procedures such as protein extraction, purification and crystallization; manipulation of different cell lines; synthesis of non-natural probes; and molecular self-assembly without chemical biology. Since chemical biology is unavoidable in almost any life science laboratory, improvements in platforms to carry out this field will aid researchers in carrying out works in a much more efficient manner. Microfluidic platforms are a robust means to reinforce the research carried out in the field by reducing the samples required and improving overall efficiency.

Generally, microfluidics is the manipulation of fluid flow in quantities from microlitres to as small as femtolitres with the help of micro-channels, valves and pumps to yield relevant results. The channels are imprinted onto a polymer platform using techniques such as soft lithography, chemical etching, hot embossing, micro-moulding, 3D printing, reactive ion etching and micromachining [1]. The most commonly used polymers are polydimethylsiloxane (PDMS), poly lactic-co-glycolic acid (PLGA), polylactic acid (PLA) and poly(methyl methacrylate) (PMMA) [2]. Glass and silicon are other non-polymer materials employed for fabrication. The materials are selected based on the properties such as tensile strength, elasticity index, plasticity, hardness, electrical and mechanical conductivity, inertness, gas permeability, liquid permeability and transparency to visible and UV light. The material used depends on the need in chemical biology. In chemical biology, microfluidics reduces the sample size significantly, requiring very little reagent while enabling easy reaction monitoring. The reactions performed in these devices are extrapolated easily for a full-scale model. The device also permits us to mimic biochemical reactions with better efficiency as the intrinsic dimensions of the device channel are comparable to that of a eukaryotic cell. The device also provides a better environment since it is easy to maintain pressure and other mechanical motion parameters.

4.2 Microfluidics in Analytical Chemistry

Analytical chemistry includes the convergence of various techniques that examine various aspects and purposes of a chemical reaction; this can be in chemistry, life science, or biochemistry. Analytical chemistry is broadly classified into separations and reactions depending on the course of action [3]. Applications of microfluidics in the field of analytical chemistry have innumerable advantages. A somewhat laborious and time-consuming protocol is performed with significant ease using very little time. This advantage is even more notable in life sciences as the reactants are usually less in quantity and costly. Hence, the above advantage increases the efficiency of the work. Another advantage of using a microfluidic platform is the possible precision control of behaviour in the system, thus enabling fastidious reactions using microfluidic platforms. Microfluidic devices can work without much human intervention, allowing the safe use of toxic reagents without human harm.

4.2.1 Microfluidic Devices in Chemical Reactions

The previous decade saw an enormous boom in the development of microfluidic devices for various purposes in research. Even though end consumers rarely use microfluidic devices commercially, several companies manufacture devices directly used in the laboratory [3–5]. Microfluidic devices are used for various applications, such as DNA analysis, chemical synthesis, enzymatic reactions and many other immunoassays.

The field of life science brings its challenges, one of which is the availability of reagents and samples. Since significantly less quantity of sample is available for studies, a platform that uses samples and reagents in the femtomolar range brings down the overall cost of the process and increases the efficiency. A microfluidic device employing silica beads and photodiodes designed to detect genetic material in the femtomolar range using sample volumes as low as 5 μL is developed [6]. Another PDMS-glass device using minimal sample volumes, employing micropillars and aptamers, was used to capture cancer cells [7]. Yang et al. demonstrated a device for isolating single cancer cells from whole blood [8]. The detection for both the above cases was done using fluorescence.

In contrast to fluorescence- and chemiluminescence-based detection, colorimetric detection is significantly simple. The costly probes are not required, and the detection devices are easier to design. A disadvantage of using colorimetry is that it cannot provide high-resolution images. However, it is practically nullified as the colorimetry devices employ a high-resolution camera-integrated smartphone allowing a point-of-care approach [6, 9]. This point-of-care approach has led to various lab-on-a-chip devices to analyse several components in the human body. Paper-based microfluidic devices are employed widely for the detection of anything from ions [10, 11] to components such as glucose and proteins [12], lactate [13], uric acid [14], total amino acid content [15], urea [16] and other biomarkers [17, 18]. The paper-based microfluidic device can detect the components mentioned above from

body fluids such as whole blood, plasma, urine, sweat and saliva [17, 19–21]. Interestingly, instead of using a smartphone, techniques are developed for reading data using a handheld device by transmitting light through paper-based microfluidic devices [22]. Researchers have employed paper-based microfluidic devices to create a system to detect multiple components from a single sample [17, 23].

4.3 Separation and Purification of Biomolecules

Chromatography and electrophoresis are analytical and separation procedures. They are widely utilized for macromolecules' macroscale separation and evaluation, namely proteins and nucleic acids. Chromatography can isolate a wide range of particles by the appropriate stationary phase and the presence of distinct ligands. In contrast, electrophoresis offers the detachment of charged species as per the ratio of molecule charge/size upon the utilization of an electric field. The two strategies provide the likelihood to recognize the structure and properties of a biomolecule. However, they have likewise tedious protocols and high costs, additionally at times require laboratory infrastructure and talented staff. On miniaturizing the two methods, such disadvantages are overcome [24].

4.3.1 Electrophoresis

Approaches of microfluidic chip electrophoresis for the separation of biomolecules.

4.3.1.1 Microchip Capillary Electrophoresis

Capillary electrophoresis (CE) has turned into a powerful and functional analysis method with benefits of electrolyte utilization of low sample volume, quick analysis time, simple coupling with other sensitive detection techniques, and good separation efficiency [25]. Distinctive separation methods for isolation and identification of different biochemicals include capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), micellar electrokinetic chromatography (MEKC), capillary isotachopheresis (CITP) and non-gel sieving capillary electrophoresis (NGS-CE) [26]. Capillary zone electrophoresis is a dominantly used separation method on microprocessors among the capillary electrophoresis for charged species [27].

MEKC separates neutral and charged species based on partitioning between micelle and background electrolyte and electrophoretic mobility. High proficiency serpentine PDMS microfluidic chips using fluorescent proteins are helpful in MEKC separation. In MEKC, functionalized ionic liquid determines the protein separation, which could be utilized as a surface modifier and supporting electrolyte to prevail the adsorption of analytes [28].

CIEF is a notable and predominant separation strategy for protein separation. It is accomplished with monolithic immobilized pH gradient materials in microchannels that are introduced to isolate ribonuclease B, α -casein and myoglobin [29]. In addition, the isolation of nucleic acids utilizing a low sample of about 25 μ L of

CITP is also an innovative microchip [30]. Another microdevice utilizing NGS-CE, loaded with short linear polyacrylamide solution, identifies the DNA fragments isolated from the gastric cancer tissue using restriction endonuclease fingerprinting. It finishes DNA detection with the help of laser-induced fluorescence (LIF) in a short period [31]. One-dimensional division mode lacks peak capacity, making it harder to examine the complex biological samples with a satisfactory resolution. Significant commitments to take advantage of a two-dimensional mode of separation on a microfluidic device for peptide blends or complex proteins analysis improve coupling MEKC accompanied by high field strength CE, which enhances the quick separation of peptides processed from a model tryptase [32].

4.3.1.2 Microchip Gel Electrophoresis

Microfluidic gel electrophoresis facilitates all sample preparing steps automatically in a miniaturized channel with reagents of diminished volumes. Furthermore, it allowed the joule heating to dissipate rapidly and advanced the improvement of gel electrophoresis [33]. In isolation of nucleic acids, agarose gel acts as a better sieving matrix for electrophoresis. Additionally, an ultrahigh-throughput way to investigate the genomic damage of cells with excellent reproducibility is developed using agarose-based microfluidic devices [34]. The isolation of DNA on polyester-toner (PeT) based electrophoresis microchip is successfully studied. On this PeT chip, channels loaded with hydroxypropyl cellulose or hydroxyethyl cellulose help isolate DNA fragments with good separation efficiency. Further studies concluded that the microdevice filled with the solutions of short straight polymer dissipated the joule heating rapidly, which prolonged the life cycle of the microfluidic device [35].

