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FOCUS ON NANOMANUFACTURING

Preparation of nanoparticles by continuous-flow microfluidics

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Abstract We review a variety of micro- and nanoparticle formulations produced with microfluidic methods. A diverse variety of approaches to generate microscale and nanoscale particles has been reported. Here we emphasize the use of microfluidics, specifically microfluidic systems that operate in a continuous flow mode, thereby allowing continuous generation of desired particle formulations. The generation of semiconductor quantum dots, metal colloids, emulsions, and liposomes is considered. To emphasize the potential benefits of the continuous-flow microfluidic methodology for nanoparticle generation, preliminary data on the size distribution of liposomes formed using the microfluidic approach is compared to the traditional bulk alcohol injection method.

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Introduction

One of the great benefits of microfluidics technology realized early in its development is that it enables fine control and manipulation of fluids and fluid interfaces. Small packets of fluids with volumes measured in the picoliter to attoliter range can be manipulated, split, or combined with precise timing. Mixing can occur by simple diffusion in an open channel, or in a patterned channel that promotes folding of the fluid layers to reduce diffusion distances. Analytes can also be separated into discrete zones using electrophoretic, chromatographic, or related focusing approaches. Moreover, fluid streams flowing toward one another can merge and form a very sharp and well-defined interface by virtue of laminar flow. Over the past 15 years, these features have been widely harnessed to enable predictable, defined, and controlled environments for chemical and biochemical reaction and synthesis.

One application of microfluidics technologies which takes advantage of these features is the synthesis, formation, and self-assembly of microscale and nanoscale particles. Nanoscale particles that have been formed using microfluidics include semiconductor quantum dots (Sounart et al. 2007), metal

colloids (Chan et al. 2003), and more recently, liposomes (Jahn et al. 2007; Jahn et al. 2004; Wagner et al. 2002) and lipid nanotubes (Brazhnik et al. 2005; Dittrich et al. 2006; Lin et al. 2005).

Quantum dots are an exciting complement to traditional fluorescent labels in in vivo and in vitro biological applications such as deep tissue imaging, assay labeling and sensing (Medintz et al. 2005; Michalet et al. 2005).

Quantum dots and metal colloids are both produced in microfluidics by miniaturization of traditional chemical synthesis methods. For both quantum dots and metal colloid formation, microfluidic synthesis has been reported to be superior to benchtop methods for producing high quality, monodisperse particles due to the ability to maintain fine control of all solution variables including reactant concentration, timing of reagent addition, and temperature.

In this paper, we examine the recent literature on continuous flow microfluidic fabrication of nanocrystals and colloidal nanoparticles, emulsions, and liposomes, and contrast the differences in their formation processes. As a specific example that reveals the benefits microfluidic technology can bring to nanoparticle fabrication, the production of liposomes using the continuous-flow microfluidic method is compared to a more traditional bulk fabrication approach. This study reveals that the microfluidic method provides substantially tighter particle distributions even after repeated cycles of membrane extrusion following the bulk method.

Nanocrystals and colloids: fast mixing

There has been tremendous interest in the development of microfluidic methods for chemical synthesis of nanoparticles for the production of nanocrystalline semiconductors with uniform and tunable distributions of size (Alivisatos 1996) and shape (Puntes et al. 2001). Although a number of bulk methods exist for the formation of semiconductor quantum dots, these techniques have exhibited problems associated with the preparation of the monodisperse formulations for applications requiring precise size distributions (Trindade et al. 2001). This issue remained problematic until the recent introduction of microfluidic methods for chemical synthesis (Jensen 2001).

The theory of colloidal formation by chemical synthesis used is well described by LaMer and Dinegar (LaMer and Dinegar 1950). It involves an initial nucleation phase followed by a continued growth by diffusion. They report that a protocol for producing highly uniform colloidal distributions should implement conditions where the concentration of the solute produced in the reaction reaches a supersaturated concentration for a short time to precipitate a monodisperse distribution of seed particles, followed by a rapid transition to a diffusion growth phase where particle size is mediated by depletion of the remaining solute.

