A Framework to Account for Sedimentation and Diffusion in Particle-Cell Interactions

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ABSTRACT

In vitro experiments provide a solid basis for understanding the interactions between particles and biological systems. An important confounding variable for these studies is the difference between the amount of particles administered and that which reaches the surface of cells. Here, we engineer a hydrogel-based nanoparticle system and combine *in situ* characterization techniques, 3D printed cell cultures, and computational modeling to evaluate and study particle-cell interactions of advanced particle systems. The framework presented demonstrates how sedimentation and diffusion can explain differences in particle-cell association, and provides a means to account for these effects. Finally, using *in silico* modeling, we predict the proportion of particles that reaches the cell surface using common experimental conditions for a wide range of inorganic and organic, micro- and nanoparticles. This work can assist in the understanding and control of sedimentation and diffusion when investigating cellular interactions of engineered particles.

INTRODUCTION

Nanoparticles are of great interest for a number of biomedical applications, ranging from imaging and diagnostics to therapeutics and regenerative medicine.¹⁻⁵ *In vitro* cell culture assays form the foundation of our understanding of how particles interact with biological systems and continue to provide important insight.⁶⁻⁹ Different physicochemical properties, including size,¹⁰⁻¹² shape,¹³⁻¹⁵ surface chemistry,¹⁶⁻¹⁹ and elasticity,²⁰⁻²² influence specific bio-nano interactions. A common confounding variable for *in vitro* studies is the difference between the amount of material added to the system (the administered dose) and the amount of material that reaches the surface of the cells (the cellular dose).²³ The cellular dose varies with time and the physicochemical properties of the particles.^{24,25} It is common for researchers to report the amount of particles added to the cell solution when communicating findings on particle-cell interactions. However, when comparing different types of particles, this can lead to under- or overestimation of cell association and downstream biological effects.^{26,27} Sedimentation and diffusion have been proposed as the primary factors that influence particle distribution in the cell solution.^{28,29} It is therefore important to understand and account for these effects when studying particle-cell interactions.

Computational modeling is a powerful complement to *in vitro* experiments for bio-nano studies.³⁰ It has been successfully used to predict the diffusion and sedimentation behavior of micro- and nanoparticles for *in vitro* toxicity studies.^{31,32} Computational models require input of accurate particle characteristics, such as size and density. For many commercially available particles, such as polystyrene or silica particles, these parameters are known. However, for more advanced particle systems, such as hydrogel particles or hollow polymer capsules, these parameters can be challenging to accurately obtain, especially at the nanoscale. For example,

hydrogel particles have different dimensions and density in their hydrated state (*e.g.*, in a cell culture medium or *in vivo*), compared to the collapsed and dried state investigated using standard atomic force and electron microscopy methods. Therefore, developing strategies to accurately quantify key particle parameters *in situ* is essential for expanding the usage of computational models to predict the biological behavior of advanced capsule and particle systems.

Here, by combining *in situ* characterization techniques (*e.g.*, cryo-electron tomography) and upright, vertical, and inverted cell culture systems with computational modeling, we present a comprehensive framework for studying the differences in particle-cell interactions induced by sedimentation and diffusion (Figure 1). We engineered core-shell hybrid particles (template present) and hollow nanostructured capsules (template absent) using a mesoporous silica (MS) templating method.^{33,34} These two particle systems are identical up until the core dissolution stage – they are made from the same materials and have similar surface chemistry – but have different density after template dissolution. Thus, we expected similar cellular responses, but different sedimentation and diffusion behavior. We characterized the hydrated *in situ* dimensions and densities of these particles using a combination of cryo-electron tomography, fluorescence spectrophotometry, flow cytometry, and super-resolution fluorescence microscopy. We used these experimentally determined physical properties as parameters in a computational model of sedimentation and diffusion, which predicted differences in cell association. These predictions were verified experimentally by challenging cells cultured in either one of two 3D-printed cell culture systems (inverted or vertical cell orientation) or a conventional multi-well cell culture plate (upright cell orientation) with our hydrogel particle systems. This provides strong evidence that sedimentation and diffusion can explain differences in particle-cell interactions. Furthermore, we demonstrate the suitability of our hydrogel capsule system for research

applications since the capsules have a similar density to water and the effect of sedimentation is therefore negligible. Finally, we provide resources that predict the proportion of particles that reach the cell surface in commonly used experimental conditions, for a wide range of commonly used particle systems, thus showing how sedimentation and diffusion can be accounted for when investigating particle-cell interactions.

