Particles under Flow: Engineering and Evaluating Nanostructured Particles in Fluidic Devices

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Short summary

Nanoengineered particles can be used to improve human health, in areas ranging from diagnostics and imaging to therapeutics and regenerative medicine, but only a few examples have made it from the lab to the clinic. The aim of this thesis is to show how flow-based devices can be used to help address some of the key challenges in the field—both in making particles and in understanding how they work—to help accelerate the development of nanostructured polymer particles.

Abstract

Nanotechnology is an important bridge between engineering and biotechnology, and has advanced the design and characterization of particles for biomedical applications. As a result, a range of delivery systems, including numerous types of polymer particles, has been generated over the last few decades.

The performance of nanoengineered particles depends on their ability to negotiate biological barriers, including avoiding premature degradation, non-specific accumulation, and eliciting unintended immune responses. Despite the tremendous potential of nanostructured particles, few have made it from the lab to the clinic. Key reasons underlying this include a lack of robust, scalable and automatable methods to nanoengineer particles, and an incomplete understanding of how to design particles with well-controlled bio-nano interactions. In this thesis, we investigate how flow-based devices (such as macro- and microfluidic devices) can be used to help address these challenges. The results are presented in three parts.

In the first part, we develop a flow-based technique for layer-by-layer assembly of nanofilms on particles. We demonstrate that multilayered particles and capsules with different sizes (from micrometers to nanometers in diameter) can be assembled on different templates using
different types of polymers. We also show the assembly of capsules loaded with doxorubicin, a drug used in cancer chemotherapy. The well-controlled, integrated, and automatable nature of the presented flow-based system provides scientific, engineering, and practical processing benefits, making it valuable for research environments and potentially useful for translating nanostructured particles into diverse applications.

In the second part, we develop a microfluidic blood capillary model and use this to investigate the flow-based deformability of nanoengineered particles. We demonstrate that two types of polymer particle systems—one produced through a mesoporous silica templating approach and one made via atom transfer radical polymerization-mediated continuous assembly of polymers—can be engineered to behave similarly to human red blood cells in this biomimetic device. The results provide insights into the structure-behavior relationship of nanostructured particles and show how aspects of an important biological barrier can be recapitulated and probed in vitro.

In the third part, we develop a flow-based method for investigating the impact of dynamic flow on bio-nano interactions. We engineer two particle systems and investigate their cell-particle interactions under dynamic flow. One particle system is used for investigating sedimentation effects under dynamic flow, and it is shown that the presented device can be used to equalize the cellular dose of particles. The second particle system consists of rod-shaped particles with different aspect ratios and is used to investigate the effect of particle shape on cellular association under dynamic flow. The results demonstrate the importance of complementing conventional in vitro assays of cell-particle interactions with more dynamic assays, and provide insights into the importance of sedimentation and shape effects on cell-particle interactions under static and dynamic flow. In summary, we show how flow-based devices can be used to engineer micro- and
nanoparticles, and investigate how they work in biomimetic and biological environments, thus addressing key challenges for using nanostructured particles in biomedical applications.

The PhD candidate has published several papers during the candidature and in part A-D below some of these are included. See last page for publication list. The full and up-to-date publication list is available via the candidate’s Open Researcher and Contributor ID (ORCID) and Google Scholar. The full thesis is available (in hardbound copy) from The University of Melbourne Library.
Part A

Engineering and Evaluating Drug Delivery Particles in Microfluidic Devices

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Review

Engineering and evaluating drug delivery particles in microfluidic devices

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ABSTRACT

The development of new and improved particle-based drug delivery is underpinned by an enhanced ability to engineer particles with high fidelity and integrity, as well as increased knowledge of their biological performance. Microfluidics can facilitate these processes through the engineering of spatiotemporally highly controlled environments using designed microstructures in combination with physical phenomena present at the microscale. In this review, we discuss microfluidics in the context of addressing key challenges in particle-based drug delivery. We provide an overview of how microfluidic devices can: (i) be employed to engineer particles, by providing highly controlled interfaces, and (ii) be used to establish dynamic in vitro models that mimic in vivo environments for studying the biological behavior of engineered particles. Finally, we discuss how the flexible and modular nature of microfluidic devices provides opportunities to create increasingly realistic models of the in vivo milieu (including multi-cell, multi-tissue and even multi-organ devices), and how ongoing developments toward commercialization of microfluidic tools are opening up new opportunities for the engineering and evaluation of drug delivery particles.

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receptor–ligand interactions at the nanometer scale can cause the release and distribution of hormones that can, ultimately, lead to organism-level changes. To work at, and understand, all of these length scales, especially at the smallest dimensions, requires a highly interdisciplinary approach [6].

In this review, we discuss current challenges facing particle-based drug delivery systems and review strategies where microfluidic technologies have been used to address some of these issues. We provide an overview of both the production and evaluation of drug delivery particles, with a focus on microfluidics as an enabling technology. Emphasis is placed on how microfluidics can complement existing technologies by providing new ways to reliably and reproducibly engineer drug delivery particles and new in vitro models that can mimic important aspects of the in vivo situation. These features of microfluidic technologies that enable detailed analysis of mechanisms that govern interactions of particles with biological systems can facilitate the correlation between in vitro and in vivo studies. Additionally, we provide an outlook of this growing interface between drug delivery and microfluidics, as well as discuss the impact of the evolution within microfluidics, from highly specialized “home-built” systems to easily accessible “off-the-shelf” instruments. This increase in accessibility is facilitating interdisciplinary work, thus accelerating the development of new and improved rationally designed drug delivery particles.

2. Drug delivery particles and challenges ahead

The objective of a drug delivery particle is to deliver a therapeutically effective agent to where it is needed, when it is needed. The archetypical example is to selectively deliver a cytotoxic compound to a tumor, at a high enough concentration and for long enough to kill the tumor, while at the same time leaving healthy tissue unharmed. A drug delivery particle can provide a different means toward realizing this, including: (i) facilitating formulation of the therapeutics; (ii) increasing specificity; and (iii) providing controlled release (Fig. 1). Multifunctional drug delivery particles therefore have the potential to enable the use of new drugs as well as to improve the performance of existing drugs.

Despite the great promise of drug delivery, significant challenges still remain. Today, several particle-based drug delivery systems exist in the clinic and others are currently undergoing clinical trials [7–9]. However, these successes should be considered against a backdrop of many different research groups around the world that have developed a plethora of diverse drug delivery systems, many of which have proven effective in in vitro studies, but with only a very few having made it successfully past preclinical studies. A reason for this is the common discrepancy seen when comparing preclinical and clinical data, where remarkable advantages in efficacy for drug carriers seen preclinically almost completely disappear when moving to humans [10], although there are examples of successful preclinical–clinical correlations [11]. This discrepancy indicates the difficulty of extracting predictive information of drug carrier behavior and performance in the clinical setting using conventional models, information that is critical for the rational design and development of drug carriers.

It may be instructive to compare this situation to the pharmaceutical industry as a whole, which is facing unprecedented challenges due to a combination of scientific, economic and legal reasons, in what has been called the “pharmaceutical industry’s grand challenge” [12,13]. A main reason for this is the high rate of expensive late-phase drug attritions, and therefore a key objective is to identify and eliminate unsuccessful drugs as early as possible. Part of the solution, as proposed by four major pharmaceutical companies, could be the development and increased usage of new and improved in vitro pharmacological profiling assays that can provide more accurate predictions of clinical performance [14]. Reliable in vitro assays with high predictive power of drug-performance in humans would not only prove valuable in preclinical evaluation, but could also inform and guide the initial research and development of new therapeutics.

To apply this to the field of drug delivery, several other factors need to be considered when evaluating engineered particles, in addition to the characteristics of the therapeutic to be delivered. Important factors affecting the behavior of drug delivery particles in a biological system include both physicochemical parameters of the particles as well as characteristics of the biological target environment [15–20]. Further,
with each new functionality added to a particle – for example imaging or targeting moieties – the biological behavior becomes even more convoluted [21]. Delineating these complex relationships between design parameters and biological performance is one of the grand challenges within particle-based drug delivery and is critical for the rational design and development of carriers.

Developing in vitro models of the complex biological setting, to evaluate intricate particle-designs, in order to delineate convoluted particle-biological interactions may seem like a daunting challenge. However, being aware of such complexity can allow common pitfalls to be avoided, and focus placed on where most impact can be made [22–25]. Ideally, a reductionist approach could be applied, where the level of complexity can be tuned, as this would facilitate the study and formation of causative relationships. The conventional strategy, however, including standard cell culture and animal models followed by clinical studies, allows only a limited reductionist approach (Fig. 2).

For example, the step from a petri dish to an animal is arguably quite large. Reasons underlying a failure in vivo following a success in vitro can therefore be difficult to investigate, explain and rectify. Therefore, a key objective is the development of in vitro models that are sufficiently complex to realistically mimic aspects of the in vivo situation, yet simple enough to enable an understanding of underlying relationships and mechanisms.

3. Microfluidics as an enabling technology

Microfluidics is a multidisciplinary field where small amounts of fluids are handled in channels with dimensions typically from tens to hundreds of micrometers [26]. At these length scales novel, and sometimes nonintuitive, properties appear. Examples include laminar flow and the relative importance of diffusion. This is because the competition between various phenomena that dictate the behavior of fluids do not scale linearly with changes in dimensions [27]. Using soft lithography, microfluidic devices that exploit these features can be designed with both high precision and relative ease [28].

The possibility of engineering controlled fluidic microenvironments opens up interesting biological applications, as many important biological processes take place at the micrometer scale. Examples include the microvasculature [29], where gases, nutrients and waste products are exchanged, as well as important functional barriers and interfaces in major organs such as the kidney and liver [30,31]. Fluidic flows are an important part of both healthy and pathological conditions. This includes not only the more obvious flow of blood and lymph in the circulatory system, but also the interstitial flow taking place in virtually all soft tissues [32]. Key aspects of the biological setting therefore include both micrometer structures as well as well-controlled fluid flows.

In a typical microfluidic set-up, basic components include a syringe pump or a pressure source, and tubing connected to a microfluidic device that is typically fitted on top of a microscope slide. Cells and/or bacteria can be added to a device to create both simpler (such as a single cell-type cultured inside a straight channel) and more complex (such as different cell-types cultured in networks of interconnected channels) environments. By changing the conditions in the channel, for example by introducing compounds and particles in the flow, biological responses can be probed [33,34].

There are several advantages of using microfluidic devices to study biological settings (Fig. 3). These include: (i) biologically relevant length scales; as discussed above, many important biological structures, as well as cells themselves, have length scales from tens to hundreds of micrometers; (ii) a high degree of control of geometry; well-developed and well-characterized microfabrication technologies, such as soft lithography [28], provides researchers with a large toolbox to manufacture specific designs in a reliable and reproducible manner; (iii) control of fluidics and the microenvironment; the intrinsic features of small volumes of liquid as well as fluid physics at these length scales [27] enable a high degree of control over the spatiotemporal environment of the cells, such as pulses or gradients of a stimulant; and (iv) real-time monitoring, microfluidic devices are typically made to fit on top of a standard microscope slide and as they are usually made of transparent glass and polymers they can be continually – and in real-time – monitored using standard microscopy techniques.

Fig. 2. Illustration of the trade-off between reductionism and complexity/realism for a few preclinical models in relation to the clinical setting (not to scale) (blue dots: in vitro, red dots: in vivo). Conventional methods typically require one to choose from models either enabling a reductionist approach or models with realistic/relevant results because of technological, economical and ethical constraints. Microfluidic models might provide opportunities to come closer to the clinical setting in relevance while retaining the power of in vitro reductionism.

Fig. 3. Probing the response and interaction of two cell types (green and blue) in interconnected chambers during exposure to two pulses of compounds (yellow and red). This is enabled by several features of microfluidic devices when applied to biomedical, which include: (i) biologically relevant length scales; (ii) designed geometries; (iii) spatiotemporal well-controlled microenvironments; and (iv) the option of real-time monitoring. These provide a means to recreate and probe important (patho-)physiological structures and conditions.
Capitalizing on these advantages, microdevices are emerging as a powerful tool for nanomedicine [35]. This includes microfluidic technologies used during both engineering and evaluation of drug delivery particles [36]. In engineering, including both synthesis and characterization, microfluidics provides new and improved methods of making particles. In the evaluation, as long as some considerations are made [37–39], microfluidics provides new and improved in vitro models, models that are starting to make an impact on our understanding of crucial interactions between drug delivery particles and biological systems.

4. Engineering drug delivery particles through microfluidics

Both the materials and the methods used to engineer drug delivery particles determine their properties. As all methods have both strengths and drawbacks, there is an ongoing process in improving existing – and developing new – techniques toward more reliable and reproducible production of particles with highly tuned properties. One example of an area where there is significant ongoing activity is the fabrication of polymer capsules [40].

Microfluidic devices offer new possibilities for the production of both micro- and nanoparticles [41–43]. Mass transport in fluids is governed by both viscous and inertial effects, with the latter being responsible for the nonlinearities that give rise to numerous instabilities, such as turbulence. However, when fluidic flows are miniaturized, as in microfluidics, some inertial effects become negligible [27]. This can be utilized to construct devices where fluidic flows interact in highly controlled and reproducible ways. This is difficult – or even impossible – to achieve at the macro scale. These types of devices have been used to create both organic and inorganic particles for biomedical applications [44,45].

In this section, we provide an overview of different microfluidic mechanisms that can be used to produce drug delivery particles. These include the microfluidic production of micro- and nanoparticles using: (i) droplets; (ii) flow focusing; (iii) microvortices; (iv) templated assembly; and (v) flow lithography (Fig. 4). We also discuss challenges in microfluidics where inherently small volumes can lead to low throughputs and approaches that are being investigated to facilitate scale-up and future industrial-scale production.

Fig. 4. Microfluidic methods to produce micro- and nanoparticles. a) Particles can be formed through droplet-formation in immiscible fluids. b, c) Flow focusing and microvortices can be used to create well-controlled liquid–liquid interfaces for nanoprecipitation of particles. d) In templated assembly, a template is moved between complementary polymer solutions to create multilayered particles. e) In flow lithography, UV light is passed through a shape-defining mask and projected down into a microfluidic channel where particles are formed through photopolymerization.
In a recent example, paclitaxel and chitosan, the latter hydrophobically modified using a lipid, were mixed under acidic conditions and then flow focused in a microfluidic device using side streams of water at basic pH, which induced nanoprecipitation and the formation of drug delivery particles [57]. Materials that require more complicated solutions, such as chlorinated organic polymer solutions and organic solvents, can also be used, for example by using glass microchannels [58]. To further improve the production of drug delivery particles, a few special geometries have been investigated in addition to the standard T-shape. This includes 3D hydrodynamic flow focusing where, in addition to the two lateral side streams, the center stream is also focused by two vertical streams [59]. Although this introduces some complexity, improved monodispersity and smaller sizes can be achieved, as well as reduced problems with aggregation that can be seen using conventional 2D focusing. Further, special geometries have been used to increase the mixing rate and the throughput. Examples include staggered herringbone micromixers [60,61] and Tesla-type mixers [62]. In a recent example, 3D hydrodynamic focusing and micromixing were combined for the on-chip combinatorial synthesis of targeted drug delivery particles for cancer therapy [63]. Drug delivery particles with different size, zeta potential, ligand density and drug loading were all synthesized on-chip in a rapid and reproducible approach to form a library of 45 variants that was subsequently screened in vitro and in vivo.