Microfluidic methods for rapid mixing and exquisite control of reagent concentration can produce the precise conditions required for nanoparticle production. These methods include hydrodynamic focusing (Knight et al. 1998), flow lamination (Bessouth et al. 1999), and fluid folding (Johnson et al. 2002) or chaotic mixing (Stroock et al. 2002).

Edel et al. (2002) reported a procedure in 2002 for controlled production of CdS nanoparticles using a microfluidic device with continuous flow conditions. They observed that as the flow rate was increased particles were more uniform, based on measurements of absorption spectra. They attributed this to reduced time for coalescence of the newly formed nanoparticles in the flow channel after formation.

Chan et al. (2003) studied in detail the formation of CdSe nanoparticles using continuous flow in microfluidic devices under varying conditions of flow rate (reaction time), temperature, and precursor concentration. Their experiments demonstrated that microfluidic systems facilitate detailed studies of formation protocols not only to optimize size, but also to fine-tune shapes (Cottam et al. 2007) and to create coated (core/shell) nanocrystals (Reiss et al. 2002; Wang et al. 2004).

It is important to note a method developed by Maeda and colleagues for formation of TiO_2 nanoparticles using a ceramic microfluidic reactor (Wang et al. 2002). This method is different from the others in that it is based on establishing a stable interface between two insoluble fluids with similar viscosity. Other reports have utilized capillary tubing in hot oil baths to form silver (Lin et al. 2004) and CdSe (Luan et al. 2007; Nakamura et al. 2004) nanoparticles. A method has also been reported that utilizes a three-step process involving capillaries in heated oil steps

combined with a microfluidic mixer intermediate step to form CdSe-ZnS composite nanoparticles (Wang et al. 2004).

Emulsions

Water droplets in oil are important for many applications such as food and drug delivery systems. They have also been shown to serve as reactors for biological and chemical reactions (Kelly et al. 2007). To create droplet emulsions having narrow size distributions on the microscale, several groups have developed production methods that utilize microfluidic systems. The theory of their formation is based on considerations of mechanical instability, which is fundamentally different from the previous discussion on colloidal formation. Although these methods do not generally result in nanometer-scale droplets, they have been used to form nanoliter reaction vessels to produce nanoparticles (Chan et al. 2005; Hung et al. 2006; Khan et al. 2004; Shestopalov et al. 2004). Christopher et al. (Christopher 2007) have recently published an extensive review of microfluidic-based droplet formation methods. Here we present a brief discussion of some of the results from this topic.

Sugiura et al. reported on the formation of both oil-in-water (Sugiura et al. 2001a) and water-in-oil (Sugiura et al. 2001b) droplets in a microfluidic structure. Droplets form by forcing the dispersion phase through a narrow channel (15 μm) into the continuous phase. A flat, disk like, shape forms on a 30 μm long terrace that extends to a drop-off. As the disk extends out to the edge of the terrace, an imbalance in the interfacial tensions between the flat disk and a sphere leads to the spontaneous formation of a spherical droplet that falls off the terrace onto a well. This process leads to a highly monodisperse collection of droplets at the 10 μm length scale.

Thorsen et al. (2001) used a microfluidic T-junction (Fig. 1a) to create micrometer-sized droplets by flowing water into a channel through which oil is flowing. In this work, they argue that droplet formation is determined by the combination of surface tension and shear forces. They use the shear force and the Laplace pressure to approximate the water droplet radius as $r \sim \sigma/\eta\dot{\varepsilon}$ where σ is the surface tension, η is the oil/surfactant viscosity, and $\dot{\varepsilon}$ is the shear rate.

Garstecki et al. (2006) presented a thorough treatment of the breakup process in the T-junction geometry in the limit of low capillary numbers, $C < 10^{-2}$ ($C = \eta v/\gamma$, where v is the mean velocity of the carrier fluid and γ is the interfacial tension). In this regime, shear stresses do not distort the droplet, but rather, the droplet breakup is driven by the balance of pressures between the two phases at the junction. They find a scaling law for the length of a water plug, L, (Fig. 1b) formed after breakup as $L/w = 1 + \alpha \cdot Q_{\text{water}}/Q_{\text{oil}}$, where w is the channel width, α a dimensionless parameter on the order of one, and Q the flow rate of the water or oil. Also of note, the droplet sizes are dependent on w thus increasing the dependence of droplet size on channel geometry.

