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Shape-dependent Biodistribution of Biocompatible Silk Microcapsules

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Abstract

Microcapsules are emerging as promising micro-size drug carriers due to their remarkable deformability. Shape plays a dominant role in determining their vascular transportation. Herein, we explored effect of shape of microcapsules on the *in vivo* biodistribution for rational design of microcapsules to achieve optimized targeting efficiency. Silk fibroin, a biocompatible, biodegradable and abundant material, was utilized as a building block to construct biconcave discoidal and spherical microcapsules with diameter of 1.8 μm and wall thickness of 20 nm. We have compared the cytocompatibility, cellular uptake and biodistribution of both microcapsules. Both biconcave and spherical microcapsules exhibited excellent cytocompatibility and internalization into cancer cells. During blood circulation in mice, both microcapsules showed retention in liver and kidney, and most underwent renal clearance. However, we observed significantly higher accumulation of biconcave silk microcapsules in lung compared with spherical microcapsules, and the accumulation was found to be stable in lung even after three days. The higher concentration of biconcave discoidal microcapsules found in lung arises from pulmonary environment, margination dynamics and enhanced deformation in blood stream. RBC-mimicking silk microcapsules demonstrated here can potentially serve as a promising platform for delivering drugs for lung diseases.

Graphical Abstract

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Associated content

Supporting Information available:

Secondary structure of biconcave silk microcapsules (Figure S1), elemental analysis of silk microcapsules (Figure S2), thickness of silk film assembled by LbL (Figure S3), CLSM images of large biconcave silk microcapsules (Figure S4), lifetimes of dye and dye-labelled silk microcapsules (Figure S5), absorption spectrum of dye and dye labelled silk microcapsules (Figure S6), biodegradation of biconcave silk microcapsules (Figure S7) and biodistribution file for 72 hours (Figure S8)

Keywords

red blood cells; silk fibroin; microcapsules; biomimetic; biconcave; layer-by-layer assembly

1. Introduction

Recent studies have shown that physical properties of particulate carriers exert a profound influence on their blood circulation dynamics,¹ biodistribution^{2–4} and cellular uptake,^{5–7} which ultimately influence their ability to carry and deliver therapeutics.⁸ Consequently, manipulating physical parameters of particulate delivery systems to optimize biological response has attracted much attention. Microparticles are developed to improve specificity and efficacy of drugs.⁹ Fabrication strategies of precisely tuning their size, shape and rigidity are explored to enhance the transport efficiency.^{10–12} Liu and co-workers found that the discoidal porous silicon microparticles enabled 5 times higher accumulation into the breast tumor mass than their spherical counterparts with similar dimensions.³

Hollow microcapsules are emerging as one of the most prominent micro-size drug carriers due to their facile fabrication, extraordinary deformability and high efficacy of drug loading.^{13–14} The complex interplay between physical parameters and biological performance has been an active area of research. Caruso and co-workers revealed that spherical microcapsules were internalized more rapidly and to a greater extent compared with rod-shaped microcapsules.⁹ Despite the constant progress in validation of microcapsules *in vitro*, their full potential *in vivo* for preferential accumulation at biological target is not well explored which prevents their efficient clinical translation.

Blood borne objects in nature provide an important insight in designing microcapsules to achieve enhanced biodistribution and blood circulation.¹³ Red blood cells (RBCs) exhibit circulatory lifetime of up to 120 days in human body and flexibility to squeeze through small capillaries during microcirculation.¹⁵ These unique features arise from the biconcave discoidal shape of RBCs, which enables reversible deformation as they pass through constrictions in the vasculature that is smaller than their diameter.^{16–17} Inspired by this nature's oxygen-carrying vehicle, synthetic micro-sized carriers mimicking the shape of RBCs with superior deformability have been demonstrated.^{13, 18–19} Gao and co-workers have developed RBC-shaped microcapsules, which could deform within glass capillary and recover their initial shape.¹⁸ In contrast, spherical microcapsules with similar size showed permanent deformation after passing through the same capillary, underscoring the importance of biconcave discoidal shape in deformability and recovery of the microcapsules.

However, there is no report about the biodistribution profile of biconcave discoidal microcapsules *in vivo* yet. Given the recognized role of mechanical properties *in vivo*, tuning the shape of microcapsules to achieve superior biodistribution is of particularly importance for rational design of therapeutic carrier.

