Colloids and Surfaces B: Biointerfaces 201 (2021) 111633

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Advances in microfluidic synthesis and coupling with synchrotron SAXS for continuous production and real-time structural characterization of nano-self-assemblies

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ARTICLE INFO

Keywords:
Nano-self- assemblies
Microfluidics
In situ characterization
SAXS

ABSTRACT

Microfluidic platforms have become highly attractive tools for synthesis of nanoparticles, including lipid nano-self-assemblies, owing to unique features and at least three important aspects inherent to miniaturized micro-devices. Firstly, the fluids flow under controlled conditions in the microchannels, providing well-defined flow profiles and shorter diffusion lengths that play important roles in enhancing the continuous production of lipid and polymer nanoparticles with relatively narrow size distributions. Secondly, various geometries adapted to microfluidic device designs can be utilized for enhancing the colloidal stability of nanoparticles and improving their drug loading. Thirdly, microfluidic devices are usually compatible with *in situ* characterization methods for real-time monitoring of processes occurring inside the microchannels. This is unlike conventional nanoparticle synthesis methods, where a final solution or withdrawn aliquots are separately analysed. These features inherent to microfluidic devices provide a tool-set allowing not only precise nanoparticle size control, but also real-time analyses for process optimization. In this review, we focus on recent advances and developments in the use of microfluidic devices for synthesis of lipid nanoparticles. We present different designs based on hydrodynamic flow focusing, droplet-based methods and controlled microvortices, and discuss integration of microfluidic platforms with synchrotron small-angle X ray scattering (SAXS) for *in situ* structural characterization of lipid nano-self-assemblies under continuous flow conditions, along with major challenges and future directions in this research area.

1. Introduction

In the development of nanocarriers for drug delivery, diagnosis and bioimaging, there is a growing interest, particularly in the last three decades, in exploring the possible use of various lipid nanoparticles (LNPs) including liposomes, solid lipid nanoparticles, and non-lamellar liquid crystalline nanoparticles, mainly cubosomes and hexosomes [1–7]. The attractiveness of such lipid nano-self-assemblies for delivery applications of drugs, antimicrobial peptides, siRNA, and DNA relies on their unique structural features and the biological relevance of their major lipid constituents [1–4,8–11]. They are well-suited to serve as nanocarriers owing to the capability of loading therapeutic compounds, particularly for solubilizing poorly water-soluble drugs, improving their bioavailability, and minimizing adverse effects [1–5]. They are also attractive for protecting the loaded drugs and peptides from degradation, and improving their distribution and intracellular penetration [1–3].

In preparation of lipid nanoparticulate formulations, including liposomes, which are the most used lipid nanoparticles in the development of drug nanocarriers, the application of most traditional batch methods is associated with poorly-controlled environmental conditions, resulting in a significant variability in nanoparticle size and size distribution [12–15]. Consequently, these methods often require typical implementation of multi-step pre/post procedures, including hydration, vaporisation of organic solvents, emulsification by means of ultrasonication or microfluidization, and post-processing steps such as extrusion [16–18]. To continuously produce nanoparticles with controllable sizes and maintain a high standard, simple and powerful

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https://doi.org/10.1016/j.colsurfb.2021.111633
Received 20 November 2020; Received in revised form 3 February 2021; Accepted 15 February 2021
Available online 18 February 2021

microfluidic synthesis methods have been developed, where control of flow and mixing conditions of injected fluids into well-defined microfluidic channels allows for their manipulation in geometrically constrained microenvironments [15,18]. Compared to conventional batch (discontinuous) methods, the high controllability of the flow and mixing conditions in these platforms allows continuous synthesis of uniform (monodispersed) nanoparticles [19,20] not only with desired morphology, surface charge, drug loading, and drug release kinetics [21,22], but also with a relatively short production time, and a minimal consumption of lipids, drugs, and chemicals [16,23,24]. In addition to such capabilities, reasonable costs of raw materials and portability of microfluidic devices are important issues to be considered [16,17,24,25]. These platforms are typically fabricated from light-weight and inexpensive raw materials.

Over the last decade, advancements in microfabrication techniques for efficient continuous production of lipid and polymer nanoparticles include development of various channel geometries and introduction of designs based on hydrodynamic flow focusing [15,26–30], droplet-based methods [31,32], and controlled microvortices [33,34]. As the nanoparticle size characteristics may affect the therapeutic outcomes and clinical translations of drug nanocarriers [4,17,18,21,23,24,35–38], microfluidics have gained a fundamental importance as platforms for the continuous production of nanoparticles with controllable sizes. For instance, it was reported that the use of monodispersed liposomes with sizes around 100 nm is associated with improved cellular uptake, and enhanced therapeutic outcomes [35,36]. Depending of the route of administration, we should be bear in mind that the mean sizes, size distributions, surface characteristics, and shapes of nanoparticles dictate the therapeutic efficacy and modulate the biological performance. For instance, it was reported that the size plays a predominant role in modulating the accumulation of nanoparticles within tumors. Here, nanoparticles smaller than 200 nm have a longer circulation time and an improved passive tumor accumulation *via* the enhanced permeation and retention (EPR) effect after extravasation through the leaky endothelium; whereas realtively larger nanoparticles can be recognized and eliminated faster from the blood circulation by the reticuloendothelial system (RES) [18,39,40].

Another interesting recent application focuses on the combination of specially designed microfluidic platforms with synchrotron small-angle X ray scattering (SAXS) for real-time detection of dynamic structural alterations occurring during the continuous production of nano-self-assemblies, or on their exposure to biologically relevant fluids, or buffers containing ions or drugs [16,20,41–44]. In addition to real-time monitoring of involved dynamic structural transitions during continuous production of lipid nanoparticles in microfluidics, this method has great potentials in different basic and applied research studies on the behaviour of nano-self-assemblies and other nano-objects under continuous flow conditions and confined geometries. For instance, it is attractive in future investigations for conducting online studies on protein aggregation, *in situ* characterization of structural alterations in nano-self-assemblies occurring due to chemical or enzymatic reactions, and online studies on encapsulation of proteins, functional foods, peptides, or drugs to nano-self- assemblies under continuous flow conditions. In addition, future integration of techniques including analytical tools and nanoparticle post-production methods into single microfluidic devices may pave the way for designing efficient microfluidic platforms that allow a precise control over the continuous production process of nanoparticles, and real-time feedback on the generated nano-self-assemblies.

In this contribution, we focus on recent advances and developments in synthesis of lipid nanoparticles and giant uni-lamellar vesicles (GUVs) by employing microfluidic platforms based on hydrodynamic flow focusing and droplet-based methods, respectively. In addition, this review provides an overview on coupling specially designed microfluidic platforms with synchrotron SAXS for *in situ* structural characterization of nano-self-assemblies under continuous flow conditions, and describes major challenges and future directions in this research area.

2. Milestones in using lipid nanoparticles in the development of drug nanocarriers

Depending on composition and lipid type, lamellar and non-lamellar liquid crystalline phases or micellar solutions can be generated on exposure of surfactant-like lipids such as phospholipids, unsaturated monoglycerides, and glycolipids to excess water at ambient temperatures [45–48]. With an attempt of gaining insight into the effects of pressure, temperature, pH, inclusion of guest additives such as drugs, peptides, and functional foods, we have witnessed in the last three decades a significant increase in number of research investigations on phase diagram constructions and structural characterization of various binary, ternary, and multi- component lipid systems [49,50]. There is also a growing interest in utilizing these self-assemblies, particularly, lamellar and non-lamellar liquid crystalline phases and their corresponding nanodispersions, including liposomes, cubosomes, and hexosomes, for drug delivery applications [51–53]. For further details on self-assembly of surfactant-like lipids on exposure to excess water, including the predominant role of bending energy on modulating the structural features of self-assemblies, and the use of critical packing parameter (CPP), known also as the molecular wedge shape factor, for predicting the self-assembled nanostructure type, the interested reader is directed to other reports on this topic [45–50].