Link et al. (2004) showed that water plugs preformed with a T-junction similar to that shown in Fig. 1 can be broken down into smaller droplets by flowing the plugs into a second T-junction (Fig. 2). Droplet breakup occurs for flow conditions where the capillary number is greater than a critical value given by, $C_{\text{crit}} = \alpha \cdot \varepsilon_o(\varepsilon_o^{-2/3} - 1)^2$, where α is a dimensionless constant which they find equals one, and ε_o is the ratio of the initial plug length, l_0, and initial channel width, w_0, such that $\varepsilon_o = l_o/\pi \cdot w_o$. The size of the two droplets formed at each junction depends on the fluidic resistance of the two side channels.

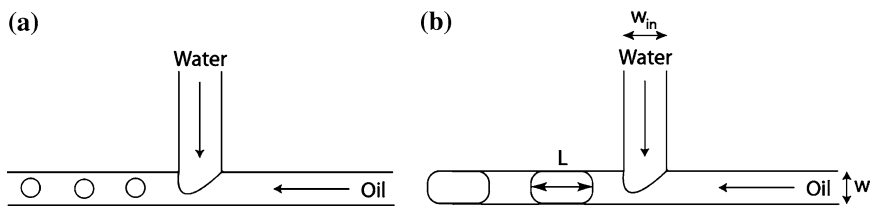

Fig. 1 Sketch of a microfluidic T-junction used to create (**a**) water droplets and (**b**) plugs in oil. Water is broken off from the vertical water stream by the perpendicular flow from the oil channel. The channel dimensions are typically on the order of 50 μm to 100 μm

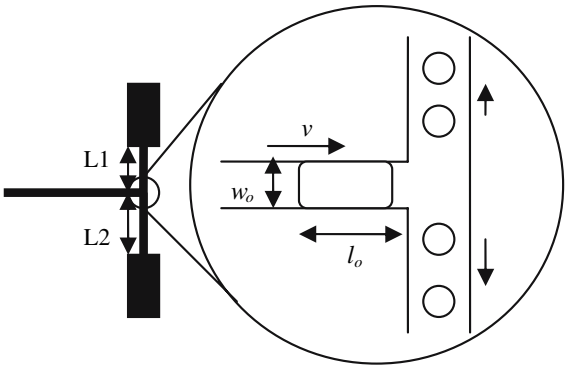

Fig. 2 Sketch of a microfluidic device used to break up larger droplets into smaller droplets. The relative size of the droplets in the upper and lower channel can be changed by adjusting the values of *L*1 and *L*2. Conditions for droplet breakup depend upon the inlet flow velocity, v, and the length of the plug, l_o, with respect to the channel width, w_o. (see (Link et al. 2004))

A different technique for creating emulsions relies on flow focusing in a microfluidic system and is described in Anna et al. (2003). The sketch in Fig. 3a shows the design used for droplet formation. The flow from the two oil channels applies pressure to the aqueous middle stream and focuses it down to a narrow stream that undergoes breakup either at or past the inlet channel into the large reservoir to the right. Figure 3b illustrates the types of droplets formed as a function of overall oil flow rate and the ratio of water to oil flow rates. This shows many different types and sizes of droplets that are smaller than the dimensions of the inlet channel.

Wu et al. (2006) use a pulsed flow approach to create droplets at predetermined locations within a microchannel. A device with a series of triangular shaped reservoirs connected by a straight narrow microchannel is initially filled with oil. Water is then forced through the channel via a syringe pump displacing the oil. After a short time the flow is stopped and the system is brought back to atmospheric pressure. This segments the aqueous stream and water droplets form in the triangular regions in the channel. Stable droplets with volumes varying from approximately 30 nL to 20 nL were formed at flow rates of 1 mL/min to 4 mL/min, respectively. Slower flow rates resulted in non-uniform droplets across the nucleation sites whereas higher flow rates produced two droplets per chamber. They also showed a strong dependence of droplet size on the size of the nucleation site in the microfluidic channel (Fig. 4).