Layer-by-layer (LbL) assembly is a simple, and now well-established technique employed to obtain shape-controlled microcapsules with precise control of wall thickness. LbL assembly-based microcapsule synthesis involves the alternate deposition of complementary species on sacrificial templates followed by the dissolution of templates.^{20–21} Sacrificial templates with sizes ranging from nanometer to micrometer enable precise control of the size, shape and morphology of the microcapsules. Kharlampieva and co-workers have demonstrated cubical, spherical and platelet capsules by LbL deposition on particulate templates with various geometries.²² Depending on the nature of components and fabrication conditions, various intermolecular interactions, such as hydrogen bonding,^{23–24} electrostatic interactions^{18, 25–26} and covalent bonding,^{27–28} are utilized to build the shell of microcapsules. Shell thickness of microcapsules can be tailored by varying the number of deposition cycles and/or the deposition conditions (e.g., concentration of the building block, pH, and ionic strength).^{23–24, 29}

Here, we utilized silk fibroin, a biocompatible and biodegradable material with programmable mechanical properties, as the building block to obtain biconcave discoidal and spherical microcapsules. Silk fibroin, extracted from cocoons of *Bombyx mori* silkworm, is a widely available and abundant protein material. Silk is a promising biomaterial owing to its excellent biocompatibility, programmable biodegradability and tunable mechanical properties.³⁰ Silk fibroin consists of a light chain and heavy chain with 12 hydrophobic blocks interspersed within 11 hydrophilic blocks. The hydrophilic regions enable facile processability of silk in aqueous solution, which makes it possible to reconstitute silk into diverse forms including thin films, foams, capsules, nanotubes, and nanowires.^{23, 31–34} On the other hand, the repetitive sequences, GAGAGS, in the hydrophobic blocks can organize themselves into β -sheet structures (nanoscale crystalline domains) via hydrogen bonding, van der Waals forces and hydrophobic interactions. The highly ordered structure provides structural stability, the resultant high mechanical strength and elastic modulus of silk fibroin.³⁵ These mechanical properties are critical for obtaining silk microcapsules with different shapes to achieve optimized biodistribution *in vivo*.

Herein, we report the first study of the shape effect of microcapsules on biodistribution *in vivo*. Biconcave discoidal and spherical silk microcapsules with identical dimensions are fabricated by silk-on-silk assembly, followed by comprehensive characterization of microscale and nanoscale structure of silk microcapsules. Cytocompatibility and cellular uptake of silk microcapsules are investigated. Furthermore, both types of microcapsules are injected to mice separately and their biodistribution profiles are compared.

2. Experimental Section

2.1. Materials

Silk Cocoons were purchased from Mulberry Farms (Fallbrook, CA). Sodium carbonate (Na_2CO_3), lithium bromide (LiBr), calcium chloride (CaCl_2), sodium hydroxide (NaOH), poly-L-lysine (PLL, Mw~368,300), polystyrene sulfonate (PSS, Mw~1,000,000), hexamethylene tetramine (HMT), triethanolamine (TEA), zinc nitride ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), dimethyl sulfoxide (DMSO), methanol, thiazolyl blue tetrazolium bromide, fluorescein isothiocyanate isomer (FITC), fetal bovine serum (FBS), antibiotics (100 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin), glutaraldehyde (GA), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), paraformaldehyde, sodium pyruvate, granulocyte-macrophage colony-stimulating factor (GM-CSF), heparin, mouse serum and hydrogen chloride acid (HCl) were purchased from Sigma-Aldrich (Saint Louis, MO). (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), Alexa Fluor 555 (AF555), Alexa Fluor 633 Wheat Germ Agglutinin conjugates (AF633-WGA), minimum essential medium, Dulbecco's phosphate buffered saline (DPBS), Hanks' balanced salt solution (HBSS), ProLong® gold antifade mountant and RPMI 1640 medium were purchased from Thermo Fisher Scientific (Waltham, MA). HUVEC (human umbilical vascular endothelium) cell line, SKBR3 (human epithelial breast cancer) cell line, JAWSII dendritic cell line, F-12K medium and McCoy's 5A medium was purchased from ATCC (Manassas, VA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution-Cell Titer 96 Aqueous One Solution kit was purchased from Promega (Madison, WI). The endothelial cell growth supplement was purchased from Corning (NY). The water used in this work is nanopure water (18.2 $\text{M}\Omega\text{-cm}$, Barnstead).

2.2. Preparation of Silk Fibroin

Silk fibroin was prepared from *Bombyx mori* silkworm cocoons using a reported protocol.³⁶ Briefly, cocoons were peeled into thin layers, which facilitated the degumming process and the dissolution of silk fibroin. Thin layers of cocoons were boiled in Na_2CO_3 aqueous solution (20 mM) for 30 minutes followed by washing three times with nanopure water. After naturally drying in air, the degummed silk fibroin was dissolved in LiBr aqueous solution (9.3 M) at 60°C for 4 hours. To remove the LiBr, the resulting solution was subjected to dialysis for 2 days at room temperature. Subsequently, the dialysate solution was centrifuged twice (9000rpm, 20 minutes) at 4°C to remove impurities and undissolved silk fibroin. The solution was stored at 4°C until further use.