As illustrated in Fig. 1, the milestones in utilizing lipid nanoparticles, particularly liposomes, as nanocarriers for drug delivery and bioimaging date back to 1964 with the introduction of liposomes by Bangham and Horne [54], and the reported first study demonstrating their use for drug delivery in 1971 [55]. Since the introduction of Doxil® [56], the first FDA-approved liposomal formulation for delivering of the anti-cancer drug doxorubicin, different liposomal formulations are FDA-approved or under clinical evaluations over the last few decades [1,2,4,5]. There are currently more than 18 FDA-approved liposomal formulations in the market for treating, among others, cancer, infectious diseases, and age-related macular degeneration [5,57]. In the development of liposome formulations as vaccines and for gene delivery applications, different liposomes based on cationic lipids (CLs) were found attractive for use [58–60]. Through electrostatic interactions with negatively charged nucleic acids, these CLs form complexes, known in literature as lipoplexes, that facilitate gene transfection *via* endocytosis [61]. Compared to viral vectors, CLs have more advantages such as low immunogenicity, low toxicity, and relatively easy production [62]. In addition to lipoplexes, there is also a growing interest in the development of lipopolyplexes from CLs. These nanoparticles consist of protamine-based polycations and DNA to overcome the major problem of inadequate DNA condensation [63]. The first generation of these lipopolyplexes was introduced more than 30 years ago [58,64]. While liposomal formulations are the most investigated lipid nanoparticles and continue to be as first choices for various drug delivery applications, other lipid nanoparticles are suggested as attractive platforms for drug delivery in the 1980s and 1990s. For instance, niosomes, which are liposomal nanodispersions based on non-ionic surfactants, were introduced in late 1980s to overcome some limitations associated with the use of conventional liposomes based on phospholipids such as pH-related toxicity, low stability, and sterilization [65–68]. Solid lipid nanoparticles (SLNs) and lyotropic non-lamellar liquid crystalline nanoparticles (LCNPs), mainly cubosomes and hexosomes, have also gained a growing attention since the 1990s. The latter lipid nanoparticles were introduced to the literature by Kåre Larsson and co-workers [69,70]. Among LCNPs, cubosomes and hexosomes are the most investigated nanoparticles. They internally consist of inverse bicontinuous cubic (Q_2) and discontinuous hexagonal (H_2) phases, respectively.

It is interesting to read these days during the pandemic on the development and urgent authorisation of use of two different mRNA

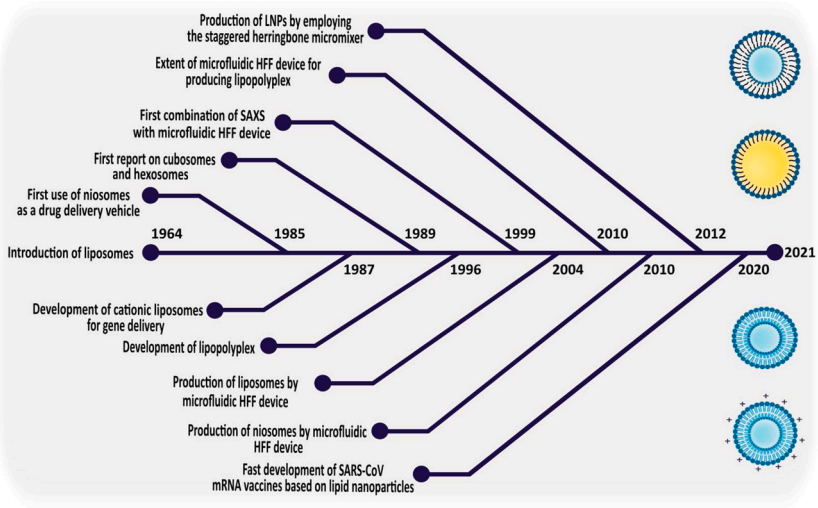

Fig. 1. Milestones in the development of lipidic nano-self-assemblies [54,58,64,65,69]. Milestones related also to first studies reporting on the use of hydrodynamic flow focusing (HFF) microfluidic platforms for continuous production and *in situ* SAXS characterization of different nano-self-assemblies are also mentioned [15,29,73–75,150].

packaged Covid-19 vaccines based on lipid nanoparticles, namely Moderna and BioNTech/Pfizer Covid-19 vaccines. This achievement is highly important when taking into account that there is no earlier approved mRNA vaccine for any disease [71]. FDA and European Medicines Agency (EMA) have granted Emergency Use Authorization (EUA) for both vaccines [72]. In the early development stage of these vaccines, other vaccines under preclinical or clinical investigations, and vaccines in preparation, microfluidic platforms are attractive for use not only for continuous production of monodispersed lipid nanoparticles, but also for controlling the flow conditions to ensure efficient mixing processes and produce relatively large quantities through use for instance of several microfluidic devices in parallel [73].

3. Microfluidic synthesis of lipid nanoparticles with emphasis on engineering aspects

3.1. Hydrodynamic flow focusing (HFF)

Although the HFF microfluidic method was developed earlier, the first report on its implementation in a controlled formation of liposomes was published in 2004 by Jahn et al. [15]. In this method, an organic solution (typically an ethanol solution) containing a single lipid or a lipid combination is passed through the center inlet channel, while an aqueous solution is injected into two side inlet channels. Thus, the center stream is hydrodynamically focused through a control of the flow conditions into a narrow sheet by the two aqueous side streams at the cross junction of the microfluidic chip. Here, it is important to take into account that the nanoparticle formation on mixing the center and side streams is a diffusion-controlled process [15]. On exposure of the organic solution to excess water, the organic solvent (typically ethanol as mentioned above) and water molecules start to mutually diffuse, leading to a decrease in the solubility of lipids under continuous flow conditions. This is associated with a simultaneous initiation of self-assembly of the lipid molecules in excess water, leading eventually to the generation of nano-self-assemblies with controllable sizes. It is worth noting, therefore, that the nanoparticle continuous production process is mainly affected by the degree of hydrodynamic focusing and the center stream width [15]. In this context, an increase in the flow rate ratio (FRR), which is defined as the flow rate ratio of the aqueous streams to the organic solution stream, is associated with a relatively high focusing degree, leading to a decrease in the center stream width [15]. In general, shorter diffusion lengths allow for controlled and reproducible mechanical and chemical conditions across the center stream width, leading to a decrease in lipid solubility and enhancing the continuous production of relatively small nanoparticles with a narrow size distribution [18]. As liposomes and other lipid nanoparticles continuously produced through a diffusive mixing, including mutual diffusion of water and water- soluble organic solvent molecules at the interface, it is possible for microfluidic synthesis in 2D HFF microfluidic platforms to estimate the required diffusive mixing time (τ_{mix}) by applying the following equation [74]:

$$\tau_{mix} \sim \frac{w_f^2}{4D} \approx \frac{w^2}{9D} \frac{1}{(1+\mathrm{FRR})^2} \quad (1)$$

where D is the solvent diffusivity, w_f is the focused stream width, and w is the microchannel width.

It was also reported that the use of multi-inlet HFF device, possessing deep channels, maintains a more homogeneous velocity profile across the channel height [18]. This allows for a more precise control over the mean nanoparticle sizes and size distributions. Koh et al. (2010) extended the application area of microfluidic HFF technology and produced lipopolyplex nanoparticles with a narrow size distribution using such multi-inlet HFF device [75]. To investigate the effect of sonication on liposome synthesis in a microfluidic channel, Huang et al. (2010) immersed the used microfluidic device in a bath sonicator during the continuous liposome formation process, and reported on a dramatic decrease in the mean sizes of liposomes as compared to liposomes prepared without sonication [27]. Here, it is worth noting that ultrasonication is one of the most commonly used post-treatment methods in conventional bulk production of lipid nanoparticles, particularly it is efficient for converting relatively large multi-lamellar lipid vesicles (MLVs) into smaller uni-lamellar vesicles (ULVs) by a cavitation that includes the formation, growth, and collapse of bubbles in a dispersed liquid [76]. It is expected, therefore, to enhance further a reduction in the mean nanoparticle sizes on combination of microfluidic device with a batch sonicator. In another study, a simple co-axial microfluidic device with two inlets and one outlet (Fig. 2A) was fabricated by using polydimethylsiloxane (PDMS) polymer and two circular glass capillaries for the continuous production of monodisperse liposomes consisting of ULVs [77]. In this device, a phospholipid-ethanol solution is passed through a core region, while a drug-containing buffer is injected and passed through an annular region. In a similar method, it was shown that the liposome size can be modulated by changing the orifice diameter of the inner capillary, where the organic phase is injected [78].

