Fabrication of biconcave discoidal silica capsules and their uptake behavior by smooth muscle cells

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1. Introduction

Hollow microcapsules have attracted much attention due to their potential applications as micro-reactors, catalysis, and especially drug delivery systems. As drug delivery systems, hollow microcapsules can be fabricated from various materials (polymers, hybrids and inorganic materials) and methods, loaded with different kinds of drugs, and may respond to diverse environmental stimuli. They can deliver drugs via many ways, such as subcutaneous, pulmonary and oral routes. For example, for pulmonary administration, 3 ways, such as subcutaneous, pulmonary and oral routes [12]. Micronized capsules or particles have special advantages in some cases. For example, for pulmonary administration, 3 μm particles can deposit deep in the alveolar region while smaller particles are exhaled. The interaction of micronized capsules or particles with macrophages is also very important because macrophages can clear particles from the circulation, and because they are potential therapeutic targets in inflammatory conditions, atherosclerosis and cancer. Thus the studies on interaction of diverse micronized capsules or particles with different cells may help people to design better drug carriers. But up to now most of the concerns about microcapsules or particles are focused on loading and release of drugs, tailored targeting surface properties and other integrated functions.

One of the important yet often neglected issues is the interaction of hollow capsules with biological systems. Recently, the physical characteristics of drug carriers, such as size, mechanical property, and shape, are attractive due to their significant influences on various biological processes. Specifically, the recognition of important influence of carriers’ shape is inspired by organisms in nature, such as the diverse morphologies of human cells (platelet and red blood cells) and unique shapes of bacteria (rods, spirals and ellipsoids) [29] and unique morphologies of human cells (platelet and red blood cells) [31], which endow these biological entities with crucial functions. The shapes of particles have great influences on many biological processes. For example, spherical and elliptical disk-like particles have obviously distinct behaviors in phagocytosis process due to their different approaching modalities to macrophages [33]. The synthesized highly stable filament-like polymeric micelles (filomicelles) can circulate up to one week after intravenous injection, which is about ten times longer than the circulation time of their spherical counterparts. Filomicelles are more persistent than any other synthetic nanoparticles, while less readily internalized by cells. Shape can greatly affect the targeting efficiency of particles, because for the particles with different shapes, they have different surface areas per unit volume, which can contact with the targeted receptors. Furthermore, shape also can affect migration dynamics of particles because of tumbling and rolling of non-spherical ones. The shape of particles also influence their distribution in vivo. For example, the discoidal silica particles accumulate more than others in most of the organs but liver, while cylindrical ones are deposited at a larger extent in liver.

The major barrier in investigating the role of particle’s shape on the biological processes is the difficulty to prepare particles with precisely controlled shape and size, thus many efforts have been given to fabricate particles with special shapes. The biconcave...
discoidal particles with the morphology similar to that of red blood cells (RBCs) have drawn much attention recently because they may mimic the superior functions of RBCs [39,40]. These particles also may have very different interactions with cells, but so far this information is absent thus needs further investigation. In this study, silica capsules with a biconcoidal discoidal shape were fabricated by a sol–gel method based on templates and their interactions with smooth muscle cells (SMCs) were primarily investigated. Silica is a non-cytotoxic material [6] and is widely used in biomedical and biotechnological fields [41–43]. The sol–gel method can be conducted on different templates [44,45], whose morphologies can be easily duplicated and maintained. Through this method, particles with different shapes and sizes but the same chemical properties can be easily obtained.

2. Experiment sections

2.1. Materials

Calcium chloride, sodium hydroxide, hydrogen chloride (HCl), tetraethoxysilane (TEOS), fluorescein isothiocyanate (FITC), dextran sulfate (DS) and 3-triethoxysilylpropylamine (APTES) were obtained from Sigma–Aldrich. Acetone and dimethyl sulphoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. Manganese sulfate (MnSO4) was obtained from Shanghai Meixing Chemical Factory Co., Ltd. Ammonium hydrogen carbonate (NH4HCO3) was purchased from Guangdong Guanghua Chemical Factory Co., Ltd. All chemicals were used as received. The water used in all experiments was prepared in a MilliPure Milli-Q reference purification system with a resistance of 18.2 MΩ cm−1.

