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Cubical Shape Enhances the Interaction of Layer-by-Layer Polymeric Particles with Breast Cancer Cells

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Abstract

Blood-borne objects display a non-spherical shape with in-flow dimensions much larger than the vascular endothelial fenestrations, yet, at the diseased state, are able to traverse through these fenestrations owing to their elasticity. The role of physical parameters including shape and elasticity in the behavior of objects found in the tumor microenvironment needs to be understood to ultimately enhance chemotherapy and minimize its side-effects. In this study, sphere and cube-shaped biocompatible elastic microparticles (EM) made *via* layer-by-layer (LbL) assembly of hydrogen-bonded tannic acid/poly(N-vinylpyrrolidone)/ (TA/PVPON) as hollow polymer shells

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and their rigid core-shell precursors (RM) are explored. In contrast to rigid 5-bilayer (TA/PVPON) core-shells, hollow shells are unrecognized by J774A.1 macrophages yet interact with endothelial and breast cancer cells. Internalization of cubical shells by HMVEC (endothelial) is 5-fold more efficient and 6- and 2.5-fold more efficient for MDA-MB-231 and by SUM159 (breast cancer cells), respectively, compared to spherical shells. The interaction of cubical (TA/PVPON)₅ shells with endothelial cells is similar under 10 s^{-1} (characteristic of tumor vasculature) and 100 s^{-1} shear rate (normal vasculature) while it is decreased at 100 s^{-1} shear rate for the spherical shells. Our data suggest that cubical geometry promotes interaction of particles with breast cancer cells, while elasticity prevents engulfment by phagocytic cells in the tumor microenvironment.

Keywords

Tumor microenvironment; shape; elasticity; particles; multilayer capsules

1. Introduction

Among the most prominent biophysical attributes of blood-borne cells are non-spherical shape and elasticity.^[1] These characteristics affect their behavior while in circulation and under different shear stresses observed in healthy and diseased vasculature, and may change as a result of biological, chemical or physical impulses.^[2] For instance, monocytes are able to extravasate through fenestrations in inflamed vasculature, which are several times smaller than their nominal diameter.^[3] Platelets have a non-spherical shape that allows them to marginate, or drift laterally, towards the blood vessel boundary in a linear laminar flow closer to the endothelial wall.^[4] The higher elasticity of metastatic cancer cell membranes in contrast to a non-invasive cell phenotype is believed to enable their efficient transport to new locations.^[5] Morphological adaptations of filamentous *Escherichia coli* bacteria enable the pathogen to evade immune cells, and its microenvironmental heterogeneities can slow or inhibit phagocytosis by macrophages.^[6] Yet, the elasticity of an object (e.g. bacteria, viruses or foreign objects) also affects interactions with cell membranes.^[7]

The shape, surface chemistry and elasticity of synthetic objects are important parameters that affect particle-biological interactions. The shape of a particle has been recognized as an important factor affecting its cellular uptake and vascular dynamics.^[8] Bio-mimetic vehicles with geometry resembling circulating blood cells have been rationally designed using mathematical modelling of their interactions with cell membrane and surfaces under flow.^[9] Elasticity of the carrier affects not only the flow characteristics, but also its recognition by cancer and immune cells.^[10]

Anti-cancer therapeutic agents encounter a series of physical, physiological and biophysical hurdles *en route* to the tumor site such as chemical and enzymatic degradation, phagocytosis by the reticulo-endothelial system, interactions with the tumor microenvironment comprising the vascular endothelium, tumor interstitial pressure and toxin clearing molecular efflux pumps.^[11] These barriers may significantly restrict the delivery of free therapeutics to the tumor site causing a fraction of the dose to be distributed to healthy tissues, resulting in severe side effects.^[12]

Synthetic therapeutic carriers hold promise to efficiently overcome one or several of these barriers and are being widely investigated for this purpose with a few examples in clinical employment from the mid 90s.^[11, 13] When injected intravenously, the delivery vehicles are transported *via* the vasculature to the tumor location where they associate with the endothelium, macrophages^[9c] or extravasate through the endothelial fenestrations of 300 nm – 1.2 μ m and release the drug to the tumor cells.^[14] The carriers can be further internalized by the tumor cells and release the drug intracellularly.^[15]