Figure 1. Controlling for variable association of advanced particle systems due to the effect of sedimentation and diffusion. (a) Scheme of the preparation of core-shell hybrid particles (SC/MS@PMA) and hollow hydrogel (PMA) capsules. (b) Experimental design of upright, inverted, and vertical orientations to investigate cell association of particles. (c) Scheme of

computational modeling to analyze particle sedimentation and diffusion, as well as orientation-dependent cell association of these particles.

EXPERIMENTAL SECTION

Materials. Tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), cetyltrimethylammonium bromide (CTAB), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), dithiothreitol (DTT), Dulbecco's phosphate-buffered saline (DPBS), Triton X-100, bovine serum albumin (BSA), sodium phosphate dibasic, triethanolamine, hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH₄F), and ammonium hydroxide solution (28-30%) were obtained from Sigma-Aldrich (Australia). Poly(methacrylic acid, sodium salt) (PMA, M_w 15 kDa, 30 wt% solution in water) was from Polysciences, Inc. (USA). Pyridine dithioethylamine (PDA) was purchased from Shanghai SpeedChemical Co. Ltd. (China). Alexa Fluor 488 Cadaverine (AF488-Cad), Alexa Fluor 647 Cadaverine (AF647-Cad), AF488 Phalloidin, Hoechst, fetal bovine serum (FBS), and Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX Supplement were provided by Life Technologies (Australia). Glass coverslips (No.1.5, diameter 18 mm) were from ProSciTech Pty Ltd (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 M Ω cm.

Synthesis of SC/MS Nanoparticles. To synthesize monodisperse spherical solid core silica nanoparticles, 100 mL of ethanol was mixed with 8 mL of water and 4 mL of ammonium hydroxide solution. After stirring for 30 min, 6 mL of TEOS was added to the above solution. The mixture was stirred for 6 h at 25 °C. To form the mesoporous shell around the above prepared silica spheres, 100 mL of the above prepared suspension was diluted with 200 mL of

water, and 30 mL of CTAB solution (110 mM in a 2:1 mixture of water and ethanol) was added quickly under vigorous stirring. After stirring for 30 min, 2.15 mL of TEOS was added to the mixture followed by stirring overnight at room temperature. To enlarge the pore size, the as-synthesized nanoparticles were washed with ethanol and water, and finally dispersed in 20 mL methanol, followed by addition of 40 mL of water and TMB (1:1 v/v). The mixture was placed in an autoclave and kept at 140 °C for four days. Finally, the as-synthesized nanoparticles were washed with ethanol and water, and dried at 80 °C overnight. The resulted nanoparticles were further calcined at 550 °C for 6 h.

Synthesis of PMA_{PDA}. PMA_{PDA} was synthesized via EDC-mediated amide bond formation between the carboxyl groups of PMA and the amine groups of PDA. In a typical experiment, a PMA solution (360 mg of 30 wt% solution, 1 equiv. of MA) was diluted into 5 mL of phosphate buffer (0.1 M, pH 7.2). The resulting solution was incubated with EDC (57.5 mg, 0.3 equiv.) with stirring for 15 min. Subsequently, PDA (33.4 mg, 0.15 equiv.) was added to the mixture and the pH was adjusted to 7.2. The reaction was allowed to proceed overnight. The resulting mixture was placed inside a dialysis membrane (molecular weight cut-off 3500, Thermo) and dialyzed extensively against water, filtered with a 0.2 µm syringe filter, and isolated via lyophilization. The degree of thiol functionalisation was characterized by measuring the absorbance of the released pyridine-2-thione ($\lambda_{\text{max}} = 343 \text{ nm}$), and then quantified from a calibration curve of PDA, which corresponded to 10 mol% modification. To label PMA_{PDA}, 30 mg of PMA_{PDA} was mixed with 15 mg of DMTMM in 1.5 mL phosphate buffer for 15 min following adding 30 µL of AF488-Cad (1 mg mL⁻¹ in DMSO). The mixture was incubated overnight, dialyzed against water, and isolated via freeze drying.