Microfluidic devices operating at higher Reynolds numbers can be designed for the formation of drug delivery particles through controlled microvortices (Fig. 4c) [64]. For example, a central stream with poly(lactic-co-glycolic) acid in acetonitrile and side streams with lipid and lipid-poly(ethylene glycol) conjugates in 4% ethanol aqueous solution can be used to produce lipid-polymer hybrid nanoparticles at high Reynolds numbers (~150) [64]. By operating at these higher Reynolds numbers, where inertial effects contribute with convective mixing in addition to the diffusive mixing, a significant increase in throughput, reportedly a 1000-fold increase compared to conventional microfluidic diffusion-based syntheses, as well as improved reproducibility and homogeneity can be achieved [64]. This method has also been used for coencapsulation of multiple drugs and imaging agents as well as for the production of lipoprotein-derived nanoparticles, the latter by having apolipoprotein A in saline in the side channels and lipids plus imaging or therapeutic agents in a solvent (ethanol, methanol and/or chloroform) in the central channel [65,66].

Templated assembly through layer-by-layer adsorption is a prominent technique through which multilayered films with tailored properties can be prepared, and this method has also been implemented in microfluidic devices [16]. Layer-by-layer buildup can be achieved by moving particle templates between complementary polymer solutions. In a microfluidic device this can be realized by having laminar streams of polymer solutions flowing side-by-side and using microfabricated structures to displace the particle templates back and forth [67]. Another strategy is to have the particle templates move through a central channel from which complementary polymer solutions are sequentially exchanged (Fig. 4d) [68]. This can be achieved by cycles of adding and withdrawing solutions, but not the templates, from the main channel using side-channels where microfabricated structures keep the particles from exiting while allowing the polymer solutions to be withdrawn [68].

Flow lithography is a photolithographic technique where a pattern is projected into a photocurable polymer to form particles (Fig. 4e). In continuous-flow lithography, shape-defined photopolymerized micro-particles can be made by passing UV-light through a transparency mask that is projected using a standard microscope objective down into a microfluidic device filled with monomer and photoinitiator [69]. The process can also be performed in a stop–start fashion [70]. Using flow lithography both the size and the shape of the particles can be tailored, as well as their degradation behavior [71]. This method has been used to encapsulate cells as well as to generate encoded particles for biomolecule analysis [72–74].

A key challenge in the translation of drug delivery particles from the lab to the clinic involves reliable and reproducible scale-up for industrial production. The inherently small dimensions and volumes of microfluidics, giving rise to the physics on which many of the advantages rest, such as laminar flow and well-controlled fluid–fluid interfaces, also pose challenges during scale-up toward industrial production [75]. Some processes, however, can be used in larger dimensions, allowing the use of millifluidic – instead of microfluidic – set-ups. Using a millifluidic system it has been demonstrated that grams of gold nanoparticles can be produced in a matter of hours, which is a thousand-fold improvement compared to common microfluidic techniques [76]. There are some microfluidic methods that can reach this high rate of production as well. One example is, as discussed earlier, by working at high flow rates and Reynolds numbers where controlled microvortices can be used to produce lipid–polymer hybrid nanoparticles, allowing a production rate of ~3 g per hour to be reached [64]. In addition to these advances toward higher rates of particle-production, a continuous fluidic platform also allows on-line implementation of other important stages, including post-synthesis functionalization and sterilization. In one example, hollow gold nanoparticles were synthesized, functionalized with poly(ethylene glycol) and sterilized with UV-light, all in the same continuous microfluidic platform [77].

In summary, microfluidics opens up the use of physics and chemistry that is difficult, or even impossible, to use at the macroscale. An important example is the relative lack of turbulence at the microscale, which enables well-defined and well-controlled fluid–fluid interfaces to be made and manipulated, thus enabling the use of interfacial processes, such as droplet formation and precipitation, in a more reliable and reproducible way. By complementing existing techniques, such as bulk emulsification and solvent displacement or centrifugation-based layer-by-layer assembly [40,78], microfluidic methods are already making an impact, as seen by the examples highlighted above. These approaches are expected to play an important role in the production of new and improved drug delivery particles.

5. Evaluating bio-interactions of drug delivery particles through microfluidics

An objective of an engineered particle-based drug delivery system is to guide the interactions between a therapeutic and a biological system. Two important aspects of this are improving the localization and kinetics of drug exposure. Ideally, a drug should be released only at the intended site of action, for example at a tumor for a cancer drug, and at a concentration and over a time frame that optimizes the therapeutic effect while minimizing toxicity. This dose–response relationship is different for different drugs but is critical for a therapeutic effect. Well-investigated examples are antimicrobial drugs, where usually one of several pharmacodynamic parameters, such as (i) peak concentration, (ii) area under curve or (iii) time over minimum inhibitory concentration, should be optimized [79]. To probe, understand, and ultimately direct these interactions is therefore of key importance in the development of drug delivery particles.

In this section, we first discuss how engineered in vitro microfluidic models can complement conventional methods. Secondly, we provide examples of how engineered particles behave under flow, including: (i) their mobility in constricted channels; (ii) their adhesion to cells; and (iii) their cellular uptake (Fig. 5). Finally, we highlight several studies where microfluidic devices have been developed to mimic human pathological conditions to investigate the behavior of engineered particles, with results subsequently validated in vivo using animal models.

Conventional cell cultures and animal models have provided tremendous insights into interactions between drug delivery particles and biological systems, and remain the bedrock on which the field stands. However, there is a growing concern that these models have some significant limitations. For example, recently the genomic and transcriptomic landscape of the HeLa cell line, arguably the most
commonly used cell line, was studied and strikingly aberrant characteristics were found [80]. Recent studies have also raised concerns on commonly used animal in vivo models and methods for the study of human conditions [81–84]. Therefore, there is a need to complement these techniques with methods that can capture other important aspects of human pathological states. Ideally these methods would allow the use of cells from more relevant cell sources that can be difficult to maintain in standard culture dishes, in environments that more closely mimic human conditions.

Microfabrication technologies, in combination with microfluidics, offer tools through which aspects of an in vivo environment can be mimicked and responses to drug exposure studied [85]. Exemplifying this are several microfluidic devices that have been developed to investigate dynamic spatiotemporal changes in drug exposure and corresponding biological responses. In one example, bacteria were grown in a microfluidic device mimicking naturally occurring bacterial niches and the heterogeneous microenvironment was studied as well as its influence on the emergence of antibiotic resistance [86]. In a similar example, drug gradients and motility of cancer cells within a microfluidic device were studied and it was shown that these aspects are important in the emergence of resistance to chemotherapy [87]. Microfluidic devices can also be used to study effects of drugs on multiple cell types in an integrated way, for example by culturing liver, colon and marrow cells in compartments connected by channels mimicking blood vessels [88]. Another multicompartiment fluidic system, mimicking the in vivo fluctuations of antimalarial drugs, has recently been devised and has demonstrated promise for the evaluation of compounds for the treatment of malaria [89]. Taken together, these studies demonstrate the potential of microfluidic models to probe dynamic drug–bio interactions, thus increasing our understanding of processes that are difficult, or even impossible, to investigate using conventional methods.

Microfluidic devices can be used to investigate the behavior of engineered particles in constricted channels. Intravenous injection is a common route for systemic drug delivery and microfluidics can be used to create models of the vasculature [90]. Using these models, particle behavior in the microvasculature and in tumor vessels can be investigated [91,92]. This includes mechanobiological aspects of the in vivo setting and the impact of particle geometry and mechanics [93,94]. By changing the shape and stiffness of a microparticle, the ease with which they can squeeze through a small capillary can be tuned [95–100]. Red blood cells are another well-studied example; for example the impact of healthy and pathological states on their behavior in flow [101]. Generally, red blood cells can squeeze through blood microcapillaries smaller than their size, but become mechanically trapped (especially in endothelial slits, ~ 0.5 to 1 μm in diameter, in the spleen) if they are stiffer [102]. This has important implications for the performance of engineered particles in vivo, as has been demonstrated using red-blood-cell shaped hydrogel particles where differences in stiffness caused differences in both biodistribution and circulation half-life in a mouse model [103].

Another important aspect of the behavior of engineered particles under flow is their interaction with cells, as they have important implications for the toxicity observed for some engineered particles [104]. An example is the discrepancy in observed toxicity between in vitro and in vivo models for quantum dots [105]. Interestingly, a difference in toxicity can also be seen when comparing static and fluidic in vitro methods [106]. A possible reason for this is sedimentation effects are much more pronounced under static than fluidic conditions, effects that have been shown to strongly influence cellular association and uptake [107].

The adhesion of engineered particles to cells under flow conditions is influenced by both design parameters and flow conditions. The use of targeting ligands can provide an “anchoring effect” to enhance the specificity. However, an increase in flow rate also decreases the number of adhered particles, even in the presence of targeting molecules [108–110]. Shape effects have also been shown to be important. Examples include particles of different geometries (spheres, disks and rods) exhibiting different adhesion profiles [111], and filaments tens of nanometer in diameter and micrometers in length demonstrating flow-aligning effects, minimizing their cell interactions [112]. By combining the knowledge of how shape-effects and targeting ligands affect cell adhesion under flow, particles with higher specific and lower nonspecific accumulation under flow can be engineered [113].

The cell response to engineered particles under different flow conditions is influenced both by differences in shear stress and by a differential particle uptake by the cells. For example, the presence of flow increases the delivery rate of liposome–DNA plasmid complexes [114], while changes in shear stress modulate the cytotoxicity of engineered particles [115,116]. This difference in cellular responses is partly due to changes occurring in cells adapting to flow conditions. It has been shown that endothelial cells undergo significant cytoskeletal rearrangements when cultured in a flow environment, which is more similar to the cell physiology in vivo compared with the static cell culture environment [117], with the formation of actin stress fibers implicated as an important mechanism affecting particle internalization pathways and profiles [118–120]. This flow-mediated modulation of particle behavior has important implications for drug delivery, as it affects how cells interact with and process engineered particles.

In addition to the studies discussed above, many of which are of a more fundamental nature, there have also been some studies where microfluidic devices that mimic pathological conditions of the in vivo situation have been developed and used to evaluate engineered particles with results validated in vivo using animal models. Here, we highlight a few examples of these studies and discuss the different steps involved.

In a study by Korin et al. [121], shear-activated drug delivery particles were designed and developed using a microfluidic model of an obstructed blood vessel and the efficacy was confirmed both in a microfluidic embolus model and in a mouse embolus model (Fig. 6). In the first step, pathological shear stresses associated with obstructed small blood vessels were identified and a microfluidic model mimicking these conditions was designed (Fig 6a). Computational fluid dynamics simulations were used to refine and verify the design, which was then realized through soft lithography (Fig 6b,c). This microfluidic device was then used to evaluate the shear-responsiveness of microaggregates (~4 μm diameter) of poly(lactic-co-glycolic acid) nanoparticles (~180 nm) under physiologically relevant shear stresses (Fig 6d). Nanoparticles were then coated with a thrombolytic drug before assembly into microaggregates, forming drug delivery particles. Following injection of emboli into another microfluidic device, these drug delivery particles were infused and shown to accumulate at, and dissolve, the blood clots (Fig 6e). In contrast, treatment with a soluble thrombolytic drug at
equivalent concentration had a negligible effect. The efficacy of the drug delivery particles was subsequently validated in an ex vivo whole-mouse-lung ventilation-perfusion model. It was shown that drug delivery particles needed a hundred-fold lower concentration for a therapeutic effect compared to soluble drugs. The therapeutic effect of the drug delivery particles was also validated in living mice by infusion of the particles, or the soluble drug, following injection of preformed fibrin clots. All seven mice in the control group (soluble drug) died within an hour while >85% (six out of seven) of mice treated with drug delivery particles survived without any symptoms (Fig. 6f). This clear increase in efficacy, combined with a rapid clearance and low diffusion, which could help to minimize unwanted bleeding and neurotoxicity, illustrates how microfluidic models can facilitate the design and development of new types of drug delivery particles, for example by targeting pathological hemodynamic states.

Microfluidic devices have also been used to evaluate nanoparticle accumulation at tumors under physiological flow conditions, providing important insights into the mobility and retention of nanoparticles in tissues [122]. In that study, multicellular tumor spheroids were first cultured in 96-well plates and then loaded into a microfluidic device (Fig. 7). Each spheroid produced, and was surrounded by, a non-uniform layer of extracellular matrix (ECM) that acted as a physical barrier between the cells and the medium. The penetration into spheroids of gold nanoparticles (NPs) functionalized with poly(ethylene glycol) (PEG) in various sizes (40, 70, 110 or 150 nm in diameter) was investigated. It was shown that the nanoparticles with smaller dimensions (40 and 70 nm) entered the spheroids and accumulated in the interstitial spaces to a greater extent than the larger ones, suggesting that size is a key factor governing tissue penetration. By functionalizing the nanoparticles (40 nm) with transferrin (Tf), the accumulation was further improved by more than an order of magnitude (both inside the spheroid and in the ECM) compared with PEG-NPs. Interestingly, Tf-NPs showed an enhanced retention compared with PEG-NPs during flushing/washing, indicating a significant “anchoring effect” by the targeting molecule. It was also shown that the tissue accumulation was responsive to the flow rate. A nine-fold increase in flow rate resulted in a two-fold increase in both PEG-NP and TF-NP accumulation. However, it was noted that both TF-NPs and PEG-NPs accumulated predominantly in the ECM surrounding the spheroid, and that the change in accumulation under different flow rates occurred almost exclusively at the spheroid’s outer layer. This indicates that diffusion is the main mechanism through which nanoparticles penetrate the spheroids. These findings on size dependence, tumor accumulation and targeting effects were further validated in an in vivo murine xenograft tumor model, suggesting that microfluidic models can be used to mimic physiological microenvironments to deepen our understanding of complex biological interactions. Moving forward, this capability could play a part in addressing...
important clinical challenges. For example, it can be envisaged that microfluidic devices could be used to help elucidate underlying mechanisms of drug resistance, as tissue distribution and drug release kinetics influence resistance, and therefore help guide future developments in particle-based drug delivery [123,124].

