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Porous, Hollow, and Ball-in-Ball Metal Oxide Microspheres: Preparation, Endocytosis, and Cytotoxicity**

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Current technology relies heavily on composite materials, but in most cases, the size of the individual components have been micrometers or larger. The development of nanotechnology is driven in part by the desire to prepare materials that are only a few nanometers in size or that are made from components in the sub-micrometer regime. Improved preparations of various examples of monodisperse,[1] porous,[2] hollow, and/or core/shell[3]metal and semiconductor nanoparticles or nanowires[4] have been developed. We report here the use of a simple and scalable technology, ultrasonic spray pyrolysis (USP),[2e,5] to prepare porous, hollow, or ball-in-ball nanomaterials (Fig. 1 and Supporting Information Fig. S1). In addition, we have investigated the cell toxicity (cytotoxicity) of these nanomaterials, in keeping with the growing concern of health effects that manmade nanoparticles could have now and in the near future.[6]

Our interest in USP stems from our long standing work on the chemical and physical effects of ultrasound.[7a,b] In liquids irradiated with high-intensity ultrasound, acoustic cavitation produces high-energy chemistry through intense local heating inside the gas phase of collapsing bubbles in the liquid.[7] There are diverse applications of such sonochemistry, including the preparation of nanostructured materials and nanoparticles. USP presents an interesting inversion of the cavitation process.[2e,5j,k] Both confine the chemical reactions to isolated sub-micrometer reaction zones, but sonochemistry does so in a heated gas phase within a liquid, while USP uses a hot liquid droplet (or resulting heated solid particle) carried by a gas flow. Thus, we view USP as a method of phase-separated synthesis, in our cases, using sub-micrometer-sized droplets as isolated chemical reactors for nanomaterial synthesis.

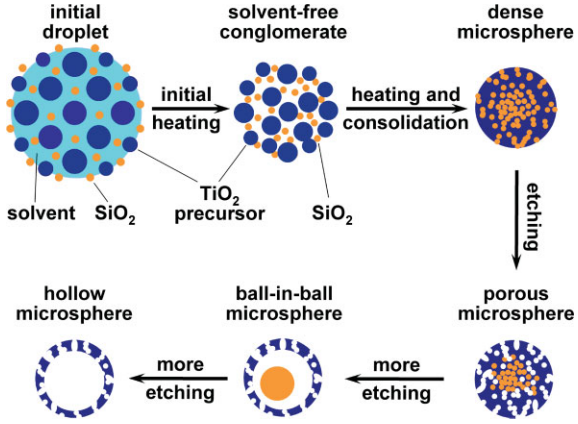

Figure 1. Schematic of the synthesis of porous, hollow, and ball-in-ball titania spheres using USP.

While USP has been used to create both titania and silica spheres separately,[2e,5b–d] there are no prior reports of titania–silica composites. We have now produced such nanocomposites, and by further manipulation, generated various porous structures with fascinating morphologies (Figs. 1 and 2 and Supporting Information Fig. S3). As described in more detail in the Experimental section, a precursor solution was nebulized using a commercially available household ultrasonic humidifier (1.7 MHz ultrasound generator), and the resulting mist was carried in a gas stream through a glass tube in a hot furnace. After exiting the hot zone, spherical particles a few hundred nanometers in size (hereon referred to as microspheres) were collected in a water-filled bubbler as an aqueous colloidal solution. The microspheres were then isolated from this solution by centrifugation. Morphology, size distribution, and composition were analyzed using scanning electron microscopy (SEM), transmission electron microscopy (TEM), scanning TEM (STEM), energy dispersive spectroscopy (EDS), and X-ray diffraction (XRD). Cytotoxicity was tested with several whole mammalian cell assays.[8] Small molecules were also delivered inside through the endocytosis of microspheres.