Fabrication of SC/MS@PMA Particles and PMA Capsules. The SC/MS particles were functionalized with amine groups by APTES modification before the preparation of PMA capsules. In this process, the SC/MS particles were dispersed in ethanol with a concentration of 30 mg mL^{-1} by sonication before ammonia and APTES were added to the suspension. The volume ratio of ethanol:ammonia:APTES was fixed at 20:1:1, and the suspension was allowed to stir overnight. PMA capsules were fabricated via the MS templating method combined with thiol-disulphide exchange cross-linking strategy according to our previously published method.³⁵ Briefly, 3 mg of amine-modified SC/MS templates were incubated with 0.9 mg of PMA_{PDA} (5 mg mL^{-1} in 100 mM acetate buffer, pH 5) under constant shaking for 6 h. Subsequently, the polymer-loaded templates were isolated by centrifugation and washed three times with acetate buffer. The pellet was dispersed in 300 μL of thiolated PMA (PMA_{SH}) solution (0.5 mg mL^{-1} in acetate buffer). Here, PMA_{SH} was freshly prepared by the incubation with 0.5 M DTT in MOPS buffer (20 mM, pH 8) for 15 min and purified with a NAP-5 Sephadex column. In this step, PMA_{PDA} was cross-linked by PMA_{SH} based on thiol-disulphide exchange at pH 5 in acetate buffer. The PMA_{SH}-loaded particles were labeled with AF488-Cad or AF647-Cad based on EDC chemistry, which resulted in fluorescent SC/MS@PMA particles. After three washing cycles consisting of centrifugation, removal of supernatant, resuspension, and dilution with water, the templates were dissolved with a 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 PMA replicas were centrifuged and washed three times with water. Centrifugation settings of $4000 \times g$ for 6 min were used for SC/MS@PMA particles, and $7000 \times g$ for 10 min were used for PMA replicas. To quantify the mass of a single particle or capsule, AF488-labeled PMA_{PDA} and PMA_{SH} were used for the fabrication.

Quantification of the Density of SC/MS@PMA Particles and PMA Capsules. The number of PMA capsules in a known volume was determined by flow cytometry (Apogee Micro Flow Cytometer). The amount of PMA in the capsule suspension was quantified by fluorescence spectrophotometry using an excitation wavelength of 490 nm and an emission wavelength of 516 nm. PMA capsules were degraded by dithiothreitol prior to the assay. The concentration of PMA at pH 7.4 in 50 mM phosphate buffer was determined using a calibration curve (Figure S1). The content of a single PMA capsule (W_1) was determined using the following equation:

$$W_1 = \frac{CV}{N_1}$$

C is the PMA concentration of degraded PMA capsules in a certain volume (V). N_1 is the number of PMA capsules before degradation counted by flow cytometry. It resulted in 1.67×10^{-15} g for one PMA capsule.

The density of a single PMA capsule (ρ_1) dispersed in water was calculated based on the contents of PMA in the shell and water in both the hollow structure and shell using the following equation:

$$\rho_1 = \frac{W_1 + \rho_{H_2O}(\frac{4}{3}\pi R_1^3 - \frac{W_1}{\rho_{PMA}})}{\frac{4}{3}\pi R_1^3}$$

R_1 is the radius of PMA capsule. ρ_{PMA} equals 1.18 g mL^{-1} . According to this equation, the density of PMA capsules was 1.06 g cm^{-3} (Table S1).

The number of SC/MS@PMA particles (N_2) in a known volume was determined by flow cytometry. The content of SC/MS@PMA (W_2) particles in a known volume was measured using

a balance after drying. The density of SC/MS@PMA particles was determined using the following equation:

$$\rho_2 = \frac{\frac{W_2}{N_2}}{\frac{4}{3}\pi R_2^3}$$

R_2 is the radius of SC/MS particles. So the density of SC/MS@PMA particle was 1.65 g cm⁻³ (Table S1).