In a study by Kim et al. [125], a microfluidic device with two compartments separated by an endothelial layer with controllable permeability, was used as an atherosclerotic endothelium model to study the translocation of engineered particles, and the results were compared to an in vivo rabbit model (Fig. 8). Permeability was monitored through transendothelial electrical resistance using four integrated electrodes. An inflammatory cytokine (TNF-alpha), involved in the pathogenesis of atherosclerosis, was infused into the device at concentrations seen clinically, which caused disruption of intercellular junctions and an increase in the endothelial permeability. Different flow rates, with different resulting shear stresses, could also be used to modulate the permeability. Lipid–polymer hybrid nanoparticles were infused into the device and their translocation across the endothelial layer was monitored. Interestingly, a significant correlation between nanoparticle translocation and endothelial permeability was observed. Rabbits with induced atherosclerotic lesions were used to study the in vivo behavior of the engineered particles and using near infrared fluorescent imaging, particle translocation was investigated both in microfluidic devices and in rabbit aortas. A high degree of correlation ($r^2 > 0.8$ and $P < 0.0001$) was observed between permeability and particle translocation for both methods, indicating the validity of the microfluidic model. Taken together, the results demonstrate that particle translocation primarily occurs at sites of increased permeability, which has important implications for targeting of drug delivery particles to atherosclerotic plaques.

Further, the similarities between the microfluidic model and the in vivo model demonstrates the potential of the former as a tool to probe the translocation of engineered particles in a highly controlled, and physiologically relevant, manner.

In an early study by Huh et al. [126], a microfluidic device was used to reconstitute the air–blood alveolar–capillary interface of the human lung by culturing human alveolar epithelial cells and pulmonary microvascular endothelial cells on each side of a membrane, facing air or liquid microfluidic channels, respectively. Physiological breathing motions were recreated by applying cyclic mechanical lateral stretching over the cells. Nanotoxicology studies were then performed by exposing the epithelial side to 12 nm silica nanoparticles. Exposure to the nanoparticles resulted in a significant increase in expression of a leukocyte adhesion molecule (ICAM-1), reactive oxygen species production, and translocation of the nanoparticles across the alveolar–capillary interface. Importantly, both the inflammatory responses and the translocation of nanoparticles were significantly augmented by the application of simulated breathing motions, an effect that could not be studied in controls using conventional transwell cultures. To validate the microfluidic model, a whole-mouse-lung ventilation-perfusion model was used. The results were in agreement with what was observed using the microfluidic model. The system presented in this study has also been used to investigate the toxicity of drugs and in the discovery of new drug candidates [127].

In summary, microfluidic devices provide new methods through which engineered particles can be evaluated. Both for more fundamental studies, such as how they behave under flow in constrained dimensions and how flow affects their interaction with cells, and in more applied studies, such as how engineered particles behave in more complicated dynamic pathological settings, including the tumor microenvironment and at the site of a blood clot. By integrating microfluidic assays with conventional in vitro and in vivo assays, a more complete understanding of the spatiotemporal performance of drug delivery particles can be reached, an understanding that is essential for the rational design of particles with highly defined drug release kinetics localized at a target site.

6. Conclusion and outlook

Engineered drug delivery particles have over the last half century moved from being primarily within the realm of science fiction to technological reality [128]. This development has been fueled by convergence of several disciplines, including biology, chemistry, engineering, materials science, and medicine. This highly interdisciplinary nature introduces some significant challenges but also provides ample opportunities, provided that researchers from disparate fields can coalesce around a common focus [129]. Illustrating this is the field of microfluidics as discussed in this review, where microfabrication techniques (commonly used by physicists) are used to recreate physiological environments (requiring biology expertise) to investigate drug delivery particles (typically designed and made by materials scientists and chemists).

A key feature of microfluidic devices when used to evaluate drug delivery particles is the ability to model physiological settings with variable complexity. The starting point can be a very simple model that captures only a part of the (patho-) physiological setting, which can then be expanded by adding components to mimic more complicated scenarios. Bioengineering techniques such as 3D cell culture and tissue engineering [130,131], as well as recent advances in applying microfluidic devices for purification and manipulation of stem cells [132–134], provide powerful tools that can help generate increasingly sophisticated and realistic models. Prominent examples are so-called “organ-on-a-chip” microdevices [135,136]. In these devices, multiple cell types are cultured in environments microengineered to reconstitute the tissue arrangement observed in specific organs. Examples of organs on which microfluidic devices have been based include the lung, liver, kidney and heart [137]. The use of these microfluidic devices holds promise for the development and translation of new therapies by allowing one to probe the behavior and response of multiple tissues, and even multiple organs, in an integrated and highly controlled and defined setting [138–141]. However, as the model becomes more and more realistic the results can become more relevant, but at the cost of more complicated operation and more convoluted behavior which can make conclusive relationships difficult to elucidate. This flexibility of microfluidic devices thus allows optimization of the trade-off between simplicity and realism to a degree not possible using conventional in vitro and in vivo methods, therefore opening up new and improved complementary assays to illuminate important relationships and mechanisms governing particle–bio interactions.

For microfluidics to continue to increase its impact on the field of drug delivery, the development toward increased and simplified access for non-experts is important. Even though there are laudable efforts to disseminate know-how (e.g. [28,142–144]), significant hurdles still
exist for researchers without prior microfluidic experience before they can apply microfluidic technologies. Part of the challenge lies in the differences in handling between a microfluidic and a standard cell culture. This means that currently existing cell culture skills and expertise might not be directly translatable to the microfluidic setting. For example, the sealed nature of typical microfluidic devices can complicate seeding and attachment of cells, and changes in media are commonly performed using fluidic-based injection instead of conventional pipetting. One can compare this to the history of flow cytometers, which started out as custom-built instruments requiring highly specialized knowledge in optics, electronics and fluids from its users, and evolved into commercialized “black box”-type instruments that only required basic knowledge of the strengths and limitations of the technology from the day-to-day user [145]. In large part due to this, flow cytometry is today one of the most widely used and powerful techniques in biomedical research. In light of this, recent developments within microfluidics toward commercially available off-the-shelf, out-of-the-box tools are highly interesting. In the present review, most of the studies discussed use devices produced by the researchers themselves, typically through soft lithography [28], but there are also several examples of studies using devices and set-ups from commercial sources. This includes a parallel plate chamber from Cytodyne (La Jolla, CA, USA) [114], a microfluidic platform from Cellix (Dublin, Ireland) [119], and a microfluidic flow chamber from Ibbid (Munich, Germany) [116]. Another example of interest is the microfluidic platform developed by CellASIC (Hayward, CA, USA) [146,147] and commercialized by Millipore (Billerica, MA, USA).

In summary, as new and more comprehensive biological in vitro models are developed and given that the commercial availability of microfluidics is increasing, microfluidics is poised to make a significant impact by enabling new and improved ways of engineering and evaluating drug delivery particles.

Acknowledgments

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References

Part B

Flow-Based Assembly of Layer-by-Layer Capsules through Tangential Flow Filtration


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Flow-Based Assembly of Layer-by-Layer Capsules through Tangential Flow Filtration

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Supporting Information

ABSTRACT: Layer-by-layer (LbL) assembly on nano- and microparticles is of interest for a range of applications, including catalysis, optics, sensors, and drug delivery. One current limitation is the standard use of manual, centrifugation-based (pellet/resuspension) methods to perform the layering steps, which can make scalable, highly controllable, and automatable production difficult to achieve. Here, we develop a fully flow-based technique using tangential flow filtration (TFF) for LbL assembly on particles. We demonstrate that multilayered particles and capsules with different sizes (from micrometers to submicrometers in diameter) can be assembled on different templates (e.g., silica and calcium carbonate) using several polymers (e.g., poly(allylamine hydrochloride), poly(styrenesulfonate), and poly(diallyldimethylammonium chloride)). The full system only contains fluidic components routinely used (and automated) in industry, such as pumps, tanks, valves, and tubing in addition to the TFF filter modules. Using the TFF LbL system, we also demonstrate the centrifugation-free assembly, including core dissolution, of drug-loaded capsules. The well-controlled, integrated, and automatable nature of the TFF LbL system provides scientific, engineering, and practical processing benefits, making it valuable for research environments and potentially useful for translating LbL assembled particles into diverse applications.

INTRODUCTION

Layer-by-layer (LbL) assembled materials and coatings are of interest because of their potential in applications ranging from optics and catalysis to energy and biomedicine. The properties of the assembled materials can be controlled through selection of the template, coating material, assembly conditions, and the assembly technology used.9−11 Using particles as templates, LbL assembly can also be used to engineer core−shell particles and capsules.12,13

Multilayered particles and capsules can be produced through several different methods and techniques, each offering distinct material and processing advantages.9 Sequential immersion of particulate templates into complementary layering solutions (e.g., oppositely charged polyelectrolytes) with intermediate centrifugation-based purification steps is the most well-studied assembly method and is one of the most commonly used.12,13 Other examples include centrifugation-free calculated saturation,14 immobilization-based techniques,15−18 atomization,19 magnetic separation,20 creaming,21 filtration,22−24 and macro- and microfluidic approaches.25−29 Recently, there has also been increasing interest in “single-step” preparation methods of polymer microcapsules, for example, using interfacial complexation or cross-linking,30,31 or using ultrasonic spraying and salt diffusion.32 Although all of these techniques have specific strengths and enable particle systems to be engineered using LbL assembly, some have restrictions on the diversity of the particles and materials that can be employed, while others require extensive manual intervention and hands-on time during the assembly process, thus making automation, scale-up, and robust and reproducible production challenging to achieve.9

Precise and highly controlled processes, with minimal to no manual intervention, can be engineered using flow-based automated closed-loop systems. Automated systems for the production of peptides33 and oligonucleotides34 have revolutionized the fields of biotechnology and biomedicine, and recent developments in automated synthesis of organic small molecules have the potential to have a similar impact.35,36 Flow-based closed-loop systems are also commonly used in industry, as they can ensure well-controlled, reliable, and cost-effective production. One method of creating a flow-based system is using filter membranes. Voigt et al. have previously demonstrated that a dead-end filtration system can be used for LbL assembly,22 but dead-end filtration is not suitable for continuous flow-based closed-loop systems (as the retentate is continuously forced against the filter and is not mobile), and filter caking can be an issue. Therefore, in high-throughput separation processes of high-value products—such as protein separation in the biopharmaceutical industry—tangential flow...
filtration (TFF; also known as cross-flow filtration) is commonly used instead of dead-end filtration. Herein, we develop a fluidic setup for LbL assembly on micrometer and submicrometer particulate templates using hollow fiber TFF (Figure 1). In this approach, polymers are mixed with particulate templates, incubated to allow layering, and then excess polymer is removed using TFF. The next layer material can then be added to the purified particles which, again after incubation, can be purified through TFF. This process can be repeated until the desired number of layers is deposited on the particles. The end products are multilayer core–shell particles or capsules following core dissolution.

**Figure 1.** Hollow fiber tangential flow filtration (TFF) LbL assembly of capsules. Particulate templates are mixed with polymer A and, after incubation, purified using TFF. The layered and purified particles can then be mixed with the complementary polymer B to create a second layer. This process of TFF purification and LbL film deposition can then be repeated, yielding core–shell particles or capsules after core dissolution.

mixed with particulate templates, incubated to allow layering, and then excess polymer is removed using TFF. The next layer material can then be added to the purified particles which, again after incubation, can be purified through TFF. This process can be repeated until the desired number of layers is deposited on the particles. The end products are multilayer core–shell particles or capsules following core removal.

Besides the TFF filter modules, the main components of the flow-based TFF system for LbL assembly are pumps, tanks, valves, and tubing, which are all standard commercially available and industrially used fluidic components. All of these components are amenable to fluidic integration, enabling the design of a fully automated system, which can provide scientific, engineering, and practical processing benefits. When the particles remain in a closed-loop system, the assembly conditions can be highly controlled. As only pumps and switching of valves are used to control both the layering and washing conditions (time, volume, turbulence, etc.), this can be used to optimize the assembly and washing conditions, which can have a direct impact on the resulting nanofilm properties and performance. Furthermore, automated systems have direct practical benefits, including minimal manual intervention required, increased robustness and reproducibility, and scalability and high throughput. These are all critical for any system that aims to be industrially relevant. In addition, closed systems can enable contamination-free (or even sterile) assembly conditions, crucial for many biomedical applications, as well as the recovery and reuse of valuable materials, such as custom-designed polymers or therapeutics. LbL assembly through TFF thereby addresses key challenges associated with the assembly of multilayered particles and capsules, through a setup that only uses standard fluidic equipment together with commercially available TFF filter modules.

### EXPERIMENTAL SECTION

**Manual TFF LbL Assembly.** Prewashing of particles and incubation with polymer to deposit the first layer (either poly(allylamine hydrochloride) (PAH) or poly(diallyldimethylammonium chloride) (PDDMAC)) were performed as for standard centrifugation-based LbL assembly (15 min under agitation) (see Supporting Information for description of standard centrifugation-based LbL assembly) using the same amount of particles. After deposition was complete, the sample was transferred into a 15 mL conical centrifuge tube, diluted to ∼15 mL with ultrapure water, and aspirated into a syringe that was then connected to the TFF filter module. One syringe was also connected to the retentate line, and a third syringe was connected to the permeate line (Figure S1 in Supporting Information). TFF was then performed by pushing the sample back and forth between the feed and retentate syringes (Video S1) until less than ∼2 mL sample volume remained. The permeate syringe was disconnected, the permeate was discarded, and 25 mL of ultrapure water was then added by back-flush through the permeate line. The washing procedure was then repeated two more times before the sample was recovered in a volume of ∼2–3 mL, transferred to a 15 mL conical centrifuge tube, mixed with an equal amount of poly(styrenesulfonate) (PSS) solution (1 mg mL$^{-1}$), giving a final polymer concentration of 0.5 mg mL$^{-1}$), and then incubated under constant agitation through rotation for 15 min. The procedure was then repeated until four bilayers had been deposited. TFF filter modules with different pore sizes were used for the different particle sizes used in this study (Table S1).

**Flow-Based TFF LbL.** Pumps, valves, tanks, and tubing were used to replace the manual handling steps in manual TFF LbL (Figures S2 and S3). Layering was performed in the system using continuous flow for 15 min in a layering loop instead of offline in a rotating conical centrifuge tube.

**Characterization.** Images were acquired using transmission electron microscopy (operating voltage of 120 kV; FEI Tecnai Spirit), scanning electron microscopy (operating voltage of 10 kV; FEI Quanta 200 field emission SEM), atomic force microscopy (tapping mode in air; NanoWizard II AFM, JPK), deconvolution fluorescence microscopy (DeltaVision, Applied Precision), and DIC and fluorescence microscopy (Olympus IX71). Particle concentration and fluorescence intensity (of particles) were measured on a flow cytometer (Apogee Micro Flow cytometer). Zeta potential measurements were performed on a Zetasizer Nano ZS (Malvern) using a clear zeta cell for a minimum of 12 runs.