4.3.2 Chromatography

Chromatographic techniques proficiently separate biomolecules in an unadulterated manner after the pre-treatment of the sample. However, it requires substantial sample volumes and is relatively time-devouring. Microfluidic chips attained consideration from the researcher community as an option to conquer the inadequacies of conventional gadgets. A microfluidic chip can be envisioned as a miniaturized device of multiplexed channels in an organized network [36]. Specifically, isolation of biomolecules is carried out by different chromatography procedures such as affinity chromatography (AC), size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX) [26]. The incorporation of stationary phases within the microchannels is crucial in chromatography for the isolation of molecules. Commonly used stationary phase materials are monoliths, resins and membranes. Nanowires (NWs) are also inserted into the microfluidic chips for biomolecule isolation along with these stationary phases. Moreover, pillar structured and surface functionalized microchannels like open channel frameworks have been utilized as stationary phases [36].

4.3.2.1 Separation of Nucleic Acids

Extraction of nucleic acids with the help of chaotropic agents, namely guanidinium thiocyanate, from various complex combinations are also extended to use in microfluidic chips. A common practice is the specific isolation of nucleic acids in an aqueous medium utilizing affinity ligands like human immunoglobulin E. The outcomes revealed that the essential requirement of affinity ligands is economically suitable and can adsorb nucleic acids selectively from complex mixtures [36].

4.3.2.1.1 Open-Channel Microfluidic Chips

Microfabrication is one of the innovations for fabricating microchannels and microstructures using various materials. Glass and silicon microfluidic chips are fabricated in a cleanroom atmosphere utilizing thin-film metallization, photolithography and chemical etching. Limitations of silicon and glass and expanding requests for the minimal expense of plastic and polymer materials have prompted the next-generation fabrication techniques. Both pillar and open structured channels are used for the stationary phase for biomolecule extraction [36].

Modification of functional groups is done on microchannel surface with cyclic olefin polymer (COP), namely diethylene glycol dimethyl ether (DEGDME), polyethylene glycol (PEG) with a neutral charge, bovine serum albumin (BSA) with a negative charge, and (3-aminopropyl)triethoxysilane (APTES) with a positive charge on the open-channel microchips. Adsorption of ssDNA atom is further examined on both altered and unaltered microfluidic chips. The negatively charged DNA follows affinity as $APTES > PEG > BSA$. If an occurrence of PEG appended COP should arise, electrostatic associations play a significant part in the affinity towards DNA. The substrate covered with BSA shows less affinity towards DNA. An increment of the microchannel length improves the DNA adsorption limit [37].

A dispensable PDMS microchip with engraved glass isolates DNA of varying lengths in a reduced electric field. This microchip effectively separated DNA fragments from mixtures and offered benefits, including re-using and separation efficiency with minimal loss [38]. Cyclic olefin copolymer (COC) and PMMA microchips are manufactured by exploring the injection moulding method. The principle of isotachopheresis (ITP) is good to isolate nucleic acids from an organic sample of salmon sperm and whole human blood. Even though the presentation of the two materials is comparative, COC needs extra pressure to move it in through the microchannel unlike other materials which spontaneously move through the channels [39].

4.3.2.1.2 Pillar Structured Microfluidic Chips

In this, augmentation of the microchannel surface region consolidates fabricating shapes in the form of pillars with the help of microfabrication techniques [36].

A silicon dioxide-coated silicon-PDMS microchip is employed to separate RNA. The dispensable PDMS cartridge contains repositories that can hold reagents, a microchannel that allows reagents' movement, and a pin valve that controls the fluid flow. The microchannel interface of the reservoir determines the bonding strength, which is explored by subjecting the microfluidic chip to an average

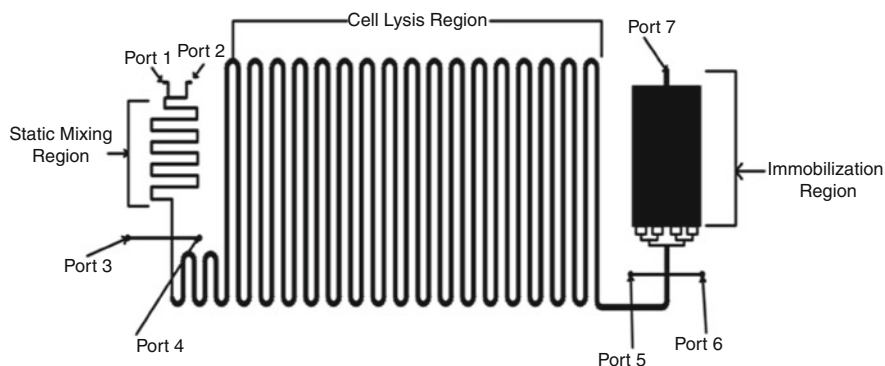


Fig. 4.1 An integrated microfluidic device for on-chip cell lysis and affinity extraction of RNA

pressure. The shortfall in liquid leakage demonstrated the bonding strength of the substrates. This chip separated dengue infection serotype 2 RNA (DEN2 RNA) from a pre-lysed infection culture [40]. A coordinated Pyrex 7740 glass microchip isolates RNA, as shown in Fig. 4.1. Crown designs are immobilized with specific oligonucleotides in the extraction chamber. Rhodamine marked IL-13 rRNAs show affinity to the oligonucleotide, affirmed by the fluorescence magnifying lens pictures. The weak bonding of RNA with oligonucleotide resulted in the effective elution of RNA on the application of heat [41].

‘Bowed edges’ inside miniature channels of PDMS microfluidic chip accomplished the fast isolation of DNA. The model combinations of minicircle DNA and linear DNA fragments demonstrated the separation efficiency along with parental DNA. Low-weighted DNA fragments passed the ridge without any hindrance, while those with more weight diverted to reach the ridge towards the contrary channel divider when the continuous injection was applied [42].

4.3.2.1.3 Resin Incorporated Microfluidic Chips

The resins for chromatography include polymers, inorganic materials and natural polysaccharides. Furthermore, the materials are modified with various chemistries to immobilize the ligands. Resins used for chromatography have a large surface area and adsorption capacity. However, their application at the analytical scale is limited due to mechanical weakness, time-consuming packing procedure, poor mass transfer and significant pressure build-up [36]. The most important strategy is to include chromatography resins such as silica resins and others into the microchannels of microfluidic devices. To firmly retain the resins, some researchers utilized ‘weir structures’ with different heights. Others used a polyethylene membrane as a ‘Frit substance’ that contained the silica resins inside the microchannel confines. An intriguing tactic during the extraction tests was the utilization of the sol-gel as an ‘interparticle glue’ to keep the resins intact, maintain consistent packing and minimize packed bed compression [36].

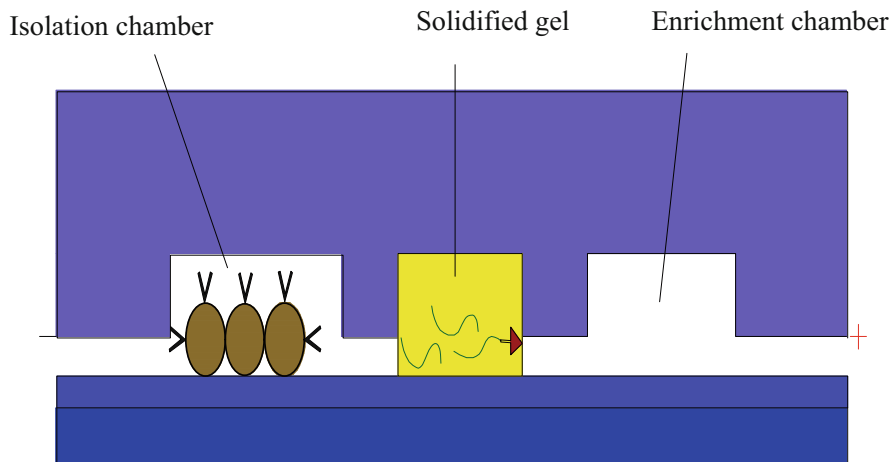


Fig. 4.2 IgE functionalized microbeads withheld within microchannels using weir structure

The affinity chromatography concept was applied in a glass-PDMS hybrid microchip for the isolation of ssDNA selectively. Separation, transfer and enrichment chambers are the conjoined chambers of microchannel on the microfluidic chip, as depicted in Fig. 4.2. The first microchannel chamber is filled with human IgE immobilized microbead to select ssDNA from a randomized ssDNA mixture. The thermally eluted ssDNA strands are transported to the third microchannel chamber through electrophoresis in the second chamber containing solidified agarose gel. Furthermore, changing the old IgE immobilized beads with new ones enhanced the efficiency of the multiple uses of the device. The benefit of this microfluidic gadget over existing gadgets is the likelihood to consolidate electrophoresis and solid-phase extraction on a solitary chip [43].