2.2. Fabrication of two kinds of templates

The biconcoidal discoidal Ca(OH)2 particles were fabricated according to a method reported previously [46]. In brief, 0.5 g DS was completely dissolved in 100 mL 0.05 M CaCl2 solution, into which 20 mL 0.5 M NaOH solution was rapidly poured. Ultrasonication was kept for 10 s. Finally, the Ca(OH)2 sediments were collected by centrifugation and washed with Milli-Q water thrice, and stored in ethanol. Spherical MnCO3 templates were synthesized by mixing MnSO4 and NH4HCO3 solutions according to reference [47].

2.3. Synthesis of silica capsules with different shapes

Biconcoidal discoidal Ca(OH)2 templates were dispersed in ethanol/water mixed solution and reacted with TEOS, yielding silica shells on the templates. In a typical procedure, Ca(OH)2 particles were dispersed in ethanol/water (5 mL:1 mL) mixed solution (≈106/mL) at pH 9 (adjusted by ammonia in a closed glass flask). Then TEOS was added into the suspension dropwise by two different methods. In the “Gradient concentration” method, different volumes of TEOS such as 10, 20, 40, and 60 μL were added at one time, and the sol–gel process was lasted for 2 h. In the “Multi-step growth” method, TEOS was added step by step, each time only 10 μL was added dropwise and the reaction was also lasted for 2 h. After each cycle, the templates were collected after washed by ethanol, and re-dispersed into a new but identical reaction system. Thus, there were 6 cycles for the addition of 60 μL TEOS. Ultrasonication was applied by bath sonication at the power level of 640 W through the reaction process. The core–shell particles were separated by centrifugation (2000 rpm, 1 min) and washed thrice with water. Finally, the silica capsules were obtained by the removal of the Ca(OH)2 templates with HCl (pH = 2), and washed with water until the supernatant was neutral. The spherical silica capsules were also fabricated by the “Multi-step growth” method following the similar procedures except the use of spherical MnCO3 templates. Their thicknesses were tuned almost same to that of biconcoidal discoidal capsules by tuning the amount of TEOS for the following cell study.

2.4. Labeling silica capsules with FITC

To label the silica shells with FITC, the particles coated with silica were washed thrice with acetone, and finally dispersed in acetone. 5% of APTES was added into the suspension, which was shaken for 2 h followed by wash with acetone. Finally, the suspension was centrifuged, and the sediment was dispersed in FITC (0.1 mg/mL in DMSO) solution, which was shaken overnight for labeling in dark. Finally, the labeled particles were collected and washed by ethanol until the supernatant of suspension was free of dye, and finally transferred into water. Then the templates were removed as mentioned in Section 2.2.

2.5. Cellular uptake of FITC–SiO2 capsules

Uptake of biconcoidal discoidal and spherical SiO2 capsules was determined by flow cytometry (FACS Calibur, Becton Dickinson BD). Smooth muscle cells (SMCs) were seeded on a 24-well plate at a density of 5 × 104 cells per well and allowed to attach for 16 h. To determine the particle uptake rate and amount, the cells were incubated with 105 FITC–SiO2 capsules per well. SMCs were cultured with regular growth medium consisting of high-glucose DMEM (Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, and incubated in a 5% CO2 incubator at 37 °C and 100% humidity. At determined time intervals (3, 6, and 12 h), the cells were washed three times with PBS and harvested by trypsination. The FITC–SiO2 capsules served as a marker for quantitative determination of their cellular uptake by flow cytometry and CellQuest Pro software. The data were calibrated according to the initial fluorescence intensity ratio between FITC–SiO2 capsules with different shapes, so that their relative uptake amounts by SMCs are comparable.

2.6. Characterizations

2.6.1. Scanning electron microscopy (SEM)

A drop of suspensions of Ca(OH)2 particles or silica capsules was applied onto a silicon wafer, and dried in air overnight. After sputtering with gold, the samples were measured using HITACHI S-4800 instrument at an operation voltage of 3 keV.