Liposomes: bulk methods

Liposomes have attracted great interest since their discovery in 1965 for a wide range of biological, pharmaceutical, and industrial applications. The liposomes' ability to encapsulate and thereby segregate aqueous components has led to a variety of applications in biological systems including quantized reagent packaging for the delivery of genes (Kikuchi et al. 1999; Templeton et al. 1997), drugs or other therapeutic agents (Abraham et al. 2005; Andresen et al. 2005; Crosasso et al. 2000; Gulsen et al. 2005; Mamot et al. 2003; Mayer et al. 2000; Pavelic et al. 2005; Ramachandran et al. 2006; Sadava et al. 2002; Schmid and Korting 1994), and as vehicles for contrast agents for enhanced imaging (Ayyagari et al.

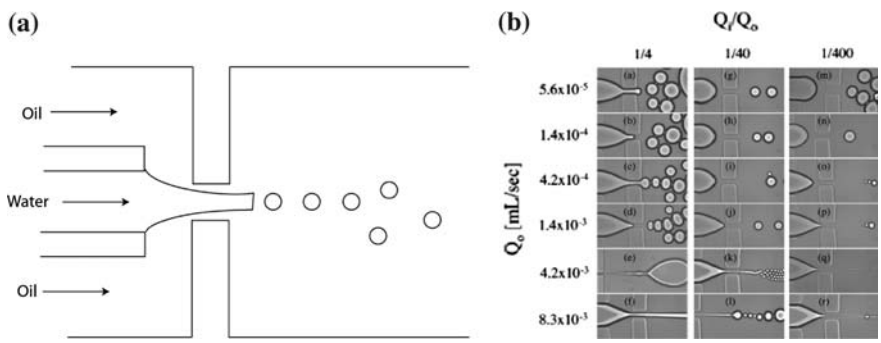

Fig. 3 (**a**) The 'flow focusing' device from ref. (Anna et al. 2003) where the oil pinches the water stream to form bubbles in the reservoir to the right. The channel dimensions are on the order of 10's of micrometers. (**b**) A collection of images taken directly from Anna et al. (2003) that shows the different types of droplets formed with this geometry. Reprinted with permission from Anna et al. (2003). Copyright 2003, American Institute of Physics

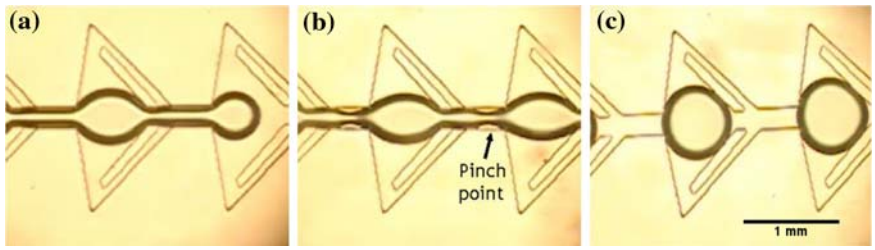

Fig. 4 Three video images that show the formation of droplets in a microfluidic device. (**a**) Water is forced into the oil-filled device. (**b**) The flow of water is stopped and pinch points form along the narrow portions of the water stream. These unstable regions break off and form droplets in video frame (**c**). Reprinted with permission from Wu et al. (2006). Copyright 2006, American Institute of Physics

2006; Martina et al. 2005; Mulder et al. 2006; Saito et al. 2005). Liposomes are especially interesting as transport vehicles for in vivo applications such as drug delivery where they are thought to achieve selective and sufficiently high localization of active drug at the disease site. While a homogenous size distribution is critical to assure a controlled drug dosage, liposome size also ultimately influences the detection and clearance rate by the complement system. Liposomes with diameters of less than 200 nm show a slower clearance rate than larger ones (Ishida et al. 2002; Litzinger et al. 1994). Other factors such as coatings are also very important and have resulted in the production of "stealth" liposomes that remain undetected by the complement system for prolonged times.

The challenge is to produce liposome formulations with a defined size for the specific application and with little size variation in their population. The methods for liposome production should also be flexible so that a protocol can be optimized for the desired concentration of encapsulated compounds and for functionalizing their surfaces. Lastly, shelf life of the formulation is of concern and so the ability for production on demand would be of interest.