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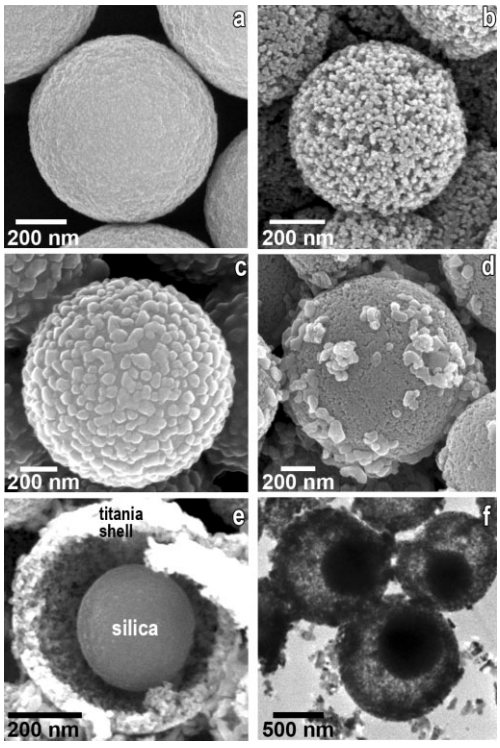

Figure 2. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of USP microspheres. a) Silica–titania composite, 1. b) 1-etched. c) Anatase phase titania with silica core and cobalt oxide nanoislands, 2. d) 2-etched; the small surface particles are cobalt oxide nanoparticles. e) SEM and f) TEM images of a ball-in-ball titania sphere, 2-etched; the small detached particles are cobalt oxide. More images are given in the Supporting Information (Figs. S1c and S2).

Mechanism of USP Preparation: Our general precursor solution was an aqueous solution containing a Ti^{IV} complex, colloidal silica nanoparticles, and (if used) a dissolved first-row transition-metal salt. This solution was transformed into a fine mist carried by air into a furnace. While inside the heating zone (700–900 °C), the water evaporates, and subsequently the remaining chemicals react in the presence of oxygen to form a metal oxide nanocomposite sphere. From SEM results, in the presence of transition-metal ions, the microspheres have bumpy 30–50 nm features on the outside of the sphere (Fig. 2c, microspheres 2, (Ti/Si/Co = 5:2:1)), whereas in the absence of transition-metal ions, they have a much smoother surface (Fig. 2a, microspheres 1 (TiO_2/SiO_2 = 1:1)). EDS reveals that the bumps on the surface of microspheres 2 are nanoislands of cobalt oxide. XRD revealed that only the anatase phase titania was formed. A comparison of the XRD patterns of commercial TiO_2 (Degussa P25) and USP titania is given in the Supporting Information (Figs. S7 and S8). In order to make porous titania microspheres, the microspheres after USP were etched with HF, a well-established technique when silica is used as a template.[3a–c,g,5j]

The average particle size was 900 nm (±400 nm). Interestingly, these spheres were built up of individual anatase phase titania nanoparticles (5–20 nm) and the amorphous silica nanoparticles (5–100 nm, as pre-selected) that were originally trapped within each droplet of the precursor mist. Currently we are looking into applications of these microspheres as supports in heterogeneous catalysis and for biological imaging and drug encapsulation where polydispersity may be a less-significant disadvantage. Polydispersity in spray pyrolysis is primarily due to droplet coalescence in the initially prepared mist, a problem especially with small-scale laboratory devices. With higher gas stream flows, longer furnaces, droplet size control, and growth/initiation rate control, good monodispersity can be obtained.[5g,k]

We can control the pore size and morphology of the resulting microspheres by varying the ratio between Ti^{IV} and colloidal silica nanoparticles (Supporting Information Fig. S3). As expected, larger pores were generated when 70–100 nm sized colloidal silica was used in place of 12 nm colloidal silica. The extent of porosity of the microspheres after etching increased as the amount of silica was increased (Supporting Information Fig. S3a and b). If the silica content was too high, however, the spherical architecture was lost as etching destroys structural integrity (i.e., titania nanoparticles are not sufficiently in contact with other titania particles to hold together, see Supporting Information Fig. S3c). Etching must be used judiciously, since titania is also etched away, but at slower rates; typically, we find that the as-prepared SiO_2-TiO_2(-M_xO_y) microspheres can be exposed to 10 % HF in ethanol at room temperature for up to 90 min.

TEM and EDS analysis (Fig. 2f and Supporting Information Figs. S9 and S10) confirmed that as the microspheres were etched they form porous structures. The EDS analysis of 1-etched (initial TiO_2/SiO_2 = 1:1) confirmed the presence of TiO_2 and the almost complete absence of SiO_2 (see Supporting Information); bulk elemental analysis showed approximately 7 wt % of Si left after etching. To our surprise, the SEM and TEM images of 2-etched (Ti/Si/Co = 5:2:1) shows that the incorporation of transition-metal ions to the precursor solution results in the synthesis of porous ball-in-ball type microspheres (Fig. 2d–f). This core/shell-type structure was best formed when Co^{II} was added. For other metal ions, such as Cr^{II}, Mn^{II}, Fe^{II}, and Ni^{II}, the resulting microspheres were a complex mixture of porous and core/shell microspheres with occasional ball-in-ball structures.