2.3. Fabrication of biconcave and spherical ZnO particles

The biconcave ZnO particles were fabricated according to a procedure reported previously.³⁷ Briefly, 25 ml PSS aqueous solution (10 mg/ml) was added into 10 ml $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solution (200 mM). After stirring for 5 minutes, 2 mmol HMT was added to the mixture followed by 10 minutes stirring. Then the mixture was transferred to a container with a closed cap and maintained at 120 °C for 3 hours. Finally, the precipitates were centrifuged and washed with nanopure water three times.

The spherical ZnO particles were prepared by hydrothermal synthesis. 3 ml TEA was added to 12 ml $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solution (40 mM) under stirring. The mixture was transferred to oil bath at 90 °C for 5 minutes with continuous stirring. Then the mixture was transferred to autoclave and maintained at 160 °C for 2 hours. The autoclave was air-cooled to room temperature. Finally, the precipitates were centrifuged and washed with nanopure water three times.

2.4. Synthesis of silk microcapsules

Biconcave ZnO particles were employed as templates to fabricate biconcave silk microcapsules, and spherical ZnO particles were employed for spherical silk microcapsules. Prior to silk coating, ZnO particles were dispersed in PLL solution (1 mg/ml) for 10 minutes on a rotating mixer (Benchmark). To remove excess PLL, the particles were centrifuged (3000 rpm, 1 minutes), and supernatant was removed and replaced with nanopore water. The centrifugation and redispersion washing cycles were repeated twice. To form silk coating on the surface, the particles were dispersed in silk aqueous solution (1 mg/ml) and left for 10 minutes on a rotating mixer. Subsequently, the particles were centrifuged (3000 rpm, 1 minutes) and washed twice to remove unbound silk. The pellet was then dispersed in methanol with ultrasonication for 10 seconds to ensure homogenous suspension. After dispersion in methanol for 15 minutes, particles were centrifuged (3000 rpm, 1 minutes) and washed twice. The silk coating and methanol treatment steps were repeated for a desired number of cycles. Subsequently, the particles were dispersed in HCl solution (pH = 1) to yield hollow microcapsules. The capsules were washed three times with nanopure water prior to using them. The total number of silk microcapsules was determined by flow cytometry (BD FACSCanto II).³⁸

To fabricate bigger biconcave silk microcapsules, biconcave $\text{Ca}(\text{OH})_2$ particles were employed as templates and fabricated according to the literature.¹⁸ After washing twice with PB buffer solution (200 mM, pH = 9), $\text{Ca}(\text{OH})_2$ particles were dispersed in PLL solution (1 mg/ml, 200 mM PB buffer, pH = 9) for 15 minutes on rotating mixer. The particles were washed with PB buffer solution (200 mM, pH = 9) twice and then were dispersed into silk fibroin solution (1 mg/ml, 20 mM PB buffer, pH = 9) for 15 minutes on a rotating mixer to ensure the adsorption of silk chains on the particles. Then ethanol (six times of volume silk solution) was added dropwise into the mixture under stirring. After centrifuging, the particles were dispersed into GA solution (20 mg/ml, 10 mM NaOH) to induce crosslinking of the silk adsorbed on particles. To stop the crosslinking process, the particles coated with silk were washed three times with NaOH solution (10 mM). In order to remove the templates, the particles were dissolved in HCl solution (80 mM). Hollow capsules were collected by centrifugation (3000 rpm, 10 minutes) and washing in water.

2.5. Fabrication of silk film

The LbL procedure on silicon wafer was similar to that employed in fabricating silk microcapsules. Piranha-cleaned silicon wafer was immersed in PLL solution (1 mg/ml) for 10 minutes on a rotating mixer. To remove excess PLL, the silicon wafer was rinsed with water thoroughly and dried under a stream of dry nitrogen. To deposit silk layer on the surface, the silicon substrate was immersed in aqueous silk solution (1 mg/ml) and left on a

rotating mixer for 10 minutes. Subsequently, the substrate was rinsed thoroughly with water to remove the unbound silk. After blowing dry under a stream of nitrogen, the silk coated substrate was subjected to methanol treatment for 15 minutes, followed by rinsing with water and blow drying. The silk coating and methanol treatment steps were repeated several times to obtain multi-layered film. The thickness of the film was measured after every layer using AFM.

2.6. Atomic force microscopy (AFM)

A drop of capsule suspension was naturally dried on a piranha-cleaned silicon wafer. AFM images were obtained using Dimension ICON (Bruker) in tapping mode. V-shaped silicon cantilever (Micromash) with a nominal tip radius of 8 nm were used for the imaging.

2.7. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analyzer

After suspension (particles or capsules) dried in the air naturally on piranha-cleaned silicon wafer, the samples sputtered with gold were observed by FEI Nova 2300 field-emission scanning electron microscope at an acceleration voltage of 10.5 kV. Elemental analysis was performed using EDX.