Another glass microchip with MagneSil™ particles isolates DNA from enormous sample volumes obtained from clinical labs. Human and mitochondrial DNA was extracted successfully from dilute whole blood and deteriorated bloodstains, demonstrating the microchip's extraction effectiveness. The overall biological sample volume is considerably low, while the concentration of DNA is high. This framework impedes the potential contamination with the co-eluted PCR inhibitors [44].

4.3.2.1.4 Monolith Incorporated Microfluidic Chips

According to the definition, a monolith is a single piece of continuous stationary phase with a strongly linked porosity network. Flow is convective due to strong pore interconnectivity, resulting in a faster mass transfer of molecules and quick separation. Monoliths are inorganic or organic based on the characterization. Monolith integration in microfluidic devices was sparked by technological advantages such as ease of introduction into microchannels as a homogeneous solution, in situ polymerization, and the absence of frits [36]. Another extra element is the likelihood to set

them up at a particular site on the microchannel by photograph-initiated polymerization helped with customized photomasks. The ideal functionality of monoliths is accomplished by utilizing two methodologies. The primary method is a multi-step measure that modifies the chemical moieties on a monolith that has been pre-synthesized. The subsequent methodology is a solitary advance interaction that includes the monomer addition functionalized with sulfonate, methyl, or carboxylate to the solution of co-monomer and crosslinker atoms before in situ polymerization [36].

A silica monolith microchip separated DNA from a low cell number sample. Here, DNA binds in an irreversible method to silica monolith when present at low concentrations. However, the increment of concentration of DNA load reduces this pattern due to the filling of reversible destinations preferentially [45]. Another coordinated microchip isolated DNA from buccal cells under chaotropic conditions uses silica monolith as the stationary phase. This chip contains low-melting temperature (LMT) agarose gel to hold the reagents for DNA extraction and polymerase chain reaction (PCR) intensification. Liquids head to the microchip utilizing the electro-osmotic pumping (EOP) rule. The main benefit of the framework is storing reagents without critical loss and appropriate processing of clinical samples [46].

4.3.2.1.5 Nanowires Incorporated Microfluidic Chips

Nanowire (NW) structures make spatial and controlled nanostructures. SnO₂ nanowires joined inside microfluidic gadgets have been utilized to extract biomolecules based on size exclusion chromatography. Self-assembly technology assisted in the production of nanowire spot array structures in the fused silica microchannels. This device is more suitable for separating long DNA molecules when contrasted with a nanowall array microchip. Three-dimensional inflexible nanowire, when integrated inside the microchannel utilizing vapour-liquid-solid (VLS) technique, showed that nanowire structures with high density have better separation efficiencies than existing nanopillar structures in proficiently isolates various sizes of DNA [36]. Protein and RNA are separated from the three-dimensional rigid nanowire microchip upon increasing the nanowire's growth cycle by reducing the pore size distribution of nanowires. This device's advantage is the likelihood of controlling pore size dispersion between nanowires by characterizing the quantity of nanowire development cycles. These chips have proficient biomolecule separation because of the presence of the SiO₂ layer and the applied electric field getting concentrated inside the pores of firm nanowire structures [47].

4.3.2.2 Separation of Proteins

4.3.2.2.1 Open-Channel Microfluidic Chips

A microchip with a microchannel coating of amine polyethylene glycol has enhanced the separation efficiency of proteins such as bovine serum albumin (BSA), myoglobin, and ribonuclease A and the suppression of non-specific protein adsorption [48]. Cyclic olefin copolymer (COC) microchip effectively reduced the non-specific adsorption of proteins via grafting of *N*-vinylpyrrolidone [27]. The

illustration is achieved by reducing the non-specific adsorption of the model molecule, BSA [49]. A super quick 2D-microchip electrophoresis along with sodium dodecyl sulphate-microcapillary gel electrophoresis (m-CGE) and microemulsion electrokinetic chromatography (m-MEEKC) isolates proteins. Furthermore, this strategy has been utilized to separate nitrosylated proteins from cerebrum tissue of Alzheimer's disease (AD) transgenic mice and epithelial adenocarcinoma cells of the human colon [36].

Similarly, capillary isoelectric focusing (CIEF) chip with m-CGE as a 2D framework working together as a hybrid microchip has been used to isolate proteins. Various proteins, including trypsinogen, BSA, β -lactoglobulin, are utilized to consider the plausibility and productivity. The utilization of low voltage and long run times impedes this microchip [50]. Another manufacture approach consolidating surface functionalization and hot embossing upheld PMMA microchannel, which isolated and preconcentrated proteins utilizing the principle of isotachopheresis (ITP). A protein model of a combination of green fluorescent protein and pacific blue marked human heart troponin I and R-phycoerythrin was utilized for anionic ITP. On the other hand, a protein combination of pacific blue named cardiac troponin I (cTnI) and fluorescein isothiocyanate (FITC) egg whites was utilized in the cationic ITP [51].

4.3.2.2.2 Resin Incorporated Microfluidic Chips

PDMS microchip loaded with mesoporous silica dots of Ia3d space group isolated biomolecules under pressure-driven liquid chromatography. This microfluidic chip proficiently isolated BSA and dextran, a neutral polysaccharide in terms of hydrophobicity [52].

4.3.2.2.3 Monoliths Incorporated Microfluidic Chips

Ethylenediamine is a feeble anion exchange ligand. When incorporated with a functionalized methacrylate monolith in a PDMS microchip, it isolated biomolecules. Utilizing BSA as a model protein, examining various parameters, including pH of the binding buffer and ionic strength, showed the expansion of ionic strength, which showed a reduced protein binding to the microchip. Under optimized conditions, an acceptable detachment of BSA and ovalbumin is accomplished [53]. An incorporated PMMA microfluidic chip separated and evaluated proteins from the human sera. In this, a thin pre-polymerized film of glycidyl methacrylate (GMA)-co-PEGDA is used to immobilize antibodies such as anti-HSP90 (heat shock protein 90), anti-cytochrome C, anti-carcinoembryonic antigen (CEA) and anti-alpha-fetoprotein (AFP). Spiked human sera determine the selectivity of the microchip in various concentrations. The serum matrix is eliminated by affinity extraction, and then the elution unveiled the spiked proteins on the analysis of on-chip capillary electrophoresis [54].

On-chip electrophoresis protein separation is developed with the help of functionalized quaternary ammonium groups. It consists of a monolith, namely 2-hydroxyethyl methacrylate (HEMA)-co-ethylene dimethacrylate (EDMA), embedded in the chip. 2-Methacryloyloxyethyl particle is photopolymerized to

accomplish the anion functionality, and this is performed on the monolith. BSA and trypsin inhibitors are effectively isolated through this method [54].

4.3.2.2.4 Nanowire

A microfluidic chip of forest of Si-nanowires (Si-NW) has been developed for the isolation of biomolecules. Furthermore, the specific partition of target analytes and desalting is included. Consolidation of the forest of Si-NW improved the geometrical surface area of the microchip. Biomolecule isolation is contemplated utilizing target analytes such as haemoglobin and enhanced green fluorescent protein (eGFP) and the ligands, namely anti-haemoglobin and anti-eGFP [55]. The majority of the high throughput productivity and processes demand purification as a pivotal downstream processing operation, which is a bottleneck step contributing up to 90% of the total production cost. Highly efficient purification techniques need to be applied for achieving a substantial yield of proteins and nucleic acids in high grade. The past two decades have seen an upsurge in cutting-edge developments of parallelization and complete mechanization of purification processes into the microminiaturized system. The microchannels hollowed out in the microfluidic chips serve as pathways for injected samples containing nucleic acids, therapeutic proteins and enzymes during multiple in-line purifications in bioanalysis. Nowadays, multiple microchips are coupled in series to clarify, pre-purify, purify and dialyze the sample in a single run, intensifying the process [24].