Hollow polymeric drug microcarriers, referred to as ultrathin multilayer capsules (or shells), can be made using the layer-by-layer (LbL) technique which involves a sequential assembly of polymers onto sacrificial substrates followed by substrate dissolution.^[16] The multilayer assembly is unique in that the capsule shape can precisely replicate the geometry of a sacrificial template and can easily impart desired elasticity to the thin (< 50 nm) polymer shell. Hollow polymeric capsules are currently being evaluated as powerful tools in cancer nanomedicine.^[17]

We have shown lately that pH-sensitive discoidal polymeric capsules can shrink 2-fold when internalized by 4T1 breast cancer cells because of acidic intracellular pH conditions.^[18] We have also demonstrated loading and release of a chemotherapeutic agent, doxorubicin, in poly(methacrylic acid) multilayer hydrogel cubes^[19] and (TA/PVPON) multilayer capsules using pH-triggered intracellular release of the drug in the former case.^[20] Herewith, we design and explore biocompatible, LbL-engineered 2 μ m microcapsules of spherical and cubical shapes made using the LbL assembly of hydrogen-bonded tannic acid/poly(N-vinylpyrrolidone) (TA/PVPON) polymers on sacrificial templates. Elastic microparticles (EM) and their rigid counterparts (rigid microparticles, RM) are compared in this work. TA is a polyphenolic antioxidant that has been used for fabrication of multilayer films, capsules, and cell surface coatings of biomedical relevance.^[21] TA and PVPON are able to form hydrogen-bonded multilayers stable in a wide pH range from 2 to 9.^[21b, 22]

TA/PVPON films have been shown to be non-toxic,^[21c] non-immunogenic,^[23] can suppress synthesis of pro-inflammatory cytokines,^[24] and function as cytoprotective material for pancreatic islets during transplantation.^[23] Very recently, we have demonstrated that biocompatible hollow particles of (TA/PVPON) of hemispherical, spherical, and cubical geometries and improved stability for drug storage and delivery can be obtained *via* the LbL assembly and used for doxorubicin encapsulation with high encapsulation efficiency enabling long-term storage in a pH range of 5 to 7.4.^[20, 21c] Herein, we investigate the interactions of (TA/PVPON) EMs and their rigid counterparts, RMs, with immune cells, breast tumor cells and endothelial cells of the tumor microenvironment and their extravasation potential through 0.8- μ m porous membranes which are morphologically similar to the fenestrations in the tumor endothelium. We find that rigid core-shells are readily detected and significantly internalized by macrophages in contrast to the elastic shells. We observe significant differences in the interactions of the cells with the 2 μ m spherical and cubical (TA/PVPON) EMs. We also study the interactions of the cubical and spherical EMs and RMs with HMVEC endothelial cells in a static environment and under 10 and 100 s⁻¹ shear rates. Comparison of the elastic shells and the rigid core-shells of similar shape and coated with the same multilayer show that 2 μ m shells can successfully permeate

through the 0.8 μm pores. These studies also imply how the variations of mechanisms found in Nature can be employed to overcome the delivery obstacles.

2. Results and Discussion

2.1 Fabrication and Characterization of Shaped Delivery Vehicles

Spherical and cubical rigid RMs and soft EMs were fabricated by the multilayer assembly of TA and PVPON as reported previously^[21c] and schematically presented in **Figure 1a**. Dissolution of the shape-imparting core in the core-shell particles led to the formation of hollow (TA/PVPON) capsules of the corresponding shape.

The spherical particles had a diameter of approximately 2 μm while the cubical particles had an edge of about 2 μm as examined by confocal microscopy and scanning electron microscopy (**Table 1**). Core-shells appeared as three-dimensional (3D) spheres and cubes (**Figure 1b-e**) while the shells collapsed upon drying and appeared slightly larger by scanning electron microscopy whereas confocal microscopy images of the shells in solution clearly demonstrated their hollow spherical and cubical shapes (**Figure 1f-i**).