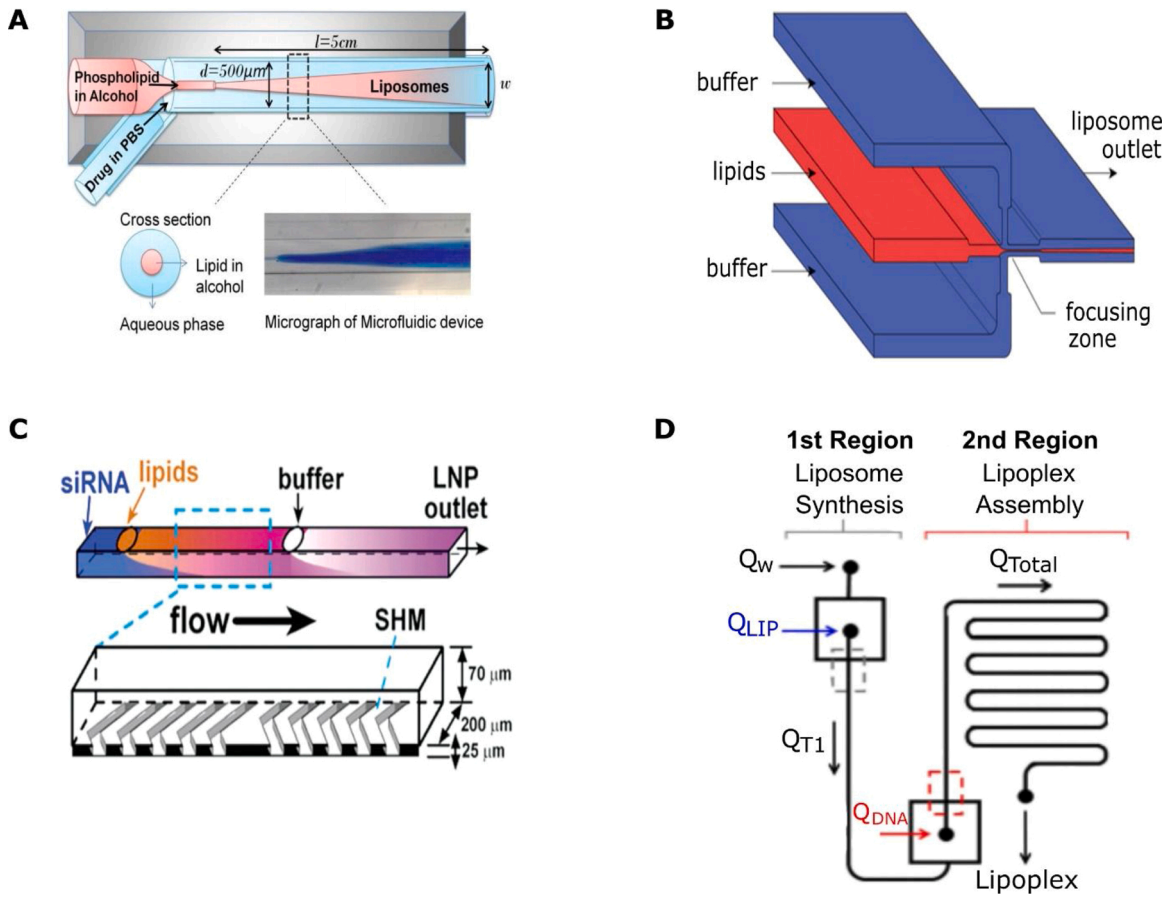

Fig. 2. **(A)** A co-axial flow focusing microfluidic device (*Reprinted with permission from Phapal and Sunthar, 2013. Copyright (2013) Elsevier)* [77]. **(B)** A vertical flow focusing (VFF) device, possessing a high channel aspect ratio (*Reprinted with permission from Chen et al., 2019. Copyright (2019) WILEY-VCH*) [94]. **(C)** Production of siRNA-loaded LNPs by using a microfluidic channel with groove structures for facilitating relatively a rapid mixing (*Reprinted with permission from Chen et al., 2012. Copyright (2012) American Chemical Society)* [115]. **(D)** A microfluidic system for one-step continuous production of lipoplexes (*Reprinted with permission from Balbino et al., 2017. Copyright (2017) Elsevier*) [123].

For the continuous production of liposomes, Pradhan et al. (2008) introduced also a simple syringe pump-driven microfluidic injection device by using laboratory instruments such as elbow connectors, syringes, and needles [79]. In this study, the use of relatively small needle at a high flow rate results in the formation of small liposomes with mean sizes of about 49 nm. This is achieved owing to the high velocity difference within the connector between the lipid stream and the aqueous stream, leading to an efficient dispersion of the lipid molecules in excess water [79]. Jahn et al. (2010) designed two microfluidic HFF devices with 10 μm and 65 μm mixing channel widths to investigate the effect of device geometry on the liposome size [80]. It was reported that liposomes with similar mean sizes (approximately 25−75 nm) were produced at different FRR values (from 6 to 48) in both microfluidic HFF devices [80]. Tessier et al. (2016) reported on the use of pseudo-Y microchannel as a more effecient platform than T-shaped microchannel for the continuous production of niosomes smaller than 91 nm [81]. Zizzari et al. (2017) reported that microfluidic HFF devices with inlets placed perpendicular to the chips provide a more efficient mixing as ethanol stream is more hydrodynamically focused as compared to microfluidic HFF device with inlets integrated into the chip with an angle of 45° [82]. In this study, liposomes with a mean size of about 92 nm were produced in the 45° chips with a higher PDI (0.35), while larger liposomes (a mean size of 120 nm) with a lower PDI (0.27) were formed in the 90° chips [82]. In two recent reports, Chang's research group introduced multi-hydrodynamic focusing techniques involving stencils consisting of a number of tiny pores (5 μm in diameter) for the continuous production of monodispersed liposomes [83], and dual-loaded liposomes with curcumin and catechin [84].

In most of 2D HFF microfluidic devices, the center stream is focused in the horizontal direction [85–87]. On the contrary, more advanced devices with 3D focusing allow for the presence of more focused and highly uniform center streams in different directions [88–91]. Therefore, 3D focusing devices may allow the formation of LNPs with relatively narrower size distributions (lower PDI values) as compared to corresponding conventional 2D focusing microfluidics. To allow 3D hydrodynamic focusing, Hood et al. (2014) reported on the design of an annular coaxial device composed of seven identical glass capillaries arranged in a relatively larger capillary, where the organic and aqueous solutions can be passed through the inner and outer capillaries, respectively [92]. This 3D-HFF system enabled the continuous production of liposomes with relatively small sizes (in the range of 53−66 nm) at a high production rate [92].