2.8. Confocal laser scanning microscopy (CLSM)

Confocal images of capsules were collected by confocal laser scanning microscope with Airyscan microscopy (LSM 880, Zeiss) equipped with a 63X oil-immersion objective lens. Silk microcapsules were dispersed in FITC solution (DMSO, 2 mg/ml) for 2 days for staining. The isothiocyanate group of FITC react with amines of the protein molecules (silk) to form a stable thiourea linkages.³⁹ After washing with water three times, the FITC-labelled silk microcapsules were mounted onto glass slides using a ProLong gold antifade mountant for imaging. The excitation /emission wavelengths were 488/515 nm.

2.9. FTIR Spectroscopy

The measurements were performed using a Nicolette Nexus 470 using a Praying Mantis Harrick that was used to obtain a diffuse reflectance IR Fourier transform spectrometry (DRIFTS). The spectra were obtained by averaging 1024 scans over 400–4000 cm^{-1} with a resolution of 1 cm^{-1} using a silicon substrate (identical to the sample except for dried silk capsules) as the background. The spectra were smoothened using a Savitzky-Golay function with 9–13 points depending on the quality of the data.

2.10. Evaluation of biodegradation in serum

Biconcave discoidal microcapsules (10^7) were dispersed in 500 μl of mouse serum, and the mixture was placed on a shaker at 37°C for 2 hours and 24 hours. The products were washed with water for three times, and finally were drop cast on piranha-cleaned silicon wafer for SEM imaging.

2.11. Cell culture

SKBR3 cells were cultured in McCoy's 5A medium supplemented with FBS (100 mg/ml) and antibiotics (10 mg/ml). Bone marrow-derived macrophages were isolated from mouse

bone marrow according to literature⁴⁰ and were cultured in RPMI1640 media supplemented with FBS (100 mg/ml), sodium pyruvate (1 mg/ml) and murine GM-CSF (20 ng/ml). JAWSII cells were cultured in minimum essential medium supplemented with FBS (100 mg/ml), sodium pyruvate (1 mg/ml) and murine GM-CSF (20 ng/ml). HUVEC were cultured in F-12K medium supplemented with FBS (100 mg/ml), endothelial cell growth supplement (50 µg/ml) and heparin (100 µg/ml). All cells were grown at 25 cm² tissue culture flasks in water jacket incubator at 37 °C, 5% CO₂ and 95% humidity.

2.12. Evaluation of cytocompatibility

Standard MTS assay was employed to determine the cytocompatibility in the presence of microcapsules. 10,000 cells (100 µl) cells were seeded per well in a 96-well plate at 37°C, 5% CO₂ and 95% humidity. After 24 hours, the mixture of fresh medium (90 µl) with suspension of biconcave/spherical silk microcapsules (10 µl) was added to each well. Cells were incubated with capsules at different ratios of capsules to cells (0:1, 1:1, 10:1, 100:1, 1000:1) for 24 hours. Afterward, 20 µl MTS solution was added to each well and incubated for 2 hours. Absorbance at 490 nm for each well was recorded using spectrophotometric microplate reader and was normalized with respect to absorbance of untreated cells.

2.13. Cellular uptake

SKBR3 cells were incubated with AF555-labelled biconcave/spherical silk microcapsules at a capsule-to-cell ratio of 100:1 for 24 h. After being washed with DPBS three time, the cells were fixed with paraformaldehyde solution (40 mg/ml, DPBS) for 15 minutes and followed by three times washing with HBSS. The cells were immersed in AF633-WGA solution (20 µg/ml, HBSS) to stain cell membrane for 20 minutes and DAPI solution (600 nM, DPBS) for 15 minutes to stain DNA prior to their analysis under a CLSM.

2.14. *In vivo* and *ex vivo* fluorescence imaging of mice

EDC/NHS chemistry was used to conjugate the LS288 with silk. 1 ml (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) solution (1.9 mg/ml) was added to 1 ml LS288 solution (1 mg/ml) followed by the addition of 1 ml N-hydroxysuccinimide (NHS) (1.1 mg/ml). After 1 hour of activation, 1 ml of the above activated dye solution was added to the mixture of 1 ml silk solution (10 mg/ml) and 8 ml water, which was subsequently used to fabricate the biconcave silk microcapsules. 0.9 ml of the above activated dye solution was added to the mixture of 1 ml silk solution (1 mg/ml) and 8.1 ml water, which was used to fabricate the spherical silk microcapsules. After 6 hours of conjugation, the dye labelled silk solution was used to fabricate silk microcapsules in the same procedure as mentioned above.

Animal studies were performed by the following protocols approved by the Washington University School of Medicine Animal Studies Committee. Balb/c mice were injected by tail vein with LS288 conjugated biconcave silk microcapsules or spherical silk microcapsules (10⁸), respectively. Mice were imaged with the Pearl imager (LiCor, Lincoln, NE) at Ex/Em of 785/810 nm channels at different points (pre injection, immediately post injection, 1 hour, 4 hours, 24 hours and 72 hours). The mice were then sacrificed at different time points and *ex vivo* biodistribution was determined. Lungs were harvested and frozen in OCT (Tissue Tek, CA, USA) for tissue analysis.