3D Printed Supports for Inverted and Vertical Particle-Cell-Association Studies. A 3D modeling software (SketchUp Make 2015; Trimble, Sunnyvale, CA, USA) was used to make the designs and 3D printers (UP Plus 2; 3D Printing Systems, Auckland, New Zealand) were used to print the designs in acrylonitrile butadiene styrene (ABS) plastic. ABS plastic has previously been used in tissue engineering scaffolds without any adverse effects on the tested cells.³⁶ For the inverted cell experiments an array of 3-by-3 cylindrical pillars (each 1 mm in diameter and spaced 5 mm apart) was used to keep the cover glass with cultured cells in the inverted position inside a standard culture-grade multi-well plate. For vertical cell culture a 3D printed block containing rectangular cavities with vertically aligned grooves in the walls were used to keep the cover glass with cultured cells in the vertical position. See Figures S2 and S3 for 3D schematics.

Cell Association Analyzed by Flow Cytometry. HeLa cells were seeded on glass coverslips (18 mm in diameter for upright and inverted orientations, and 15 mm in diameter for vertical orientation) in 12-well plates (8×10^4 cells per well) and allowed to adhere overnight. Cells were then incubated with AF488-labeled SC/MS@PMA particles or PMA capsules in upright, inverted or vertical orientations for 24 h at 37 °C with 5% CO₂, in a ratio of 5000:1

particles:cells. Cells were washed three times with DPBS and harvested by trypsinization, followed by centrifugation at 300 g for 5 min. The cell pellet was resuspended in 300 μ L of DPBS and analyzed by flow cytometry. The fluorescence intensity of cells associated with particles or capsules was determined by acquisition of AF488.

Cell Imaging. HeLa cells were seeded on glass coverslips (18 mm in diameter) in 12-well plates (8×10^4 cells per well) and allowed to adhere overnight. Cells were then incubated with AF647-labeled SC/MS@PMA particles or PMA capsules in upright or inverted orientations for 24 h followed by three washing steps with DPBS. Cells in each well were fixed with 200 μ L of 4% paraformaldehyde for 10 min at 25 °C followed by washing with DPBS, and incubated with 400 μ L of permeabilization buffer (0.1% Triton X-100 and 1% BSA) for 5 min followed by washing with DPBS. Cells were stained for actin with AF488 Phalloidin (5 μ L of stock solution in 200 μ L of PBS containing 1% BSA, 20 min incubation), and for DNA with Hoechst (5 μ g mL⁻¹ in DPBS, 10 min incubation). Cells were washed three times after each staining. To lessen photo bleaching, DPBS in each well was removed and 100 μ L of Vectashield (Vector Labs) was added. Super-resolution fluorescence images were acquired via a DeltaVision OMX SIM microscope (Applied Precision) operating in “Structured Illumination Microscopy” mode to obtain an approximate resolution of 110 nm in X/Y and 250 nm in Z. Deconvolution fluorescence images were acquired in “Conventional” mode.

Virtual Reference Environment. A virtual reference environment³⁷ to run all of the simulations in this work is freely available at:

https://github.com/uomsystemsbiology/particle_dosimetry_reference_environment.

The code for our simulations is freely available at:

https://github.com/uomsystemsbiology/particle_dosimetry.

Partial Differential Equation (PDE) Details. The complete PDE model used to generate concentration profiles is as follows, including boundary conditions and initial values:

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \vec{v} \cdot \nabla c$$

$$c(x, 0) = c_0 \forall x$$

$$c = 0 \text{ (at cell boundary)}$$

$$D \nabla c = \vec{v} c \text{ (at non-cell boundary)}$$

where D = diffusion coefficient, v = sedimentation velocity, and $c(x, t)$ = particle concentration at point x at time t . The 1D form of this PDE is sufficient to generate concentration profiles for the upright and inverted cell orientations, as there is no variation along the horizontal. For the vertical orientation, a 2D form of this PDE is necessary. Solutions were found using numerical integration.

Parameter Determination. The bulk diffusion coefficient D is given by the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta r}$$

where k_B = Boltzmann constant, T = temperature, η = viscosity of media, and r = radius of particle. This equation calculates the single particle diffusion coefficient assuming hard spherical particles and laminar flow. Particles considered in this work were approximately spherical, and

the Reynolds number for nanoparticles in stable, non-moving (non-turbulent) media is low. Finally, the single-particle diffusion coefficient is expected to be a reasonable approximation of the bulk diffusion coefficient due to the low volume fraction of particles ($\ll 1$) within our solutions.