**Statistics.** The zeta-potential measurement graphs provide the mean value (data points) and standard deviation (error bars) of the peaks, as provided by the instrument software (Zetasizer software, Malvern). Flow cytometry fluorescence intensity experiments were performed in triplicates, and graphs provide the mean value (data points) and standard deviation (error bars) of the median fluorescence intensity as determined using a flow cytometry analysis software (FlowJo) (Figure S4). Particle counting was performed in triplicates, and graphs provide the mean value (data point) and standard deviation (error bar) of the particle counts as determined by the concentration reported by the instrument software (Apogee Histogram) multiplied by the dilution factor and the sample volume. Additional experimental details are available in the Supporting Information.
RESULTS AND DISCUSSION

As a first example, silica particles (2.39 μm in diameter) were layered through standard incubation (in a rotating tube) with polymer and then washed using a TFF filter module (0.2 μm pore size) using a manual setup where syringes are used to move the particle suspension through the filter, while collecting the permeate in a third syringe (Figure S1 and Video S1). The polymers used were the well-established model LbL polyelectrolyte pairs of PAH/PSS or PDADMAC/PSS. Zeta-potential analysis showed charge reversal in each layering step, as expected when layering with oppositely charged polymers (Figure 2a). Fluorescence microscopy imaging of particles layered with fluorescein isothiocyanate (FITC)-labeled PAH indicated low background levels of free polymer after TFF washing (Figure 2b). Quantification of remaining polymer in the system further demonstrated successful washing and removal of free polymer (Figure S5) with the free polymer signal decreasing to background levels after washing with ten wash volumes (as suggested by the guidelines from the manufacturer of the TFF filter modules). Successful washing was also indicated by the absence of any visible formation of precipitates when introducing the oppositely charged polymer into the system. (The used polymer pairs can easily aggregate and form complexes if significant amounts of free polymers are available in solution at the same time.) Atomic force microscopy (AFM) imaging demonstrated that air-dried capsules folded and formed creases (Figure 2c), which is typical for polyelectrolyte capsules. Wall thicknesses of ~12 nm were obtained from these AFM images, corresponding to ~1.5 nm thickness per layer (Figure 2d). The root-mean-square roughness of the film was ~2–3 nm. The values for both layer thickness and roughness are similar to values reported for capsules of the same polymer pair prepared using similar conditions in centrifugation-based assembly.9

After confirming that microparticles can be layered, the layering of submicrometer-sized particles was investigated. Silica particles (519 nm in diameter) were layered with four bilayers of PAH-FITC/PSS (Figure 3). The fluorescence intensity of the particles was measured after layering, and the observed increase in fluorescence intensity indicates that PAH-FITC was successfully deposited on the particle surface. Following core removal, deconvolution fluorescence microscopy demonstrated that capsules were formed. The coating of two other sizes of submicrometer particles (889 and 177 nm in diameter) was also investigated. After layering, transmission electron microscopy (TEM) was used to investigate the film structures of the core–shell particles and the capsules (Figure 4). The difference in contrast between the surface and the core for the core–shell particles indicates that the particles were successfully coated with a thin polymer film on the order of nanometers in thickness, which is in agreement with the AFM measurements (Figure 2c,d). Successful formation of capsules after core removal further demonstrates successful layering (Figure 4).

After confirming that TFF can be applied for the assembly of submicrometer-sized particles and capsules using a manual TFF setup, we designed a flow-based system to investigate the possibility of automation. The system was designed using two fluidic loops: one used during incubation with the polymer for layer deposition and one used during washing with the TFF filter (Figure 5a). Zeta-potential analysis and fluorescence

![Figure 2](image_url)  
Figure 2. Microparticles can be layered using LbL assembly and TFF washing. (a) Zeta-potential as a function of the number of layers. Layer 0 represents the bare silica particles (2.39 μm in diameter) before layering. Odd layer numbers are PDADMAC, and even layer numbers are PSS. (b) Fluorescence microscopy image of SiO₂ particles coated with (PAH-FITC/PSS)₄ multilayers in solution. (c, d) AFM image and corresponding cross section of air-dried (PDADMAC/PSS)₄ capsules. The position of the height profile in (d) is indicated with a white line in (c). Dotted line at 25 nm in (d) indicates approximate double wall thickness.

![Figure 3](image_url)  
Figure 3. Submicrometer-sized particles can be layered using LbL assembly and TFF washing. Increase in fluorescence intensity as a function of layer number measured using flow cytometry. Layer 0 represents bare particles before layering. Odd layer numbers correspond to PAH-FITC. (Inset) Deconvolution microscopy image of four bilayer PAH-FITC/PSS capsules obtained from silica templates (519 nm).
microscopy measurements demonstrated that capsules could be successfully assembled (Figure 5b,c).

Next, we investigated the possibility of centrifugation-free assembly of drug-loaded capsules. Silica templates are typically dissolved using hydrofluoric acid (HF) treatment, and the TFF filters used only have limited compatibility with HF (according to the manufacturer’s chemical compatibility chart). To circumvent this and to enable centrifugation-free assembly, we used porous calcium carbonate templates that have both high loading capacity and can be easily dissolved in mild conditions (e.g., using acetate buffer or ethylenediaminetetraacetic acid (EDTA))40-43 compatible with the TFF filters (Figure 6a). The porous calcium carbonate templates (∼5 μm in diameter) were loaded through incubation with doxorubicin (DOX), a drug used in cancer chemotherapy, and then added into the TFF LbL system. The calcium carbonate templates themselves are not highly charged, but after a bilayer had been deposited the difference in zeta-potential for each layer increased, and the particles become positively or negatively charged after each layering step, as expected when using oppositely charged polyelectrolytes for LbL assembly (Figure 6b). After the deposition of four bilayers, the core was dissolved by washing with EDTA solution, and capsules were obtained (Figure 6c). Differential interference contrast (DIC) and fluorescence microscopy demonstrated that capsules containing both PAH-FITC and DOX had been successfully assembled.

Finally, we investigated the yield of the TFF LbL assembly system and compared it to the yield achieved from “standard” centrifugation-based LbL. Silica particles (889 nm in diameter) were layered with four bilayers of PAH/PSS with three centrifugation-based washing steps in each. Alternatively, particles from the same batch were layered with four bilayers of PAH/PSS (same batch as used for centrifugation-based LbL) using the TFF LbL system. The number of particles remaining after each layer was determined using flow cytometry (Figure 7).

Centrifugation-based LbL assembly and TFF LbL assembly showed similar behavior in particle loss after deposition of each layer, and the total particle count decreased approximately an order of magnitude for both systems (total particle count decreased from ∼10^{10} to ∼10^{9}). The multiple washing steps involved in centrifugation-based assembly (three washing steps per layer multiplied by eight layers equals 24 washing steps) means that even if one recovers 90–95% of particles per washing step, the total loss still adds up to around 80–90% (0.9^{24} ≈ 0.1), a fact well-known for LbL systems and multistep assembly.9,22 An advantage of many closed-loop systems is that
they can aid in minimizing losses. To investigate the reason for particle loss during TFF LbL assembly, we disassembled the filter modules and imaged them using scanning electron microscopy (SEM; Figures S6–S9). The images show a highly porous network of the filters and that this network consists of both micrometer- and nanometer-sized structures. When comparing a filter before and after use (Figures S8 and S9), it is clear that particles coat the inner surfaces of the filter and that this significantly contributes to a loss of particles. The inherent propensity of widely used LbL materials, such as the polyelectrolytes used in this study (PAH and PSS), to coat most surfaces is what makes the technique so useful but it can also cause losses when particles and polymers adsorb onto other surfaces, such as filter membranes (a property that has been exploited to make functional membranes\textsuperscript{43–44}). In this study, we reused the same filters for several batches of particles with intermediate washing using just ultrapure water, although the filters are compatible with ethanol or sodium hydroxide. We have discovered that simple back-flush through the permeate line of the filter aids in particle recovery, which is why the presented system includes this in the design. Prefiltering of polymer solutions (prior to addition to the particle templates) to remove any dust and contaminating particulate matter can also aid in increasing filter module performance. The TFF filter modules used in the current study are commercially available and use hydrophilically modified (proprietary) poly(ether sulfone) membranes. Using other membrane chemistries and/or pretreating the membranes may also increase performance.\textsuperscript{45–51}

A standard incubation time of 15 min per adsorption step was used for both the flow-based system and for manual centrifugation-based assembly, so even though the washing time was decreased (from typically 15 min for centrifugation-based assembly to <5 min for flow-based assembly), the total time per layer is still limited by the adsorption incubation time. However, for the flow-based system this can potentially be circumvented, as highly controlled flow conditions can allow for quicker layering (e.g., a 1 min deposition step was used in a fluidized bed system\textsuperscript{50}). Furthermore, for centrifugation-based assembly all steps were performed manually, while for the flow-based system only simple operations such as switching of valves and the addition of washing buffer were performed by the user. These operations have the potential to be fully automated (as routinely undertaken in industry), thus creating an integrated and automated closed-loop flow-based LbL system (Figure S3).

■ CONCLUSIONS

This study demonstrates the use of TFF for flow-based LbL assembly on particulate templates to generate multilayered particles and capsules. A wide size range of particles, from micrometer to submicrometer in diameter, were layered with polymer thin films. The film properties of the assembled capsules, as assessed using AFM, TEM, DIC, and fluorescence microscopy imaging as well as AFM thickness and roughness measurements, are similar to those prepared by conventional centrifugation-based LbL assembly. Through judicious choice of layering material, template, and pore size of the TFF filter module, TFF LbL assembly has the potential to be used for even smaller templates than the 177 nm reported here, which can be of interest for certain applications. Further, by using calcium carbonate templates that can be easily dissolved in conditions compatible with the TFF LbL assembly system, we demonstrated centrifugation-free assembly of drug-loaded capsules. The possibility to perform all LbL assembly steps, including core dissolution, inside a flow-based closed system, combined with the potential for scaled-up, fully automated, and controlled multilayer particle and capsule production, makes TFF LbL assembly not only valuable for research environments
but also possibly useful for translating research into applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b02099.

Additional experimental details, figures, and schematics (PDF)

Movie demonstrating manual TFF washing using syringes and a TFF filter module (AVI)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Flow-Based Assembly of Layer-by-Layer Capsules through Tangential Flow Filtration

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SUPPORTING METHODS

Materials. Poly(styrene sulfonate) (PSS, \(M_w \sim 70\) kDa), poly(allylamine hydrochloride) (PAH, \(M_w \sim 58\) kDa), poly(diallyldimethylammonium chloride) (PDADMAC, \(M_w \sim 200-350\) kDa), fluorescein isothiocyanate (FITC), ethylenediaminetetraacetic acid (EDTA), hydrofluoric acid (HF), NaCl, HCl, NaOH, CaCl₂, Na₂CO₃ were obtained from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin HCl (DOX) was purchased from OChem Incorporation (Chicago, IL, USA). Ultrapure water with a resistivity greater than 18 MΩ·cm, from an inline Millipore RiOs/Origin purification system (Millipore; Billerica, MA, USA) was used. Silica particles with diameters 2.39 µm, 1.04 µm, 889 nm, 519 nm, and 177 nm were obtained from Microparticles GmbH (Berlin, Germany). The components for the flow-based tangential flow filtration (TFF) system were purchased from Spectrum Labs (KrosFlo Research Ili Tangential Flow Filtration System; Spectrum Labs; Rancho Dominguez, CA, USA) which included tubing (size 14, Masterflex, Pharmapure). TFF filter modules made of modified (proprietary) polyethersulfone with a pore size of 300 kDa, 750 kDa or 0.2 µm were obtained from Spectrum Labs (Microkros, 20 cm² surface area). The product code for one of the typical TFF filter modules used is C02-E20U-05-N. Full technical specifications (module type, effective length, membrane type, MWCO rating, full dimensions, packaging conditions, technical drawings and schemes etc.) are available online from the manufacturer (Spectrum Labs, http://www.spectrumlabs.com/). PAH, PSS and PDADMAC solutions were prepared at 1 mg mL⁻¹ with 0.5 M NaCl and pH was adjusted to ~ 6.0–6.5 using HCl or NaOH. FITC was added into a 1 mg mL⁻¹ solution of PAH and brought to high pH (~ 9-10) for an hour to allow for a roughly 1:500 functionalization with FITC. The pH of PAH-FITC was then returned to ~ 6.0–6.5 before use. PSS-stabilized porous CaCO₃ particles with a diameter of ~5 µm were produced as previously described.¹ The same batches of particles and polymer solutions were used for all comparative experiments.
Table S1. TFF filter modules and particles. The TFF filter modules used in this study had three different pore sizes, two of them supplied by the manufacturer with molecular weight cut-off (MWCO) values and one supplied with a pore size value in micrometers. The manufacturer provides a “pore size chart” (available on the manufacturer’s webpage: http://www.spectrumlabs.com/) that can be used to approximate dimensions of pore sizes for filters with MWCO values. “Suitable particle diameter” is determined using the guideline that particles need to have a diameter of at least around five times the diameter of the pores to be efficiently retained.

<table>
<thead>
<tr>
<th>TFF filter module</th>
<th>Typical diameter of pores according to “pore size chart”</th>
<th>Suitable particle diameter</th>
<th>Template sizes used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 kDa</td>
<td>~ 20 nm</td>
<td>≥ 100 nm</td>
<td>177 nm</td>
</tr>
<tr>
<td>750 kDa</td>
<td>~ 60 nm</td>
<td>≥ 300 nm</td>
<td>889 nm, 519 nm</td>
</tr>
<tr>
<td>0.2 µm</td>
<td>~ 200 nm</td>
<td>≥ 1 µm</td>
<td>2.39 µm, 1.04 µm</td>
</tr>
</tbody>
</table>

Core removal. Calcium carbonate cores were dissolved with EDTA (200 mM, pH 8.0) or sodium acetate buffer (40 mM, pH 4). Silica cores were dissolved with 5 M hydrofluoric acid (HF). Caution: HF is dangerous and should be handled with caution. Capsules were then washed three times with ultrapure water.

Centrifugation-based LbL. Centrifugation-based LbL assembly was performed using standard methods. Specifically, the procedure was:

1. 300 µL of 50 mg mL⁻¹ silica particles (889 nm) was placed in a 1.7 mL microcentrifuge tube and mixed with 700 µL of ultrapure water.

2. Sample was washed three times with ultrapure water:
   a. Centrifuge: 1200 rcf, 30 s
   b. Supernatant was carefully aspirated (to not disturb the pellet) until ~ 50-100 µL solution remained.
c. The particles were resuspended in the remaining solution and then mixed with 900 μL fresh ultrapure water.

3. After the last washing step, 1 mL of PAH solution was added to the particles.

4. Polymer was allowed to adhere to surface of the particles for 15 min with constant agitation through rotation.

5. Particles were washed three times with ultrapure water (as step 2 above).

6. Step 3-5 were repeated with PSS solution, then again with PAH solution etc., until four bilayers had been deposited. One deposition cycle of PAH and PSS constitutes a bilayer.

7. The finished particles were washed three times with ultrapure water (as step 2 above).

**Flow-Based TFF LbL.** See Figure S2 for the schematic to be used with these instructions, Figure 5 for a simplified overview and Figure S3 for additional details for a fully automated system.

1. Make sure the loop has been washed/rinsed thoroughly.

2. Load the surge tank with particle suspension. Typical amount of particles used: 300 μL, 50 mg mL⁻¹. Pre-wash and/or sonicate particles before starting if necessary.