Although HHF microfluidic systems offer remarkable advantages over conventional bulk methods, these systems still suffer from relatively low-throughput. This is mainly due to application of limited volumetric flow rates in the continuous process [93]. This drawback restricts their uses in preclinical and clinical applications, where relatively larger quantities of nanoparticles are needed [93,94]. To overcome this limitation, vertical flow focusing (VFF) devices with focusing channels that have high aspect ratios (height/width ratios) were developed and allowed nanoparticle microfluidic synthesis in larger quantities by applying larger volumetric flow rates than those of 2D HFF microfluidic devices [93]. Compared to 2D HFF microfluidic devices, the lipid stream (a stream of a single lipid (or a lipid combination) dissolved

in an organic solvent) in VFF devices is focused by aqueous streams above and below it (Fig. 2**B**) [94]. Different studies have shown that VFF devices enabled a high throughput production of monodispersed liposomes with tunable sizes (ranging from 80 to 200 nm) [93,94] at high production rates of 95 mg/h and 4 mg/min, respectively. As compared to the production rate of 2D HFF microfluidic devices (<10 mg/h) with typical aspect ratios of 0.5:1 and 5:1, the VFF devices provide more attractive platforms for producing larger quantities of nanoparticles with controllable sizes [93]. In another study, Michelon et al. (2017) [95] introduced a different definition for the channel aspect-ratio (a width/height ratio instead of a height/width ratio) [93,94], and reported on the continuous production of monodisperse liposomes with a significantly low PDI at a hight production rate and a high aspect- ratio.

In addition to the introduction of VFF platforms, it was reported on the use of a double HFF microfluidic device with two consecutive flow-focusing regions that has a higher productivity level as compared to conventional single HFF platforms [96,97]. Such a high productivity level can be achieved through an increase in the diffusion surface area (contact zone) between the organic and aqueous solutions (enhancement of mass transfer), and an application of relatively high total flow rates [96,97]. As the operating regimes of microfluidic devices are typically characterized by laminar flows with low Reynolds numbers (generally smaller than 1), mixing the injected fluids is dominated by diffusion instead of turbulence [98,99]. However, the diffusion process is an inherently slow process and generally requires the use of relatively long microchannels to attain sufficient mixing [100,101]. With an attempt of introducing more efficient mixing conditions, a range of micromixing strategies have been proposed in the literature [102–108]. For instance, Hong, Choi and Ahn (2004) reported on the design of a passive micromixer by using modified Tesla structures, which provide sufficient mixing at relatively high flow rates by dividing one of the fluid streams into two substreams that are merged again [109]. Such repeated stream combinations of dividing and recombining steps lead to the creation of chaotic advections and provide efficient mixing of the injected fluids [109]. Following this method, it was possible to produce monodisperse nanoparticles with sizes ranging from 35 to 180 nm by using a Tesla mixer [110]. Even though the Tesla-type microfluidic structure is attractive for the formation of monodisperse hybrid lipid−polymeric nanoparticles, microvortices may provide more efficient platforms for achieving a high-throughput nanoparticle synthesis: continuous nanoparticle generation with a high productivity level and a high reproducibility [34]. The use of microvortices enabled up to 1000 times higher productivity than Tesla structures owing to more efficient diffusive and convective mixing conditions [34]. By varying microvortice designs, it was possible at defined polymer−lipid compositions and concentrations to control and optimize nanoparticle sizes and size distributions. The development of hybrid lipid-polymer NPs is associated with the formation of a lipid shell covering nanoprecipitated polymeric-core containing encapsulated drug. As compared to control and lipid-free polymeric NPs, it was reported that the shell formation in generated hybrid nanoparticles is important to enhance the formulation's shelf life by improving stability against aggregation even in the presence of high salt concentrations as compared to the control sample (naked drug encapsulated polymeric NPs) [110].

In this contribution, we focus merely on lipid and lipid-polymer hybrid nanoparticles. However, it is still important to take into account that polymeric soft nanoparticles, including vesicles and polymersomes are attractive for drug delivery applications [111]. For further details on stability, size, and other physical properties of polymeric vesicles and other polymeric nanoparticles as compared to lipid nanoparticles, the readers are directed to a recent review [111]. Therein, liposomes and polymersomes were compared and contrasted for a better priori choice and design of vesicles. In addition, the authors reviewed different aspects, which have been studied and developed for each of them. Even if polymersomes are tougher and more stable vesicles, liposomes are closer mimics to eukaryotic cell membranes as fluidity is a key parameter of cell membranes and stability does not always rhyme with cell mimicry. They also mentioned that lipid-polymer hybrid vesicles have gained interest as a good compromise between liposomes and polymersomes for a greater control and adaptability of physicochemical properties to any desired functionality and applications, optimal for cell mimicry [111].

In recent years, the staggered herringbone micromixer (SHM) has become popular for the continuous production of LNPs with controllable sizes as the grooves on the microchannel floor provide an efficient mixing through an induction of a chaotic advection between the fluid streams [112,113]. Such presence of a chaotic flow profile leads to an enhancement of the diffusion and a simultaneous rapid decrease in lipid solubility [19]. Previous studies reported on the use of SHM for the production of liposomes and siRNA-encapsulated liposomes with sizes in the range of 20−100 nm [29,114,115] (Fig. 2C). Kastner et al. (2015) [116] and Joshi et al. (2016) [117] reported on the continuous production of drug-loaded liposomes by using SHM. Maeki et al. (2015) [118] found that an increase in SHM cycle numbers is associated with a decrease in the LNP size, and leads to a narrower nanoparticle size distribution. It was also reported that the chaotic micromixer depth plays an important role in modulating the nanoparticle sizes and size distributions [119]. Li et al. (2017) [120] demonstrated the use of SHM in a single-step process for the synthesis of conjugated monodisperse LNPs with a narrower size distribution (PDI of 0.129) as compared to a multi-step bulk mixing method that leads to nanoparticles with PDI of 0.216. The influence of the emulsification method on the lamellarity of liposomes was also discussed in a recent report [38]. It was found that nanoparticles produced by using SHM structure are uni-lamellar vesicles (ULVs), whereas those produced using the conventional thin-film hydration batch method are multi-lamellar vesicles (MLVs) [38].

In addition to single-stage microfluidics such as Tesla mixers, SHM, and microvortices, internalisation of different microstructures (such as curved microchannels and double spiral microchannels), or increasing the mixing stage number could be considered to enhance the mixing efficiency during the continuous production of LNPs [121]. For instance, hollow-structured hybrid liposomes were produced in a one-step procedure without the need to add any additional stabilizer by using a three-Y-shaped-stage microfluidic chip [122]. In another example, two-stage microfluidic platform with two consecutive flow-focusing regions was designed for one-step continuous generation of cationic liposomes (CLs) and plasmid DNA/cationic lipoplexes (pDNA-CLs) [123] (Fig. 2D). In this study, CLs were produced in the first focusing region, while lipoplexes were formed in the second region. This method allows possessing a longer mixing channel for promoting proper DNA condensation into the lipid bilayers, and provides a sufficient mixing performance at relatively low focusing [123]. In addition, a multi-stage microfluidic device with mixing regions, including a buffer exchange (membrane dialysis) was successfully used for a rapid continuous production of liposomes with an efficient remote loading of amphipathic drugs [124]. This is considered an important achievement as these nanoparticles can be continuously produced within a very short time (3 min) as compared to conventional multi-step bulk methods that could require at least few hours for their preparation.

3.2. *Droplet-based microfluidics*

Different microfluidic platforms that are attractive for continuous production of nanoparticles with controllable sizes are described above. In addition to microfluidic synthesis of monodisperse NPs and their uses as drug nanocarriers, it is worth noting that microfluidics are attractive for use in other applications including generation of relatively large particles for living cells' encapsulation. Here, we briefly describe droplet microfluidic techniques and employed methods for generation of relatively large particles, including giant unilamellar vesicles (GUV). In contrast to HFF microfluidic systems, droplet-based microfluidic platforms focus with the use of immiscible fluids on the generation of

discrete volumes in the form of droplets [87,125]. The different droplet generation strategies, including hydrodynamics (T- and Y-junctions, flow-focusing, and co-flowing), pneumatic pressure, and optical and electrical techniques, focus on the formation of single or multiple dispersed lipid droplets (typically emulsions) with defined droplet sizes, structures and compositions, which serve as templates for the continuous synthesis of LNPs in the droplet-based devices [121,125,126]. In the production of such relatively large self-assemblies such as GUVs, the employed flow rates and the geometries of the microfluidic channels play an important role in modulating their sizes [127–129].