2.15. Histological analysis of lung tissue

Excised lung tissues were flash-frozen in OCT (Tissue Tek, CA, USA) and stored at -20°C . The lungs were sliced at a thickness of $10\text{ }\mu\text{m}$ (Cryocut 1800, IL, USA). Fluorescence imaging was performed with an Olympus BX51 upright epifluorescent microscope (Olympus America, PA, USA). A cy7 49007 (Chroma Technology Corp, VT, USA) filter cube was used for excitation at $710/75\text{ nm}$ and emission collection at $810/90\text{ nm}$. Hematoxylin & eosin (H&E) staining of excised lung tissues was used for histologic validation of tissue types.

3. Results and discussion

The biconcave discoidal shape of RBCs provides a large surface area-to-volume ratio and thus allows RBCs to achieve significant deformation and recovery to their initial shape after passing through capillaries while maintaining a constant surface area.¹⁸ This shape is an essential design parameter in realizing microcapsules that mimic the flow behavior of RBCs. We used template-assisted method for the fabrication of silk microcapsules. Particularly, we employed biconcave discoidal ZnO microparticles as templates, which closely resemble the shape of RBCs. These ZnO microparticles were synthesized by a facile one-step hydrothermal method using poly(sodium 4-styrenesulfonate) (PSS), which selectively adsorbed on nanoplatelets and introduced an intrinsic dipole field resulting in the formation of ZnO microparticles with biconcave discoidal shape.³⁷ Unlike other commonly used templates such as silica and polystyrene microparticles, which require harsh chemicals (*e.g.* hydrofluoric acid)^{23, 26} or organic solvents (*e.g.* tetrahydrofuran)^{19, 24} to dissolve templates, ZnO templates obviate the need for hazardous reagents as they can be dissolved under mild acidic conditions.

We fabricated biconcave silk microcapsules through LbL deposition of silk fibroin from aqueous solution on biconcave discoidal ZnO templates (Figure 1A). Spherical silk microcapsules were fabricated by the same approach except that spherical ZnO microparticles were employed as templates (Figure 1C). The diameter of biconcave and spherical ZnO microparticles was measured to be $\sim 1.8\text{ }\mu\text{m}$. Considering that the ZnO microparticles and silk fibroin are negatively charged under the deposition conditions (neutral pH), we have employed cationic poly(lysine) (PLL) as a primer layer.²⁴ Subsequently, negatively charged silk fibroin was adsorbed on the positively charged templates followed by methanol treatment (see Experimental section for details). It is known that silk fibroin in freshly prepared aqueous solution exhibits amorphous random coil secondary structure and forms silk I, which is characterized by crank-shaft conformation. Both amorphous silk and silk I are water soluble. Upon exposure to methanol, ethanol, water vapor, heat and/or mechanical strain, silk I transforms to silk II, which is water insoluble and extremely stable.^{41–42} Silk II is characterized by β -pleated sheet structure stabilized by inter-chain hydrogen bonding.²³ The 12 hydrophobic blocks with repeating sequences on the heavy chain of silk enable subsequent deposition of silk-on-silk through hydrophobic interactions between the silk chains. Silk deposition followed by methanol treatment was repeated multiple times to prepare silk microcapsules with desired shell thickness. Finally,

the cores were dissolved in hydrochloric acid solution (pH = 1) to yield hollow silk microspheres.

After the removal of ZnO core, the secondary structure of hollow biconcave silk microcapsules was studied using Fourier transform infrared (FTIR) spectroscopy (Figure S1). The amide I band (1600–1700 cm^{-1}) corresponding to the peptide backbone in the FTIR spectrum reveals the secondary structure of silk fibroin.⁴³ The absorption band at 1656 cm^{-1} corresponds to the random coil structure, while the two absorption bands at 1623 cm^{-1} and 1700 cm^{-1} indicate the formation of crystalline β -sheets.⁴⁴ Upon drying on a silicon substrate, both biconcave discoidal and spherical silk capsules collapsed with random folds because of local instabilities and wrinkles as a result of capillary force,^{45–46} which are typical features for hollow capsules (Figure 2A, B, D, E). We performed energy dispersive X-ray (EDX) characterization of biconcave and spherical silk microcapsules. The amount of Zn in both microcapsules was almost undetectable, indicating the complete dissolution of the ZnO templates (Figure S2). Both microcapsules were naturally dried on silicon substrates, resulting in a strong signal corresponding to Si. The Au signal can be ascribed to Au sputtering. The thickness of both dry microcapsules was measured to be ~40 nm by atomic force microscopy (AFM), which indicated the wall thickness to be ~20 nm (Figure 2B, E). The distinct globular texture on the surface of both dried microcapsules was observed in AFM images, which corresponded to the characteristic morphology of silk nanolayers with partial silk II structure. To prove that the microcapsules were assembled in a step-wise manner, we have investigated the evolution of the thickness of silk film on a silicon wafer during the LbL assembly. The thickness was found to increase linearly with the layer number, indicating the layer-by-layer assembled structure as opposed to “burst” deposition (Figure S3).