3. Add polymer A to suspension. Typical value: 4 mL of polymer A (e.g., PAH).

4. Install the surge tank.

5. Adjust valve-1, 2 to put layering loop in service. Washing loop should be completely isolated at this step.

6. Adjust the set point of pump flow rate. Typical value: 50 mL/min.

7. Start the pump.

8. Monitor the feed and retentate pressure for some seconds to make sure the layering loop is properly in service.

9. Record the time. Let the system run for a desired period of time. Typical value: 15 min layering.

10. Disconnect the pump suction line and let the loop discharges in surge tank via return line. As a measure of an empty system, feed line pressure should reach its minimum value. Typical value: 0.1 psig.
11. Stop the pump.

12. Connect the suction line and normalize the loop.

13. Re-adjust the valve-1, 2 to put washing loop in service.

14. Check both valve-3 and permeate valve to be open.

15. Start the pump.

16. Inject wash solution in surge tank for dilution. Typical value: 10mL of ultrapure water.

17. Check the permeate stream to see if polymer separation rate is reasonable.

18. Monitor the level of liquid in surge tank. Wait until the level reaches to a desired volume. Typical value: 10-30 s.

19. Stop the pump.


21. Inject wash liquid via permeate stream. Typical value: 12.5 mL of ultrapure water. This volume should be collected in surge tank.

22. Open valve-3.

23. Start the pump. Repeat the process from step 17 if washing is not adequate. Typical value: two more times.


25. Depending on the volume of particle suspension to be recovered, use a small syringe and connect it to filter backwash/permeate stream (typical: 3 mL) and proceed with backwash and particle collection.

26. If required: take sample for zeta potential measurement, flow cytometry measurement etc.

27. Go to step 3 and repeat the process for polymer B (e.g., PSS). If 2× concentration of polymer is used add equal amount to particle suspension to achieve 1× concentration.

**Quantification of PAH-FITC in permeate.** A calibration curve for PAH-FITC was prepared through serial dilutions. The permeate from the TFF LbL system was collected continuously during
the washing step into 96-well plates. The polymer concentration was then analyzed using a multimode microplate reader (Infinite 200 PRO NanoQuant; Tecan, Männedorf, Switzerland).
**SUPPORTING FIGURES**

**Figure S1.** Manual washing using a TFF filter module. The particle suspension is pushed back and forth using the left and right hand syringes. Permeate is collected in the third (upper) syringe.

**Figure S2.** Lab-scale testing of flow-based TFF LbL system. See “*Flow-based TFF LbL*” (Supporting Methods) for details. See Figure S3 for fully automated TFF LbL system.
Figure S3. Design for fully automated TFF LbL system. Filter A is used for washing after layering of polymer A (e.g., PAH) and filter B is used for washing after layering with polymer B (e.g., PSS), the use of two filters minimizes the build-up of LbL films inside the filter. See Figure S2 for design for lab-scale testing.
Figure S4. Analysis of fluorescence intensity of PAH-FITC/PSS layered particles using flow cytometry. (a) Particles are first identified and gated using scatter signals that exclude aggregates and debris. Around 90% of the total event count was inside the gate defining particles here. (b) The FITC signal from PAH-FITC (odd numbered layers) was then analyzed on the gated particles.
Figure S5. Removal of free polymer in solution during washing in the TFF LbL system. Silica templates 889 nm in diameter were layered with PAH-FITC and PSS, as outlined under “Flow-based TFF LbL” in Supporting Methods. (a) Quantification of PAH-FITC in permeate during washing after the first PAH layer (procedure described in Supporting Methods). Additional washing buffer is added first through dilution of the working volume and then through back-flush steps (as described in Supporting Methods). The amount of washing performed is expressed as “wash volumes”, a common term in continuous-flow filtration and diafiltration, which is defined as volume of wash buffer added divided by the batch volume. (b) Quantification of PAH-FITC in permeate during washing after the second PAH layer (PAH/PSS/PAH, i.e., layer 3). (c) Quantification of PAH-FITC remaining in supernatant after TFF LbL washing of PAH/PSS/PAH-coated particles. Note that the detected amount is virtually at the background/noise level (compared to control sample of ultrapure water). Additional information regarding washing efficiency, recommended wash volumes etc. when performing TFF can be found in the manual and the application notes for the “KrosFlo Research IIi TFF System” available from the manufacturer’s webpage: http://www.spectrumlabs.com.
Figure S6. Scanning electron microscopy (SEM) image of cross-section of side-wall of a TFF filter module fiber with pore-size 0.2 µm. The inset is a schematic of approximately where (black box) on the fiber the SEM was obtained. Full technical specifications (including technical drawings/schematics) of filter modules are available from the manufacturer (Spectrum Labs, http://www.spectrumlabs.com/). Scale bar is 50 µm.
Figure S7. SEM image of cross-section and “outside” (permeate side) of a TFF filter module with pore-size 0.2 µm. Full technical specifications (including technical drawings/schematics) of filter modules are available from the manufacturer (Spectrum Labs, http://www.spectrumlabs.com/). Scale bar is 50 µm.
**Figure S8.** SEM image of unused TFF filter with pore-size 0.2 µm, close-up of lumen side. Full technical specifications (including technical drawings/schematics) of filter modules are available from the manufacturer (Spectrum Labs, [http://www.spectrumlabs.com/](http://www.spectrumlabs.com/)). Scale bar is 10 µm.
Figure S9. SEM image of used TFF filter with pore-size 0.2 μm, close-up of lumen side. Silica particles coating the surfaces in the filter are clearly visible. Full technical specifications (including technical drawings/schematics) of filter modules are available from the manufacturer (Spectrum Labs, http://www.spectrumlabs.com/). Scale bar is 10 μm.
**Video S1.** Demonstration of manual TFF washing using syringes and a TFF filter module. See Figure S1 for labels for each component. This video is available free of charge via the Internet together with the article at http://pubs.acs.org.

**References**


Part C

Super-Soft Hydrogel Particles with Tunable Elasticity in a Microfluidic Blood Capillary Model

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Nanostructured polymer particles can be designed and engineered with a wide range of properties and can provide new and improved ways to diagnose and treat diseases. The performance of nanostructured polymer particles in a biological setting is governed by their ability to interact with and address biological barriers. Important factors determining this behavior include chemical and physical parameters, such as surface chemistry, size, shape, and elasticity.

Different strategies have been investigated to avoid degradation and excretion of nanostructured particles. One prominent technique is the coating of particles with “stealth” materials such as poly(ethylene glycol) (PEG) or “self” peptides. A high density stealth coating could improve penetration of large polymer particles and prolong their circulation time. However, an incomplete or insufficient coating can limit the efficacy of such approaches. A current challenge is accurately quantifying the surface coverage of stealth coatings on polymer particles. One possible way to address this challenge is to assemble particles solely made of materials such as PEG. Other important aspects of particle design are softness and deformability. Many mammalian cells have the remarkable capacity of reversible deformation. For example, human red blood cells (RBCs) have a mean diameter of ~7.5 µm and routinely traverse tissue blood capillaries with diameters that can be smaller than 5 µm. Aged RBCs become rigid and lose this ability, which is reported to induce their clearance from the blood by splenic filtration. Therefore, this flexibility is one of the key properties that enable long circulation of RBCs, and the reduced deformability is a characteristic of several pathological states. It has been reported that the stiffness of nanoparticles plays an important role in their in vivo behavior: softer particles typically have longer circulation times and lower splenic accumulation. Particles made of nanostructured materials that combine stealth properties with tunable mechanical properties are therefore of great interest for biological applications. Microfluidic devices have been recently used to mimic in vivo environments for studying the biological behavior of engineered particles.

In the last few years, there have been several important steps made toward engineering soft particles with stealth properties. Using mixtures of diacrylated PEG with a photoinitiator and other acrylated monomers, templated or mask-based photopolymerization have been used to prepare hydrogel particles with tunable elasticity. Such properties were achieved by varying the amount of diacrylated PEG used in prepolymer mixtures, as determined through electromechanical measurements on the bulk material and/or deformability behavior of particles inside designed microfluidic networks. Another route that has been explored is the use of layer-by-layer assembly to engineer particles that mimic RBCs. Although these studies represent important advances, the particles were made of several components, including non-stealth components. In addition, the elasticity of the particles related to their behavior in microfluidic microchannels were only measured on the bulk material, rather than the particles. Furthermore, direct comparisons to the deformability behavior of RBCs in microfluidic channels were not reported.

Previously, we reported a robust approach for preparing polymer replica particles using a mesoporous silica (MS) templating method, where the template is removed following polymer infiltration and cross-linking. This versatile method has been used to prepare particles of different size, shape, chemical composition, and elasticity, which have the capacity for both cargo encapsulation and delivery. However, previous studies have not reported the preparation of stealth polymer replica particles with tunable elasticity.

Herein, we engineer PEG hydrogel particles with tunable elasticity and with a similar size to human RBCs via a MS templating method, followed by investigation of particle deformability using atomic force microscopy (AFM) and a microfluidic blood capillary model (Scheme 1). The particles consist solely of PEG, where 8-arm-PEG functionalized with amine (8-arm-PEG-NH₂) and succinimidyl carboxyl methyl ester (8-arm-PEG-NHS) were used as building blocks (Scheme 1b). The elastic modulus of the PEG particles is quantified using liquid colloidal-probe atomic force microscopy (CP-AFM, Scheme 1c). Our method results in “super-soft” PEG particles, with more than 40-fold lower modulus than what has been previously reported for PEG-based hydrogel particles, and 130-fold lower modulus than human RBCs. Further, using a microfluidic blood capillary model that mimics dimensions and pressure differ-
entials of the in vivo environment, we investigate the deform-
ability behavior of these particles in microchannels and dem-
onstrate that this behavior can be tuned to be similar to that
of RBCs. This work demonstrates the nanoengineering of
mechanically tunable super-soft hydrogel particles made solely
out of the well-recognized stealth material PEG, which can
provide improved ways of influencing and directing particle-
cell interactions, biodistribution, and ultimately biological
performance.

For the construction of PEG particles, MS particles with an
average size of 7.5 µm were used as templates (Figure 1a). To
facilitate efficient PEG loading, 8-arm-PEG-NH₂ was used for
infiltration into the MS templates based on electrostatic inter-
actions between PEG and the templates. The PEG particles
were obtained through cross-linking the amine groups with
8-arm-PEG-NHS, followed by removal of the MS template
(Scheme 1 a). The left scheme shows aspects of typical human
blood capillaries. The right scheme shows a microfluidic blood capillary
model, mimicking aspects of blood capillaries for investigation of capil-
lary behavior of PEG particles.

For the construction of PEG particles, MS particles with an
average size of 7.5 µm were used as templates (Figure 1a). To
facilitate efficient PEG loading, 8-arm-PEG-NH₂ was used for
infiltration into the MS templates based on electrostatic inter-
actions between PEG and the templates. The PEG particles
were obtained through cross-linking the amine groups with
8-arm-PEG-NHS, followed by removal of the MS template
(Scheme 1 a). Cross-linker concentrations from 0.5 to 4 mg mL⁻¹
were used, while concentrations below 0.5 mg mL⁻¹ did not
yield intact particles. For fluorescence imaging, Alexa Fluor
488 carboxylic acid, succinimidyl ester (AF488)-labeled 8-arm-
PEG-NH₂ was used for the assembly of fluorescent PEG par-
ticles. Figure 1b shows the obtained PEG particles dispersed
in aqueous solution with a diameter around 7–9 µm, which is
similar to that of RBCs (Figure 1c). The fluorescence intensity
of the PEG particles increased when a higher concentration of
cross-linker was used, as observed in Figure 1d. We speculate
that more 8-arm-PEG-NH₂ was cross-linked in the particles as
the concentration of 8-arm-PEG-NHS increased. That is, highly
cross-linked PEG particles contained more polymer material.
This is further supported by AFM and TEM imaging results
(Figure S1). AFM images show a general trend of increasing
height (from 25 ± 10, 45 ± 20, 300 ± 80 to 820 ± 100 nm) of the
air-dried PEG particles with an increase of the 8-arm-PEG-NHS
concentration from 0.5 to 4 mg mL⁻¹, while TEM images show
that particles prepared with a higher concentration of 8-arm-
PEG-NHS exhibit higher contrast.

In addition to the material type, the elasticity is also
dependent on the material density. As the relative amount and/
or density of PEG in each particle changed according to the
amount of cross-linker used, we expected that the elasticity of
the PEG particles would also vary with the cross-linker concen-
tration. To measure the elasticity of the PEG particles, CP-AFM
experiments were carried out. High-resolution force maps
were performed on single PEG particles with different cross-
linking densities. CP-AFM measurements were performed on
individual PEG particles dispersed in 20 mM phosphate buffer
on a glass slide. An array of force curves were then collected
over a 25 × 25 µm area, and then modified to give force-defor-
mation information. Due to adhesive contact between the col-
loidal probe and particle, as well as possible viscoelastic effects
and associated hysteresis, only the approach force curve was
analyzed (Figure 2a). It was found that the Young’s modulus (Eᵧ)
of the PEG particles could be finely tuned by adjusting
the cross-linker concentration (Figure 2b), which is consistent
with our previous findings that the Eᵧ of poly(methacrylic acid)
and poly(L-glutamic acid) particles increases with increasing

![Scheme 1.](image-url)
cross-linker concentration. However, the obtained PEG particles were super-soft with a tunable $E_Y$ from 0.2 to 3.3 kPa (stiffness range from 0.3 to 3 mN m$^{-1}$), which is extremely low compared with previously reported polyelectrolyte particles (2–23 kPa), protein particles (~4 kPa) or PEG-based particles (7.8–64 kPa). We hypothesize that this is due to the low material density within the cross-linked structure.

$E_Y$ values determined using JPK data processing software were arranged into a matrix and then graphically represented in three dimensions (Figure S2a–d). Based on the repetitive nature of the force mapping with little variability in eluated modulus and height, it is seen that the PEG particles deformed elastically. From the force maps, $E_Y$ values for the substrate have been removed for clarity, and it can be seen that there is a homogeneous region in the center of the particles, with decreasing modulus toward the edges due to edge effects during compression. Additionally, the diameter of the PEG particles is much larger than that of the dispersed particles (7–9 µm), which can be explained by the radius of curvature (12.5 µm) for the spherical probe used (Figure S2e–h).