The sedimentation velocity v is given by Stokes' law:

$$v = \frac{2g(\rho_p - \rho_m)r^2}{9\eta}$$

where g = acceleration due to gravity, ρ_p = density of particle, ρ_m = density of the medium, r = radius of particle, and η = viscosity of media. This equation makes similar assumptions to the Stokes-Einstein equation. Other parameters used are as follows: $T = 310.15 \text{ K}$, $\eta = 0.00101 \text{ P}$, and $\rho_m = 1 \text{ g cm}^{-3}$.

Cellular Dose and Association Determination. Cellular dose E is equivalent to the amount of material removed from solution in our simulation, thus it can be calculated as follows:

$$E = \int_S c_0 dx - \int_S c(x, t) dx$$

where $\int_S$ refers to integrating over the entire solution. We can set $c_0 = 1$ and thus calculate the relative cellular dose, or percentage of material removed from the system.

To compare cellular dose and association with different particles, orientations, and experiments, it is necessary to scale E to the “total fluorescence” TF that each solution starts with, which can be calculated as:

$$TF = \text{Concentration of particles} \times \text{Volume of system} \times \text{Fluorescence per particle}$$

(Fluorescence is included as the commonly used experimental measure of association.)

Fluorescence per particle is a somewhat poorly defined concept, as fluorescence measurements are commonly given as “arbitrary units”. In this work we assume that the relationship between measured fluorescence intensity and number of associated particles is linear, and that the ratio of fluorescence intensity measured for particles in solution holds when particles are associated with cells. Thus, we can use the relative fluorescent intensity of our particle systems (measured in solution) as the fluorescence per particle. Raw values for theoretical cellular dose are given in Table S2.

Finally, to predict cellular association from our scaled cellular dose estimations, we chose one orientation as a reference and normalized all doses to that orientation. This is equivalent to assuming that cellular association is proportional to cellular dose. We chose the upright PMA orientation as our reference orientation for normalization.

Characterization. TEM (FEI Tecnai G2 Spirit, operated at 120 kV) was used to examine the particle morphologies. The TEM samples (1 μ L) in water were placed onto strong carbon film coated copper grids (ProSciTech Pty Ltd, Australia) and allowed to air-dry. For cryo-electron microscopy, sample vitrification was performed using an automated plunge freezing robot (Vitrobot). For 2D imaging and tomography, the samples were studied using a FEI Tecnai G2 Spirit TEM operated at 120 kV. For cryo-electron tomography, a total number of 61 low-dose TEM images were acquired every 2° over $\pm 60^\circ$ tilt range using a Gatan 626 cryo-transfer TEM tomography holder. The accumulated electron dose for tomographic acquisition was $72 \text{ e}/\text{\AA}^{-2}$. The alignment of the image series was performed using the FEI Inspect 3D software and the

reconstruction was done using a total variation minimization reconstruction technique³⁸ (TVM). 3D visualization and quantification analysis of 3D reconstructed data was accomplished using Amira and MATLAB software. The surface areas and porosities of the MS particles were measured by a Micromeritics TriStar surface area and porosity analyzer at 77 K, using nitrogen as the adsorption gas. The surface area was calculated using the Brunauer-Emmett-Teller (BET) method, and the pore diameter distributions were derived from the desorption branch by the Barrett-Joyner-Halenda (BJH) method. The degree of PDA functionalisation was quantified via absorbance readings at 343 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Australia). Particle counting and cell association were performed using Apogee flow cytometer (Apogee A50 Micro). Fluorescence spectrophotometry were carried out on a Horiba Fluorolog-3 Model FL3-22 spectrofluorometer (Jobin Yvon Inc., USA) equipped with a HgXe lamp (increment size, 1 nm; excitation and emission slit widths, 5 nm). Size distributions and zeta-potential measurements of particles and capsules were measured with a Malvern Zetasizer Nano ZS. Fluorescence microscopy images of PMA capsules 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).