4.3.3 Solid-Phase Extraction

Solid-phase extraction (SPE) is a versatile domain used alone or often integrated with other processes like a polymerase chain reaction inside a microfluidic device, as depicted in Fig. 4.3. Here, the microchambers moulded from polymers PDMS, PMMA, or silicon wafers consist of a single inlet hole or multiple inlet holes, extraction domain, where the rinsing-elution phases take place and, the outlet port.

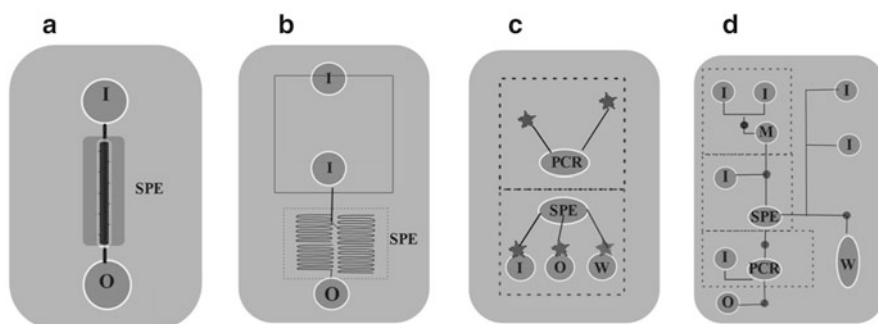


Fig. 4.3 Schematization of chip structures from a simpler to a more complex design. (a) Chip with a simple linear structure. (b) Chip with a coil-shaped microchannel. (c) Chip with electrokinetic motion. (d) Chip with a multi-domain design

A single inlet hole is preferred when samples are preloaded with a syringe, while multiple port devices usually have an automatic pump system. The microchip structure may vary according to the sample composition, type and extraction protocol. A typical design has a single microchannel, a T-shaped or convoluted channel (providing a greater surface area and mixing) packed with a capturing material in the form of filtering units, matrices, or functionalized surfaces [56]. The most widely used solid supports are silica based in the form of beads, immobilized particles, micro-posts, micropillars, columns, sol-gels and hybrid sol-gel/silica bead phases. Apart from these, phases that have gained considerable significance are organic polymer monoliths, photoactivated polycarbonates and aluminium oxide membranes [57]. A significant drawback of this approach is the limited surface area available for target analyte adsorption.

Nucleic acid purification is crucial because of the minute target analyte, complex samples, and excessive fragmentation. Any of these factors may introduce contamination in the downstream DNA or RNA, eventually entangling the amplification process, blotting techniques and assays [58]. Notably, this has been overcome, and a high elution yield has been attained by fabricating expendable microfluidic devices using micro-solid-phase extraction (μ SPE). The microchambers are designed with chitosan functionalized on microfabricated posts using crosslinker (3-glycidyloxypropyl)trimethoxy silane (GPTMS). Ethanol is passed through the chambers to increase surface wettability and later to wash off excess crosslinkers. A second wash with aqueous NaCl removes excess chitosan. A series of elution buffers mediate the entrapment and release of nucleic acid, so purification is based on the pH value. Thereby, at a low pH value, chitosan interacts electrostatically with negatively charged DNA due to its positive charge (below pK_a 6.5). Higher pH values regain their deprotonated state when the interaction is controlled through buffer exchange [58].

Another typical usage of solid-phase extraction in nucleic acid purification has been demonstrated in convergence with hand-operated microfluidic devices. Here, the implement is multi-layered, that is, a cover layer, thin membrane layer, pneumatic channel and fluidic channel layer. The pneumatic and fluidic layers are fabricated using photolithography, the cover layer by curing PDMS on top of the silicon wafer, and the membrane layer by spin coating a blend of the curing agent and PDMS in the ratio of 1:7. The plasma treatment results in an assembled layer, which is then incubated, and silica microbeads are injected via injection hole and sealed. The device incorporates a reciprocator, switching valves and microfluidic dispenser, which is finger actuated. The valves are operated using buttons in a programmed order. The first button allows the injection of reagents into the microbead column. The second button initiates the agitation of silica microbeads, in turn generating a reciprocating flow. Washing buffer and elution buffer are subsequently added during the process. The technique has been shown to accelerate the recovery rate by increasing the number of washes and agitation and decreasing silica beads' size [59]. These device designs are flexibly applied for the purification of both RNA and DNA.

Another interesting methodology followed for RNA capture and purification is centrifugally controlling the microfluidic device employing solid-phase extraction. The design consists of tetra-ethoxy orthosilicate treated glass microbeads as RNA capture beads forming a matrix with four reservoirs for washing buffer, elution buffer, RNA sample and collecting the sample. The washing and elution reservoirs are joined to microchannel containing bead beds by capillary and siphon valves. The RNA sample, washing buffer and elution buffer loaded into the reservoirs reach the bead bed by revolution per minute (RPM) control. After successfully capturing RNA (a high capture yield), the purified sample is collected in the final reservoir [60].

A combination of electrophoresis and magnetophoresis or solely one of the two can aid purification in microfluidic devices, especially DNA. The chip consists of the following wells: a positive electrode, sample input, elution and negative electrode wells. Solid-phase reversible immobilization magnetic beads coated with carboxyl can selectively adhere the DNA onto its surface and transfer it to the purified buffer upon the action of the external magnetic field. On the other hand, unbound and unwanted DNA molecules (primers and adapters) are driven in the opposing direction by velocity proportional to the external electric field and are removed successfully from the bead cluster. Magnetophoresis alone cannot achieve this velocity by simple diffusion, which is the major drawback of the method. Both magnetic and electric fields simultaneously can migrate the beads from one well to another [61].

4.3.4 Aqueous Two-Phase System

One of the best and most common approaches used for purification and enrichment of proteins and nucleic acids is the aqueous two-phase system (ATPS), also known as the aqueous biphasic system, where water is the primary solvent replacing other organic solvents typically utilized in the conventional liquid-liquid extraction, thus keeping native conformations of analyte intact. The aqueous phases can undergo parallel, slug, or droplet flow patterns depending upon which microchannels are shaped. A Y-shaped inlet (2-inlet) for concurrently injecting two aqueous phases (Fig. 4.4) or a ψ -shaped (3-inlet) microchannel with a third channel for the induction

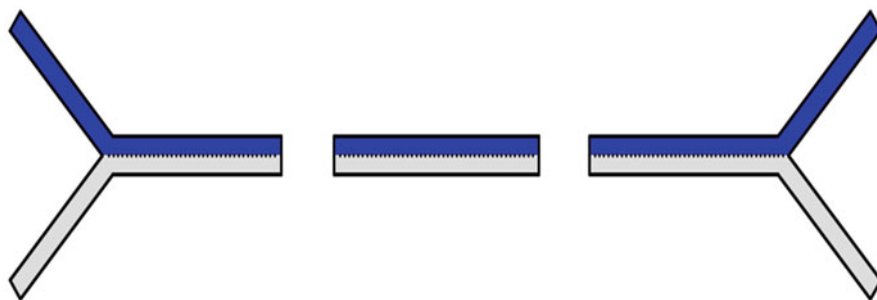


Fig. 4.4 Laminar flow—two inlets and two outlets

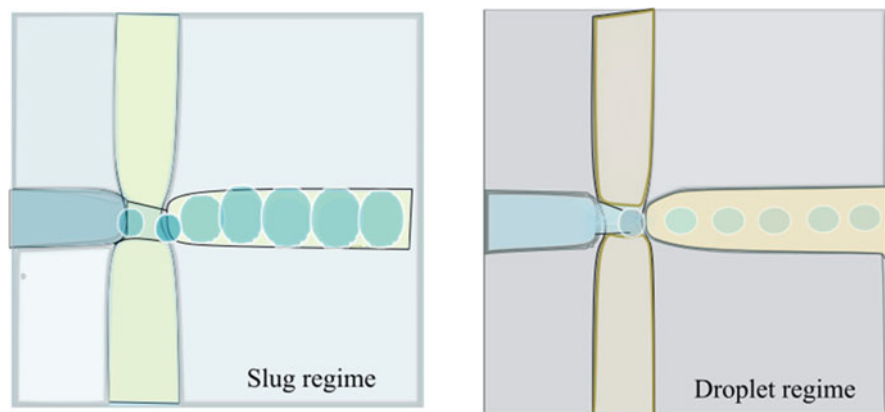


Fig. 4.5 Segmented flow—X-shaped configuration

of a second interface is typical for the laminar parallel flow pattern. Thus, a significant advantage of this pattern is highly efficient mass transfer due to the greater interfacial area and phase separation at the channel exit resulting in the recovery of target analyte-based phase and discard of impurity-based phase. A second conformation is the T shaped or X shaped (Fig. 4.5). Two immiscible phases merge simultaneously, producing instant slugs or droplets due to surface tension and shear forces between the two aqueous phases containing immiscible compounds, causing non-linearity. Here, the surface-to-volume ratio is much higher than the parallel flow. It can also incorporate membranes or micropillars to stabilize co-flowing streams. The two phases maintain a stable interface over the entire length of microchannels due to the interplay of Stokes flow and moderate interfacial tension [62].