Human RBCs with a $E_Y$ of ~26 kPa can easily pass through capillaries with dimensions smaller than their size and have a long circulation time in vivo (about 120 days). As the PEG particles are very soft (up to 130 times softer than RBCs), it was of interest to examine if they can behave like RBCs in hydrodynamic flow (e.g., narrow blood capillaries). To investigate the deformability of PEG particles in narrow capillaries, we designed a microfluidic blood capillary model. Finite element simulations (COMSOL Multiphysics 4.3) were performed using different parameters and the design was refined until physiologically relevant results were obtained (Figure 3a). Here, pressure drops (2–20 mbar) were established over the microchannels, which are comparable to the in vivo pressure drops across typical human blood capillaries (~1–10 mbar). The parameters for the simulations of...
flow in the microfluidic device are listed in Table S1. The refined and validated design was then realized using soft lithography (Figure 3b) and consisted of a high-pressure and a low-pressure side connected by many microchannels in parallel, similar to the in vivo environment where a high-pressure arterial side is connected to a low-pressure venous side through capillary networks. This four-point device design (two inlets, two outlets) facilitates the establishment of well-controlled differential pressures and pressure drops across the microchannels (as the pressure on each side can be tuned independently). Figure 3c shows a scanning electron microscopy (SEM) image of the central microchannels (width 5 µm, height 12 µm) in the microfluidic device.

To test the deformability behavior of PEG particles in capillaries, the inlet pressures were chosen based on the simulations to achieve physiologically relevant pressure drops across the microchannels. The PEG particles were injected from the high pressure inlet and only buffer was used in the low pressure inlet. The highly cross-linked PEG particles had trouble passing through the microchannels, especially at lower pressure differentials (Figure 4a). In contrast, lowly cross-linked PEG particles could more easily deform to fit inside the microchannels and pass through (Figure 4b). Interestingly, no damage to the spherical shape was observed when comparing PEG particles before and after passing through the microchannels. This indicates that PEG particles can pass through microchannels that are smaller than the particle diameter through reversible elastic deformation. As a comparison, human RBCs were also tested in the microfluidic blood capillary model. They could also pass through the microchannels and recover their shape, as expected (Figure 4c).\[8,22\]

To quantify the deformability behavior of the PEG particles with different cross-linking densities in microchannels, a ratio of the number of particles that passed through the microchannels to the number of particles that did not was calculated. Particles that can easily pass through will have a high ratio while particles that do not pass through at all will have a ratio of zero. Each measurement was performed in triplicate with more than 100 particle/RBC trajectories analyzed for each replicate. In total, over 20 000 trajectories were analyzed. The results demonstrate a clear inverse correlation between amount of cross-linker used and the ease with which particles can pass through the microchannels (Figure 4d). This correlates well with the AFM data (Figure 2), where a decrease in cross-linker used resulted in a decrease in \( E_Y \) corresponding to an increase in softness, which here translates to an increased capacity to deform to pass through the microchannels. Further, the PEG particles pass through the microchannels more easily when the pressure drops increased, as expected. Virtually none of the control PEG particles with the hard MS template still remaining (MS@PEG) could pass through the microchannels, even at the highest pressure differential used, as expected. Interestingly, when the cross-linker concentration decreased to 0.5 mg mL\(^{-1}\), the lowly cross-linked PEG particles with an \( E_Y \) of 0.2 kPa behaved similar to RBCs in the microfluidic blood capillary model. It should be noted that even though the \( E_Y \) of highly cross-linked PEG particles is still lower than that of RBCs, they do not pass through the capillary model as well as RBCs. This is due to a combination of geometric differences between biconcave-discoidal RBCs and spherical PEG particles with distinct internal volumes, along with effects from the RBC cytoplasmic viscosity and contrasts in mechanical deformation of the respective membranes.\[20,23\] Nevertheless, the obtained PEG particles demonstrate tunable elasticity and they can be made to behave similar to RBCs under physiologically relevant conditions in a microfluidic blood capillary model, thus making them potentially useful for biomedical applications, such as targeted drug delivery.

In summary, super-soft hydrogel particles made of 8-arm-PEG were successfully fabricated via a MS templating method. These PEG particles were well dispersed in aqueous solution and had a size similar to RBCs. The cross-linking density of the PEG particles increased as the concentration of cross-linker used increased. It was found that the \( E_Y \) of the PEG particles, as quantified with CP-AFM, could be tailored from 0.2 to 3.3 kPa by increasing the cross-linker concentration from 0.5 to 4 mg mL\(^{-1}\). A microfluidic blood capillary model was used to investigate the behavior of PEG particles in a physiologically relevant flow environment. It was found that the deformability behavior and ability to pass through microchannels could be tuned through the cross-linking
concentration, and that this behavior could be tailored to be similar to that of human RBCs. The reported PEG particles represent a new generation of soft hydrogel particles for investigating particle behavior in biological environments, which have the potential to provide new insights for the design and development of improved drug delivery carriers for biomedical applications.

**Experimental Section**

**Materials**: 8-arm-PEG-NH$_2$ (40 kDa) and 8-arm-PEG-NHS (10 kDa) with hexaglycerol core structure were purchased from JenKem Technology USA Inc (China) and Creative PEGWorks (USA), respectively. MS particles (Separose SX 200, average diameter 7.5 µm, pore size 20 nm) were obtained from Tessek Ltd (Czech Republic).

**Fabrication of PEG Particles**: MS particles were incubated overnight in 8-arm-PEG-NH$_2$ solution (5 mg mL$^{-1}$ in phosphate buffer) under constant shaking. After washing, the PEG particles were dispersed in 8-arm-PEG-NHS solution at different concentrations (0.5, 1, 2, or 4 mg mL$^{-1}$ in phosphate buffer) and incubated for at least 2 h. After washing, the MS templates were removed with 2 M HF/8 M NH$_4$F solution (pH ∼5). Caution! HF is highly toxic. Extreme care should be taken when handling HF solution. The resultant PEG particles were washed and dispersed in water.

**Characterization Methods**: Images were acquired using transmission electron microscopy (Philips CM120 BioTWIN), atomic force microscopy (NanoWizard II AFM) and fluorescence microscopy (Olympus IX71). Fluorescence intensity of PEG particles were measured on an Apogee Micro Flow Cytometer. Mechanical characterization of the PEG particles was performed using a Nanowizard II AFM equipped with a fluorescence microscope (Leica DM14000B). The microfluidic model, mimicking aspects of human blood capillaries, was made through soft lithography.\[3] Full experimental details are available in the Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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Supporting Information


Super-Soft Hydrogel Particles with Tunable Elasticity in a Microfluidic Blood Capillary Model

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Supporting Information

Super-Soft Hydrogel Particles with Tunable Elasticity in a Microfluidic Blood Capillary Model

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Materials: Trichloro-(1H,1H,2H,2H-perfluorooctyl)-silane (TCPFS), Pluronic F127, Dulbecco’s phosphate buffered saline (DPBS, pH 7.4), hydrofluoric acid (HF, 48 wt.%), ammonium fluoride (NH₄F), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (Australia). 8-arm-PEG-NH₂ (40 kDa) and 8-arm-PEG-NHS (10 kDa) with hexaglycerol core structure were purchased from JenKem Technology USA Inc (China) and Creative PEGWorks (USA), respectively. MS particles (Separon SGX 200, average diameter 7.5 µm, pore size 20 nm) were obtained from Tessek Ltd (Czech Republic). Alexa Fluor 488 carboxylic acid, succinimidyl ester (AF488) was provided by Invitrogen (Australia). SU-8 2015 and SU-8 developer were obtained from MicroChem (USA). Polydimethylsiloxane (PDMS) base and curing agent (Sylgard 184) were obtained from Dow Corning (USA). Silicon wafers were obtained from WRS Materials (USA). Core samplers (Harris Uni-Core) and cover glass slides were obtained from ProSciTech (Australia). The water used in all experiments was prepared in a three-stage Millipore Milli-Q Plus 185 purification system and had a resistivity greater than 18.2 MΩ cm.

Fabrication of PEG particles: 10 mg of MS particles were washed with phosphate buffer (100 mM, pH 8) and incubated in 800 µL of 8-arm-PEG-NH₂ solution (5 mg mL⁻¹ in phosphate buffer) under constant shaking overnight. Subsequently, the particles were isolated by centrifugation and washed three times with phosphate buffer. The pellet was dispersed in 650 µL of 8-arm-PEG-NHS solution at different concentrations (0.5, 1, 2, or 4 mg mL⁻¹ in phosphate buffer) and incubated for at least 2 h, to cross-link the infiltrated PEG to form
networks in the MS particles. After three washing cycles with water, the MS templates were removed with 2 M HF/8 M NH₄F solution (pH ~5). *Caution!* HF is highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.

The resultant PEG particles were washed three times with water and redispersed in water. For fluorescent imaging, 8-arm-PEG-NH₂ was pre-labeled with AF488. Briefly, 50 mg of 8-arm-PEG-NH₂ was incubated with 50 µL of AF488 in 1 mL of methanol for 6 h under stirring. The mixture was dialyzed extensively against water inside a MINI Dialysis Device (molecular weight cut-off 3500 Da, Thermo) and isolated via lyophilization, which resulted in yellow powder.

**Force Spectroscopy Measurements and Analysis:** Mechanical characterization of the PEG particles was performed using a Nanowizard II AFM (JPK Instruments AG, Berlin, Germany) equipped with a fluorescence microscope (Lecia DMI4000B).¹ The tipless cantilever (MLCT-O10, Bruker AFM Probes) was calibrated on a cleaned glass substrate to calculate the Inverse Optical Lever Sensitivity (InvOLS), and the spring constant was determined using the thermal tune method as described in literature.² The resultant spring constant was evaluated as 0.15 N m⁻¹. For fabrication of the CP-modified cantilever, a spherical glass bead (12.5 µm radius, Polysciences Inc., USA) was attached to the tipless cantilever using an epoxy resin (Selleys Superstrength, Australia) via careful manual manipulation using AFM and associated optics, and left overnight. Prior to measurement, the cantilever and the attached glass bead was cleaned using oxygen plasma for 3 min.

For the force spectroscopy measurements, particles were dispersed in 20 mM phosphate buffer and allowed to settle onto a glass slide. The cantilever was then fully immersed in phosphate buffer and the InvOLS of the cantilever was measured again in buffer (30 nm V⁻¹). Next, a force map of 16 × 16 force spectra were collected over a 25 µm × 25 µm area on single immobilized particle, which was visualized using a fluorescence microscope. A piezo approach velocity of 500 nm s⁻¹ was utilized for all measurements to avoid hydrodynamic
effects. A force set-point (i.e., maximal load) of 1 nN was utilized. Raw AFM voltage–displacement data were processed using JPK data processing software (v.4.4.28) to subtract the zero-force baseline, shift the data along the deformation axis to zero the initial contact point, subtract the effect of cantilever compliance, and extract data points. The $E_Y$ was then determined from the first 100 nm of the deformation using the Hertz relationship for a spherical indenter. The radius of the particle was also taken into account when determining the effective system radius. To achieve representative $E_Y$ values for the PEG particles, at least five different particles were analyzed, and four central data points from the force map were used to generate the mean $E_Y$. Modulus values determined using JPK data processing software were subsequently arranged into a matrix for graphical representation in three dimensions.

**Microfluidic Blood Capillary Model:** A microfluidic model mimicking aspects of human blood capillaries was designed based on previously reported characteristics, and the model was realized using soft lithography. A master pattern was designed using computer-aided design software (AutoCAD 2013; Autodesk, USA) and simulations were performed using a finite element analysis, solver and simulation software (COMSOL Multiphysics 4.3, USA) to refine and validate the design. Assumptions for the simulations were: water at 20 °C and 1 atm incompressible flow and no-slip boundary conditions for all surfaces except the inlets, where inlet pressures were defined, and the outlets, where the pressure was set to zero (ambient pressure). Meshing was performed using the in-built “physics-controlled mesh”-engine using one of the four finest element sizes, out of nine possible levels. Simulations were repeated for multiple parameters and input pressures, and the designs were refined until physiologically relevant pressure drops (~1–10 mbar) could be established over the microchannels.

A chrome mask was made from the refined design (Bandwidth Foundry, Australia). A master was produced by spin coating (4000 rpm, 30 s; Laurell Technologies, USA) of an
epoxy-based negative photoresist (SU-8 2015) on top of a silicon wafer (4 inch diameter, 0.5 mm thickness) according to the manufacturer process guidelines to obtain a ~10 µm thick layer. After a soft baking step, UV patterning was performed by bringing the chrome mask into contact with the wafer and exposure to UV light (~130 mJ cm⁻²; UV flood light source, ABM, USA). Following post exposure baking, unexposed SU-8 was removed using developer and the remaining SU-8 was hard baked. Before soft lithography, the wafer was ‘anti-stick’ treated by placing a droplet of TCPFS on a glass slide next to the wafer inside a desiccator (~100 Torr, 1 h) following oxygen plasma treatment (500 mTorr O₂, 90 s, high RF level) of the wafer using a plasma cleaner (Harrick Plasma, USA). Soft lithography was performed by pouring a 10:1 w/w mixture of PDMS base and curing agent on top of the silicon wafer. After degassing (desiccator, ~100 Torr, 1 h), the PDMS was heat-cured in an oven (~70 °C, overnight). The patterned and cured PDMS was peeled off the wafer and holes were punched (1.20 mm Harris Uni-Core) over the inlets and outlets. A microscope cover glass slide and the patterned PDMS was treated by oxygen plasma and brought into contact to form a sealed microfluidic device. Reservoirs were made over the inlets using the luer-tip of plastic syringes that were cut-off using a scalpel and glued on top of the device using PDMS.

**Microfluidic Experiments:** The reservoirs were filled with PBSP (DPBS with 0.1 mg mL⁻¹ Pluronic F127) using a syringe and a needle and the device was then placed inside a desiccator (~100 Torr, 1 h) to fill the device and remove any trapped air. The liquid in one of the inlet reservoirs was replaced with sample solution and both inlet reservoirs were then pressurized by connecting to a pressure controller (MFCS-EZ; Fluigent, France) using standard luer-connection liquid chromatography components (Upchurch Scientific, USA) and placed on top of a standard fluorescence microscope (IX71, Olympus, Japan). During particle testing, the sample solutions consisted of ~1000 particles per µL in PBSP. For blood tests, 5 µL of blood was obtained from the finger of one of the authors, using a medical-grade finger-pricking lancet purchased from the local pharmacy and used as instructed by the pharmacist,
and directly mixed with 495 µL PBSP with 1.5 mg mL⁻¹ EDTA added. The different pressures used for the two inlets were chosen based on the simulations to achieve physiologically relevant pressure drops over the microchannels. Videos of particle trajectories were recorded for each pressure differential and with each sample.

Characterization Methods: Transmission electron microscopy (TEM) analysis was carried out with a Philips CM120 BioTWIN instrument operated at 120 kV. AFM images were acquired with a NanoWizard II AFM with intermittent contact mode. Aqueous particle suspensions were air-dried onto mica and tapping mode cantilevers with a spring constant of 46 N m⁻¹ (MikroMasch) were used. Fluorescence microscopy images were taken using an Olympus IX71 inverted fluorescence microscope equipped with a DIC slider (U-DICT, Olympus), the corresponding filter sets, and a 60× oil immersion objective (Olympus UPFL20/0.5NA, W.D. 1.6). Fluorescence intensity of PEG particles were measured on an Apogee Micro Flow Cytometer at an excitation wavelength of 488 nm. Successful patterning on silicon wafers, for soft lithography, was confirmed using a surface profilometer (Ambios XP200, Ambios Technology, USA), bright-field microscopy (Olympus), and scanning electron microscopy (SEM, Philips XL30, Netherlands).

