Dynamic Flow Impacts Cell–Particle Interactions: Sedimentation and Particle Shape Effects

Mattias Björnmalm, Matthew Faria, Xi Chen, Jiwei Cui, and Frank Caruso*

ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Supporting Information

ABSTRACT: The interaction of engineered particles with biological systems determines their performance in biomedical applications. Although standard static cell cultures remain the norm for in vitro studies, modern models mimicking aspects of the dynamic in vivo environment have been developed. Herein, we investigate fundamental cell–particle interactions under dynamic flow conditions using a simple and self-contained device together with standard multiwell cell culture plates. We engineer two particle systems and evaluate their cell interactions under dynamic flow, and we compare the results to standard static cell cultures. We find substantial differences between static and dynamic flow conditions and attribute these to particle shape and sedimentation effects. These results demonstrate how standard static assays can be complemented by dynamic flow assays for a more comprehensive understanding of fundamental cell–particle interactions.

INTRODUCTION

The human body is in constant but well-controlled flux. This includes the flow of a wide range of nutritional, waste, and signaling substances in blood, lymph, and interstitial fluid at flow rates that range from decimeters per second (large blood vessels)\(^2,3\) to micrometers per second (interstitial convection).\(^4,5\) The ability to recapitulate aspects of these dynamic biological environments in vitro is therefore of interest both for probing the biology and for evaluating fundamental bionano interactions of nanoengineered particles aimed at biomedical applications.\(^6–9\)

The application of particles in biomedicine is, in large part, governed by their bionano interactions, with both the properties of the particles and the biological environment being of importance.\(^10–13\) In vitro cell cultures have been and continue to be an integral part in research efforts aimed at elucidating fundamental bionano interactions. However, the static nature of conventional assays (e.g., standard multiwell plates) captures only part of the dynamic in vivo environment. Some commercial systems for flow-based cell cultures exist, although these are typically made for specific suspension cell types or for specific assays, such as leukocyte recruitment assays.\(^14\) Additionally, it has been demonstrated that the effects of diffusion and sedimentation in standard in vitro assays can cause large differences in the cellular dose of different particles.\(^15–19\)

Microfluidic devices have proven to be a powerful tool to complement conventional assays.\(^16,20,21\) Examples include probing tissue transport,\(^22,23\) blood flow behavior,\(^24\) deformability,\(^25–28\) and shape effects of engineered particles under flow.\(^29–31\) Microfluidic systems are highly suitable for well-controlled laminar flow environments, but these reflect only part of the in vivo milieu, and laminar flow can be challenging to combine with more dynamic/chaotic flow and mixing.\(^32,33\) Furthermore, the widespread adoption of microfluidic devices remains limited, possibly because the skills and expertise required for designing and developing these devices are different from those required for standard cell cultures.\(^16\)

Herein, we present a simple, self-contained, and programmable mixing device (compatible with standard multiwell plates) for investigating fundamental cell–particle interactions under dynamic flow conditions (Figure 1). We engineer two particle systems and investigate the effects of particle shape and sedimentation on cell–particle association under dynamic flow conditions. Substantial differences in how particles interact with cells under dynamic flow conditions, compared to standard static cell cultures, are found, and possible explanations are discussed.

EXPERIMENTAL SECTION

Materials. Dulbecco’s phosphate-buffered saline (DPBS), hydrofluoric acid (HF, 48 wt %), \(1\times(\text{dimethylamino})\text{(proppyl)-3-ethylcarbodiimide (EDC)}, \text{ammonium chloride} (\text{NH}_4\text{F}), +(4,6\text{-dimethoxy-1,3,5\text{-triazo-2-yi}})-4\text{-methylmorpholinium chloride (DMDM)}, \text{dithiothreitol (DTT)}, \text{poly(N-vinylpyrrolidone) (PVPPON), thiazolyl}