Conventional liposome formation batch methods have uniformity issues related to nanoparticle sizes and shapes, which are attributed to the random nature of bilayer foldings. To overcome this major limitation, droplet-based microfluidic devices can be used as attractive platforms for the continuous production of LNPs with high uniformity and encapsulation efficiency through the synthesis of single or double emulsion droplets as templates [87,130,131]. For instance, it was reported on the use of monodispersed water-in-oil (W/O) type single-emulsion droplets as templates in a single-step shear- focusing process based on microfluidic droplet generation system for the continuous generation of GUVs loaded with different and relatively large biological substances (macromolecules), ranging from cancer cells and yeast cells to micron-sized beads, and nanosized proteins [132]. They have also potential uses for drug and gene delivery applications [132]. In one of these reported studies [132], the lipid emulsion droplets were produced within the microfluidic channel by emulsifying an aqueous phase containing target species in the presence of a lipid phase consisted of a binary phospholipid/oleic acid mixture (Fig. 3A). Subsequent to the injection of these lipid emulsion droplets produced by microfluidic chip into an aqueous medium containing ethanol as a bench- top dissolution mixture, the binary lipid mixture was rapidly dissolved in ethanol and excess ethanol was then removed. On exposure to excess water, the concentration of ethanol becomes below the solubility limit of the lipids (phospholipids and oleic acid), leading eventually to their self-assembly into vesicles. The yield and encapsulation efficiency of these vesicles are dependent on the flow rate of ethanol solution containing lipids and ethanol concentration in the bench-top dissolution mixture. Regarding the generation of double emulsion templates, although various microfluidic techniques have been described, some of them are worth mentioning. For instance, double emulsion templates were generated in microfluidic devices with two consecutive cross-junctions, enabling hydrodynamic flow focusing [131], or in 3D axisymmetric flow-focusing devices that provide a constricted inner flow in all directions [133] (Fig. 3B). These methods rely on the removal of the dispersed oil from the double emulsion droplets by using a solvent extraction process, resulting in the formation of vesicles [31,87,133]. For the generation of oil-in-water (O/W) emulsion droplets, the most important aspect in the microfluidic device designed by Teh et al. (2011), which consists of two consecutive cross-connections, is related to a selective hydrophilic treatment of the

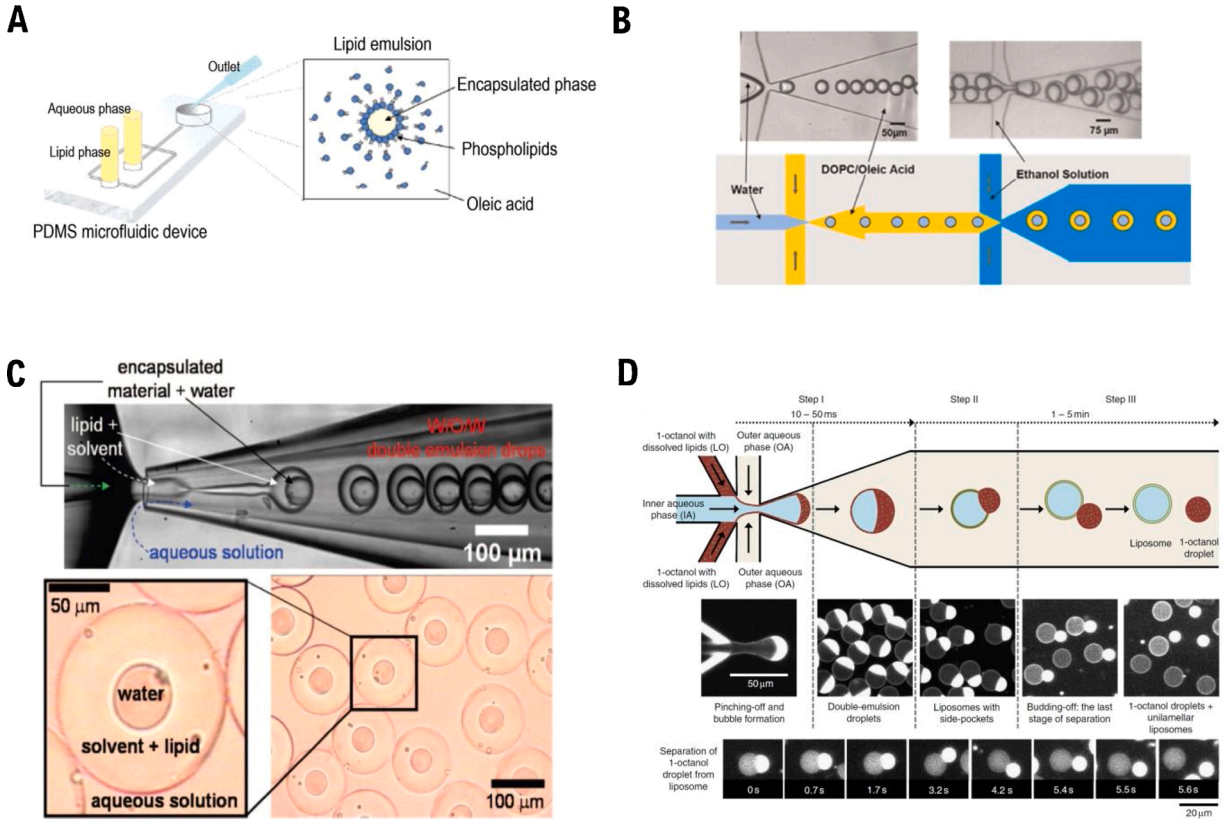

Fig. 3. **(A)** Schematic representation of single emulsion-templated vesicles. When the lipid single-emulsion solution produced by microfluidic platform is injected into an aqueous solution containing ethanol, phospholipids rearrange themselves around the emulsion to assemble into lipid vesicles (*Reprinted with permission from Tan et al., 2006. Copyright (2006) American Chemical Society*) [132]. **(B)** W/O/W double emulsion droplets as templates are fabricated inside PDMS-based microfluidic channels with double junctions. The selective hydrophilic treatment of the PDMS microfluidic channel surface was achieved by coating with PVA providing complete wetting of the walls (*Reprinted with permission from Teh et al., 2011, with the permission of AIP Publishing*) [131]. **(C)** Schematic representation for the formation of phospholipid-stabilised W/O/W double emulsions in a glass microcapillary device (*upper*), and an image of the generated double emulsion droplets enveloping an aqueous core surrounded by a solvent shell containing phospholipids (*down*) (*Reprinted with permission from Shum et al., 2008. Copyright (2008) American Chemical Society.*) [31]. **(D)** Schematic representation of on-chip production of liposomes using octanol-assisted liposome assembly (OLA) technique (*upper*), and corresponding fluorescence images showing production process (*middle and down*) (*Reprinted with permission from Deshpande et al., 2016, an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (*http://creativecommons.org/ *licenses/by/3.0/)*) [32].