We speculate that the role of the metal ions is to induce a phase separation within the aerosol particles during the reactions that occur as the particles are rapidly heated.[5] The role of Co^{II} in the phase-separation process is still under investigation. We hypothesize that the Co^{II} ions preferentially react with the titania precursors to form a cobalt–titania phase in which silica is less soluble. Thus, a shell of cobalt–titania is formed around a separated silica–titania core. On further heating, islands of cobalt oxide migrate to the outer surface. In keeping with this hypothesis, the external nanoislands of cobalt oxide do not form below 700 °C, and the cobalt remains contained in the titantia matrix (incorporation of Co^{II} into titania has been recently noted[9]). Over 900 °C, Co_3O_4 nanoislands start to disappear as they react with the TiO_2

ADVANCED MATERIALS

matrix to form $CoTiO_3$. This process is distinctive because the color of the product changes from dark brown to jade green (Supporting Information Fig. S8c and d). It is also possible that the gases formed from high-temperature decomposition of the precursor counteranions[5e,10] (e.g., acetate, lactate) may play some unknown role in the phase separation.

Cytotoxicity and Endocytosis of Microspheres: Toxicity studies with cells revealed that silica and titania nanocomposites have substantially lower cytotoxicity compared to metal and semiconductor nanoparticles.[6,11,12] In particular, anatase titania microparticles, amongst all metal oxides, show the lowest toxic response in cell studies.[13] Cytotoxicity was tested with a standard in vitro WST-1 assay and PC12 cells (Fig. 3).[8] As prepared, 1 and 2 were tested for PC12 cell viability; PC12 cells adopt a neuronal phenotype and are normally used as an initial screen for potential dementia-related pharmaceuticals.[8b] PC12 cells were simply treated with our microspheres and compared to H_2O_2 oxidative damage. The results show little cytotoxicity from our microspheres, similar to previous results on nanoparticulate oxides.[12,13] Further PC12 cell-culture studies were conducted for up to six days. Optical microscope images suggest that there is no difference between the control cells and the test cells (Supporting Information Fig. S4). Morphology, growth kinetics, and growth patterns are all similar.

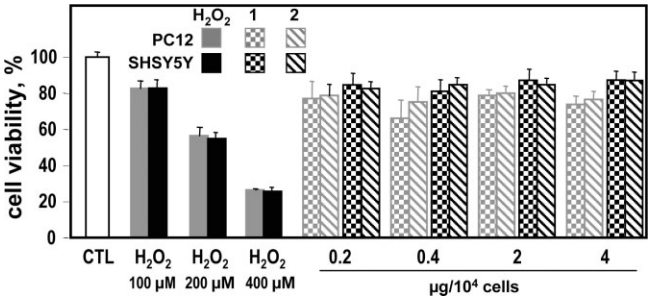

Figure 3. Cytotoxicity of USP microspheres to mammalian whole cells, compared to H_2O_2 exposure, using the WST-1 assay for cell viability. Two types of microspheres were examined, 1 in checkered and 2 in hatched lines. Two cell lines were used: PC12 (black bars) and SHSY5Y (gray bars). WST-1 assays. CTL = control. 0.2 µg of microspheres/10^4 cells ~ a number ratio of 16 microspheres/cells. Cell culture microscopic images are available in the Supporting Information.