Received: August 31, 2016
Revised: October 7, 2016
Published: October 17, 2016
blue tetrazolium bromide (MTT), penicillin-streptomycin, and ammonium hydroxide solution (28–30%) were obtained from Sigma-Aldrich (Australia). Poly(methacrylic acid, sodium salt) (PMA, Mr 15 kDa, 30 wt % solution in water) was from Polysciences, Inc. (USA). Alexa Fluor 488 (AF488) Cadaverine, AF488 wheat germ agglutinin (WGA), Alexa Fluor 633 (AF633) hydrazide, Hoechst 33342, a LIVE/DEAD viability/cytotoxicity kit, fetal bovine serum (FBS), and Dulbecco’s modified Eagle’s medium (DMEM) with a GlutaMAX supplement were purchased from Life Technologies (Australia). A MycoAlert mycoplasma detection kit was purchased from Lonza (Australia). Thiol-functionalized PMA (PMA_SH) was prepared as described previously.18 Electronic components, including an IR sensor, servo, and wires were obtained from a local electronics retailer (Jaycar Electronics, Australia). Rechargeable battery packs (5 V, 16 800 mAh capacity) were obtained from Gorilla Gadgets (USA). The water used in all experiments was prepared in a three-stage purification system and had a resistivity greater than 18.2 MΩ cm.

Synthesis of Hybrid Core–Shell PMA Particles and Capsules. The details of particle synthesis and characterization have been described previously.18 Briefly, solid core/mesoporous shell (SC/MS) silica templates were prepared and infiltrated with polymer after pore enlargement. To facilitate the loading of negatively charged PMA into the porous shells of the SC/MS templates, 3-aminopropyltriethoxysilane was used to introduce primary amine groups through a postgrafting method. After polymer infiltration into the porous shell of the templates, the PMA was cross-linked using a thiol–disulfide exchange strategy. Well-dispersed PMA_SH capsules with negligible aggregation were obtained after dissolution of the silica templates in HF and removal of the sacrificial PVFON layers by washing at pH 7.4. The capsules were fluorescently labeled by incubation with AF633 hydrazide and DMTMM.

Characterization. For hybrid core–shell PMA particles and capsules, transmission electron microscopy (TEM) and cryo-electron tomography were used to examine the particle morphologies, dimensions, and densities, as described previously.18 TEM samples (1 μL) in water were placed onto strong carbon-film-coated copper grids (ProSciTech, Australia) and allowed to air dry before TEM analysis. (FEI Tecnai G2 Spirit, operated at 120 kV). For cryo-electron microscopy, sample vitrification was performed using an automated plunge freezing robot (Vitrobot). For imaging and tomography, the samples were studied using a FEI Tecnai G2 Spirit TEM operated at 120 kV and processed as described previously.18 For spherical and rod-shaped PMA_SH capsules, the dimensions were determined using TEM analysis (FEI Tecnai G2 Spirit, operated at 120 kV), as described previously.34 Particle counting and cellular association studies were performed using an ApoCyt flow cytometer (ApoCyt A50 Micro). Fluorescence microscopy images of particles and capsules were obtained using an Olympus IX71 inverted fluorescence microscope. Confocal microscopy was performed using a Nikon A1R+ laser scanning confocal microscope. Results of super-resolution fluorescence microscopy and atomic force microscopy analysis have previously been reported for the particle systems used in this study.18,34

Dynamic Flow Device. See Figures S1–S3 in the Supporting Information for photographs of the device. The multiwell plate holder was machined out of a 14 cm × 9.5 cm × 1 cm metal plate using a milling machine. The support structure was also assembled from metal plates, with one of them machined to hold the servo (Figures S2 and S3). The Arduino was programmed (Supporting Information) with the software and instructions provided. An IR sensor was used to provide the Arduino with feedback on the position of the multiwell plate holder to keep the tilting stable over time (e.g., adjusting for any drift in the servo). The Arduino, battery pack, IR sensor, and servo were connected using standard electrical wire and a solderless breadboard. A mixing frequency of 1 s per cycle was chosen for the proof-of-concept study reported herein. During each cycle, the liquid in each well moved from side to side (Figure 1B–D). For the 12-well plates that were used herein (where each well has a diameter of around 2 cm), this corresponds to an average flow rate of ~2 cm s⁻¹. This flow rate is within the range of physiological flow rates present in the human body, which range from decimeters per second (large blood vessels)² to micrometers per second (interstitial convection).⁵ Although this proof-of-concept study focuses on one average flow rate (to facilitate the comparison of multiple particle systems under dynamic mixing), other average flow rates can be obtained by tuning the servo (the tilting frequency) and by using well plates with other geometries and dimensions.

Cell Culture. NIH/3T3 cells were kept in culture (DMEM with 10% FBS) in a standard humidified incubator (37 °C, 5% CO₂) and passaged before confluence. Cells were cultured without antibiotics and with no sign of bacterial infection. The cell cultures also tested negative for mycoplasma infection (MycAlert kit).