To ensure the consistency in the surface properties of the EMs and RMs, we compared the (TA/PVPON)₅ capsules with the corresponding cores coated with (TA/PVPON)₅ multilayers. Thus, the negative ζ -potential values of the particles as measured by Zeta Phase Analysis Light Scattering indicated that all systems were comparable in their surface charge. The Young's moduli of the systems (**Table 1**) indicate that the EMs are comparable to platelets in elasticity.^[25-26]

2.2 Interaction with Tumor Cells and Cells of the Tumor Microenvironment

When drug carriers are systemically introduced, they first encounter immune cells or monocytes (macrophage precursors), and then are transported through the vasculature where they come across endothelial cells and gradually accumulate in diseased tissue in the proximity of tumor cells.^[15a] Hence, we evaluated the interactions of the shells and core-shells with macrophages, endothelial cells and cancer cells.

Modeling studies have predicted that elasticity of particles plays an important role in the uptake by macrophage cells.^[7] The particle shape did not seem to be important for macrophage internalization of the rigid particles when J774A.1 murine macrophages were incubated with the spherical and cubical core-shells, and both shapes were equally internalized within 15 min of incubation (**Figure 2a-c**). In contrast, their EM counterparts interacted significantly less with the macrophages compared to the corresponding core-shells. For instance, after 2 h-incubation, cubical EMs were internalized 14-fold less by the macrophages than the cubical core-shells (**Figure 2d**). Similarly, spherical EMs were taken up 10-fold less than their rigid counterparts (**Figure 2e**). This dramatic difference in macrophage internalization between RMs and their elastic polymeric replicas was observed even at 15 min and 1 h, implying that the particle rigidity is among the main factors determining the delivery vehicle recognition and uptake by macrophages (**Figure 2**).

The HMVEC endothelial cells could internalize both cubical and spherical shells (**Figure 3a-b**) as observed using confocal microscopy. However, the cubical (TA/PVPON)₅ EMs interacted significantly better than the spherical after 2, 6 and 24 h (**Figure 3c**). This trend reversed in the case of rigid core-shells with 5-fold better interaction of the spherical core-shells with HMVEC cells compared to the cubical core-shells after 24 h-incubation (**Figure 3d**). The spherical RMs were internalized more than their corresponding EMs at all 3 time-points (**Figure 3e**) which agrees with the previous observation that particle rigidity favors endothelial cell internalization.^[18] However, in the case of cubes, the shells interacted with the cells almost 2-fold better than the corresponding core-shells (**Figure 3f**). This difference, however, developed only by 24 h of incubation and can be attributed to the fact that cubes must overcome a higher cell membrane wrapping energy barrier than spheres.^[27] The adhesion strength for a cube should be 3-fold more than that of a sphere to compensate the membrane deformation energy at the upper edges of the cube.^[27-28] Yet, for a softer cubical shell, the cell membrane deformation energy might be less than that for a rigid cube which could be the reason for the observed difference.

For drug carriers in circulation, flow conditions may affect their interactions with endothelial cells. For instance, endothelial cells, when adapted to continuous flow, exhibited actin stress fiber formation which inhibited endocytosis of antibody-functionalized particles, in contrast to the endothelial cells not adapted to flow and devoid of actin stress fibers.^[29] The endothelial cell monolayer in microfluidic channels was subjected to particle flow at two shear rates – 10 and 100 s⁻¹. Shear stresses greater than 100 s⁻¹ are characteristic for normal vasculature, while those of 10 to 100 s⁻¹ are observed for capillaries feeding tumors.^[30] In flow, the EMs appear as 3D spheres and cubes rather than flattened objects as they appear in the SEM micrographs after drying. There was no significant difference between the two shapes in association with the cells at 10 s⁻¹, while at 100 s⁻¹, the spherical shells associated with the cells significantly less than the cubical ones (**Figure 4**). Importantly, the cubical capsules exhibited a similar HMVEC associating behavior for both tested shear rates.