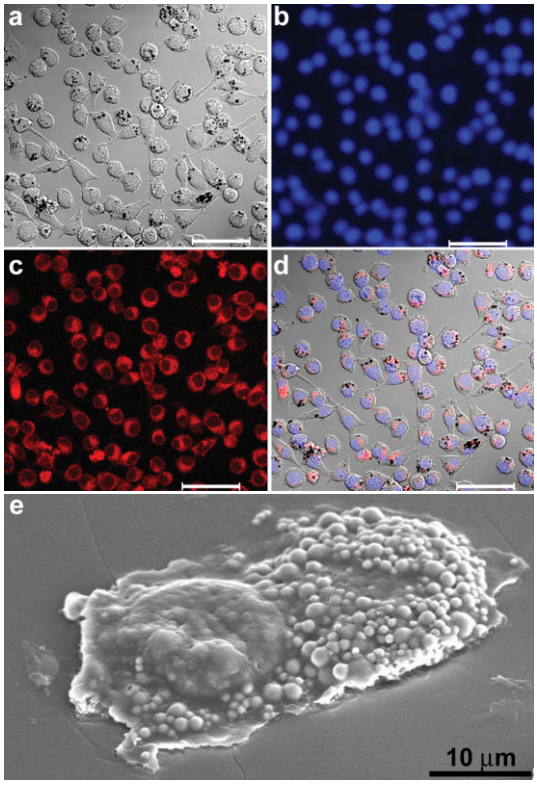

Figure 4. Confocal microscopy and SEM images of endocytosized USP microspheres. a) Optical image with BV2 macrophage cells and microspheres 1 engulfed. b) 4′, 6-Diamidino-2-pheyl indole (DAPI) stained nucleus. c) Microspheres 1 with adsorbed rhodamine shown inside the cells. d) Overlay image of a–c. Magnification is identical in all cases; scale bar is 50 µm. e) SEM of a single BV2 macrophage cell with engulfed microsphere 1, showing the localization of the microspheres in the cytosol and not in the nucleus.

Due to our interest in using USP-generated silica and titania nanocomposite microspheres as drug delivery agents, we further examined microsphere cytotoxicity using a macrophage cell line BV2 (murine)[8c] as well as a neuroblastoma cell line SHSY5Y (human)[8d] (Fig. 4 and Supporting Information Fig. S6). In vitro WST-1 cytotoxic assays were made on BV2 cells (Supporting Information Figs. S5 and S6). They were grown together with microspheres 1, 1-etched, 2, 4, and 4-etched for 4 h with no apparent toxicity.

Other results suggest the possibility that the cobalt oxide coated microsphere 2 might eventually become cytotoxic: cobalt metal-coated microspheres, prepared by exhaustive H_2 reduction of microspheres 2, do show some toxicity (Supporting Information Fig. S5).

As a second test for cytotoxicity and to test the possibility of delivering small molecules inside cells, microspheres 1 and 2 (onto which rhodamine had been adsorbed) were grown with BV2 cells for two days. Confocal microscopy (Fig. 4) shows delivery of the dye-covered microspheres into the cytosol, but without further penetration into the cell nucleus. Cytotoxicity in BV2 cells is very low, similar to that with PC12 cells. A third test for cytotoxicity was run using SHSY5Y cells, which confirmed again that microspheres 1 and 2 show very low levels of cytotoxicity (Fig. 3). 1-Etched and 4-etched show similarly low cytotoxicity (Fig. S6). We believe, therefore, that USP silica and titania microspheres are sufficiently non-cytotoxic to be worth investigating as drug-delivery systems.

To this end, we used a fluorescent drug called dehydroevodiamine hydrochloride (DHED, a potential drug for treatment of Alzheimer's disease[14]) to tag the microspheres, and found excellent delivery to the cytosol (and not to the nucleus). Again, no acute cell toxicity was observed.

Surface and Interior Modification of Porous Microspheres: Zeta-potential measurements of particle charge were made

for 1, 2, and 4 with values of –34.4, –35.2, and –24.8 mV, respectively. After etching, however, the particle charges decreased due to preferential removal of negatively charged silica, and the zeta potentials became less negative: zeta potentials of 1-etched, 2-etched, and 4-etched were –11.9, +4, and –17.2 mV, respectively. Surface charges can provide for easy surface modification by layer-by-layer (LBL) adhesion[15] of biocompatible polyelectrolytes with opposite charges. After confirming the charges, a cationic polymer (poly(diallyldimethyl ammonium chloride) (PDDA) was put on 4-etched. As confirmed both by SEM and STEM the pores can be filled in (Fig. 5), suggesting that this method will permit facile surface modification for applications targeting drug carriers with similar morphologies. STEM elemental map analysis shows that the polymer infiltrated the pores (Supporting Information Fig. S11).