2.6.2. Fourier transform infrared spectroscopy (FTIR)

FTIR was measured on a Vector 22 spectrophotometer (Bruker optics, Switzerland) using a KBr pellet. Particles were completely dried in oven before measurement.

2.6.3. X-ray diffraction (XRD)

The as-prepared samples were dried at 60 °C overnight for XRD (RIGAKUD/MAX-C) characterization. Spectra were recorded from 10° to 80° at a scanning rate of 2°min−1 and a step size of 0.02°.

2.6.4. Transmission electron microscopy (TEM)

The silica capsules were washed with water thrice and then with graded ethanol/water mixed solutions. The sample was embedded into epoxy resin and ultramicrotomed into thin sections, which were transferred onto a carbon film-coated copper grid and observed by a Philips Tecnai-10 TEM.
2.6.5. Confocal laser scanning microscopy (CLSM)
Confocal images were taken with Leica TCS SP5 confocal scanning system equipped with a 100× oil immersion objective. A drop of FITC–SiO₂ capsules suspension was applied onto a glass slide for visualization after they were precipitated.

3. Results and discussion
3.1. Fabrication of biconcave discoidal silica microcapsules

Firstly, the biconcave discoidal Ca(OH)₂ particles were synthesized according to the literature [46]. The size of the Ca(OH)₂ particles can be easily tuned by the concentration of CaCl₂ and NaOH, from 3 µm to larger than 10 µm. As shown in Fig. 1a, the particles are of uniform size and low polydispersity (3.5 ± 0.3 µm). The shape of the particles is biconcave discoidal referring to both the ones standing on their sides (solid arrows) and the one lying (dashed arrows). This phenomenon is consistent with the donut-shaped silica particles reported recently [39]. The EDX spectrum shows the existence of sulfur element (Fig. 1b), resulting from the doped DS in the particles. According to the sulfur detection measurement, the DS amount in the particle was calculated to be 5.9%.

Then, the ammonia-catalyzed sol–gel method was used to fabricate the silica shells on these biconcave discoidal Ca(OH)₂ particles. This process does not need any surfactant and can be conducted at room temperature. Due to the slight solubility of Ca(OH)₂ in water, the volume ratio between ethanol and water was set as 5:1 to prevent severe dissolution of Ca(OH)₂ particles. Ammonia catalyzed hydrolysis and condensation (pH = 9) of TEOS was chosen for the silica coating via “Multi-step growth” method. During the entire hydrolysis and condensation process, ultrasonication was used to avoid aggregation of particles [48].

Then the obtained particles were characterized by FTIR first. The two strong peaks in the spectra at 3640 cm⁻¹ and 1463 cm⁻¹ of Ca(OH)₂ particles in Fig. 2a are attributed to stretching and vibration modes of the structural hydroxyl groups. Three new peaks (1093 cm⁻¹, 799 cm⁻¹, 471 cm⁻¹) appear in Fig. 2b (silica-coated Ca(OH)₂) and Fig. 2c (silica capsules), which can be assigned to the anti-symmetric stretching vibration, symmetric stretching vibration and bending vibration of Si–O–Si bond, respectively. They are typical FTIR spectrum characteristics of amorphous silica [45]. The X-ray diffraction peaks of Ca(OH)₂ (Fig. 3a) and silica-coated Ca(OH)₂ (Fig. 3b) correspond to each other perfectly both in peak position and in relative intensity, except the tiny difference shown in the inset, which is a broad diffraction peak (18–28°) centered around 23°. This broad diffraction peak comes from the as-synthesized amorphous structure of silica shells [49,50], which is the only strong peak shown on the curve for hollow silica capsules (Fig. 3c). There is no crystalline impurity formed during the fabrication process. These results demonstrate the presence of silica shells on Ca(OH)₂ templates and silica capsules are obtained after templates removal.

Fig. 1. SEM image (a) and EDX spectrum (b) of Ca(OH)₂ particles.