References:

Figure S1. a-d) AFM and e-h) TEM images of PEG particles dried in air. The cross-linker concentrations were 0.5, 1, 2, and 4 mg mL$^{-1}$ (from left to right), respectively. The height bars in (a-d) are 30, 60, 350, and 900 nm, respectively.
Figure S2. a-d) 3D $E_Y$ maps and e-h) relative height images for PEG particles in 20 mM phosphate buffer. The cross-linker concentrations were 0.5, 1, 2, and 4 mg mL$^{-1}$, respectively.
Table S1. Simulation results of flow in the microfluidic blood capillary model.

<table>
<thead>
<tr>
<th>Pressure at high pressure inlet, mbar</th>
<th>Pressure at low pressure inlet, mbar</th>
<th>Average pressure drop across central microchannels, mbar</th>
<th>Flow rate at high pressure outlet, µL min⁻¹</th>
<th>Flow rate at low pressure outlet, µL min⁻¹</th>
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Part D

Structure Governs the Deformability of Polymer Particles in a Microfluidic Blood Capillary Model


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Structure Governs the Deformability of Polymer Particles in a Microfluidic Blood Capillary Model

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Supporting Information

ABSTRACT: Particle stiffness is a design parameter that affects bionano interactions, including biodistribution kinetics and cellular processing. Herein, we develop soft polysaccharide (hyaluronic acid, HA) replica particles and capsules with tunable stiffness and sizes similar to human red blood cells (RBCs) via atom transfer radical polymerization-mediated continuous assembly of polymers (CAP ATRP) and investigate their stiffness and deformability using colloidal-probe atomic force microscopy (CP-AFM) and a microfluidic blood capillary model, respectively. We demonstrate that HA replica particles and capsules with comparable nanoscale stiffness exhibit significantly different behaviors in a microfluidic blood capillary model. HA capsules behaved as RBCs, while HA replica particles had difficulty passing through the capillaries. These results (i) demonstrate how flow-based deformability measurements can be used to complement nanoscale stiffness measurements and (ii) provide important insight into the role of particle structure on the flow-based deformability of soft replica particles and capsules in a physiologically relevant microfluidic model.

Engineered nanostructured particles are of interest due to their potential application in various fields, including drug delivery, nanoreactors, sensors, and artificial organelles. The performance of engineered particles in biological environments is governed by their ability to negotiate biological barriers, which is determined by both the biological target environment and the physicochemical properties of the particles, including size, shape, surface chemistry, and stiffness.

Stiffness and deformability are particle characteristics that have recently gained increasing interest, as they have been shown to strongly influence the cellular interaction and processing of engineered particles. An example of reversible deformability found in nature is that of human red blood cells (RBCs), which routinely and repeatedly deform to pass through blood capillaries smaller than themselves. Inspired by this, several types of particles with varying stiffnesses and shapes have been engineered and subjected to constricted channels to investigate their deformability. Soft particles with high deformability were found to deform to pass through constricted environments, a behavior that correlated with extended circulation times in vivo. Although these studies provide important insights into how particles of various materials can be engineered to have different mechanical properties and biological behavior, the effect of the internal structure of soft polymer particles on deformability behavior remains to be explored.

Herein, we use a recently developed templating technique, called continuous assembly of polymers (CAP), to engineer polymer capsules (i.e., with a hollow interior) and porous replica particles (i.e., with a structured interior) with tunable stiffness and similar size to RBCs and investigate their deformability through liquid colloidal-probe atomic force microscopy (CP-AFM) and a microfluidic blood capillary model (Scheme 1). The replica particles and capsules were both made of hyaluronic acid (HA) and could be engineered with tunable stiffness, as assessed by CP-AFM. The flow-based deformability of the replica particles and capsules was then investigated using a microfluidic blood capillary model, and interestingly, a clear difference was observed between capsules and replica particles, even though both particle types had similar nanoscale stiffness. These results provide key insight into the importance of internal particle structure on the overall physical and mechanical properties and highlight how high-resolution CP-AFM results can be complemented by large-scale flow-based deformability measurements to facilitate the rational design of soft particles with enhanced performance in physiological environments.

To prepare HA capsules and replica particles, atom transfer radical polymerization-mediated CAP (CAP ATRP) was used. HA, a natural polysaccharide, was chosen to construct the capsules and replica particles due to its high biocompatibility, nonimmunogenicity, and targeting ability, which are important for any future biomedical applications. HA capsules with similar size to RBCs were prepared via CAP ATRP of methacrylate-functionalized HA (HA-AEMA, see Scheme S1a in Supporting Information) on sacrificial SiO2 particles (7.0 μm)

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in diameter) and subsequent template removal. The CAPATRP step number was altered from 1 to 4 to afford HA capsules (HA_{C1}, HA_{C2}, HA_{C3}, and HA_{C4}, respectively) with different wall thickness and stiffness, as reported previously.\textsuperscript{14} HA capsules were further labeled with an Alexa Fluor 488 N-hydroxysuccinimide ester to enable fluorescence imaging. The capsules were imaged using fluorescence, differential interference contrast (DIC), and deconvolution microscopy, as well as transmission electron microscopy (TEM) and AFM (Figure 1a1–e1 and Figure S1). The HA capsules were well dispersed in aqueous solution with a hollow structure and a diameter of \(\sim 7 \mu m\). All HA capsules (HA_{C1}–HA_{C4}) showed a collapsed structure with folds and creases in the air-dried state, as commonly observed for hollow capsules.\textsuperscript{38} In addition, AFM analysis of HA capsules showed a linear increase in capsule wall thickness from \(\sim 4.5 \text{ nm (HA}_{C1}\text{)}\) to \(\sim 15.9 \text{ nm (HA}_{C4}\text{)}\) (Figure S2) with the number of CAPATRP steps, which is consistent with our previous study of HA film growth on 2.4 \(\mu m\) SiO\(_2\) particles.\textsuperscript{14}

For construction of HA replica particles, one CAPATRP step of HA-AEMA was performed on mesoporous silica (MS) templates (average diameter \(\sim 7.2 \mu m\)). MS particles were functionalized with an ATRP macroinitiator (Scheme S1b) and subsequently incubated with HA-AEMA and ethylene glycol dimethacrylate (EGDMA) solution for 2 h prior to CAPATRP by mixing with PMDETA, NaAsc, and CuBr\(_2\) (Scheme 1b). Various amounts of HA-AEMA (HA to MS weight ratio of 10\%, 20\%, 40\%, and 80\%) were used to construct replica particles with different HA densities upon removal of MS templates, yielding HAP_{1}, HAP_{2}, HAP_{3}, and HAP_{4}, respectively. The obtained replica particles were characterized, as shown in Figure 1a2–e2 and Figure S3. The HA replica particles were well dispersed in aqueous solution and had a size of \(\sim 6–8 \mu m\). However, these particles had a different morphology after air-drying, compared with the HA capsules (Figure 1c1): unlike HA capsules, which showed a fully collapsed structure, HA replica particles maintained more of their spherical shape. This has been observed for other polymer replica particles constructed by surface-initiated ATRP\textsuperscript{39} or through a polymer infiltration-cross-linking method.\textsuperscript{40} AFM analysis also showed that the height (thickness) of air-dried HA replica particles increased, from \(\sim 690 \text{ nm (HAP}_{1}\text{)}\) to \(\sim 1200 \text{ nm (HAP}_{4}\text{)}\), as the initial HA to MS weight ratio increased (Figure S2b), indicating a higher polymer density in HA replica particles prepared using a larger amount of HA-AEMA. Deconvolution microscopy images confirmed the hollow interior of the capsules and a filled interior of the replica particles, demonstrating the difference in internal structure (Figure 1d,e and Figure S4).

**Figure 1.** Structural characterization of HA_{C3} capsules (a1–e1) and HAP_{3} replica particles (a2–e2). (a) DIC microscopy, (b) TEM, (c) AFM, and (d) fluorescence deconvolution microscopy (single z-plane) of HA_{C3} capsules and HAP_{3} replica particles, respectively. (e) The fluorescence intensity profiles of the red line marked in d1 and d2, respectively. a1, a2, d1, and d2 are capsules/particles in solution. b1 and b2 are capsules/particles under vacuum, and c1 and c2 are capsules/particles in the air-dried state. Scale bars are 10 \(\mu m\) in a, c, and d and 5 \(\mu m\) in b.
For particles with the same material composition, the material density and/or amount can be used to tune stiffness.\textsuperscript{15,41−43} In the current study, the amount of HA-AEMA used during CAP\textsubscript{ATRP} was varied, and high-resolution nanometer-scale deformation measurements (100 nm deformation) were made using CP-AFM on individual capsules and replica particles in phosphate-buffered saline (PBS) solution.\textsuperscript{44,45} It was found that capsule stiffness increased almost linearly with increasing HA layer number, with the measured stiffness of capsules ranging from \(~4.6 \text{ (HAC1)}\) to 13.6 mN m\textsuperscript{−1} (HAC4, Figure 2a). The stiffness of HA replica particles could also be finely tuned by changing the concentration of HA-AEMA in the CAP\textsubscript{ATRP} step, with values ranging from \(~2.4 \text{ (HAP1)}\) to 21.3 mN m\textsuperscript{−1} (HAP4, Figure 2b). The observed trend is consistent with a previous report where an increase in cross-linker amount led to an increase in particle stiffness.\textsuperscript{27} Moreover, the stiffness range measured for the replica particles was similar to that for the HA capsules.

The flow-based deformability of the particles was investigated in a microfluidic blood capillary model (Figure 3). HAC3 and HAP3 had similar nanoscale stiffness (11.1 and 11.4 mN m\textsuperscript{−1}, respectively) and were therefore compared using a recently introduced microfluidic blood capillary model.\textsuperscript{27} This model was designed using physiologically relevant dimensions and pressure drops across capillaries. The capsules and replica particles were injected from a high-pressure inlet, and only buffer was injected from a low-pressure inlet. The four-point device design facilitates the establishment of well-controlled physiologically relevant differential pressures and pressure drops across the capillaries, as each inlet can be tuned independently (Figure 3a). The HAC3 capsules could deform and passed through the capillaries (Figure 3b) with no visibly damaged capsules seen in either of the outlets, thus indicating that the capsules could pass through the capillaries via reversible elastic deformation (Figure 3c). However, a different behavior was observed for the HAP3 replica particles, as they had difficulty passing through the capillaries, especially at lower pressure differentials.

![Figure 2](image2.png)

**Figure 2.** Stiffness of (a) HA capsules with a different number of HA layers and (b) HA replica particles prepared using different HA-AEMA to MS weight ratios, as determined by CP-AFM analysis.

![Figure 3](image3.png)

**Figure 3.** (a) Illustration of available flow paths for HA capsules and replica particles in the microfluidic blood capillary model, overlaid on a SEM image of the PDMS microfluidic device. (b) Time-lapse fluorescence microscopy images of a HAC3 capsule passing through a capillary. (c) Fluorescence microscopy image of capsules at the outlets of the device after passing through capillaries (pass-through) or staying on the same side (sample side). Note that the capsules were not visibly damaged. (d) Time-lapse fluorescence microscopy images of HAP3 replica particles at the entrance of capillaries at lower capillary pressure drops. The pressure drop across the capillaries is \(~6\text{ mbar}\) in b and d. The nonspherical ("drawn out") shape of the capsules observed in some of the time-lapse frames is due to the acquisition rate of the camera. Spherical capsules are again observed when the capsules are no longer in motion (as seen in (c)). Scale bars are 100 μm in a and 10 μm in b–d.
To quantify the differences found in the flow-based deformability behavior of the different particles, particle trajectories in the device were analyzed, and a ratio of the number of particles that passed through the capillaries to the number of particles that stayed on the sample side was calculated (Figure 4). Particles that can easily pass through will have a high ratio, while particles that do not pass through will have a ratio of zero. Each measurement was performed in triplicate with more than 100 trajectories analyzed for each replicate. In total, over 10,000 particle trajectories were analyzed. The controls behaved as expected, with RBCs easily passing through the capillaries and the ratio increasing with increasing pressure drop, while none of the SiO2@HA particles (i.e., with core remaining) could pass through, resulting in a ratio of zero, even at higher pressure drops. The capsules (HA11−HA21) could all pass through the capillaries with relative ease and exhibited deformation-based trajectories in the microfluidic blood capillary similar to the RBCs. However, the replica particles (HA31−HA41) had difficulty passing through the capillaries, even at higher pressure drops. Even when comparing the capsule with the highest nanoscale stiffness (HA3) to the replica particle with the lowest nanoscale stiffness (HA4), it was observed that the capsule had a significantly higher ratio, despite the fact that the capsules were almost 6-fold higher in nanoscale stiffness. As the main difference between the two particle types is their internal structure, where both particle types were made of the same materials and engineered to have the same stiffness at the nanoscale, this demonstrates the importance of particle structure on flow-based deformability in the microfluidic blood capillary model. These results also show the importance of investigating stiffness at different length scales, both at the nanoscale (e.g., using CP-AFM) and at the microscale (e.g., using microfluidic models), to obtain a more complete understanding of the deformability of soft microparticles.

In summary, HA replica particles and HA capsules were nanoengineered through CAPTR. Both particle types were well dispersed in aqueous solution and were of similar size to each other and to human RBCs. The nanoscale stiffness of both particle types was investigated using CP-AFM, and it was demonstrated that both replica particles and capsules could be engineered to have similar nanoscale stiffness, with capsule stiffness ranging from 4.6 to 13.6 mN m−1 and replica particle stiffness from 2.4 to 21.3 mN m−1. A microfluidic blood capillary model was then used to investigate the behavior of both particle types in a physiologically relevant flow environment. It was found that the flow-based deformability behavior and the ability to pass through the capillaries for capsules were similar to those of RBCs, while replica particles could not easily pass through the capillaries, even at higher capillary pressure drops. These results highlight how flow-based deformability measurements can complement nanoscale stiffness measurements and demonstrate the impact of particle structure on the overall deformability behavior of particles, thus underlining particle structure as an important parameter that, together with particle stiffness, has the potential for fine-tuning particle behavior in flow-based environments, both in vitro and in vivo. These results can therefore facilitate the rational design of particle systems with improved ability to negotiate biological barriers at multiple length scales, which is important for advancing the use of engineered particles in biomedical applications such as imaging, diagnostics, and drug delivery.

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**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.5b00591.

Full experimental details, supporting scheme, and supporting figures (PDF)

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### Author Contributions

These authors contributed equally. The manuscript was written through contributions of all authors.

### Notes

The authors declare no competing financial interest.
ship (FS11200025, F.C. and G.G.Q.) schemes, as well as the Australian Government through an Australian Postgraduate Award (M.B.). We acknowledge Martin P. van Koeverden for providing the ATRP macroinitiator and Junling Guo, Katelyn T. Gause, and Dr. Biao Kong for helpful discussions. This work was performed in part at the Materials Characterisation and Fabrication Platform (MCFP) at The University of Melbourne and the Victorian Node of the Australian National Fabrication Facility (ANFF).