Viability Testing. Cells (80 000) in 1 mL of DMEM with 10% FBS were added to each well of cell-culture-grade 12-well plates and allowed to adhere overnight (37 °C, 5% CO₂) in the standard static setup (multwell plate in incubator without mixing). After overnight incubation, the multiwell plate was placed in the multiwell plate holder of the mixing setup (Figure S1), and mixing was started. Tilting had been preprogrammed to achieve liquid movement seen in Figure 1B,C (close to maximum tilting without any liquid spilling out of the well), and each cycle lasted ~1 s. The whole self-contained mixing setup was kept on top of a plastic document holder (A4 size) on a shelf inside the humidified incubator (37 °C, 5% CO₂). After 24 h of incubation under dynamic flow conditions, the mixing was stopped and the cells were stained using a LIVE/DEAD staining kit and evaluated using PMA_SH/PVFON through layer-by-layer assembly⁵⁵ under acidic conditions (pH 4), followed by the cross-linking of PMA_SH thiol groups through oxidation. Well-dispersed PMA_SH capsules with negligible aggregation were obtained after dissolution of the silica templates in HF and removal of the sacrificial PVFON layers by washing at pH 7.4. The capsules were fluorescently labeled by incubation with AF633 hydrazide and DMTMM.
fluorescence microscopy. The experiment was also repeated with the LIVE/DEAD kit replaced by an MTT assay with readout performed using an Infinite M200 microplate reader (Tecan, Switzerland). In both experiments, a static control multwell plate was included in which the cells were treated the same way as for the mixing setup but kept static instead of under dynamic flow during incubation.

**Cell–Particle Experiments.** Cells (80 000) in 1 mL of DMEM with 10% FBS were added to each well in a cell-culture-grade 12-well plate and allowed to adhere overnight (37 °C, 5% CO₂) in a standard static setup (multwell plate in incubator without mixing). After overnight incubation, the medium was replaced with 1 mL of the particle dispersion for each well, and the multwell plate was then placed in the multwell plate holder of the mixing setup (Figure S1) and the mixing was started. For PMA hybrid core–shell particles and capsules, a cell-to-particle ratio of 1 to 5000 was used. For PMASH spherical and rod-shaped capsules, a cell-to-particle ratio of 1 to 100 was used. The particle dispersions consisted of particles/capsules (at the cell-to-particle ratios listed above) added to DMEM supplemented with penicillin, streptomycin, and 10% FBS. Tilting had been preprogrammed to achieve the liquid movement seen in Figure 1B,C (close to maximum tilting without any liquid spilling out of the well), and each cycle lasted ~1 s. The whole self-contained mixing setup was kept on top of a plastic document holder (A4 size) on a shelf inside the humidified incubator (37 °C, 5% CO₂). After 24 h of incubation, the cells were washed three times with DPBS and harvested by trypsinization. Following centrifugation (300g, 5 min) the cell pellet was resuspended in 300 μL of DPBS and analyzed by flow cytometry. At least 10 000 cells were interrogated for each sample, and experiments were performed at least in duplicate. For PMA capsules and core–shell particles, the median and median absolute deviations of the AF488 signal (from associated particles) for the cell population were recorded (Figure S4). The fluorescence intensity was normalized between the two particle types by measuring the fluorescence intensity of the particles without cells (e.g., the fluorescence intensity of core–shell particles was 1.2 times the fluorescence intensity of the polymer capsules). For the PMAS₃₁₅ spherical and rod-shaped capsules, the percentage of cells positive for particles (assessed using AF633 signal from particles) was recorded (Figure S5), as reported previously.

## RESULTS AND DISCUSSION

The mixing device was made from a holder for a multwell plate attached to a servo controlled by a microcontroller (Figures S1–S3 in the Supporting Information). The whole self-contained device fits inside a plastic document holder (A4 paper size). When investigating cell viability, no substantial difference was observed between dynamic and static control cell cultures (Figure 2). All particle types remained intact (with no visible damage) after exposure to dynamic cell culture conditions.

The effects of sedimentation and diffusion can be a significant factor when comparing cell–particle interactions using standard static assays. We engineered core–shell particles and polymer capsules based on mesoporous silica templating to investigate these effects under dynamic flow conditions (Figure 3). SC/MS silica templates (250 nm in diameter) were loaded with PMA, and after cross-linking, the template was dissolved. The particles were made in a single batch, with the primary difference being that the silica template remains for one (core–shell particle) and has been dissolved for the other (polymer capsule). Therefore, the main difference between these particles is their effective densities (Table 1) and hence their sedimentation behavior.