RESULTS AND DISCUSSION

The nanoparticle systems developed in this study were hybrid core-shell particles and hollow hydrogel capsules prepared from solid core/mesoporous shell (SC/MS) silica templates, which were prepared based on a previously published method.³⁹ Transmission electron microscopy (TEM) revealed that these particles were monodisperse with a diameter of ~250 nm (Figure S4a). To facilitate polymer infiltration, the mesopores were enlarged with a mixture of 1,3,5-

trimethylbenzene, water, and ethanol.⁴⁰ After pore enlargement, the SC/MS particles retained their size as well as monodispersity, and had a shell thickness of ~38 nm and a mesopore distribution of 3-10 nm (Figure 2a-d). To load negatively charged poly(methacrylic acid) (PMA) into the shells of the SC/MS templates, primary amine groups were introduced on the surface using 3-aminopropyltriethoxysilane through a postgrafting method.⁴¹ The loaded PMA was cross-linked in accordance with a thiol-disulphide exchange strategy (Figure S5) to form hybrid core-shell particles (denoted as SC/MS@PMA particles).³⁵ PMA capsules were obtained by dissolving the SC/MS templates. The main difference between SC/MS@PMA particles and PMA capsules is therefore the presence or absence of the silica templates.

Figure 2. Fabrication and characterization of SC/MS@PMA particles and PMA capsules. (a) Scanning electron microscopy image, (b) TEM image, (c) a volume rendering visualization of the reconstructed structure from cryo-electron tomography measurements, and (d) pore size distribution of SC/MS particles after pore enlargement. (e) TEM, (f) an orthogonal slice through

the 3D reconstruction of the particles obtained by cryo-electron tomography, (g) fluorescence microscopy image, and (h) size distribution of PMA capsules obtained by dynamic light scattering measurement.

The PMA capsules preserved their structural integrity, and showed few folds and creases in a dry state (Figure 2e), while keeping spherical structure in water (Figure S6). Cryo-electron tomography measurements indicated that the diameter of the PMA capsules was ~200 nm in water (Figure 2f), approximately 20% smaller than the template nanoparticles, which is due to shrinkage of capsules after removal of the templates.³⁴ In addition, the shell thickness of the PMA capsules was ~30 nm, which is ~8 nm thinner compared to that of the SC/MS particles. The structure of the capsules was further examined by TEM using ultramicrotomed sections (90 nm thin slices) of the capsules immobilized in resin (Figure S7), with the capsule shell thickness of ~32 nm being in agreement with the cryo-electron tomography results. These PMA capsules were well dispersed in aqueous solution with a narrow size distribution and negligible aggregation (Figure 2g,h). To quantify the density of single particles or capsules, fluorescently labeled PMA was used as a building block. We combined several experimental techniques to determine the densities of single capsules, a full explanation of which is given in “Quantification of the Density of SC/MS@PMA Particles and PMA Capsules” in the Experimental Section. In brief, we determined the concentration of PMA in a known volume using fluorescence spectrophotometry, and determined the concentration of particles in a known volume using flow cytometry. By combining these techniques, we determined the mass of PMA in a single capsule. By combining this mass with the known volume of hydrated capsules (determined by cryo-electron tomography), we could estimate the relative fraction of water in capsules, and thus estimate the total mass and density of capsules. The density of PMA capsules was similar to

water, while SC/MS@PMA particles were ~1.5 times more dense than PMA capsules (Table S1).

To determine cell association in different cell culture orientations, standard multi-well plates were used for upright orientation and two 3D printed cell culture systems were designed for the inverted or vertical orientations (Figure 1b, and Figure S2 and S3). HeLa cells were seeded on glass coverslips before being added to the different systems. No difference in cell viability was observed between the different orientations for cells cultured in media for 24 h (Figure S8).

Cell association was assessed by incubating the same number of fluorescently labeled SC/MS@PMA particles or PMA capsules with HeLa cells in the different orientations for 24 h followed by investigation with flow cytometry, deconvolution microscopy and super-resolution microscopy (Figure 3 and Figure S9).⁴² For SC/MS@PMA particles, the fluorescence intensity of HeLa cells cultured in the upright orientation was substantially higher compared to the inverted and vertical orientations (Figure 3a), which indicates that more particles are associated with cells in the upright orientation. Interestingly, the orientation-dependent cell association that is observed for the SC/MS@PMA particles was not observed for the PMA capsules (Figure 3d). This can be potentially attributed to the higher sedimentation rate of SC/MS@PMA particles because of their higher density compared to PMA capsules. As a result, the amount of particles reaching the cells for SC/MS@PMA particles in the upright orientation was higher than that in either inverted or vertical orientations. The fluorescence microscopy measurements confirmed the results observed by flow cytometry (Figure 3 and Figure S9). This demonstrates the importance of considering orientation-dependent effects while studying particle-cell interactions of advanced nanoparticle systems, and that engineering particles with similar density to water

(*e.g.*, PMA capsules) can minimize the effect of sedimentation and the differences between administered dose and cellular dose.