Here, the partition occurs due to the preferential interactivity of the analytes and impurities towards respective phases, which sometimes is supplemented by external electric field imposition. Among various two-phase systems, polymer-salt and polymer-polymer are the most conventional aqueous combinations. Recently, polymer-surfactant, surfactant-salt combinations have proven to be successful in protein purification. The polymer-salt is favoured due to its more significant polar gradient between two phases, lower viscosity and faster attainment of thermodynamic equilibrium. These have been markedly applied to purify BSA, bacteriorhodopsin, immunoglobulin G (IgG), green fluorescent protein (GFP), β -galactosidase, protein A, insulin, genomic DNA and several other biomolecules [24]. ATPS is a high-throughput technique on a micro-scale rather than bench-scale liquid-liquid extraction. For instance, PEG/detergent (SDS) ATPS allows successful partitioning of proteins, by complementary solubilization, from impurities. As soon as the crude protein sample enters the central channel of a 3-inlet serpentine microchannel, it is hydrodynamically concentrated by the surrounding flowing streams and the contaminants and the unwanted proteins migrate to the PEG phase. In contrast, the proteins of hydrophobic nature remain in the detergent phase. The flow rate of two

aqueous phases is controlled by microsyringe pumps [63]. The loopholes of inherent partitioning can be circumvented by genetically tagging the desired protein, which can powerfully drive proteins into the PEG phase [62].

4.3.5 Bioinspired Biomolecule Purification

A bioinspired biomolecule purification is a promising approach inspired by naturally functioning biological systems, such as natural sponges and kidneys, to purify proteins such as β 2-microglobulin and lysozyme. The design of a serpentine microfluidic channel consists of two different materials, a porous membrane that separates two channels, that is, a sample channel from the dialysate channel, and the heterostructured nanoporous particles fixed at the bottom of the dialysate channel. The former functions as the filtering module for the sample liquid in the sample channel, and the latter is an active sorbent, both clearing out the macromolecules of any impurities. The porous membrane is usually super hydrophilic, sorbent and amphiphilic, such as polyamide membrane and polyacrylic acid-polystyrene divinylbenzene nanoporous particles. The sorbent interacts with the impurities through electrostatic, hydrophilic and hydrophobic forces [64].

4.3.6 Miniaturized Free-Interface Diffusion Devices

Hansen et al. developed the first microfluidic device for high-throughput screening of protein crystallization settings by miniaturizing and parallelizing the free-interface diffusion (FID) approach. This glass or the PDMS system includes valves and pumps (up to 480 valves in the current version) and can perform 144 crystallization experiments on the same chip in 25 nL reaction chambers. Since PDMS is gas permeable, it is possible to prime all of the wells with the help of only a few microlitres of the protein sample by manually pipetting different crystallizing agents. When the valves are opened, FID commences with well-defined interfaces in all of the chambers. The crystallizer must be small since molecular diffusion is the only way to mix the crystallizing chemicals and proteins. There are no buoyancy-driven convection instabilities at such small length scales that would cause unwanted mixing and disrupt crystal formation. Furthermore, lithography allows for accurate construction of the crystallization chamber geometries, allowing for experimentation with mixing kinetics without affecting the final state of equilibrium [65].

4.3.7 Droplet-Based Microfluidics

Ismagilov et al. proposed employing two-phase flow microfluidics to screen crystallization conditions of proteins. These devices take advantage of an inert oil stream's capacity to create aqueous nanolitre-sized droplets [66]. These droplets, which contain specific concentrations of crystallizing chemicals and proteins, operate as

miniature crystallizers with precise volume control (from about 0.1 to 100 nL) [67, 68]. The flow rates of numerous aqueous streams (protein, crystallizing chemicals) are continually altered before the droplets are formed. Using a low-frequency droplet formation (2 Hz for 7.5 nL droplets), they can control the concentrations within a 15% level of certainty. Once flow rates are stopped, various droplets with different crystallization conditions are created, which can be incubated either in the PDMS chip or in an external glass capillary. These approaches can be used to investigate various circumstances utilizing tiny amounts of eaten proteins (a few litres) [69]. The type of oil is a critical consideration in such research. For lengthy periods, per fluoroalkanes oils (PFP) prevent water from exiting the droplets (up to a few weeks), making the droplets ideal micro-batch reactors [68]. However, vapour-diffusion tests replicate water-permeable oils. Researchers held arrays of droplets with different compositions and varying chemical potential differences, which concentrates the protein plugs [70].

Later, they presented a more straightforward technique based on stored droplet arrays. Several 15 nL plugs holding various aqueous reactants are manually made in PFP and stored in a polytetrafluoroethylene (PTFE) tube. Gas plugs divide these droplets to prevent coalescence and water permeation between them. When put into a glass capillary, this array can be preserved for months. The array is initially kept in a PTFE tube and subsequently inserted into a PDMS T-junction to screen crystallization conditions. This chip allows the array to be combined with a protein stream flowing at a set rate. In the settings examined by these authors, the protein stream combines within the array's distinct plugs, and the resultant droplets are collected in a glass capillary for incubation. Using only 1 L of a model protein, these researchers examined 48 distinct crystallization settings offered by a commercial sparse matrix [70].

When crystallization hits are discovered through random screening, the goal is to optimize the crystallization by making minor adjustments to the circumstances. Zheng et al. suggested a hybrid method that included the ideas from the earlier works to accomplish simultaneous screening and optimization in nanolitre droplets [67]. The goal is to store an array of big drops (100–150 nL) containing various precipitants [70]. To avoid coalescence and pollution, all of the droplets get separated once more with the help of gas plugs. After that, the array is placed in a PDMS chip and flows under pressure. When paired with a protein and a buffer stream, the big plugs create lengthy segments that act as continuous streams, forming smaller droplets (15 nL) whose composition may be altered by continuously changing the flow rates. For incubation, these plugs are placed in a PTFE tube once more. Zheng et al. used a hybrid approach to accomplish as much as 1300 crystallization trials in 10 nL plugs using only 10 mL of sample, demonstrating that this technology is acceptable for handling membrane protein solutions. However, this approach necessitates a high level of control over the microfluidic characteristics of the droplet fluxes. To begin, a PDMS chip stores droplet arrays and manipulates them as lengthy streams. Second, to adjust the composition, constant modifications in flow rates must be implemented. Because thousands of distinct reactors can be produced in less than 20 min, indexing the droplets is critical. In Zheng's work, droplet indexing is accomplished by continuously adjusting the flow rate of the carrier fluid, which

results in a continuous change in their volume. Finally, to avoid evaporation during long-term storage in the PDMS chip, the input array and output droplets are held in long PTFE tubes [67, 70].

There are a few issues to consider while dealing with droplets. First, the PDMS device elasticity and injection systems frequently cause extended transients with pressure-driven flows, resulting in the consumption of significant amounts of samples (several litres) before stable droplet flows (to know the concentrations, this stability is required). However, more importantly, problems frequently arise when it comes to the mechanics of droplet production. Indeed, it necessitates excellent surface chemistry control and is highly dependent on various factors, such as viscosities, channel geometries and surface tensions. Even while recent research has captured the underlying principles of droplet formation, a thorough knowledge of droplet formation remains elusive. It may thus be difficult for a group lacking a solid understanding of two-phase flows and microfluidic expertise to form droplets, manage their traffic and merge them, mainly when dealing with membrane protein solutions or solutions with a wide range of characteristics (surface tensions, viscosity etc.). Ismagilov et al. contributed significantly to droplet microfluidics and the chemistry of the surfactants and oils employed in the studies. Indeed, they found fluorinated oils that were practically impervious to water and created oligoethylene glycol-capped perfluorinated surfactants that hindered protein adsorption at the droplet interface to replicate perfect micro-batch conditions. They also employed perfluoroamines, which have a high surface tension, to address membrane proteins.