These results agree with the earlier findings that the contact surface area of the particles at which maximum adhesion occurs is generally higher for non-spherical particles than for spherical ones possessing similar volume. This leads to a higher probability of a vessel wall contact and subsequent better adhesion for non-spherical particles when compared with spherical particles of the similar volume and under similar conditions.^[31] We also explored the interactions of the cubical and spherical particles with 4T1, MDA-MB231, and SUM159 breast cancer cell lines. When aggressive murine 4T1 breast cancer cells mimicking human stage IV breast cancer were incubated with the particles, cubical core-shells were internalized 1.8-fold more than the spherical ones after 24 h (**Figure 5a**). MDA-MB231, a human breast cancer epithelial cell line, preferentially internalized cubical over spherical core-shells, and cubical over spherical capsules after 2, 6, and 24 h. At 24 h, cubical core-shells were internalized 8.8-fold more than the spherical ones, whereas cubical capsules were internalized 6 times more efficiently than the spherical capsules (**Figure 5b**). Lastly, the human breast cancer cell line SUM159 showed a distinct affinity for cubical capsules and internalized significantly more cubical shells over spherical shells and core-shells after 2 h of incubation (**Figure 5c**). In 24 h, the cells internalized cubical core-shells

and shells 4- and 2.6-fold more than spherical RMs and EMs, respectively (Figure 5c). The observed preferential uptake of cubical particles by the cancer cells is probably due to the significantly larger contact area of the flat surface of the cubical capsule particle which, in this case, became crucial in the process of internalization by epithelial cells. Recent modeling studies of a cell lipid membrane wrapping around rigid cubes and spheres suggested that the particle adhesion to the cell membrane followed by the membrane wrapping around the particle is governed by the adhesion energy gain for particle-cell membrane contact and the energy cost for the cell membrane deformation.^[27] The greater adhesion strength for a cube may compensate the deformation energy of the membrane at the upper edges of the cube. Also, as suggested above for the interaction of the cubical and spherical RMs and EMs with HMVEC cells, slightly softer cubical EMs may require less energy for cell membrane deformation and a subsequent better uptake of the cubical EMs by cancer cells unlike less soft spherical EMs (Table 1). A similar trend was reported by Liu et al. where flexible micrometer-sized hydrogel particles were taken up by HepG2 cells at a higher rate than less elastic particles^[10d] Moreover, in a recent study by Parak and colleagues, the more flexible spherical multilayer capsules were shown to be better processed by cells in contrast to their more rigid (with a thicker shell) counterparts.^[10c] The cubical EM shells were not taken up by macrophages because of their low rigidity compared to the rigid core-shells and were remarkably active with endothelial and cancer cells (because of the shape), and can further be investigated as a promising carrier for delivery of cancer therapeutics as well as a tool for understanding the physical interactions in the tumor microenvironment.

2.3 Evaluation of Particle Extravasation Potential

We have previously shown that cubical and discoidal multilayer hydrogel hollow capsules can bulge and change shape in response to pH changes.^[19, 32] The volume of the discoidal shells decreased 2.9-fold when pH was changed from 7.4 to 4.^[19] Tumor microvasculature has fenestrations ranging from 300 nm to 1.2 μm , depending upon the microenvironment and the tumor type.^[33] These fenestrations, the vascular permeability and hydraulic conductivity are significantly higher in tumor than in normal tissues,^[34] serving as a basis for Enhanced Permeation and Retention (EPR) effect.^[14] Previous studies with particles having sizes below 200 nm show that they extravasate in tumor tissue from the large pores in tumor vessels, and vascular permeability decreases with an increase in the size of the transported rigid species with cutoff size below 200 nm.^[35] We explored if the elastic (TA/PVPON) polymer shells could squeeze through the pores 2-3 times smaller than the shell diameter and maintain their dimensions.