We hypothesized that differences in cellular dose would explain differences in cell association between our particles and capsules. To make quantitative predictions of cellular dose that accounted for bulk effects and particle removal, we implemented a partial differential equation model of sedimentation and diffusion:⁴³

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \vec{v} \cdot \nabla c$$

where c is particle concentration, D is bulk diffusion coefficient, and v is sedimentation velocity. We calculated D and v using the experimentally determined radii and densities (Table S1), Stokes' law, and the Stokes-Einstein equation.

Figure 3. Cell association with particles or capsules in different orientations. (a,d) Comparison of fluorescence intensity of HeLa cells after 24 h incubation with (a) SC/MS@PMA particles or (d) PMA capsules in upright, inverted, or vertical orientations. (b,c,e,f) Deconvolution microscopy images with maximum intensity projection of HeLa cells after 24 h incubation with (b,c) SC/MS@PMA particles or (e,f) PMA capsules in the (b,e) upright or (c,f) inverted orientations. Blue, green and red represent nuclei stained with Hoechst, actin stained with Alexa Fluor 488 (AF488), and particles or capsules labeled with Alexa Fluor 647 (AF647), respectively. Scale bars are 10 μm.

We assumed that our particles were initially uniformly dispersed. At the cell boundary, we assumed every particle contacting the boundary was removed, consistent with evidence that cell membrane-particle interaction forces are many orders of magnitude higher than diffusive or

sedimentary forces.⁴⁴ We assumed no particle flux at non-cell boundaries. Cell association was taken to be proportional to the amount of particles that contact the cell boundary. We predicted relative cellular association for each combination of particle system and orientation (Figures S10 and S11). These predicted associations demonstrate strong agreement with experimental results (Figure 4).

Figure 4. Comparison of experimental vs predicted cellular association. Comparison of experimental and predicted cell association. The data from SC/MS@PMA particles and PMA capsules are in orange and green, respectively. All values given are relative association, calculated by normalizing to cell association of PMA capsules in the upright orientation. Of particular note is the disparity between upright orientations – this is the standard assay used to determine nanoparticle efficacy or toxicity.

Previously, more complex mathematical models of nanoparticle mechanics have been proposed – for instance, accounting for agglomeration.³¹ For the particle system that we have used here, agglomeration of the particles has been shown experimentally to be negligible.

Therefore we employ a model that requires only two physical parameters to be specified – particle radius and density – and we suggest that this model is appropriate for all systems that have achieved common goals of advanced particle engineering – high monodispersity and low aggregation.

This model could successfully predict the orientation-dependent cell association of the investigated hydrogel particle systems. However, there remain physical processes not accounted for, including electrostatic effects, hydrodynamic interactions, and convection forces; understanding these may prove useful for further improving predictive accuracy.⁴⁵ Additionally, the model might be extended to incorporate additional particle-cell interactions, which depend on particle concentration at the membrane and physicochemical properties of both the cell membrane and the particle. Although this would begin modelling intracellular or internalized dose, which is dependent on cellular dose, this requires further experimental investigation to better understand the underlying biology of nanoparticle-cell uptake.