Fig. 2. FTIR spectra of (a) Ca(OH)₂ templates, (b) Ca(OH)₂ particles with silica shells, and (c) silica capsules.

Fig. 3. XRD patterns of (a) Ca(OH)₂ templates, (b) Ca(OH)₂ particles with silica shells, and (c) silica capsules. The inset is magnified region from 16° to 30°.
The successful formation of silica shells on the Ca(OH)₂ particles was further identified by the obtained hollow capsules after templates removal (Fig. 4a and b). As shown in Fig. 4a, some capsules are standing (white dashed arrows) while others are lying (white solid arrow) on the substrate. The inset in Fig. 4a exhibits a magnified image of a capsule. The non-spherical hollow silica structures were also reported previously, such as flake silica capsules templated on gibbsite platelets [44]. These results demonstrate that coating of silica on different templates via the sol–gel reaction is a powerful way to fabricate capsules with different morphologies. The hollow nature of the obtained silica capsules was further confirmed by TEM (Fig. 4c and d). The capsules have

![Fluorescent CLSM image (a) and bright field image (b) of biconcave discoidal silica capsules. TEM images of ultrathin section of individual biconcave discoidal capsule on a “Lying” state (c) and a “Standing” state (d). The inset in (a) shows a magnified image of a capsule. The amount of added TEOS is 20 µL for (a and b) and 60 µL for (c and d) via “Multi-step growth” method. The solid and dashed arrows in (a) indicate the capsules in “Lying” and “Standing” states, respectively.](image1)

**Fig. 4.** Fluorescent CLSM image (a) and bright field image (b) of biconcave discoidal silica capsules. TEM images of ultrathin section of individual biconcave discoidal capsule on a “Lying” state (c) and a “Standing” state (d). The inset in (a) shows a magnified image of a capsule. The amount of added TEOS is 20 µL for (a and b) and 60 µL for (c and d) via “Multi-step growth” method. The solid and dashed arrows in (a) indicate the capsules in “Lying” and “Standing” states, respectively.

![SEM images of silica capsules fabricated by (a–d) “Gradient concentration” method, and (e–h) “Multi-step growth” method. The volume of TEOS were (a and e) 10, (b and f) 20, (c and g) 40, and (d and h) 60 µL, respectively. Scale bar: 2 µm.](image2)

**Fig. 5.** SEM images of silica capsules fabricated by (a–d) “Gradient concentration” method, and (e–h) “Multi-step growth” method. The volume of TEOS were (a and e) 10, (b and f) 20, (c and g) 40, and (d and h) 60 µL, respectively. Scale bar: 2 µm.
Figure 4 represents the "lying" and "standing" capsules, respectively, which are in good agreement with the CLSM images (Fig. 4a).

3.2. Tuning the shell thickness of the silica capsules via two different methods

Tuning the thickness of the silica shells is an effective way to obtain intact capsules and may further regulate their mechanical property. Here, the thickness of silica capsules was first regulated by adding different amounts of TEOS into the Ca(OH)$_2$ suspension in basic ethanol/water mixed solution via the so-called Gradient concentration method [51]. Fig. 5a and b clearly reveal the hollow structure of the capsules due to their broken and collapsed morphology after drying. However, the morphology of the silica capsules changes to intact discoidal structure in the dry state due to the enhancement of mechanical properties when the amount of TEOS increases to 40 kmol (Fig. 5c) and 60 kmol (Fig. 5d). The thin sections of silica capsules with different TEOS amounts were observed via TEM (Fig. 6a–d). Through these images, the shell thickness was quantified (Fig. 7). As expected, the shell thickness increases gradually from 39 ± 5 nm to 104 ± 7 nm for 10 μL and 60 μL samples, respectively. However, there are some secondary silica particles with a size of 100–200 nm. Many of these nanoparticles are remained in the samples even after 3 times washing. These nanoparticles are free silica particles nucleating homogeneously in solution but not heterogeneously on the templates due to the insufficient surface areas of templates [44,52]. The formation of free silica nanoparticles in solution consumes a part of TEOS, resulting in only small increase in the shell thickness.