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(20) Björnmalms, M.; Yan, Y.; Caruso, F. J. Controlled Release 2014, 190, 139–149.
Supporting Information

Structure Governs the Deformability of Polymer Particles in a Microfluidic Blood Capillary Model

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Materials

Hyaluronic acid sodium salt (HA, $M_w$ 47 kDa) was purchased from Shandong Freda Biopharmaceutical Co., Ltd. (China). 2-aminoethyl methacrylate hydrochloride (AEMA), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), ethylene glycol dimethacrylate (EGDMA), $N,N,N',N',N''$-pentamethyldiethylenetriamine (PMDETA, 99%), copper(II) bromide (CuBr$_2$, 99%), sodium ascorbate (NaAsc, $\geq$ 98%), $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide (EDC), $N$-hydroxysuccinimide (NHS, 98%), hydrofluoric acid (HF), ammonium fluoride (NH$_4$F), branched polyethyleneimine (PEI, $M_w$ 25 kDa), sodium phosphate dibasic, sodium phosphate monobasic monohydrate, Dulbecco phosphate-buffered saline (DPBS), and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich and used as received. Sodium acetate (NaOAc) was purchased from Merck. Nonporous SiO$_2$ particles (50 mg mL$^{-1}$, average diameter 7.0 ± 0.3 µm) were purchased from Microparticles GmbH (Berlin, Germany). Mesoporous silica (MS) particles (Separon SGX 200, average diameter 7.2 µm, pore size 20 nm, pore volume 0.8 mL g$^{-1}$, surface area 300 m$^2$ g$^{-1}$) were obtained from Tessek Ltd (Czech Republic). Alexa Fluor 488 carboxylic acid, succinimidyl ester (AF488-NHS) was purchased from Invitrogen. Polydimethylsiloxane (PDMS) base and curing agent (Sylgard 184) were obtained from Dow Corning (USA). The water used in all experiments was obtained from an inline Millipore RiOs/Origin system and had a resistivity greater than 18.2 MΩ cm.

Characterization Methods

Proton nuclear magnetic resonance ($^1$H NMR) spectroscopy was conducted on a Varian Unity 400 MHz spectrometer, using deuterated water (D$_2$O) as the solvent and a sample concentration of approximately 4 mg mL$^{-1}$. Differential interference contrast (DIC) and
fluorescence microscopy images of HA capsules and particles were obtained using an inverted Olympus IX71 microscope equipped with a DIC slider (U-DICT, Olympus), a UF1032 fluorescence filter cube, and a 60× oil immersion objective (Olympus UPFL20/0.5NA, W.D1.6). Deconvolution microscopy images of HA capsules and particles were acquired using a DeltaVision (Applied Precision) microscope with a 60× 1.42 NA oil objective with a standard FITC/TRITC/CY5 filter set. Transmission electron microscopy (TEM) images were taken using a FEI Tecnai G2 Spirit instrument with an operation voltage of 120 kV. Atomic force microscopy (AFM) experiments were performed with JPK NanoWizard II BioAFM. Typical scans were performed in intermittent contact mode with MikroMasch silicon cantilevers (NSC/CSC). The film thickness of the capsules and height of replica particles were analyzed using JPK SPM image processing software (version V.4.4.29).

**Force Spectroscopy Measurements and Analysis**

Mechanical characterizations of the HA capsules and replica particles were performed using a Nanowizard II AFM (JPK Instruments AG, Berlin, Germany), as described previously.\(^1\) The tipless cantilever (MLCT-O10, Bruker AFM Probes) was calibrated on a cleaned glass substrate to calculate the Inverse Optical Lever Sensitivity (InvOLS), and the spring constant was determined using the thermal tune method as described in literature.\(^2\) The resultant spring constant was evaluated as 0.06 N m\(^{-1}\). For the fabrication of the CP-modified cantilever, a spherical glass bead (27 µm radius, Polysciences Inc., USA) was attached to the tipless cantilever using an epoxy resin (Selleys Superstrength, Australia) via careful manual manipulation using AFM and associated optics, and left overnight. Prior to measurements, the cantilever and the attached glass bead was cleaned using oxygen plasma for 3 min.
For the force spectroscopy measurements, capsules and replica particles were dispersed in PBS and allowed to settle onto a PEI-modified glass slide. The cantilever was then fully immersed in PBS and the InvOLS of the cantilever was measured again in PBS (22.9 nm/V). Next, a force measurement was performed on a single immobilized capsule or replica particle, which was visualized using an optical microscope (Leica DMI4000B). A piezo approach velocity of 500 nm s\(^{-1}\) was utilized for all measurements to minimize hydrodynamic effects. A force set-point (i.e., maximal load) of 4 nN was utilized. Raw AFM voltage–displacement data were processed using JPK data processing software (v.4.4.29) to subtract the zero-force baseline, shift the data along the deformation axis to zero the initial contact point, subtract the effect of cantilever compliance, and extract data points. The stiffness of the capsules and replica particles was then evaluated as the linear gradient of the recorded force vs. deformation curves in the nanoscale deformation regime (100 nm). To achieve representative stiffness values for the capsules and particles, at least 10 different capsules or replica particles were analyzed to generate the mean stiffness.

**Synthesis of Macrocrosslinker and Macroinitiator**

Methacrylated hyaluronic acid (HA-AEMA, Scheme S1a) and ATRP macroinitiator P(METAOTs-co-BIEM) (Scheme S1b, METAOTs: 2-(methacryloyloxy)ethyl trimethylammonium toluene sulfonate, BIEM: 2-(2-bromoisobutyryloxy)) were synthesized using previously reported protocols.\(^3\)

Briefly, DMTMM (0.80 g, 2.89 mmol) and AEMA (0.25 g, 1.50 mmol) was added to a 50 mL solution of HA (1.00 g, 2.49 mmol -COOH) in phosphate buffer (50 mM, pH 7.2). The reaction mixture was stirred for 3 days at 37 °C. Then, the AEMA-modified HA was
purified by dialysis against water, followed by lyophilization for 48 h. Yield: 88%, 0.91 g. 
AEMA functionality: 18 mol%.

P(METAOTs-co-BIEM) was prepared by free radical copolymerization of METAOTs 
with BIEM. Briefly, METAOTs (1.37 g, 4 mmol), BIEM (1.12 g, 4 mmol), and AIBN (13.4 
mg, 0.08 mmol) were dissolved in 7.2 mL of DMSO and degassed by argon bubbling. The 
solution was allowed to react at 100 °C for 2.5 h, and subsequently quenched by cooling in 
liquid N₂ and exposure to air. The reaction mixture was diluted with 5 mL of methanol and 
precipitated into 150 mL of cold acetone/diethyl ether (15:1 v/v). Finally, the precipitated 
white powdery polymer was isolated by centrifugation and redissolved in water, followed by 
lyophilization. Yield: 42%, 1.04 g. BIEM percentage: 48 mol%, $M_w$ 7.7 kDa.

**Fabrication of HA Capsules**

HA capsules were prepared in two steps including electrostatic interaction-mediated ATRP 
macroinitiator adsorption and following CAP_{ATRP} of HA-AEMA. Firstly, 200 µL of 
negatively charged SiO₂ particle suspension (50 mg mL⁻¹, 7.0 µm) was centrifuged (1000 g, 
30 s) and washed with water (3 × 1 mL). The particles were then incubated in 100 µL of 
ATRP macroinitiator solution (1 mg mL⁻¹) in NaOAc buffer (50 mM, pH 5.5) containing 
0.5 M NaCl with constant shaking for 1 h at room temperature, isolated by centrifugation 
(1000 g, 30 s) and washed with water (3 × 1 mL). Subsequently, the initiator-functionalized 
particles were dispersed in 600 µL of an aqueous stock solution containing HA-AEMA 
macrocrosslinker (23.0 mM AEMA), EGDMA crosslinker (3.3 mM), PMDETA (2.9 mM), 
CuBr₂ (1.0 mM) and NaAsc (19.3 mM). The mixture was agitated with an orbital shaker at 
room temperature for 2 h. Then, the particles were isolated by centrifugation (1000 g, 30 s), 
and washed with water (3 × 1 mL). This process represents the typical procedure for single
HA layer formation and it was repeated multiple times to afford multilayered HA-coated SiO\textsubscript{2} particles in so-called reinitiating and CAP\textsubscript{ATRP} steps.

The aforementioned CAP\textsubscript{ATRP}-assembled HA-coated SiO\textsubscript{2} particles were spun down (1000 g, 30 s) and resuspended in 1 mL of PBS (pH 7.4), then 8 µL of AF488-NHS in DMSO (1 mg mL\textsuperscript{-1}) was added. The mixture was allowed to react in the dark for 2 h with constant shaking at room temperature. AF488-labeled HA-coated SiO\textsubscript{2} particles were then washed with PBS (3 × 1 mL) and finally redispersed into 500 µL of PBS (pH 7.4).

HA capsules were obtained by mixing the HA-coated SiO\textsubscript{2} particle suspension (500 µL in PBS) with 1 mL of ammonium fluoride (13.3 M) buffered HF (5 M) at a volumetric ratio of 2:1 to remove the SiO\textsubscript{2} template. [Caution! HF solution is highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.] The capsules were subsequently centrifuged (1500 g, 5 min) and washed thoroughly with PBS (3 × 1 mL).

**Fabrication of HA Replica Particles**

HA replica particles were prepared via one CAP\textsubscript{ATRP} step using the same procedure for the fabrication of HA capsules, but with slight changes. Firstly, 2.5 mg of MS particles (average size: 7.2 µm) were dispersed and washed with water (3 × 500 µL), then incubated in 250 µL of ATRP macroinitiator solution (5 mg mL\textsuperscript{-1}) in NaOAc buffer (50 mM, pH 5.5) containing 0.5 M NaCl with constant shaking for 1 h at room temperature, isolated by centrifugation (1000 g, 30 s) and washed with water (3 × 500 µL). Subsequently, the initiator-functionalized particles were dispersed in 120 µL of an aqueous solution containing different amounts of HA-AEMA macromacrosslinker (0.25 mg, 0.5 mg, 1 mg, and 2 mg) and EGDMA crosslinker.
(EGDMA to AEMA molar ratio of 1:10), and incubated for 2.5 h to allow the HA-AEMA adsorbs into the pores of MS particles. Afterward, 3.6 µL of PMDETA, CuBr\textsubscript{2} and NaAsc solution in water was added into the system to afford a final concentration of 2.9 mM, 1.0 mM and 19.3 mM, respectively. The mixture was agitated with an orbital shaker at room temperature for 2 h. The HA-coated MS particles were then isolated by centrifugation (1000 g, 30 s) and washed with water (3 × 500 µL).

The aforementioned HA-coated MS particles were spun down (1000 g, 30 s) and resuspended in 200 µL of PBS (pH 7.4), then 1.5 µL of AF488-NHS in DMSO (1 mg mL\textsuperscript{-1}) was added. The mixture was allowed to react in the dark for 2 h with constant shaking at room temperature. Afterward, the AF488-labeled HA-coated MS particles were washed with PBS (3 × 500 µL) and finally redispersed into 200 µL of PBS (pH 7.4).

HA replica particles were obtained by mixing the HA-coated MS particle suspension (200 µL in PBS) with 250 µL of ammonium fluoride (13.3 M) buffered HF (5 M) at a volumetric ratio of 2:1 to remove the MS template. [Caution! HF solution is highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.] The replica particles were subsequently centrifuged (1500 g, 5 min) and washed thoroughly with PBS (3 × 500 µL).

**Preparation of Microfluidic Blood Capillary Model**

A recently introduced microfluidic blood capillary model mimicking dimensions (5 µm in diameter and 50 µm in length) and pressure drops of human blood capillaries was prepared as described previously.\textsuperscript{4} Briefly, a master pattern was designed using computer-aided design software (AutoCAD 2013, Autodesk, USA) and simulations were performed using a finite
element analysis, solver and simulation software (COMSOL Multiphysics 4.3, USA) to refine and validate the design. The design was then transferred to a silicon wafer using a chrome mask and a negative photoresist and then transferred into PDMS through soft lithography.\(^5\)

**Microfluidic Experiments**

The microfluidic experiments were performed as described previously.\(^4\) Briefly, the three non-sample reservoirs were filled with PBSP (DPBS with 0.1 mg mL\(^{-1}\) Pluronic F127), and the device was then connected to a pressure controller (MFCS-EZ, Fluigent, France) and placed on top of a standard fluorescence microscope (IX71, Olympus, Japan). During particle testing, the sample solutions consisted of \(~1000\) replica particles or capsules per \(\mu\)L in PBSP. For red blood cell (RBC) tests, 5 \(\mu\)L of blood was obtained from the finger of one of the authors, using a medical-grade fingerpricking lancet purchased from the local pharmacy and used as instructed by the pharmacist, and directly mixed with 495 \(\mu\)L PBSP with 1.5 mg mL\(^{-1}\) EDTA added. The different pressures used for the two inlets were chosen based on the finite element simulations to achieve physiologically relevant pressure drops over the microchannels. Videos of particle trajectories were recorded for each pressure differential and with each sample.

**References**


Scheme S1. Chemical structure of (a) HA-AEMA macrocrosslinker (AEMA substitution degree of 18 mol%) and (b) P(METAOTs-co-BIEM) ATRP macroinitiator.

Figure S1. DIC microscopy (a1–a3), fluorescence microscopy (b1–b3), TEM (c1–c3) and AFM (d1–d3) images of HA capsules (HAC1, HAC2, and HAC4) after 1, 2, and 4 CAP_{ATRP} steps, respectively. SiO2 particles (7.0 µm in diameter) were used as templates. Scale bars are 10 µm for DIC and fluorescence microscopy images, and 5 µm for TEM and AFM images. HAC3 can be seen in Figure 1.
Figure S2. (a) Wall thickness of HA capsules with different numbers of HA layers, and (b) height of HA replica particles prepared using various HA to MS weight ratios, as determined by AFM analysis.
**Figure S3.** DIC microscopy (a1–a3), fluorescence microscopy (b1–b3), TEM (c1–c3) and AFM (d1–d3) images of HA replica particles (HA\(_{P1}\), HA\(_{P2}\), and HA\(_{P4}\)) after one CAP\(_{ATRP}\) step using different amounts of HA-AEMA macrocrosslinker. HA to MS weight ratios: 10% (HA\(_{P1}\)), 20% (HA\(_{P2}\)), and 80% (HA\(_{P4}\)). MS particles (average diameter of 7.2 µm) were used as templates. Scale bars are 10 µm. HA\(_{P3}\) can be seen in Figure 1.

**Figure S4.** 3D deconvolution microscopy images of (a) HA\(_{C3}\) capsules and (b) HA\(_{P3}\) replica particles. Scale bars are 10 µm.
References


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