4.3.8 Engineered Micro-batch Experiments

Another method is to use lithography techniques to microfabricate micro-batch. Juárez et al. created a ten by ten array of 5 μL volume-per-element device etched in a silicon wafer early on. Because silicon has excellent thermal conductivity and linear temperature gradients (usually between 15 and 35 $^{\circ}\text{C}$), the array of wells is silicon coated. With only 250 μL of protein solution pipetted into the wells manually, a microfabricated plate can screen for different temperature settings resulting in diverse nucleation and growth mechanisms. The fluids are not handled microfluidically, and microfabrication is solely employed to create precise geometries in specific substrates [71]. Zhou et al. recently presented a microfluidic solution for fluid handling, which does not require the usage of syringes or valves. The authors microfabricate a well-array (usually 150 wells of 20 nL) in PDMS, glass, or PMMA on a variety of substrates (poly(methyl methacrylate)). PDMS microchannels are constructed and oriented above these wells to prime them [72]. When PDMS is previously degassed, as recommended by Hansen et al., aqueous solutions plugged at the device's inlets spontaneously fill the chambers. Only 5 nL of protein sample fill all of the wells. Now, without using any syringes or valves, an array of crystallization conditions is constructed by aligning chambers filled with different precipitants using the same way. Even while the valves provide for more refined control of the mixing kinetics, mixing between precipitants and proteins happens via an accessible interface diffusion (FID) pathway, as in the

device of Hansen et al. [65]. Evaporation is vital in the above chips since extensive incubation durations are frequently necessary. Evaporation is prevented for months by layers of paraffin oil covering wells engraved on glass slides to overcome these challenges.

4.3.9 Determination of the Microfluidic Phase Diagram to Optimize Crystallization Conditions

In general, crystallization hits are found by searching for reactants that crystallize other proteins at random. The crystals' quality must then be improved by optimizing the conditions. Hansen et al. proposed a microfluidic method that consists of roughly three steps to justify such a strategy [73]. First, large-scale screening of a protein's solubility is carried out in a parameter space that includes multiple buffers and crystallizing agents, as well as varied protein concentrations. When instantaneous precipitation is detected, this solubility fingerprint reveals promising conditions. The kinetic extent of the metastability limit is determined by this screening, which is also known as super solubility rather than thermodynamic solubility [74]. In a second step, detailed phase diagrams around the favourable circumstances reported before are constructed to determine the target protein's super solubility limit. Conditions slightly below super solubility are suitable for the crystallization of the protein. Crystallization studies are then carried out under these circumstances, utilizing either the microfluidic FID described above or traditional micro-batch or vapour-diffusion techniques.

The creation of a microfluidic formulator has enabled this ambitious plan. Based on the PDMS multilayer technique, this complex device combines a microfluidic multiplexor with a rotary mixer. This chip creates mixtures from 32 reactants (proteins, polymers, salts etc.) in a 5 nL ring at precise known concentrations. After a few seconds, the rotary device mixes the reactants, and image analysis determines whether or not precipitation happens. In one day, about 4000 titration tests may be completed with only 8 L of a protein material. The formulator chip is also exploited to screen phase diagrams around the most promising conditions observed during the solubility fingerprint measurement. Each phase space, which has 72 possible combinations, is completed with only 100 nL of protein sample and aids in defining the metastable region's extent. This method has been used to crystallize a variety of proteins that are notoriously difficult to crystallize. In comparison to the traditional random procedure, this method considerably improves the possibility of producing crystals. It is also worth mentioning the device's high-throughput capabilities, which require less sample (a few litres) for more tests (thousands of titrations per day) [75, 76].

4.3.10 Kinetics-Based Passive and Active Control

Kinetics is critical in obtaining high-quality crystals for X-ray observations. It is especially true in the crystallization of protein, which is prone to metastability due to various polymorphs, habits and other non-equilibrium structures (e.g. clusters, gels) [77]. Microfluidics provides excellent potential for precisely following defined kinetic routes. The primary concern for long-term storage is evaporation, especially in microfluidic systems (droplets, micro-wells). Volume loss and concentration evolution induce incorrect calculation of supersaturations which may derail the whole crystallization process. While creating a crystal is frequently the primary purpose of a study, knowing the exact circumstances is necessary to replicate the crystallization conditions in other systems, such as vapour-batch diffusion. The preferred method to solve the evaporation problem is to promote it rather than prevent it. More precisely, by interacting microsystems with an osmotic bath, it is feasible to control the chemical potential by regulating the exchanges between the microsystem and the bath. As a result, incorporating an exchange membrane into microsystems is a critical challenge for technological advancement. While polymeric membranes are used in many microsystems for dialysis, concentration, filtration and other purposes, the role of a PDMS membrane is of particular relevance because it is the basic material of many microfluidic chips. PDMS, although being hydrophobic, is permeable to gas and liquids such as water. Since the 1960s, it has been widely employed in the industry for pervaporation. Evaporation through PDMS can be a severe drawback for long-term storage in PDMS microsystems devoted to protein crystallization, and humidity around the chip must be regulated. In addition, osmotic regulation across PDMS membranes is applied in various microsystems, with the most advanced combining three modules: formulation, addressing and concentration/dissolution cycles. During a crystallization experiment, osmotic regulation allows for fine control of the kinetic routes followed in the phase diagram [78].

The phase chip, developed by Fraden's group, is used to screen the phase diagram of multi-component aqueous systems efficiently. The two fundamental advances of this droplet-based microsystem are docking droplets into wells and contacting them through a thin layer of oil with a PDMS membrane and an osmotic bath beneath. Dry gas or water at a set chemical potential, adjusted with a dissolved salt (NaCl), flows through this osmotic bath. The authors of the two papers closely examine the device's functioning conditions: they can swell or concentrate droplets with great kinetic control. A typical swelling/shrinkage time is about 1 h. However, it varies depending on the ionic intensity of the bath, which may be quantified using a simple model. These osmotic conditions investigate phase diagrams of aqueous solutions such as polymer and salt in water; the latter system is effectively investigated with homogeneous phases, and liquid-liquid breakdown is seen. The chip is then used to study the crystallization of model proteins with neat kinetic control. In particular, the pathway used in the experiments allows for the formation of many nuclei (high supersaturations). The majority of them are then dissolved by osmotic swelling of the drop to select only a few of them, eventually producing good quality crystals in

terms of X-ray scattering. It is thus conceivable to decouple protein crystal nucleation and growth using an osmotic control [79, 80].

In a large-scale version of FID with valves that control the diffusion process and a membrane that provides osmotic regulation, analogue results based on the osmotic exchange were produced. It crystallizes a variety of proteins and figures out what the best conditions are. Furthermore, the experimental settings alter crystallization tendencies. One of the chip's key advantages is that it directly allows cryoprotectants to protect the crystal from X-ray damage. Lau et al. provide demonstrated membrane-controlled crystallization platform. A formulation stage, a droplet injector and a two-phase storage module are all integrated into this sophisticated device. The formulator estimates the limit of metastability by monitoring the appearance of a precipitate in a 5 nL combination of protein and reagents. This chip also enables the creation of nanolitre droplets from designed mixes and their storage in a storage module. The latter links to a membrane exchange zone that ensures the plug's chemical potential remains constant, unlike earlier device iterations where evaporation was an issue. The key strength is the real-time feedback on precipitation and the additional kinetic tuning of crystallization by active osmotic bath management [81].

Droplet-based crystallization tests are useful for manipulating a kinetic pathway via membrane exchange. The exchange of droplets in a train over the oil separating them is analogous but less effective. It is conceivable to alternate solution droplets crystallizing with droplets of brine utilized as a reservoir to concentrate the protein solution via diffusion over the oil spacer. The latter is crucial, as is the space between droplets, regulating the (slow) maturation process. However, in contrast to membrane exchange, the evolution is unidirectional, and the concentration process is not under active control. Aside from evaporation, droplets are also used in a kinetic process for crystal development known as seeding. It effectively separates the nucleation of seed from its subsequent growth in a separate bath with optimal circumstances. In general, high supersaturation is necessary for seed nucleation, whereas low supersaturation is necessary for orderly development, and the two conditions are frequently incompatible. Ismagilov's group uses this technique to stimulate the production of seeds in highly saturated protein and precipitant mixtures (nucleation conditions) and then transport the droplets into larger ones where the solution is diluted to reach orderly growth conditions [82]. Before lowering the supersaturation, the number of seeds can be optimized by adjusting the incubation time. As a result, the two phases are no longer linked, and problematic proteins can now be crystallized.