We hypothesized that dynamic flow could be used to equalize the cellular dose of particles by maintaining a well-mixed solution and preventing particles from settling. To investigate this, the two particle types were incubated with cells under both standard static and dynamic flow conditions (Figure 4). We have previously investigated how these particles interact with cells in 3D printed cell culture systems, and we have observed that the capsules (density ∼1.06 g cm⁻³) had substantially less cellular association than did core–shell particles (density ∼1.65 g cm⁻³), which was explained by differences in sedimentation behavior (with differences in...
Table 1. Size, Shell Thickness, and Average Density of Hybrid Core–Shell PMA Particles and Capsules

<table>
<thead>
<tr>
<th>sample</th>
<th>diameter (nm)</th>
<th>shell thickness (nm)</th>
<th>average density (g cm$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>core–shell particles</td>
<td>250 ± 10</td>
<td>38 ± 3</td>
<td>1.65</td>
</tr>
<tr>
<td>capsules</td>
<td>200 ± 10</td>
<td>30 ± 2</td>
<td>1.06</td>
</tr>
</tbody>
</table>

Figure 4. Dynamic flow conditions reduce sedimentation-based effects of cell–particle association. (A) Cell–particle association, as assessed by using flow cytometry after the incubation of NIH/3T3 cells with fluorescently labeled core–shell particles or capsules under either static or dynamic flow conditions (particle diameter ∼0.2 μm, Table 1). Data points represent the median and error bars indicate the median absolute deviation of the fluorescent cell population. (B) Scheme illustrating a possible explanation for the observed reduction in cell–particle association where dynamic flow conditions introduce mixing that substantially reduces sedimentation effects.

stiffness having no discernible effect). This is also visible here (Figure 4A, solid lines). Interestingly, however, for dynamic flow conditions these effects are not observed, and the cellular association for particles and capsules is similar. This is in agreement with the hypothesis that dynamic flow conditions introduce mixing that reduces or eliminates the effects of sedimentation (Figure 4B). If the liquid in a well of a 12-well plate on average moves a full well diameter during one cycle (Figure 1B–D), then this corresponds to an average flow velocity of ∼20 mm s$^{-1}$, which is several orders of magnitude higher than the sedimentation velocities for these particles (<1 μm s$^{-1}$). Further details (including computational modeling) on the sedimentation and diffusion of these particles were reported previously.

Inspired by reports of how particle shape can influence cellular association, especially under flow, we investigated how soft polymer capsules with different aspect ratios (ARs) interact with cells under dynamic flow conditions. Three types of PMA$_{SH}$ capsules with different ARs (spheres, 670 nm diameter, AR = 1; short rods, 310 nm × 2 μm, AR = 7; long rods, 300 nm × 7 μm, AR = 24) were engineered using a silica templating method (Figure 5, Table 2, and Table S1). These capsules were incubated with cells under both standard static conditions and dynamic flow conditions (Figure 6). The strong fluorescence signal and relative size of these capsules led us to select the percent cellular association as our metric for particle comparison. However, in general, the percent cellular association is semiquantitative, and caution is advised when using it to compare nanomaterials. Under static conditions (solid lines, Figure 6A), particle-shape-dependent cellular association is visible, with cellular association being higher for the rods (∼90–100%) than for the spheres (∼60%) after 24 h.

The higher degree of association seen for long rod-shaped particles could be due to their larger dimensions (Table 2) and the rods (AR = 7) capsules in solution. Inset show higher magnifications of the indicated regions. See Figure S6 for images of long rod capsules (AR = 24). Note that TEM is performed on air-dried capsules in vacuum and fluorescence microscopy is performed on hydrated capsules in water. Scale bars are (A, B) 1.25 and (C, D) 5 μm.