PDMS microfluidic channel surface by coating with poly(vinyl alcohol) (PVA) [131]. In this method, W/O emulsion droplets are generated within the first untreated hydrophobic flow-focusing regime, and this stream is then sheared at the second hydrophilic treated orifice to produce water-in-oil-in-water (W/O/W) double emulsions in the presence of the external aqueous solution containing ethanol. It is possible in this method to produce vesicles with an enhanced colloidal stability and a precise control of their sizes through a precise control of the microfluidic channel geometry and flow parameters. In another study focusing on double emulsion templates [133], a 3D flow focusing method was employed by using a corona discharge for designing a novel surface patterning feature. This allows for a spatial control of the wettability of PDMS surface. By combining surface functionalization for reducing surface wetting requirements with an axisymmetric flow focusing, this microfluidic device was capable of generating double emulsions as templates for liposome formation [126,133]. Monodisperse GUVs with a high encapsulation efficiency were also produced by extracting a mixture of volatile organic solvents (toluene and chloroform) from double emulsion droplets in a coaxial glass microcapillary device, combining a flow-focusing with a co-flow geometry [31] (Fig. 3C). Although PDMS droplet generators allow a precision control of the intricate geometry, and provide a high reproducibility, tapered and aligned glass capillary tubes are the embodiment of 3D axisymmetric flow focusing devices [31,133]. In this method, the phospholipids are dissolved in a volatile organic solvent that is immiscible with an aqueous medium and form the shell of W/O/W double emulsion templates, which are converted into vesicles by removing the volatile solvent present in the oil phase through evaporation [31]. Within this microfluidic device, the aqueous innermost phase is injected through a tapered capillary, whereas the organic middle phase containing phospholipids and the aqueous outermost phase are injected through the outer coaxial region, and the outer coaxial region from the opposite side, respectively [31]. W/O/W emulsion droplets are generated, when the hydrodynamically focused innermost and middle streams rupture at the orifice of the collection tube [31,87,134]. By evaporating the organic solvents from the middle layer, phospholipids are forced to rearrange themselves on the double emulsion templates, resulting in the generation of GUVs (typically 20–150 μm in size) [31,87]. Actually, this method has been more successfully applied for the production of polymersomes [135,136], which are vesicular structures composed of bilayers of amphiphilic diblock copolymers since phospholipid bilayers are too fragile to undergo the solvent evaporation process, without rupturing and destabilization. Attention must be paid to the implemented experimental strategies. For instance, a slow evaporation of the organic solvent (or mixtures of organic solvents) by using a loosely sealed container, a solvent removal by dialysis with aid of an anodized alumina filter as a dialysis membrane, or use of highly concentrated glycerol solutions can be employed for improving the stability of fragile phospholipid vesicles during solvent extraction [31,137,138]. Despite the attractiveness of the W/O/W double emulsion templates technique, they have some common disadvantages, including an inability to remove the associated oil or the solvent remnants from the dispersed lipid bilayers in quick and efficient manner [32].

Deshpande et al. (2016) proposed a new and robust microfluidic method based on a double-emulsion-droplet-based approach, known as *octanol-assisted liposome assembly* (OLA) (Fig. 3D), in which conventional oil-based or alkane-based lipid-carrying phases are replaced with 1-octanol [32]. In the study, suitability of different alcohols as lipid-carrying organic phases was evaluated with an attempt of accelerating the solvent extraction procedure, and a marked improvement of the required time for full extraction of oleic acid was reported when using 1-octanol as a lipid carrying organic phase [32]. On contrary to the highly time-consuming (more than 10 h) conventional process of ethanol extraction [32,139], OLA method allows within few seconds for the development of an intermediate complex containing two distinct phases from double-emulsion droplets, which acts as a prominent pocket of 1-octanol containing excess lipids and an inner aqueous core surrounded by a lipid bilayer. Following this method and through a successful integration of OLA platform with optofluidic transport assay, it was reported on the continuous production of liposomes and the quantification of drug permeability across biomimetic liposome membranes on the same device [139].

4. Coupling synchrotron SAXS to microfluidics for real-time detection of dynamic structural alterations

Small-angle X-ray (SAXS) and neutron (SANS) scattering methods are powerful and well-established techniques to characterize soft matters including liquid crystalline phases and micellar solutions [5,7,44,47,140–146]. In particular, they are the techniques of choice in the literature for gaining insight into the influence of various factors including temperature, pressure, lipid composition and type, and aqueous (or biological) medium composition [145–147]. They provide important information on the effect of loading hydrophilic or hydrophobic guest molecules (including drugs, functional foods, and peptides) on the structural features of self-assemblies under equilibrium and non-equilibrium conditions [11,148–158]. Both SAXS and SANS are also attractive for use to characterize micellar solutions including microemulsions, and gain insight on the shape of micelles, and their inter-droplet interactions and even charge [159–162].

There is also a growing interest in coupling these scattering techniques (particularly SAXS) with a stopped-flow apparatus, an infra-red (or UV) light source, or optical tweezers, among others, for improved understanding of dynamic structural transitions and involved structural mechanisms during formation of nano-self-assemblies or in response to exposure of already prepared nano-self-assemblies to an external stimulus [42,142,157,163–167]. Among these investigations, it is worth mentioning the attractiveness of coupling synchrotron SAXS with stopped-flow devices for gaining important dynamic structural information on relatively fast processes, involving significant structural alterations or structural transitions that occur within few hundreds of milliseconds [142,165,168–170]. Such investigations are also important for detecting intermediate phase states with short lives, and exploring their roles in structural transition pathways. For instance, this method was used to shed light on dynamic structural transitions, occurring on rapid exposure of negatively charged lipid nanoparticles to buffer containing calcium ions [165,170]. It was employed for gaining insight into the involved kinetic pathways, and the ultrafast structural dynamics during complexation of cationic liposomes with plasmid DNA (pDNA) [168]. However, conventional stopped-flow devices are often expensive and complex that require relatively large volumes of samples and considerable amounts can be wasted, particularly when utilizing inefficient designs [171]. It is possible to overcome such limitations by combining microfluidic platforms with synchrotron SAXS (or SANS) owing to various advantages including automation with a precise flow control, high-throughput nanoparticle production within short time scales, minimization of sample consumption, and maintenance of a high level of accuracy and reproducibility [16,150,172–175]. Thus, in addition to the attractiveness of microfluidics for the continuous production of monodispersed lipid or polymer nanocarriers for drug delivery [176], such combinations with scattering techniques allow gaining important structural information on the formation mechanism of these nano-self-assemblies [16,41,42,44,150,167,175,177].

In this section, we focus and highlight recent advances on coupling suitable microfluidics with synchrotron SAXS for continuous production of nano-self-assemblies. This method allows for simultaneous characterization of the involved dynamic structural changes or phase transitions, and it is an important tool for determination of short-lived intermediate phases under confinement. For instance, we discuss dynamic structural alterations and transitions on a rapid mixing of micellar solutions, and on exposure of charged nanoparticles to buffers containing divalent ions. In addition, we highlight the importance of such

experimental set- ups as attractive tools for investigating the structural features and dynamics in response to variations in lipid composition of emulsions during the digestion process. Different representative examples on combining microfluidic platforms with synchrotron SAXS are presented in Table 1.

A schematic illustration for a typical experimental setup of synchrotron SAXS combined with a microfluidic device is shown in Fig. 4**A**. The X-ray beam crosses the chip along the *z*-axis, perpendicularly to the flow direction, and the continuous flow conditions reduce the probability of radiation damage, but at the same time, a reduced scattering signal to background is obtained [42,167]. In such a set-up, a high flux monochromatic beam travels through the microfluidic channels and hits a small volume of fluid and then the scattered X-rays are captured typically by a two dimensional (2D) detector, providing a scattering pattern with structural information on the investigated sample. Here for gaining structural information in both temporally- and spatially-resolved manners, SAXS measurements are conducted at different positions along the center microfluidic microchannel after mixing the fluids that are injected through the center and two sheath (side) microchannels. These positions correspond to durations (retention times or reaction times) along the center microfluidic microchannel. In addition to the aforementioned dynamic structural investigations, this *in situ* real-time tracking characterization method may provide important information on nucleation (early dynamic self-assembly process) and nanoparticle growth [16,42,156,180–182].