There are a variety of methods available to create liposomes in bulk solution processes (e.g., alcohol injection (Batzri and Korn 1973; Kremer et al. 1977; Szoka and Papahadjopoulos 1980; Wagner et al. 2002), membrane extrusion (Szoka and Papahadjopoulos 1980), detergent dialysis (Szoka and Papahadjopoulos 1980), and sonication (Maulucci et al. 2005; Szoka and Papahadjopoulos 1980)), but in all cases they are formed by the self-assembly of phospholipid molecules in aqueous solution to form a lipid bilayer membrane that encapsulates an aqueous core. Comparison of liposome populations produced from those different techniques reveals a great deal of variability in terms of mean size and population homogeneity, but the tightest population distributions are most often obtained from multi-step methods that include an initial self-assembly followed by a post-processing step (most often, sonication or membrane extrusion).

Kuribayashi et al. showed electroformation of giant liposomes in microfluidic channels (Kuribayashi et al. 2006). Specifically, a polymethylvinylsiloxane sheet containing the microchannel is sandwiched between two indium tin oxide (ITO) glass plates which serve as transparent electrodes. A thin lipid film is dried at the bottom of a microchannel, hydrated with deionized water by capillary filling, and subsequently exposed to an alternating current (ac) signal producing giant unilamellar vesicles. Electroformation of liposomes in this manner resulted in giant unilamellar liposomes (liposomes composed of a single bilayer membrane) in contrast to giant multilamellar liposomes (liposomes composed of many bilayer membranes) that are produced by gentle hydration of lipid films without the electric field. Liposomes prepared by electroformation were overall larger with a mean liposome diameter of about 12 μm compared to those produced by gentle hydration without applying an ac field with a mean liposome diameter of about 5 μm. This is thought to be due to liposome fusion caused by the vibrational energy induced by the ac field.

In another study Wagner et al. used the fluidic crossflow ethanol injection method to produce homogenous liposome populations (Wagner et al. 2002). The crossflow injection technique utilizes two welded stainless steel tubes that form a cross. At the

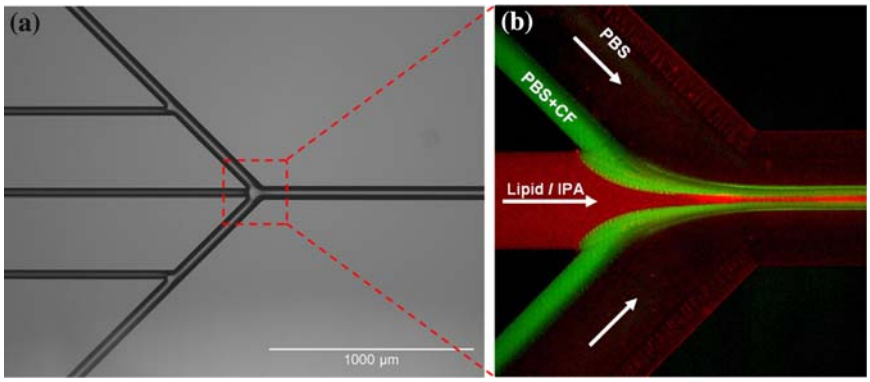

Fig. 5 (**a**) Bright-field micrograph showing the 5-inlet design to generate liposomes of variable size and size distribution. The solute to be trapped within the liposomes is closely confined to the interface of lipid self-assembly. (**b**) Confocal fluorescent micrograph of a hydrodynamically focused lipid/isopropyl alcohol (IPA) stream containing red fluorescent $DiIC_{18}(3)$. Green carboxyfluorescein (CF) is dissolved in aqueous phosphate buffered saline (PBS) and confined with non-fluorescent PBS to the interface where spontaneous lipid self-assembly is likely to occur. The arrows indicate the direction of fluid flow in the microchannel network

connecting point a hole with a diameter of 150 µm to 250 µm serves as an injection point for a lipid/ethanol mixture. This method is an improvement to the traditional ethanol injection method in which a lipid/ethanol mixture is manually slowly injected into rapidly vortexing aqueous buffer. The crossflow injection method provides better control and reproducibility compared to manual injection of the lipid. In this method, the ethanol/lipid stream is injected obliquely into the water stream. One part of the ethanol/lipid stream forms a non-miscible interface with the stainless steel tubing and the other forms an active liquid miscible interface with the buffer. Liposomes form at the miscible buffer/ethanol interface. The liposomes produced with the crossflow injection method vary in diameter between 200 nm and 500 nm.