Confocal laser scanning microscope (CLSM) was employed to further characterize the structure of silk microcapsules in solution. Fluorescein isothiocyanate (FITC) was used as a label to visualize the biconcave/spherical silk microcapsules. Characteristic “spot-in-the-center” of circle, which corresponded to the biconcave shape, was observed for RBC-shaped capsules (Figure 2C). As expected, no such feature was noted for spherical capsules (Figure 2F). Due to the limited resolution of CLSM and associated difficulty in acquiring a three-dimensional (3D) image of the biconcave microcapsules with diameter of 1.8 μm , we fabricated larger biconcave silk microcapsules with 20 μm in diameter by template-assisted assembly described above. CLSM revealed the biconcave discoidal structure of these larger microcapsules orientated in different ways with respect to the imaging axis (Figure S4A, B, C). The capsules showed intact circle with a spot in the center from the top view (Figure S4A) and “bowknot” structure from the side view (Figure S4B). The capsules exhibited a clear biconcave discoidal shape from the side view following a 3D reconstruction of the z stack (Figure S4D). These results demonstrate that the template-assisted fabrication approach employed here can yield size-controlled biconcave discoidal silk capsules.

Next, we explored the interactions of silk microcapsules with cells *in vitro*. SKBR3 cells, human epithelial breast cancer cells, were incubated with biconcave and spherical silk microcapsules, which were labeled with Alexa Fluor 555 (AF555) for visualization of their cellular uptake. After 24-hour incubation, strong AF555 fluorescence was observed in the

cytoplasm of the cells, suggesting that spherical and biconcave silk microcapsules were successfully internalized by the SKBR3 cells (Figure 3).

Two immune cells, bone marrow derived macrophages and JAWSII dendritic cell line, are chosen to evaluate the cytocompatibility of both microcapsules, because they are the first line of defense with any intravenously administered substance.³ Due to the interaction of silk microcapsules with vascular wall during their circulation in blood, human umbilical vein endothelial cell line (HUVEC) is also chosen to test the toxicity of both silk microcapsules. The cell viability was quantified after incubation with silk microcapsules using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.^{40, 47} The immune cells and HUVEC were incubated with various concentrations of capsules (capsule-to-cell ratio ranging from 1:1 to 1000:1) for 24 hours at 37 °C. No significant cell death was observed with both capsules within the concentration range tested, indicating the excellent biocompatibility of silk microcapsules (Figure 4). The remarkable cytocompatibility of silk capsules owes to the biological origin and biocompatibility of the silk fibroin. In fact, numerous reports in the past have described the biocompatibility and programmable biodegradation of silk in various forms.^{24, 48–51}

To investigate the biodistribution profile of the two different types of silk microcapsules in rodents, microcapsules were labeled with near infrared (NIR) dye, LS288,^{52–53} for imaging *in vivo*. In order to avoid the detachment of dye from silk microcapsules, we used EDC/NHS chemistry to covalently conjugate the dye to the silk microcapsules. We measured the lifetime of the fluorophore to confirm its covalent conjugation to silk as lifetime of the fluorophore critically depends on the chemical structure of dye (Figure S5).⁵⁴ The lifetime of free dye was found to be 0.47 ns. When dye was physically mixed with biconcave silk microcapsules, the lifetime was found to be 0.45 ns, which was similar with free dye, suggesting no change in the chemical structure. However, the lifetime of the dye was 0.71 ns after covalent conjugation with silk microcapsules using EDC/NHS chemistry and such difference in lifetime implied successful covalent conjugation between the dye and silk.

Free dye, dye-labelled biconcave and spherical silk microcapsules (1×10^8 microcapsules dispersed in 100 μ l buffer solution) were injected via lateral tail vein of Balb/c mice (N = 3 per group). In all three groups, the concentration of dye was maintained constant, which was confirmed by optical absorption intensity (Figure S6). No specific targeting moieties were introduced to their surface. Noninvasive fluorescent images were acquired at 4- and 24-hour post injection. To validate uptake of both silk microcapsules seen in noninvasive fluorescence imaging, major organs and tissues were imaged *ex vivo* at the 24-hour time point and fluorescence was quantified (Figure 5K). *Ex vivo* imaging showed similar fluorescence signal from these organs, illustrating the correlation between noninvasive *in vivo* imaging and *ex vivo* validation. NIR fluorescence corresponding to the free dye was not observed in whole body (Figure 5A) or any organs (Figure 5J) at 24-hour time point after injection. On the other hand, intense signal was noted in whole-body images and some organs (lung, spleen, kidney and liver) after the injection of the dye-labelled silk microcapsules at 24 (Figure 5B, C, J) and even 72 hours (Figure S8). Considering that the free dye is rapidly cleared from mice within 24 hours, the fluorescence signal observed after 24 hours and 72 hours can be ascribed to the dye-labelled silk. Both biconcave and spherical