Since it incorporates optimized geometries (made by lithography) with small length scales, microfluidics eventually allows passive action on the kinetics through a smooth control of the mass transfers. The microfluidic FID is a good example. The geometry of the connecting chambers can tailor the path taken by the FID on its journey to the ultimate mixed state. In a recent study, researchers created channel networks to mimic counter-diffusion crystallization studies in a microfluidic format. The crystallization conditions gradients are calculated based on the channel geometries. Mixing of the compounds occurs only through molecular diffusion in all of these devices due to the tiny length scales, and the channel network's

architecture allows it to act on the mixing kinetics. Taljera et al. developed an evaporation-based crystallization platform to detect and optimize protein crystallization conditions using similar concepts. Microlitre-sized droplets carrying specified amounts of protein and reactants evaporate through a well-defined geometry channel in this device. The channel geometry allows for the fine control of the evaporation rate and, as a result, the kinetic pathway through the phase diagram. These scientists recently demonstrated that kinetic parameters (nucleation, crystal development) might be calculated from such experiments using kinetic models employing such tight control of evaporation and, therefore, on drying rates [83].

4.3.11 Crystal Harvesting Versus On-Chip X-Ray Diffraction

After obtaining a protein crystal, it must be harvested and mounted for X-ray measurements. Crystals are frequently flash-frozen in liquid nitrogen to reduce X-ray radiation damage and reduce thermal noise that causes undesired background. Crystals are usually immersed in a cryoprotectant solution before freezing to prevent them from shattering during this perilous process. Because the diffraction characteristics of the crystals can be altered throughout the harvesting and cryoprotection processes, these processes may be difficult to control. Since these microfluidic devices are not irrevocably sealed, standard crystal harvesting is often possible in the PDMS chips stated previously. However, *in situ* X-ray diffraction studies show promise in reducing harvesting disturbances. X-ray analysis may be performed directly on-chip in the PDMS devices created by Hansen et al., as shown recently. Cryoprotectant solutions were diffused into the crystal wells over time, and parts of the PDMS chip holding the crystals were frozen using liquid nitrogen immersion [84].

Crystal harvesting is *a priori* a highly risky process in droplet-based systems, and *in situ* data collection appears to be necessary. Zheng et al. show direct X-ray diffraction in the capillaries used to retain the droplets, but they also mention significant X-ray damage due to the lack of cryocooling. The same group is now looking into the advantages and disadvantages of *in situ* data collection in droplet-based systems. This reference thoroughly discusses critical topics such as capillary material (glass versus PTFE), absorption and diffraction background due to the oil phase, and radiation damages. These authors also mention that X-ray analysis on several droplets containing identical crystals may be useful in overcoming the challenges mentioned above.

4.3.12 Challenges

1. Microfabrication technologies such as high-resolution stereolithography, soft lithography and 3D print processes fabricate microfluidic chips. The majority of chips are for one-time usage only. They require specialized equipment (integrated pumps and valves, mixers, micro-fabricated micro-channels, or chambers

specially designed for multiple arrivals of pressurized gas). On the other hand, microfabrication procedures can be costly and time-consuming, necessitating specialized infrastructure and significant handling.

2. Chip materials now in use, such as silicone, hydrogels, glass and elastomers, are not entirely compatible with all crystallization solvents. For example, the most commonly used substance, PDMS (polydimethylsiloxane), is incompatible with organic solvents like acetone. Furthermore, owing to the permeability of the polymer, the solvent may evaporate.
3. It is difficult to change the arrangement because of the mandated channel network geometry, making it less accessible to non-experts.
4. Liquid qualities such as surface tension and flow viscosity are essential in microfluidic liquid handling. As a result, for each working fluid, system calibration is required. It can become quite problematic when dealing with liquids with high viscosity (i.e. polyethylene glycol) or low surface tension (i.e. detergent used for membrane protein solubilization). It is also the fundamental reason why surfactants are used in so many of today's droplet procedures. Surfactants, on the other hand, can interact with the solute molecules, affecting crystallization.

4.4 Microfluidics in Molecular Self-Assembly

Self-assembly in microfluidic devices is a route taken when it comes to the bottom-up approach in the field of nano- or micro-fabrication. Molecular self-assembly is the property of various individual components to self-form spontaneously into the desired shape. This shape gives the compound its function. In general, molecular self-assembly points to the spatial assemblage of nucleic acids or proteins (folding of proteins). However, polymers are made to fold in three dimensions to obtain the requisite structure [85]. Nanoparticles made from DNA have immense potential for various applications such as drug delivery and as a sensing element in various biosensors. Electrostatic interactions are responsible for the self-assembly of these components. This section explains the strategies for developing self-assembled structures using microfluidic devices.

Five main attributes determine the self-assembly of a system [86]. (1) The components involved in the self-assembly. The individual components interacting may be similar or practically opposing. However, the result of their interaction forms a more stable state than their individual existence. (2) The interaction or reaction during the self-assembly. The interactions are generally Coulomb and van der Waals forces, hydrogen bonds, or other hydrophobic interactions. Self-assemblies have coordination bonds. Apart from chemical bonds, complementarity in the interacting molecules' shapes also plays a critical role in self-assembly. (3) Adjustability of assembled molecules is critical in forming stable and ordered structures. The ability to disrupt bonds and reform new bonds in itself helps in obtaining structures with lower entropy. The ability to reversibly bond would imply that the force trying to disrupt the bond is comparable to the forces keeping them associated. (4) The environment in which the components react is critical. An ideal environment is

required for self-assembly to occur. (5) Ability of the interacting molecules to move. At least one of the interacting molecules should be mobile (although, in most cases, all the interacting molecules are in motion). Forces such as thermal motion, Brownian motion, gravitational force, friction and inertia become relevant to the required motion of the interacting particles. The attributes mentioned above are relevant in the self-assembly of particles as small as molecules to as large as galaxies [87].

Free-flow self-assembly and hydrodynamic co-flowing self-assembly are two main strategies for understanding self-assembly in microfluidic devices. The self-assembly occurs in a free-flowing system with the assistance of capillary interactions. Fluidic force, surface force and molecular force are responsible for the self-assembly process. Hydrodynamic co-flowing can produce three different flows: laminar flow, droplet flow and plug flow [88]. The type of flow depends on the capillary number and water fraction. The laminar flow coerces many structures, such as liposomes [89] and scaffolds [90]. Self-assembly of peptides has been known to man for a long time. Modifying peptides to obtain specific structures, ideal for various scientific techniques, has been performed in bulk systems. The same work can be done on a chip with microlitres of liquids. Toprakcioglu et al. has demonstrated a microdroplet array-based system for self-assembly of peptides [91]. Thousands of water-in-oil droplets determine the aggregation kinetics for proteins associated with Alzheimer's disease. Diphenylalanine was also employed to produce microfibrils using the same droplet-based method. A self-assembled monolayer on PDMS using 1-undecyl-thioacetate-trichlorosilane is developed for protein array experiments [92].

DNA origami is the technique in which many short strands of single-stranded DNA (ssDNA) self-assemble to form a two-dimensional or three-dimensional structure. Individual strands of DNA are intelligently designed to form unique pairs with another strand. The sequence of the strands determines the shape of the structure formed by DNA origami. A microfluidic device can be designed so that the process of repeated self-assembly of DNA can be performed. This microfluidic DNA origami 'factory' has reservoirs for DNA, linker ssDNA, sample holder etc. It will also employ fluid flow control components such as valves, pumps, separators and mixers. There will be provision for the control of temperature [93]. Applications of self-assembled DNA origami know no bounds. The nanostructures developed using these DNA strands find application in areas such as biosensing [94], catalysis [95], drug delivery [96], therapeutics [97], molecular motors [98], nanorobots [99] and scaffolds for tissue culture [100].