Liposomes: microfluidic continuous flow

Our group first reported on the controlled formation of sub-micrometer sized liposomes in a microfluidic channel network via hydrodynamic focusing (Jahn et al. 2004). The liposomes are formed by a diffusively driven process when a stream of lipids dissolved in an organic solvent such as isopropyl alcohol (IPA) is hydrodynamically sheathed between two oblique buffer streams in a microfluidic channel. The lipid stream is hydrodynamically focused into a very narrow sheet with a thickness varying from a few micrometers down to sub-micrometers depending on the respective volumetric flow rate (VFR) ratios (the ratio of buffer volumetric flow rate to lipid volumetric flow rate). The laminar flow conditions facilitate diffusive mixing at the two miscible liquid interfaces predictably diluting the alcohol concentration below the solubility limit of lipids initiating lipid self-assembly into small unilamellar vesicles. In our initial study, we had reported diameters of liposomes produced using this method can be controlled from 50 nm to 150 nm.

According to a theory by Lasic et al., lipids dissolved in an organic solvent transform into intermediate bilayer phospholipid fragment (BPF) structures (essentially sheets of phospholipids bilayers) (Lasic 1988). Continuous diffusion of water and alcohol reduces the solubility conditions of the lipid which causes thermodynamic instabilities at the edges of BPFs inducing bending and closing of the BPF upon itself forming vesicles. Our data show that liposome size remains approximately constant over VFRs ranging from 30 µL/min to 200 µL/min as long as the ratio of VFR between the aqueous buffer and the organic solvent remains constant. However, the diameter of the vesicles changes considerably when the VFR ratio is changed (Jahn et al. 2007). Changes in the VFR ratio result in variable stream widths of the focused solvent stream. As the ratio of the VFRs decreases the stream width of the organic solvent increases, therefore providing a central region with higher organic solvent concentration. Assuming that

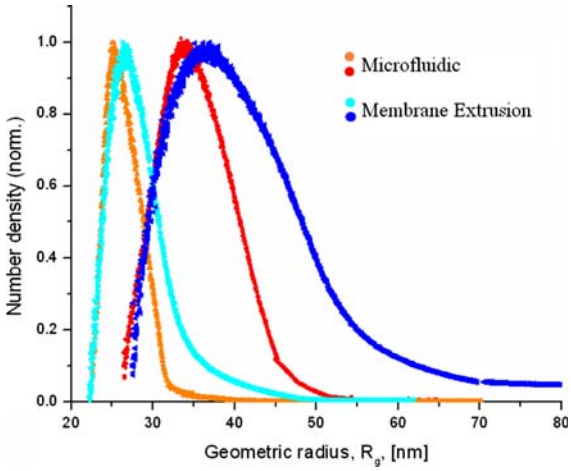

Fig. 6 Comparison of liposome populations obtained using the microfluidic method and macroscale injection methods. Two different populations of liposomes are compared from each technique. In order to compare population distributions, we show liposomes that were prepared from each method that resulted in approximately similar mean size of the liposome populations. The liposome diameter distribution data was obtained with multi angle laser light scattering after sample fractionation of four different samples. The buffer-to-alcohol ratios in the data shown are ● 20:1 and ● 50:1 with a mean geometric radius, R_g, and one standard deviation of 34.9 nm ± 4.7 nm and 26.6 nm ± 3.2 nm respectively for the microfluidic method, and ● 10:1 and ● 50:1 with an R_g of 42.8 nm ± 10.4 nm and 29.7 nm ± 6.1 nm respectively for the macroscale method

liposomes are formed in our microfluidic systems via a BPF intermediate structure, we hypothesize that a higher solvent content can potentially stabilize BPFs and allow larger congregation of lipids to yield larger BPFs resulting in larger vesicles and broader distribution. At higher VFR ratios the solvent stream is focused into a more confined jet whose alcohol content is more dilute. The lower alcohol concentration limits BPF formation resulting in smaller and more homogenous liposome populations (Jahn et al. 2007). This is consistent with our measurements. We emphasize that at this time it is experimentally impossible to observe the formation of these intermediate BPFs; however, this explanation supports our experimental findings and is consistent with previous theories of vesicle formation. The liposome formation process cannot be directly visualized because of the high flow rates in the microchannel with peak flow velocities of up to 1 m/s and Reynolds numbers of up to 100. Additionally vesicle sizes are well below the limit of optical resolution.