Table 2. Dimensions of Spherical and Rod-Shaped PMA$_{SH}$ Capsules

<table>
<thead>
<tr>
<th>capsule</th>
<th>length (nm)</th>
<th>width (nm)</th>
<th>length/width</th>
</tr>
</thead>
<tbody>
<tr>
<td>sphere</td>
<td>670 ± 60</td>
<td>670 ± 60</td>
<td>1:1</td>
</tr>
<tr>
<td>short rod</td>
<td>2020 ± 150</td>
<td>310 ± 30</td>
<td>6.5:1</td>
</tr>
<tr>
<td>long rod</td>
<td>7150 ± 480</td>
<td>300 ± 40</td>
<td>23.8:1</td>
</tr>
</tbody>
</table>
conditions at 6 and 15 h. These differences decrease over time, and for the long rods both methods result in nearly 100% of cells having associated with rods after 24 h. For the short rods, the largest difference in cellular association between dynamic flow conditions and static conditions is observed after 6 h (∼78% for static compared to ∼35% for dynamic), and even after 24 h, the cellular association observed for dynamic flow conditions does not fully reach the cellular association observed for static conditions (∼80% for dynamic flow conditions compared to ∼92% for static conditions). The differences observed between particle types with different ARs indicate that shape effects are important for cellular association under dynamic flow. Although the rod-shaped particles show similar cellular association after 24 h, considerable differences are observed during earlier time points (especially after 6 and 15 h; Figure 6), indicating the importance of not only the overall shape (sphere versus rod) but also the AR of the rod shape.

The results observed here are different than those previously observed for filomicelle particles in a microfluidic device. In that study, cellular association and uptake decreased for particles with larger AR whereas cellular association increased herein for particles with larger AR. In addition to the two different particle types used (filomicelles versus layer-by-layer assembled polymer particles), these differences may be explained by the different flow-based devices used. In the filomicelle study, a microfluidic device with laminar flow was used, which caused particles with larger AR to align with the flow so that larger particles could “flow past” the cells, thus decreasing cellular association. In contrast, the dynamic mixing device used herein produces chaotic mixing, which does not give rise to this type of laminar flow alignment.

The behavior of rod-shaped particles and spheres under dynamic flow is likely influenced by the multiple modes in which rod-shaped particles contact and interact with cells. Although spheres are symmetrical and thus contact cell membranes in the same way, rod-shaped particles can be connected to the cell surface end-first, side-first, or somewhere in between (Figure 6B–D). When a rod-shaped particle is connected to the cell end-first such that it protrudes from the surface of the cell, it is more vulnerable to disturbance and detachment due to the external flow. There is evidence that during the endocytosis of particles with a high aspect ratio (i.e., rods), particles transition through an end-first orientation. This may explain why rods see a substantial drop in cellular association under dynamic conditions. The soft nature of the polymer capsules and the dynamic cellular interface further complicate these interactions (e.g., it has been shown that mechanical properties can influence cellular association and uptake), and additional studies are needed to fully understand the processes involved. Confocal laser scanning microscopy was also performed but did not show any substantial differences between the samples (Figure S7); this is not unexpected because these were obtained from cells after they had been removed from the mixing device, fluorescently labeled, and washed. Ideally imaging should be performed in situ during dynamic flow, and we plan to explore this in future designs.

### CONCLUSIONS

We investigate fundamental cell–particle interactions under dynamic flow conditions using a simple and self-contained device for creating dynamic flow conditions in standard multwell cell culture plates. We used this device to investigate the effects of particle shape and sedimentation on cellular association for two particle systems, and we show that dynamic flow can be used to reduce sedimentation-based effects by comparing two almost identical particle types (excluding density), thus providing a new experimental platform for comparing particles. Furthermore, we use three polymer capsules with different aspect ratios and show that the geometry can significantly affect the cellular association and that this effect is different under dynamic flow conditions compared to that under static conditions. Shape effects under flow-based conditions have proven important for targeting tissues in vivo and finding ways to investigate these effects in vitro have the potential to provide new insights valuable for the design and development of improved particle systems for biomedical applications. Taken together, these results demonstrate a simple and rapid way to investigate fundamental biointeractions of engineered particles in vitro and provide insight into how sedimentation and particle shape effects affect cellular association under dynamic flow conditions.

### ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.6b03216.

Supporting figures and code for the microcontroller (PDF)
Schillingford, J. P.; Braunwald, E. Measurement of Instantaneous Microscopy Imaging. (MCFP) at the University of Melbourne and the Victorian number CE140100036). This work was performed in part at the Materials Characterisation and Fabrication Platform (MCFP) at the University of Melbourne and the Victorian National Fabrication Facility (ANFF). We acknowledge Paul Brannon for help with confocal microscopy imaging.

REFERENCES