We have shown that cellular dose is a critical determinant of cellular association, and thus may strongly influence all downstream cellular responses. Therefore, determination of the proportion of particles that reaches the cells is a necessary step to quantify and compare the efficacy of particle systems. We sought to address the questions: “How much do dosage effects influence results?” and “What is the relative dose delivered to cells for a given particle?” To address the former, we have used our model to provide “quick reference” graphs, which demonstrate the predicted disparity in cellular dose (*i.e.*, <5%, <10%, <20%) for upright and vertical cell orientations (Figure 5). The approximate (bulk) density of several materials is indicated so that the graphs can easily be used to estimate the importance of dosage effects for commonly used particle systems. Several particles,

including gold nanoparticles and calcium phosphate nanoparticles for which experimental data is available,^{28,46} are labeled in Figure 5. We find broad agreement with our predictions and the reported importance of orientation-dependent effects in these studies. Further, the low influence of cell culture orientation on cellular dose of polymer hydrogel capsules reported here (with an average diameter of 200 nm), being similar to 15 nm gold nanoparticles,²⁸ shows the advantage of hydrogel systems with low density for minimizing the effect of sedimentation on cell association in *in vitro* studies. To address the latter question (“What is the relative dose delivered to cells for a given particle?”), we used our computational approach to predict the proportion of particles that reach the cells for a wide range of organic and inorganic particles with different sizes and densities (Figure 6). This figure allows researchers to control for the effect of sedimentation and diffusion and facilitates direct comparison between different nanoparticle systems.

Figure 5. Percentage difference for upright vs vertical cellular dose for a range of different particles. To address the question “How much do dosage effects influence results?” Graphs display the disparity in predicted cellular dose between vertical and upright cell orientations for a range of different particles. Bulk density estimations for commonly used materials are labeled with vertical lines (polystyrene (PS), polylactic-co-glycolic acid (PLGA), polyglycolic acid (PGA), silica, titania, iron oxide, silver, and gold). All values shown are for 24 h incubation in 5 mm cubes. (a) Scheme of how comparison is made. (b) Low density materials, including many organic nanoparticles. SC/MS@PMA particles (core-shell) and PMA capsules (capsule) used in this study are labeled with dots. (c) High density materials, including many inorganic nanoparticles. We include four particles described in literature.^{15,25} These particles are labeled as follows in their respective papers: Cho-1: 15 nm Gold, Cho-2: 54 nm Gold, Cho-3: 100 nm Gold, Giger-1: 190 nm Calcium Phosphate. Hydrodynamic diameter and apparent density is used where available.

Figure 6. Proportion of particles that reach the cell for a variety of different theoretical particles.

To address the question “What is the relative dose delivered to cells for a given particle?” and allow comparison of different particle systems. (a) Scheme of experiment that was computationally modeled. (b) Table listing the proportion of particles that reach the cells with 1 (dark green) and 0 (dark red) indicating that all and no particles reach the cells, respectively. At smaller dimensions diffusion ensures an even and high cellular dose while at larger dimensions sedimentation starts to dominate. This gives rise to the trend of low cell dose observed for intermediate dimensions. Cellular dose is calculated *in silico* for the standard upright cell orientation, with a 24 h incubation in 12-well plates, with particles initially fully dispersed in 2 mL of liquid media. Code provided can predict cellular dose for other experimental/particle conditions.

CONCLUSIONS

We have shown the importance of considering orientation-dependent effects on cell association for advanced particle systems and provide a comprehensive framework to assess and account for these effects. Hybrid core-shell particles and hydrogel capsules were designed and characterized by advanced microscopy and spectrophotometry techniques. We designed, developed and made accessible the blueprints for two 3D printed cell culture supports that, combined with

conventional multi-well cell culture plates, can be used in the assessment of orientation-dependent effects of particle systems. Decreasing particle size or density helps to minimize the influence of sedimentation on cellular dose or cell association. Hydrogel particles with hollow structures (*e.g.*, PMA capsules) can be engineered with an effective density close to water, which substantially reduces their sedimentation, even for relatively large dimensions (>100 nm diameter), thus making them suitable for studies that rely on conventional upright assays, such as standard multi-well plates and associated systems (*e.g.*, robotic and high-throughput systems). We also implemented computational models to quantitatively determine the proportion of particles that reaches the cells for different particle types and cell orientations. We used these models to provide guidelines to evaluate the impact of these effects for a range of commonly used nanoparticle sizes (0-1000 nm diameter) and materials (densities ranging from 1 to 20 g cm⁻³). Our intention is that this framework and these references can be used as a standard for contrasting different particle systems and facilitate investigations into bio-nano interactions of particles.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

Additional figures and tables (PDF).

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Notes

The authors declare no competing financial interest.

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