For drug delivery applications, the encapsulation efficiency needs to be controlled as well as size and size distribution. In our multiple inlet configuration in Fig. 5 we reduce the sample consumption by confining the water-soluble substance to be encapsulated to the immediate vicinity of the alcohol stream where liposomes formation is expected to occur. This minimizes sample consumption without adversely affecting the liposome encapsulation efficiency. Furthermore, the multiple inlet design allows us to alter the concentration of the solute to be encapsulated from an initial starting concentration via controlled diffusive mixing. This enables control over the loading efficiency of particles into liposomes in a continuous flow mode.

Comparison of microfluidic and bulk techniques

Adjusting the flow conditions provides a tool to modify the average liposome size and size distributions. Liposomes formed by hydrodynamic focusing produce very narrow size distributions which increase as the mean liposome size increases. In summary, with hydrodynamic focusing very narrow size distributions are achievable that can be skewed towards broader distributions by injecting larger volume fractions of organic solvent into the aqueous buffer. For high volumetric flow rate ratios post processing steps to control the size can be obviated (Jahn et al. 2007).

The formation of vesicles in the microchannel by hydrodynamic focusing is comparable to the bulk alcohol injection process. In a recent publication (Jahn et al. 2007) we demonstrated that slight changes in the mixing conditions result in a substantial change of the mean liposome diameter and size distribution. Standard alcohol injection into a vortexed aqueous buffer does not allow for controlled mixing conditions, resulting in polydisperse liposome populations. Here, we briefly compare liposome formation by hydrodynamic focusing in a microchannel with the traditional bulk alcohol injection method.

Figure 6 compares the distribution of radii for liposomes produced with hydrodynamic focusing in a microfluidic channel and those produced by a macroscale bulk IPA injection method. The samples were prepared with identical lipid composition and concentration. In the macro-scale IPA injection method,

lipids were injected into an agitated scintillation vial containing phosphate buffered saline solution and subsequently post-processed by eleven membrane extrusions using a commercially available membrane mini-extruder according to vendor specifications.

In all cases, the microfluidic approach generates a narrower distribution when small liposomes are desired. For both methods, as the average size of the liposome population increases, the width of the size distribution increases. In the macroscale method, agitation during the injection process is critical to facilitate rapid mixing and narrow liposome size distributions. As larger IPA amounts are injected agitation becomes a critical parameter to obtain quality liposome formulations. The liposome size distributions grow increasingly polydisperse with a larger mean size when agitation is neglected during the injection process. Post-processing of the original liposome population with membrane extrusion reduces the polydispersity and results in liposomes that are comparable, but still more polydisperse than microfluidically generated liposomes. Similarly, hydrodynamic focusing in a microfluidic channel produces liposomes with a very narrow size distribution, but obviates post-processing steps that affect the diameter.

Conclusions

The characteristics of micro- and nanoparticle formulations produced by continuous flow microfluidic systems have distinct advantages over traditional bulk methods that motivate their adoption by the broader scientific community. The exquisite control of flow and mixing conditions in microfluidics led to improved homogeneity of particle size distributions and the control of particle size in a reproducible fashion. The monolithic structure of microfluidic systems often allows for simple implementation of particle formation procedures in lab-on-a-chip applications with integrated real time analysis of the generated particles. The continuous formation of particles in a microfluidic system obviates disassembly and assembly processes often required in traditional bulk technologies and allows for dynamic control of flow and mixing parameters to tailor particles to one's need. Continuous flow microfluidic systems have the potential to become a standard technology that allows for particle formulations with unprecedented homogeneity and fine control over the critical process parameters that are difficult to achieve in bulk techniques.

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