The diagram in **Figure 6a** shows the experimental setup. The track-etched membrane with 0.8 μm pore size used in the study resembled the morphology and architecture of the fenestrations in the tumor endothelium. **Figure 6 (b and c)** shows that 90% of spherical and 80% of cubical shells 2.5-fold larger than the membrane pore size penetrated across the fenestrations under pressure (automatically controlled by a flow pump) of less than 18 psi, while the core-shells were unable to cross it. Confocal microscopy of the flow-through showed that the shells maintained their size and shape (**Figure 6b Insets**). Previous studies with elastic non-hollow polymeric particles made via particle replication in a non-wetting

template (PRINT) process have shown that filamentous particles with 80 nm diameter and various lengths containing low concentration of polyethylene glycol diacrylate and high concentrations of tetraethylene glycol monoacrylate were able to cross through 200-nm pores under manual pressure of 40 psi.^[36]

3. Conclusion

In this work we evaluated the effect of physical characteristics of the rigid particles and their elastic polymer replicas on their interactions with cells of the tumor microenvironment. Biocompatible elastic shells were not internalized by J774A.1 macrophages regardless of their shape, and efficiently extravasated through a membrane with pore diameter 2.5 times smaller than the particle size. In contrast, their rigid counterparts were efficiently taken up by the phagocytic cells. The cubical shape played a crucial role when considering the interactions of the shells with endothelial HMVEC and especially 4T1, MDAMB231, and SUM159 breast cancer cells. Cubical shells exhibited highly increased internalization in the three tested breast cancer cell lines, pointing towards the importance of mechanical stimuli in the process of uptake. There was no significant difference between cubical and spherical particles in association with endothelial cells under 10 s^{-1} shear rate which is characteristic of tumor vasculature. Our data show that elastic cubical capsules possess important biological characteristics which can warrant their further development for cancer therapy.

4. Experimental Section

Materials

Poly(ethyleneimine) (PEI, average M_w 25,000), poly(N-vinylpyrrolidone) (PVPON, average M_w 1 300 000 g mol^{-1}), tannic acid (TA, M_w 1700 g mol^{-1}), 2-propanol, 46 wt% hydrofluoric acid, manganese sulfate monohydrate and ammonium bicarbonate were purchased from Sigma-Aldrich. Silicon dioxide particles of $2.0 \pm 0.1 \text{ }\mu\text{m}$ were obtained from Polysciences Inc. Ultrapure de-ionized water with a resistivity of 0.055 $\mu\text{S/cm}$ was used (Siemens, USA). Monobasic and dibasic sodium phosphate (Fisher Scientific, USA) were used for preparation of polymer solutions unless otherwise noted.

Synthesis of cubic sacrificial templates

Cubic manganese carbonate cores were synthesized as described previously^[19, 21c]. Briefly, fresh nano-seed solution was prepared by mixing 0.04 g NH_4HCO_3 and 0.02 g MnSO_4 in 200 mL DI water. Then, 100 mL of the nano-seed solution was added to 500 mL of 6 mM MnSO_4 containing 0.5% 2-propanol. Then 500 mL of 0.06 M ammonium bicarbonate solution with 0.5% of 2-propanol were poured into the nano-seed solution. The mixture was immediately heated at 80°C for 30 min to result in $2 \text{ }\mu\text{m}$ cubic manganese carbonate particles. The MnCO_3 cubic particles were collected by filtration through $0.45 \text{ }\mu\text{m}$ Whatman filters before being rinsed with DI water several times and dried at room temperature ($\sim 25^\circ\text{C}$). Scanning electron microscopy (SEM) analysis of the cubic templates was performed using a FEI QuantaTM FEG microscope at 10 kV.