silk microcapsules exhibited high fluorescence in the bladder and residual fluorescence in the kidney and liver at 4-hour post injection (Figure 5B, C). This result suggests rapid clearance by mainly renal pathways rather than hepatobiliary clearance.

Although silk microcapsules with 1.8 μm in diameter are too big for glomerular filtration in kidney, it is quite possible that the biologically degraded products of silk microcapsules have been cleared by kidney. To study the biodegradation of silk microcapsules, biconcave discoidal silk microcapsules were dispersed in mouse serum and the morphological change with time was characterized using SEM. Considering that the spherical silk microcapsules are fabricated in exactly same way except that the shape of templates is different, we have investigated the biodegradation of only biconcave discoidal silk microcapsules. After incubation in serum for 2 hours, we observed that the biconcave silk microcapsules remained intact. On the other hand, after 24-hour incubation in serum, we observed only debris (*i.e.* no capsules), indicating that silk microcapsules were readily degraded in serum after 24 hours (Figure S7).

By the 24-hour time point, fluorescence of microcapsules was not detectable throughout the body except from some reticulo-endothelial organs such as the liver, spleen and lung, minimizing the potential for long-term systemic toxicity. Long-term systemic toxicity was investigated in mice at 24-hour and 72-hour time point after microcapsules injection, and no adverse effects were observed. *Ex vivo* fluorescence imaging and biodistribution were studied after 72 hours, and no abnormal lesions were observed in either lung tissue and other organs (Figure S8). These observations suggest negligible toxicity of silk microcapsules. Noteworthy was the strong fluorescent signal detected in lung, but weak signal was observed in the liver and spleen even at the 72-hour time point (Figure S8). Our results support previous report that particles with size ranging from 2 to 5 μm can accumulate readily within capillaries of the lungs.⁵⁵ Indeed, such biodistribution is a characteristic feature of many microparticles *in vivo*. Nanoparticles without any surface modification tend to accumulate in liver and spleen, but such propensity decreases with increasing the size to micrometer scale and higher retention in lung occurs. In the systemic circulation, particles are transported along the vasculature and accumulate in various organs through size-dependent mechanism: nanoparticles are inclined to be internalized by Kupffer cells in liver and splenic macrophages, whereas microparticles are more likely to be trapped in the capillaries of lung.

To gain further insight into the spatial distribution of the biconcave and spherical microcapsules in lung, histological tissue sections were stained with hematoxylin & eosin (H&E) and their NIR fluorescence were imaged (Figure 5F, G, H, I). For both microcapsules, the fluorescence was well distributed and detectable in different sections of tissue. No blockage of blood vessels or alveoli was noted, which was consistent with absence of any adverse reactions and survival of all mice after 3 days. Although past reports indicated that 2–3 μm silicon beads resulted in total occlusion of capillaries,⁴ the deformable hollow silk microcapsules exhibited significantly different behavior. Silk microcapsules exhibit remarkable deformation, which has a profound effect on their flow within blood vessels. In the microfluidic blood capillary model, hollow capsules with 7 μm in diameter

were found flow through capillaries with 5 μm in diameter, while solid particles with the same shape and diameter could not easily pass through the capillaries.³⁸

For the accumulation in lung, both longitudinal and tissue section imaging showed much higher uptake with biconcave microcapsules than spherical silk microcapsules at 24 hours. The net sum of fluorescence intensity of biconcave silk microcapsules was three times stronger than spherical silk microcapsules in lung (Figure 5K). The main mechanisms for particulate accumulation in organs are cellular uptake and attachment to endothelial surfaces. Numerous *in vitro* studies demonstrate that discoidal microparticles can effectively evade internalization by different types of cells. Gao and co-workers found that biconcave discoidal silicon microcapsules exhibited lower internalization into muscle cells than their spherical counterparts.⁵ Kharlampieva and co-workers also reported less uptake of discoidal microparticles by macrophages and cancer cells.¹³ Considering the lower interaction between cells and biconcave discoidal silk microcapsules, higher concentration of biconcave discoidal microcapsules in lung should be mainly ascribed to their adhesion to vascular walls, which has been predicted by mathematical models and flow chamber experiments for discoidal microparticles.⁵⁶ Discoidal particles exhibit the unique tumbling and margination dynamics that enables favorable interaction with pulmonary vasculature, so they are more likely to bind and adhere to endothelium in the blood capillary channels than spherical particles.⁵⁷ Discoidal particles can drift laterally towards the wall and stick to the vascular wall compared to spherical particles in the blood stream.⁵⁸ The hydrodynamic force on discoidal microcapsules is smaller compared to that on spherical microcapsules, which makes it hard for biconcave discoidal microcapsules to detach from vascular wall.⁵⁹ In an addition, the contact area between the discoidal microcapsules and the walls of the blood vessels is larger compared to that of spherical microcapsules with similar diameter, resulting in easier binding of basal planes of the biconcave discoidal microcapsules to the endothelial walls.⁴ Furthermore, the biconcave discoidal microcapsules with large surface area-to-volume ratio enable reversible deformation, which allows increased circulation time in blood and extravasation through constricted environment.^{18, 60} These shape-enabled mechanical properties contribute to highly efficient localization of the biconcave discoidal microcapsules in lungs compared to spherical microcapsules.