Self-assembly of polymeric nanoparticles and microparticles is an exciting and ever-evolving field. The application of ternary polymeric particles and spherical Janus in various optical and electronic devices has significantly increased. These particles can be produced with the help of microfluidic devices. Nie et al. demonstrated the creation of Janus particles using a microfluidic device. The parallel flowing immiscible monomers can be emulsified at controlled rates and cured using a UV source (Fig. 4.6) [101]. Latest studies have shown that Y-type microchannel produces Janus particles with significant and sharp interphases [102, 103]. A collaboration of sophisticated techniques such as surface-enhanced Raman scattering and

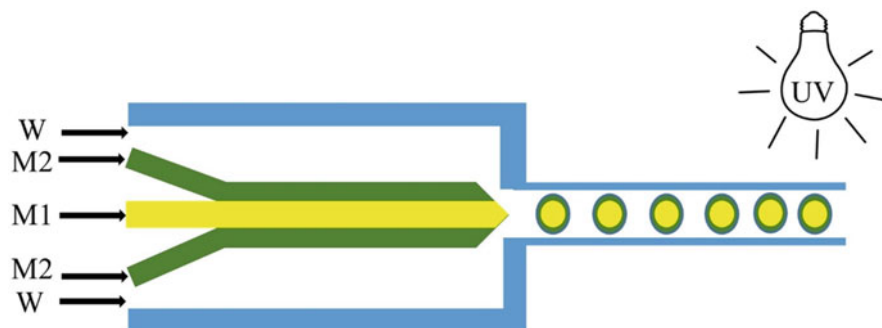


Fig. 4.6 Ternary droplets developed by emulsification of monomers M1 and M2 in aqueous solution of SDS

3D printing has employed Janus particles as a substrate for the quantitative detection of various biomedical analytes [104]. The self-assembly of polymeric nanoparticles does not stop with the Janus. Hydrophobically modified chitosan is used to make nanoparticles with the help of a T-shaped channel. The flow rate and mixing regime is controlled to produce a chitosan-based carrier for the transport of Paclitaxel. Paclitaxel is mixed with the chitosan in the device as a core flow to create drug encapsulated chitosan nanoparticles [105]. Another example of polymeric microparticle self-assembly is the synthesis of the amphiphilic microparticle. The device has a Y-shaped channel wherein the patterns for wedges are embedded. A hydrophilic stream is introduced through one channel, and a hydrophilic stream is introduced through the other channel. The wedge patterns are such that half of the wedge is made with a hydrophobic substance while the other half is a hydrophilic substance. The wedges, due to the positioning of the hydrophobic and hydrophilic layers, self-assemble to form a particle [106].

The latest studies employ self-assembly and microfluidics to their core to produce particles specifically of different applications. Nanoprecipitation using the microfluidic device creates polymeric nanoparticles for drug deliveries [107]. Permanent micromagnets are formed by self-assembly using microfluidics to apply cell sorting [108]. MacFarlane et al. developed π -conjugated polymers for electronic applications [109]. Polymeric self-assembled micelle was developed by microfluidic application for nanoscale drug delivery [110].

4.5 Microfluidics in Chemical Biology: Interesting Applications

Chemical biology and microfluidics hand in hand form an incredible platform of which applications have no bounds. Directly or indirectly, it spans almost all the fields of science. In this section, some of the exciting applications of chemical biology are elaborated.

The flow of micro amounts of fluid is a typical feature of a microfluidic device. These laminar flows are carriers of many components such as proteins, small molecules, growth factors, enzymes and other substances [111]. Flows in microfluidic devices enable the creation of a chemical gradient, exploited for various applications. Microfluidic devices with a continuous gradient of epidermal growth factors, platelet-derived growth factors, and fibroblast growth factor 2 differentiate neural stem cells [112, 113]. The development of cells in a natural environment depends on a gradient of substances such as proteins and small molecules rather than the discrete amounts provided *in vitro*. It is difficult to mimic these types of concentration gradients on a macroscale. O'Grady et al. developed a platform using hydrogel channels to supply many morphogens to the system [114]. Although this study is at a macroscopic level, it can be easily translated to a microfluidic device [115]. The chemical concentration gradient can be linear or temporal. Lin et al. developed a microfluidic device that can generate different chemical gradients by controlling the flow of solutions entering the mixer [116], giving better flexibility in developing devices that can model various diseases. Microfluidic devices can also be used to provide pH gradients. This has been used to create microfluidic devices for making chitosan membranes [117]. A temperature gradient is yet another characteristic gradient that is important, especially in the field of biology. Shah et al. demonstrated a microwave-based microfluidic device that can give rapid temperature gradients [118].

Advances in fabrication techniques and enhancement of wisdom in chemical biology have led to the creation and development of complicated devices such as the polymerase chain reaction (PCR) on a chip. In 1998, Kopp et al. developed a continuous-flow PCR on a glass microchip with various time zones [119]. Since this demonstration, many researchers have worked tirelessly to develop many improved versions of PCR-on-chip. The improvement may be in terms of engineering or based on its application. Ahrberg et al. broadly categorized PCR as space domain PCR, time-domain PCR, isothermal nucleic acid amplification, digital PCR and commercial PCR [120]. A space domain PCR is a device where the sample is transported through a defined microfluidic channel, wherein the temperature depends on the channel's position in the device. The channels can be serpentine [121, 122], radial [123], oscillatory [124] and heating/cooling systems [125]. A time-domain PCR has a temperature change through heating or cooling while keeping the sample stationary. The heating or cooling can be done by using various tools such as infrared rays [126], lasers [127], or even fans [128]. In isothermal nucleic acid amplification, as the name suggests, the amplification occurs at a constant temperature. Loop-mediated isothermal amplification was demonstrated using multiple primers [129–131]. Digital PCR divides a bulk sample into thousands of sub-samples [132]. The amplified sub-samples have a wide range of applications. It can find specific scarce nucleotides [133]. Thus, digital PCRs detect DNA contaminants in minor quantities [134]. Multiplex PCR is yet another application of digital PCR [135, 136]. Commercial PCR devices are devices with PCR unified into commercial thermal cycles [137, 138]. The research curiosity and advancement in engineering techniques have bought PCRs with improved efficiency and less

complex designs. Devices are further being refined to meet the required applications. Currently, self-propelled continuous-flow PCR [139] and droplet-based digital PCR [140] are developed for various applications.

Single-cell sequencing of DNA and RNA is a promising area with a wide range of applications. The significant outcomes from microfluidics are identifying rare anomalies in cells, studying cell subpopulations, identifying pathogens and studying various diseased cells. Digital microfluidics creates platforms that allow flow based on the design of wells and channels (passively) [141]. Smart-seq2 [142] detects RNA. Zilionis et al. established a fast and efficient method using droplet microfluidics [143]. Hydrogel beads with barcoding primers immobilize the cells. The cells are lysed following which reverse transcription is used to bar code the RNA in it. After this step, all the materials come together once the cells are broken open. Now, cDNA library processing for next-generation sequencing follows. Pellegrino et al. demonstrated a similar single-cell DNA sequencing [144]. Using microfluidic platforms enables comparatively easy sequencing of tens of thousands of individual cells in a matter of hours with very little human intervention. It has direct application in the detection of cancers at very early stages.

4.6 Conclusion

An era is at the horizon where microfluidics is inevitable to the field of science. Microfluidics has found applications in nanotechnology, medicine, chemistry, material science and physics. The use of microfluidics has brought about a system where devices are fabricated with significant dexterity. It has enabled the development of user-friendly systems that put the life of researchers at ease. Microfluidics in chemical biology is proliferating as it requires only minute quantities of samples and reagents, which increases the device's functionality. Even though microfluidics in chemical biology is a significant asset, it comes with its challenges. A major drawback in using microfluidics in chemical biology is the adsorption of material onto the channel walls. Since the surface area of the wall is notably more considerable, the possibility of adsorption is more. Research is happening at a solid pace to develop novel materials to circumvent this issue. Another critical disadvantage in using microfluidics is fouling. Fouling can reduce or even completely block the flow in a channel. Studies are conducted in designing devices that evade this issue. Even though there are disadvantages to using microfluidics for chemical biology, they are belittled regarding the advantages these devices provide. With the motivated and robust research in the field, the cons will be addressed and microfluidics will be the future in the sciences.

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