Fabrication of multilayer (TA/PVPON) capsules of cubic and spherical shapes

Hollow hydrogen-bonded spherical capsules were prepared by coating 2 μm silica particles with PEI(TA/PVPON)₅ multilayer coating followed by particle dissolution, where subscript denotes the number of bilayers. Specifically, poly(ethylene imine) was allowed to adsorb on the particles as the first layer from 1 mg mL^{-1} aqueous solution for 10 min followed by sequential adsorption of TA/PVPON multilayers. For that, 1.5 mL of 10% particle suspension was pelleted in a 1.5 mL Eppendorf centrifuge tube and washed two times with 0.01 M sodium phosphate rinsing solutions at pH=3.5. Then, PVPON was allowed to adsorb onto particle surfaces from 0.5 mg mL^{-1} of 0.01 M sodium phosphate solution at pH=3.5 for 10 min followed by the deposition of TA layer from 0.5 mg mL^{-1} of 0.01 M sodium phosphate solution at pH=3.5 for 10 min. After each deposited layer, particles were centrifuged for 3 min at 2000 rpm and washed two times with the rinsing solution. Alternating coating of particles with the polymers was continued until 5 bilayers were deposited. Silica cores were dissolved in 8% hydrofluoric acid to yield hollow polymeric PEI(TA/PVPON)₅ capsules. For cubical capsules, the deposition process was similar and PEI(TA/PVPON)₅-coated MnCO_3 cores were dissolved using 0.1 M EDTA solution (pH=7). After that, capsules were dialyzed in DI water for 2 days. To visualize the rigid core-shells or soft hollow capsules of cubic and spherical shapes, PVPON was fluorescently labelled with Alexa Fluor carboxylic acid succinimidyl ester 488 nm or 532 nm as described previously (Molecular Probes).^[23] Confocal images of the core-shells and capsules were obtained with Zeiss LSM 710 confocal microscope equipped with a 63x oil immersion objective. To observe the particle shape, a drop of the particle suspension was added to Lab-Tek chambers (Electron Microscopy Sciences), which were filled with deionized water and imaged.

Determination of Particle Concentration

10 μL of each particle stock solution was loaded into a Hausser Bright-Line Hemocytometer used for cell counting (Hausser Scientific Company, PA, USA). The samples were allowed to settle overnight and then counted using a standard upright microscope. The number of particles per mL^3 of the stock was obtained as an average of the number counted per $\text{mm}^2 \times 10^4$. Appropriate dilutions of the stock were made when necessary.

Determination of Particle Zeta Potential

Zeta potential of 10^7 - 10^8 particles suspended in 850 μL 10 mM phosphate buffer (pH 7.4) was analyzed using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). Each sample was taken in a disposable zeta cuvette and the measurements were done in triplicate with 12 runs each. The zeta potential values and electrophoretic mobility were calculated as an average of three measurements of multiple runs.

Characterization of Particle Shape and Size by SEM

Size and shape of the particles were studied by SEM. Surfaces of $5 \times 5 \text{ mm}^2$ silicon wafers were cleaned with absolute ethanol and adhered onto SEM aluminum specimen mounts using conductive carbon tape. 10 μL of an appropriate concentration of the particles was placed on top of the silicon chips and dehydrated for two nights in a desiccation chamber.

The samples were sputter coated with platinum of density 19.72 g/cm^3 , using a high resolution sputter coater 208HR (Cressington Scientific Instruments, MA, USA). The samples were analyzed with the ultra-high resolution Nova NanoSEM 230 (FEI, OR, USA) at 10 kV.

Fluorescence Characterization of Particles

The systems were all fluorescently tagged with Alexa Fluor dihydrazide sodium salt 488 nm or 532 nm, to analyze their interactions with cells. The (TA/PVPON)₅-coated particles and (TA/PVPON)₅ capsules were observed using the 100x objective of Nikon A1 Confocal Imaging System and Nikon Fluorescent Microscope.