Recently, Ghazal et al. (2017) used a HFF microfluidic platform combined with synchrotron SAXS for real-time monitoring of continuous production of MLVs that are started to evolve within less than 0.43 s of contact between ethanol solution containing binary lipid mixture and buffer (Fig. 4**B**) [16]. Briefly, the continuous production of these vesicular nanostructures involves a rapid exposure of ethanol solution consisting of binary phytantriol (PHYT)/glyceryl dioleate-PEG12 (DO-PEG12) mixture to two side streams of phosphate buffer solution (PBS) containing 1 wt% Pluronic F127. The involved rapid mixing leads to the diffusion of ethanol into excess buffer until its concentration becomes below the solubility limit of both lipids, triggering therefore their fast self-assembly into planar bilayers. To ensure successful SAXS experiments, it was important to maintain a tighter SAXS beam focus confined between the walls of the microfluidic channel as scattering from these walls may significantly decrease the SAXS data quality [16]. In another study, Otten et al. (2005) reported also on the combination of synchrotron SAXS with a HFF microfluidic device for investigating the effect of loading DNA on the structural features of MLVs based on a binary lipid mixture of 1,2-dioleoyl-3- trimethylammonium-propane (DOTAP) and dioleoylphosphatidylcholine (DOPC) [178]. Kim et al. (2018) also combined synchrotron SAXS with a staggered herringbone mixer (SHM) for the continuous microfluidic synthesis and *in situ* characterization of small siRNA-free and siRNA-loaded cubosomes (cuboplexes) with mean sizes of about 75 nm [26]. These continuously produced nanoparticles outperform commercially available delivery vectors, as well as liposomes. In a recent report, Ghazal et al. (2016) [20] employed a compatible X-ray HFF microfluidic platform based on off ;-stoichiometric thiol-ene (OSTE) in combination with synchrotron SAXS for studying the effect of exposing negatively charged cubosomes to buffer containing Ca^{2+} ions. As shown in Fig. 4C, this experimental set-up allowed investigating the real-time dynamic structural transitions from nanoparticles (cubosomes) enveloping internally an inverse bicontinuous cubic phase of the symmetry *Pn3m* to nanoparticles with an internal biphasic feature of coexisting inverse bicontinuous cubic *Im3m* and *Pn3m* phases at different positions along the center microfluidic channel (corresponding to reaction times in the range of 0.5–5.5 s). Here, it is worth mentioning that the attractiveness of OSTE-based microfluidic devices for SAXS investigations lies on their unique properties including optical transparency (including a reduced level of X-ray scattering background), and tunable surface properties [20,183]. Komorowski et al. (2020) [43] recently reported also on coupling synchrotron SAXS with a compatible cyclic olefin copolymer (COC) microfluidic platform (or a stopped-flow apparatus) to study the adhesion of vesicles based on binary lipid mixtures of DOPC and dioleoylphosphatidylserine (DOPS) in the electrostatic strong-coupling regime in the presence of divalent ions. They found that the obtained results are in good agreement with the strong coupling theory, which is employed to predict the adhesion state. It was evident that the divalent ion-induced adhesion state in highly charged bilayers under flow conditions is only of transient nature with a subsequent transformation of the adhering vesicles to multi-lamellar vesicular (MLV) structures. In spite of the attractiveness of coupling microfluidics to synchrotron SAXS for investigating structure and dynamics of such nano-self-assemblies with reasonable temporal and spatial resolution, it is still important to consider that the application of relatively tighter beam focus is

Table 1
Coupling synchrotron SAXS with microfluidic platforms for the following investigations: (i) real-time monitoring of the involved dynamic structural alterations during the continuous production of nano-self-assemblies, and (ii) real-time feedback on dynamic structural events, occurring on exposure of already prepared nano-self-assemblies to ions, or due to enzymatic reactions.

Microfluidic synthesis/ use	Lipid Composition[a]	Microfluidic Platform	Comments	Ref.
Multilamellar vesicles (MLVs)	PHYT/DO-PEG12	Hydrodynamic focusing (HFF) Kapton and thiol-ene microfluidic platforms	A fast continuous production of monodispersed vesicles that start to evolve within fractions of a second	[16]
Cubosomes	PHYT/DOPG	Hydrodynamic focusing (HFF) thiol-ene microfluidic platform	Dynamic structural alterations on exposure of negatively charged cubosomes to a buffer containing Ca^{2+} ions	[20]
Vesicles	DOPC:DOPS	Cyclic olefin copolymer (COC) microfluidic platform	Adhesion of lipid vesicles in the electrostatical strong-coupling regime that is induced by divalent ions	[43]
Lamellar and non-lamellar nanoparticles	Citrem/SPC	Hydrodynamic focusing (HFF) Kapton microfluidic platform	Rapid nonlamellar-lamellar liquid crystalline transitions on mixing normal and inverse micellar nanosystems	[41]
Vesicles	DOTAP:DOPC	Hydrodynamic focusing (HFF) and diffusive mixing microfluidic platforms Kapton–steel–Kapton system	Dynamic behaviour of DNA intercalation into multilamellar vesicles (MLVs)	[178]
Emulsions	PHYT/TB PHYT/TC	Hydrodynamic focusing (HFF) microfluidic platform (sample T-junction microcapillary or custom-built chip)	Dynamic structural transitions during digestion of two enzyme-sensitive emulsions	[179]
Micelles and liquid crystalline phase	Poly(isoprene-*b*-ethylene glycol) copolymer	Hydrodynamic focusing (HFF)	Self-assembly of an amphiphilic copolymer under continuous flow conditions. The study reports on the involved rapid dynamic structural transitions from micelles to a liquid-crystalline *Fm3n* phase	[180]

[a] PHYT: phytantriol; DO-PEG12: glyceryl dioleate-PEG12; DOPG: 1,2-dioleoylphosphatidylglycerol; DOPC: dioleoylphosphatidylcholine; DOPS: dioleoylphosphatidylserine; SPC: soy phosphatidylcholine; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; TB: tributyrin; TC: tricaprylin.