It is noticeable that no free silica nanoparticles were formed when 10 μL of TEOS was added (Fig. 5a). Therefore, a "Multi-step growth" method was adopted to avoid the formation of free silica nanoparticles. In this process, only 10 μL of TEOS was added at each step for the sol–gel reaction. As expected, no free silica nanoparticles were formed even at high amount of TEOS (60 μL) (Fig. 5e–h). The morphology of the silica capsules is similar to that of the “Gradient concentration” method. Only the thinnest capsules collapse (Fig. 5e), while all others keep good biconcave discoidal shape after drying (Fig. 5f–h). The shell thickness quantified from TEM images (Fig. 6e–h) increases sharply compared with that of the “Gradient concentration” method (Fig. 7). For example, the shell thickness (286 ± 49 nm) is nearly 3 times of that obtained by the “Gradient concentration” method when the capsules were fabricated with 60 μL TEOS. This result suggests that the newly added TEOS is mostly condensed on the template surface, forming the thicker silica shell in the “Multi-step growth” method.

3.3. Cellular uptake of silica capsules with different geometries

It is known that the shapes of particles affect strongly their interactions with cells [19,53–55]. The biconcave discoidal silica capsules have unique shape which resembles RBCs and their interactions with cells are unknown. In order to compare the shape...
effect, biconcave silica capsules and spherical silica capsules, with similar sizes and thicknesses, were fabricated by the “Multi-step growth” method. The obtained biconcave discoidal and spherical silica capsules (Fig. 8a) have a size of $3.5 \pm 0.3 \mu m$ and $3.4 \pm 0.2 \mu m$ with a shell thickness of $101.3 \pm 19.3$ nm and $108.4 \pm 26.5$ nm, respectively. They were labeled by FITC for flow cytometry measurement. The zeta potentials of labeled discoidal biconcave and spherical silica capsules are $14.7 \pm 1.1$ mV and $8.5 \pm 0.3$ mV, respectively. Smooth muscle cells can internalize both capsules. Fig. 8b reveals that the cellular fluorescence intensity increases along with the prolongation of incubation time regardless of the capsule shape. However, the uptake rates are different. The spherical capsules are internalized more quickly than the biconcave discoidal ones, i.e. nearly 1.6 folds higher in 12 h. Due to the relatively strong mechanical strength, the internalized capsules can keep intact without obvious change on their original morphology (Fig. 8c and d), and are located around the cell nucleus (Fig. 8c3 and d3). At this moment, the exact uptake mechanism is not clear and more detailed biological experiments are ongoing. But according to our previous study [56], the pretty big polyelectrolyte capsules ($\sim 4.2 \mu m$), even bigger than the capsules here, also can be internalized by SMC cells. The internalization of the microcapsules is mediated by macropinocytosis and caveolae-mediated endocytosis [56]. Here the similar pathways may be also involved.

Previous study found that micron-size poly(lactic-co-glycolic acid) (PLGA) particles with a spherical shape were internalized by endothelial cells more rapidly than elliptical disks [53]. Again in another study, the endocytosis of gold nanorods mediated by receptor significantly reduced with an increased aspect ratio [57]. The recent study revealed that the shape-dependent cellular internalization behavior of particles was probably related to their different uptake mechanisms [58]. However, this is not always the case. For example, nanoparticles with an aspect ratio of 3 (150 $\times$ 450 nm) were internalized by Hela cells almost 4 times faster than the more symmetric low-aspect-ratio ones [59]. The intrinsic reason for this discrepancy is not clear yet, although it is likely tired with the cellular endocytosis pathways, compositions of particles, and cell lines. Further detailed experiments are ongoing.

4. Conclusions

In summary, biconcave discoidal silica capsules were fabricated by hydrolysis of TEOS on biconcave discoidal Ca(OH)$_2$ templates under alkaline condition in ethanol/water mixed solution. Their thickness can be effectively tuned by the “Multi-step growth” method, without the formation of free nanoparticles. The biconcave discoidal capsules were internalized much slower than their spherical counterparts by SMCs.

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References