Analysis of Interaction of the Particles with Cells

J774A.1 macrophage, 4T1, MDAMB231 and SUM159 breast cancer cells were obtained from American Type Culture Collection, VA, USA. HMVEC cells were obtained from Lonza Walkersville Inc., MD, USA. J774A.1, MDAMB231 and SUM159 were cultured in DMEM-10% FBS (Gibco, Life Technologies, NY, USA), HMVEC cultured in EBM2-MV (Lonza Walkersville, Inc., MD, USA) and 4T1 in MEM (Gibco, Life Technologies, NY, USA). All cells were seeded at a density of 25×10^4 cells/ well except J774A.1 macrophages which were seeded at 50×10^4 cells/ chamber- well in 8-chamber polystyrene slides and allowed to attach overnight. The following day, the standing media was aspirated and the cells were incubated with media containing the particles at [5 particles/cell]. All cells except J774A.1 were treated with the particles for 2, 6 and 24 h while J774A.1 were treated for 15 min, 1 and 2 h. At the end of the time period, the media containing the particle suspension was aspirated out of each well, washed twice with phosphate-buffered saline (PBS) and fixed with methanol-free 4% paraformaldehyde (PFA).

For flow cytometric analysis, 2×10^5 cells were seeded per condition in multiwell plates, and treated similarly. At each time point, the cells were trypsinized (HMVEC, 4T1, MDAMB231, SUM159) or scraped (J774A.1), fixed and fluorescently labeled in suspension.

Fluorescent Staining and Preparation of Cells for CLSM and ImageStream^X Analysis

The fixative was removed and the cells were washed twice with PBS and permeabilized with 0.1% Triton® X-100 (Sigma-Aldrich, MO, USA) for 5 min. The cells were washed twice with PBS and incubated with Alexa Fluor Phalloidin 488 or 650 (Molecular Probes Inc.) for 30 min. The phalloidin solution was aspirated, the cells were washed thrice with PBS, and the slides were sealed with coverslips using ProLong Gold antifade reagent mounting medium with DAPI (Molecular Probes Inc.). The mounted slides were allowed to dry and Cytoseal XYL (Richard-Allan Scientific, MI, USA) was applied along the edges of the slide. The cell interactions with particles were analyzed with the Nikon A1 Confocal Imaging System (Nikon Instruments Inc., NY, USA). The samples for image-based flow cytometry were washed and labeled similarly but in suspension. The percentage of cells with internalized particles was acquired using ImageStream^X (Amnis Corporation, WA, USA) and analyzed using the IDEAS software (Amnis Corporation, WA, USA).

Interaction of Endothelial Cells with Particles under Physiological Shear Stress

The interaction of the spherical and cubical capsules with the endothelial cells in physiological conditions were studied using the microfluidic BioFlux™ 200 System (Fluxion Biosciences). The microfluidic channels were coated with fibronectin and HMVEC were seeded at a density to form a monolayer when incubated overnight at 37°C and 5% CO₂. After 2-3 h when the cells attached, media was replenished. The following day, 10⁷ capsules/mL was flowed through the endothelial monolayers at shear rates 10 s⁻¹ and 100 s⁻¹ for 2 h at 37°C and 5% CO₂. Fresh media was then flowed through to remove any unbound/non-interacted capsules and the system was further incubated for 4 h. Cells in the channels were fixed and fluorescently labeled for confocal analysis as mentioned in the previous section. Percentage of the cells associated with the capsules in a field of view at the 2 different shear rates was plotted.

Extravasation Potential of Particles

The extravasation of the 2-μm rigid particles and capsules through 0.8 μm fenestrations was tested by passing them through Whatman™ Nuclepore™ Track-Etched Polycarbonate Membranes with 0.8 μm sized pores (GE Healthcare Life Sciences). Approximately 10⁴ to 10⁵ particles of each type were flowed using a mini-variable flow pump at a flow rate of 5 mL min⁻¹ and pressure below 18 psi. The pre- filter and post-filter particle solutions were taken in chamber slides, allowed to settle overnight, imaged, and the number of the particles was counted using the ImageXpress Micro- High Content Screening (HCS) System (Molecular Devices, CA, USA). The percentage of the particles having passed through the membrane pores was calculated. Confocal microscopy of the collected flow through was performed to confirm if the particles were intact. SEM of the membrane surface after filtration was done to detect blocked pores, trapped or broken capsules, if any.

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Figure 1.