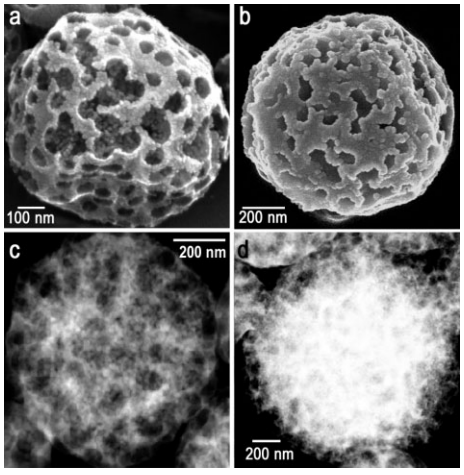

Figure 5. Electron microscopy images of 4-etched before and after layer-by-layer treatment. a) SEM of 4-etched and b) SEM of 4-etched with PDDA. c) STEM of 4-etched and d) STEM of 4-etched with PDDA.

In a second method of microsphere modification, the as-prepared oxide microspheres can be decorated with other smaller oxide nanoparticles. Furthermore, the oxide nanoparticles can themselves be modified, for example, by pre-adsorption of drug molecules. For instance, DHED was adsorbed onto silica nanoparticles, which were then adsorbed into and onto 4-etched microspheres. As shown in Figure 6b, the deposition of DHED–silica fills in the pores of the microspheres nearly completely. STEM clearly confirms that the individual microspheres do have silica nanoparticles attached to them (Supporting Information Figs. S12 and S13). Even when no adsorbed molecules are present on the silica nanoparticles, silica nanoparticles can still be put into the porous oxide microspheres. This means that the pore filling is not dependent on DHED and suggests that this method may prove to be a quite general formulation for other drug carriers with similar morphologies.

Surface areas[16] increase after the silica nanoparticle template was dissolved using wet chemical techniques and thus generating the pore structure (e.g., Fig. 1b and d); see Supporting Information Table S1. Porous titania microspheres (ca. 1 μm diameter) can have surface areas as high as 138 $m^2 g^{-1}$. The pore size is basically determined by the size of the template silica, for example, for 70–100 nm silica nanoparticles (Fig. 5a and c) pores are ≥ 70 nm as confirmed by SEM. Using smaller sized commercial silica nanoparticles (e.g., as small as 5 nm), titania and metal-doped titania microspheres with huge surface areas can be produced; these may have useful applications as supported heterogeneous catalysts.

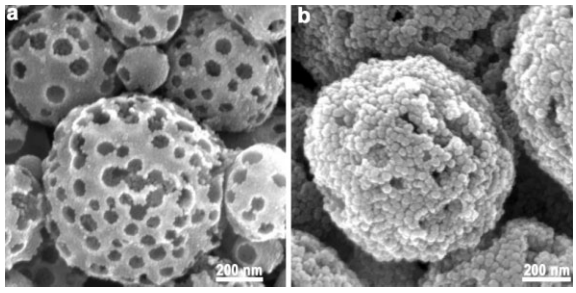

Figure 6. SEM image of 4-etched before and after deposition of nanoparticles. a) SEM of 4-etched and b) 4-etched after deposition of DHED-adsorbed 20 nm silica colloid (images after deposition of nontreated 20 nm silica are essentially identical).

In conclusion, we have demonstrated that porous, hollow, and ball-in-ball titania microspheres can be synthesized by using an inexpensive high-frequency ultrasonic generator (i.e., a household humidifier). By varying the silica to Ti^{IV} ratio and silica particle size, the morphology and pore size were controlled. With the introduction of a second metal ion, we were able to generate a core/shell-type structure in a one-pot preparation. To screen these nanomaterials for biological use (e.g., as drug carriers), we conducted cell-viability assays. USP silica and titania microspheres are sufficiently non-cytotoxic to be worth investigating as drug-delivery systems.

Experimental

Materials: All chemicals were handled in air and are available commercially. Silica colloid Ludox, titanium(IV)bis(ammonium lactato) dihydroxide, PDDA, Co_3O_4 nanopowder, TiO_2 nanopowder, and cobalt acetate were purchased from Aldrich Chemicals. Hydrogen fluoride (49 %) was purchased from Fisher Scientific. Silica colloid Snowtex was purchased from Nissan Chemicals. Poly(dimethylsiloxane) (PDMS), Sylgard 184 kit was purchased from Dow Corning. Dehydroevodiamine HCl was synthesized and supplied by Jeil Pharmaceutical Company, Korea. TiO_2 P25 was kindly supplied by Degussa, Germany. Water was purified and filtered using a Barnstead Nanopure system.