The shape-dependent lung accumulation provides insights into designing better drug delivery carriers to treat lung diseases. After loading with anti-cancer drug, biconcave discoidal silk microcapsules can potentially serve as a platform for chemotherapy in lung cancer. The prolonged residence in lung may result in alveoli macrophages uptake. Antigen and adjuvant can be incorporated into biconcave silk microcapsules to induce immune stimulus to achieve efficient immunotherapy for lung cancer. Apart from lung cancer therapy, oxygen carrying materials such as perfluorocarbons and anti-inflammation drugs can also be integrated with biconcave microcapsules to treat patients with lung ischemia reperfusion injury(LIRI) resulting from lung transplantation or cardiopulmonary bypass.

4. Conclusions

In summary, we have fabricated biconcave discoidal and spherical silk microcapsules to explore the shape effect on biodistribution. Extensive microscopy characterization (using

scanning electron microscope, atomic force microscope and confocal laser scanning microscope) confirmed the biconcave discoidal structure of silk microcapsules, and wall thickness of capsules to be ~20 nm. Both biconcave and spherical microcapsules exhibited excellent cytocompatibility and ability to be internalized by cancer cells. Biodistribution studies after intravenous administration in mice revealed both microcapsules in liver and kidney, and most of the capsules underwent renal clearance. However, owing to pulmonary vascular microenvironment and biconcave discoidal shape exhibiting intrinsic dynamic forces and superior deformability, we observed significantly higher accumulation of biconcave silk microcapsules in the lung compared with spherical microcapsules, and the accumulation was found to be stable in lung even after three days. Considering the unique biodistribution of biconcave silk microcapsules, future studies will involve biofunctionalization (for specific targeting) and drug loading of those microconstructs to investigate their therapeutic efficacy for lung diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic illustration showing the synthesis of silk microcapsules using layer-by-layer assembly employing ZnO particles as templates. The process involves coating ZnO templates with PLL, followed by the repeated cycles of adsorption of silk, methanol treatment to induce β -sheets, followed by template dissolution. SEM images of biconcave (B) and spherical (C) ZnO microparticles.

Figure 2.

SEM images (A) and AFM image (B) of naturally dried biconcave silk capsules on silicon substrate. (C) CLSM image of biconcave silk microcapsules dispersed in water. SEM images (D) and AFM image (E) of naturally dried spherical silk capsules on Si substrate. (F) CLSM image of spherical silk microcapsules dispersed in water.

Figure 3.

CLSM images of SKBR3 cells incubated with biconcave or spherical silk microcapsules for 24 hours. (A)(E) The cell nuclei were stained with DAPI (blue). The biconcave (B) and spherical (F) silk microcapsules were dyed with AF555 (red). (C)(G) The cell membrane was stained with AF633-WGA (yellow). Merged image of DAPI, AF555 and AF633-WGA, white arrows indicate the internalized biconcave (D) or spherical (H) silk microcapsules.

Figure 4. Viability of bone marrow-derived macrophages (A), JAWSII dendritic cell line (B) and HUVEC cell line (C) after incubation with different concentrations (expressed as capsule-to-cell ratio) of biconcave or spherical microcapsules for 24 hours.

Figure 5. Representative whole-body mice fluorescence images at 24-hour post-injection with free dye (A), biconcave silk microcapsules (B) and spherical silk microcapsules (C). NIR fluorescence (10×) and H&E stained images of free dye (D)(E), biconcave silk microcapsules (F)(G) and spherical silk microcapsules (H)(I) in lung tissue, subsequently. The tissue was stained with H&E and the same areas were imaged to correlate with the fluorescence data. (J) Relative fluorescence intensity analysis of different organs and tissues at 24-hour time point. (K) *Ex vivo* biodistribution of silk microcapsules at 24-hour time point. Mice were sacrificed at 24-hour time point to get *ex vivo* fluorescence intensity for each organs and tissues. N = 3.