(a) Rigid RMs (core-shells) and EMs (shells) are produced using layer-by-layer assembly of tannic acid (TA) and poly(N-vinylpyrrolidone) (PVPON) on sacrificial inorganic manganese carbonate and silicon dioxide cores of cubic and spherical shape, respectively. Confocal microscopy images of cubical (b, c) and spherical (d,e) RM (core-shells) (b-e) and their cubical (f, g) and spherical (h, i) EM replicas (soft shells, f-i) produced *via* dissolution of the cores. The scale bar is 5 μm .

Figure 2.

Confocal microscopy images of J774A.1 macrophages with (a) internalized spherical RMs (S-RM core-shells) and (b) sparsely associated but non-internalized spherical EMs (S-EM shells). Scale bar is 5 μm. (c-f) Quantification of J774A.1 macrophages internalizing spherical (S) and cubical (C) core shells (C-RM) and shells (C-EM) as measured by image-based flow cytometry (Amnis ImageStream^x). The experiments were carried out in triplicate and >4000 cells were analyzed in each experiment (Mean ± SEM, *p 0.017; **p 0.004; ***p 0.002).

Figure 3.

Confocal microscopy images of HMVEC endothelial cells with internalized (a) spherical and (b) cubical (TA/PVPON)₅ EM (shells). Scale bar is 5 μ m. (c-f) Quantification of HMVEC cells internalizing spherical (S) and cubical (C) RM (core-shells) and EM (shells) after 2, 6, and 24 hours of incubation. The experiments were carried out in triplicate and >4000 cells were analyzed in each experiment (Mean $\pm$ SEM, *p 0.007; **p 0.01; ***p 0.045; ****p 0.008; *****p 0.07).

Figure 4.

Interaction of (TA/PVPON)₅ EM (shells) with the HMVEC endothelial cells under physiological flow conditions showing no significant difference in the internalization of the cubical shells (C-EM) at 100 and 10 s^{-1} shear rates when the particles were flowed for 2 hours. The experiments were carried out in triplicate and >250 cells were analyzed in each experiment (Mean $\pm$ SEM). There is a significant difference in the cell association between the spherical and cubical shells (S-EM and C-EM) at 100 s^{-1} ; *p 0.005.

Figure 5.

Internalization of spherical (S) and cubical (C) RM (core-shells) and EM (shells) by (a) 4T1 (*p 0.05; **p 0.01; ***p 0.005), (b) MDAMB231 (*p 0.05; **p 0.01; ***p 0.005), and (c) SUM159 (*p 0.02; **p 0.01; ***p 0.05) breast cancer cells. The experiments were carried out in triplicate and >4000 cells were analyzed in each experiment.

(a)

(b)

Figure 6.

Extravasation of spherical and cubical (TA/PVPON)₅ RM (core-shells) and EM (shells). (a) Schematic diagram shows the mesh filtration experiment. Scanning electron micrographs of the membrane surface, after subjecting the shells to flow through show that the core-shells retained on the surface, whereas the shells have crossed through the 0.8-micrometer pores. The insets show the EM (shells) that have passed through the pores and maintained their geometry. Scale bar is 5 μm . (c) Percentage of the particles able to traverse through the

pores. No RM (core-shells) passed through. The experiments were carried out in triplicate and >30,000 events were analyzed in each experiment (*p 0.0027; **p 0.0002).

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Table 1

Physicochemical characterization of the systems.

System	Particle Diameter ^{a)} , (μm)	E ^{b)} , (MPa)	ζ-Potential ^{c)} , (mV)
Spherical core-shell (S-RM)	2.03 ± 0.03	> 1×10 ⁴	-16 ± 4
Spherical shell (S-EM)	3.04 ± 0.47	4.30 ± 0.40	-10 ± 1
Cubical core-shell (C-RM)	2.40 ± 0.10	> 1×10 ⁴	-27 ± 9
Cubical shell (C-EM)	2.86 ± 0.20	0.61 ± 0.08	-9 ± 4

^{a)} Particle diameter was measured using SEM

^{b)} Young's moduli of the capsules, E, was obtained from previous reports [25]

^{c)} ζ-Potential was determined using phase analysis light scattering (Mean ± SD).