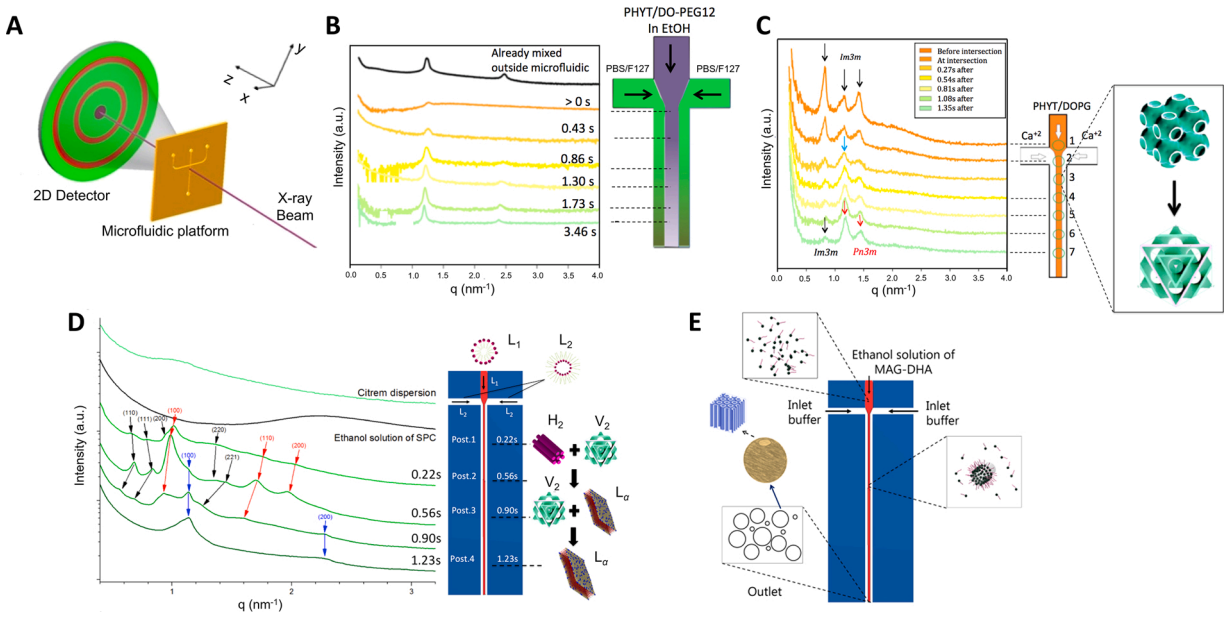

Fig. 4. **(A)** Schematic representation of an experimental setup coupling synchrotron SAXS with X-ray compatible microfluidic device. **(B)** A microfluidic platform combined with synchrotron SAXS was used for monitoring the continuous production of multilamellar vesicles (MLVs). SAXS patterns at different positions (corresponding different reaction times) along the microchannel after mixing of the center and side microchannels are presented. Inset: Schematic illustration for such combination with SAXS is shown (*Adapted with permission from Ghazal et al., 2017)* [16]. **(C)** Time-resolved SAXS experiments at room temperature, displaying the dynamic structural transitions in the interiors of phytantriol/dioleoylphosphatidylglycerol (DOPG) cubosomes stimulated by an influx of PIPES buffer containing Ca^{2+} ions. Each signal on the left corresponds to the relevant position (reaction time) along the center microchannel. Inset: Schematic illustration for the detected dynamic structural changes in cubosomes' interiors (*Adapted with permission from Ghazal et al.2016)* [20]. **(D)** Coupling microfluidic device with synchrotron SAXS for investigating lipid transfer on rapid exposure of citrem nanoparticles to an ethanol solution of soybean phospholipid (SPC). Inset: Schematic illustration for the hydrodynamic focusing (HFF) with corresponding reaction times and the detected phases based on SAXS data analysis *(Adapted with permission from Khaliqi et al., 2017)* [41]. **(E)** Schematic illustration for the hypothetical molecular re-distribution of docosahexaenoic acid monoglyceride (MAG-DHA) and its self-assembly in excess buffer, leading eventually to the microfluidic synthesis of hexosomes *(Adapted with permission from Yaghmur et al., 2019)* [184].

associated with compromising SAXS data quality [43].

In a novel method for the continuous production and *in situ* structural characterization of lamellar and non-lamellar liquid crystalline nanoparticles, normal and inverse micellar solutions were rapidly mixed and investigated by synchrotron SAXS coupled to a compatible HFF microfluidic platform [41]. As shown in Fig. 4D, a fast transition from micelles to more ordered lamellar and non-lamellar liquid crystalline phases was detected after about 0.22 s of exposure of an ethanol solution of soy phosphatidylcholine (SPC) to citrem nanodispersion consisting of nanoparticles with internal inverse micelles (L_2 phase). In addition to this microfluidic synthesis method for the continuous production of lamellar and non-lamellar liquid crystalline nanoparticles, the same microfluidic platform as shown in Fig. 4**E** was used to produce directly F127-stabilized hexosomes based on docosahexaenoic acid monoglyceride (MAG-DHA) with narrow size distribution and mean nanoparticle sizes (depending on the applied flow conditions) in the range of 108–138 nm [184]. Clearly, microfluidics in combination with synchrotron SAXS can be used to continuously produce structurally tunable nano-self-assemblies with narrow size distributions through precise control of mixing conditions and flow parameters [41,175].

Aside from the aforementioned examples, combination of synchrotron SAXS (or SANS) with microfluidics is also attractive for other applications that mimic flow conditions under confinement and/or mimic simultaneous variation in lipid compositions in real-world situations such as digestion of lipid formulations. For instance, Hong et al. (2019) [179] recently combined a HFF microfluidic platform (T-junction microcapillary or custom-built HFF microfluidic chip) with synchrotron SAXS to investigate *in situ* the structure and dynamics of two enzyme-sensitive emulsions during their digestion. The involved enzymatic reactions were associated with a colloidal transformation from emulsions (known also in literature as emulsified microemulsions, EMEs) enveloping internal inverse micelles (L_2 phase) to nanoobjects enveloping internally an inverse bicontinuous cubic phase of the symmetry *Pn3m* coexisting with an inverse hexagonal phase (H_2). The results were consistent with previous SAXS findings on the formation of colloidal lamellar and non-lamellar liquid crystalline nanoparticles during digestion of model food-grade emulsions and triglycerides containing food products [185–189].

The given examples highlight the importance of combining microfluidics with advanced techniques such as SAXS or SANS for monitoring in real-time the dynamic structural transitions in soft matters, and gaining further insight into the formation mechanism of nano-self-assemblies under confinement and continuous flow conditions. With the possibility to reduce further the microfluidic background scattering and develop more advanced versatile X-ray compatible 2D or 3D microfluidic devices addressing specific needs, these systems may serve as promising platforms for structural characterization of nano-self-assemblies during their continuous production and in response to alterations in the composition of already prepared lipidic or polymeric nanoparticles due to enzymatic or chemical reactions.

5. Advances and future directions

There is a growing interest in designing microfluidics for continuous production of drug nanocarriers with controllable sizes. In this contribution, we exclusively focus on the continuous generation of drug-free and drug-loaded lipid nano-formulations with relatively small nanoparticle sizes and narrow size distributions. The attractiveness of microfluidics has been demonstrated by presenting various examples and the drug delivery implications of the continuously produced nano-self-assemblies have briefly been discussed. In addition, we present new approaches for real-time structural analysis of lipid nano-self-assemblies under flow conditions through combination of microfluidics with synchrotron SAXS or SANS. As demonstrated by the

presented examples, almost all recent investigations focused on coupling microfluidics with synchrotron SAXS. Owing to the fundamental importance of such investigations, we expect that this research area will continue to receive a great attention in the near future and other state-of-art techniques than SAXS and SANS will be combined with microfluidics for various analytical and quality control investigations. Further studies on the combination of SAXS and SANS with more advanced and even sophisticated microfluidic platforms, including 3D devices, are also expected. The main attention will not only be paid on *in situ* structural characterization of lipid nano- self-assemblies during their continuous production or when they are exposed to biologically relevant media, but also on the introduction of single compatible microfluidic platforms with high mixing efficiencies to achieve both high quality dynamic structural analysis and high nanoparticle size controllability. This is an important aspect, as the used 2D HFF device in our recent investigations [16,20,41,184] was found to be suitable for dynamic structural analysis but not so efficient at generating nanoparticles with improved monodispersity.

Through precise fluid control and highly efficient microscale mixing, microfluidics not only allows for the continuous production of nanoparticles with tunable size features and high monodispersity through high-throughput and reproducible manners, but also has the potential to improve drug encapsulation efficiency and therapeutic outcomes of nanoparticles as compared to those produced by conventional batch methods [12,18,21,23,35,36,190]. In addition, microfluidics can play role in enhancing the colloidal stability and improving further the therapeutic outcomes of lipid nanoparticles by modulating their physicochemical properties, and modifying their surfaces through PEGylation and targeting ligands.

Other important aspects of microfluidic synthesis include the possible use of small amounts of lipids and other additives in the continuous production process, and reproducibility. However, they should be easy-to-operate, and limitations related to scalability and suitability to organic solvents should be considered in future investigations. Here, it is worth mentioning that batch methods provide typically easy scalability and the yielded volumes of nanoparticles are relatively greater as compared to those produced using conventional microfluidics. In this respect, scaling up and increasing nanoparticle production rates can be achieved through parallel arrangements of microchannels and microfluidics and development of new fabrication paradigms [190] and new advanced 3D devices. As continuous production of multifunctional nanoparticles and surface nanoengineered nanoparticles are rarely investigated in this research area [21], future studies should also focus on the development of advanced microfluidic platforms and evaluating their roles in modulating the physicochemical properties of such nanoengineered and multifunctional nanoparticles.

Taking into account the multidisciplinary nature of this research area, it is important to involve scientists from different backgrounds (including engineering, biology, material science, and physics), and initiate academia-pharmaceutical industry collaborations. Introduction of monodispersed drug nanocarriers with improved therapeutic outcomes by employing efficient microfluidic platforms may contribute and accelerate their clinical translation.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

AY and OYC acknowledge the INP grant (Ref. no.: 8073-00022B) from the Danish Agency for Science and Higher Education for initiating and exploring new networking and collaboration opportunities between Danish and Turkish research institutions.

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