Synthesis of Microspheres (*see Figs. 2–6*): Microspheres 1 refer to silica–titania nanocomposites containing 12 nm silica, $TiO_2/SiO_2 = 1:1$, and after HF etching, 1-etched. Microspheres 2 refer to anatase phase titania with silica cores (from 12 nm silica) and cobalt oxide nanoislands (Ti/Si/Co = 5:2:1), and after HF etching, 2-etched. Microspheres 3, 4, and 5 refer to silica–titania nanocomposites with 70–100 nm silica at Si/Ti = 1:5, Si/Ti = 1:1, and Si/Ti = 8:1, respectively. See Supporting Information for more details.

Porous Microspheres, *1*: Ludox HS-40 (0.02 mol, 12 nm silica), titanium(IV) bis(ammonium lactato) dihydroxide (0.02 mol), and purified

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water (50 mL, Barnstead Nanopure ion exchange) were mixed and nebulized. The furnace temperature was set at 700–900 °C with an air flow rate of 1 standard liter per minute (SLPM). A Sunbeam 1.7 MHz household ultrasonic humidifier (with a cost of < $30) was used with minor modification to nebulize the reaction solution through a Teflon membrane into a glass apparatus and through the furnace. After 6 h of collection into water-filled bubblers, the white colloidal particles were obtained by centrifugation at $8000 \times g$. The products were washed with purified water at least three times and sampled for analysis.

Hollow and Ball-in-Ball Microspheres, *2*: Ludox HS-40 (0.00866 mol, 12 nm silica), titanium(IV) bis(ammonium lactato) dihydroxide (0.02 mol), $Co(OAc)_2$ (0.004 mol), and purified water (50 mL, Barnstead Nanopure ion exchange) were mixed and nebulized. The furnace temperature was set at 700–900 °C with an air flow rate of 1 SLPM. For nebulization, a Sunbeam 1.7 MHz household ultrasonic humidifier was used. After 6 h of collection into water-filled bubblers, the grey colloidal particles were obtained by centrifugation at $8000 \times g$. The products were washed with purified water at least three times and sampled for analysis.

Etching Experiments: For all etched microspheres, removal of silica was done with 10 % HF in ethanol at room temperature. After 1 h, the spheres were centrifuged and washed four times with purified water.

Microscopy and X-Ray Analysis: SEM was carried out on a Hitachi S-4700; TEM and STEM on a JEOL 2010-F; optical microscopy images were collected using an Olympus IX50 microscope. XRD patterns were collected using a Rigaku D-Max diffractometer using Cu Kα radiation ($\lambda = 1.5418$ Å). Confocal microscopy was recorded on a Leica SP2 (UIUC) or Zeiss 510 (SNU).

Cell Toxicity Assays: Cytotoxicity was measured using a 96-well WST-1 assay (Roche) [8a]. PC12 (neuronal) cells [8b] were cultured in 10 % fetal bovine serum (FBS, Roswell Park Memorial Institute) media at 37 °C under a 5 % CO_2 atmosphere prior to use. After 4 h of incubation with the microspheres, control wells were treated with H_2O_2, and 1 h later, the tetrazolium salt WST-1 was added. Viability analysis was carried out using an ELISA reader from Molecular Devices (V_{max} kinetic microplate reader, 450 nm) 1 h later. BV2 (microglial cells) and SHSY5Y (neuroblastoma) cells were grown in 5 % and 10 % FBS Dulbecco's Modified Eagle Medium media at 37 °C under a 5 % CO_2 atmosphere prior to use [8c,d].

Particle cytotoxicity was additionally measured using endocytosis experiments with BV2 cells. Microspheres 1 and 2 were incubated with cells (microspheres/cells = 320) and after two days all the microspheres were observed using confocal microscopy to be inside the cytosol of the cells (Fig. 4).

SEM Sample Preparation of Microsphere-Engulfed Macrophages: BV2 cells were incubated with a known number ratio of particles to cells in a 24-well plate at 37 °C under 5 % CO_2. Prior to cell introduction, the wells were filled with PDMS (thickness ≈ 5 mm), which is already widely used as a substrate [17] for cell growth. After one day of incubation, the PDMS was carefully removed and dried under vacuum for one day. SEM was carried out after sputtering the PDMS with gold. The result of this novel microscopic method is given in Figure 4e.

Zeta Potential and Surface-Area Measurements were performed with a Malvern Zetasizer 3000, Malvern Instruments, Worcestershire, UK and a Nova 2200e, Quantachrome Instruments, Boynton Beach, FL.

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