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7 Understanding the effects of nanocapsular
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ABSTRACT

The physicochemical properties of nanoparticles (size, charge and surface chemistry, *etc.*) influence their biological functions often in complex and poorly understood ways. This complexity is compounded when the nanostructures involved have variable mechanical properties. Here we report, for the first time, the synthesis of liquid-filled silica nanocapsules (SNCs, ~ 150 nm) having a wide range of stiffness (with Young's moduli ranging from 704 kPa to 9.7 GPa). We demonstrate a complex trade-off between nanoparticle stiffness and the efficiencies of both immune evasion and passive/active tumor targeting. Soft SNCs showed 3 times less uptake by macrophages than stiff SNCs, while the uptake of PEGylated SNCs by cancer cells was independent of stiffness. In addition, the functionalization of stiff SNCs with folic acid significantly enhanced their receptor-mediated cellular uptake, whereas little improvement for the soft SNCs was conferred. Further *in vivo* experiments confirmed these findings and demonstrated the critical role of nanoparticle mechanical properties in regulating their interactions with biological systems.

Keywords: nanoparticle, nanocapsule, stiffness, cellular uptake, targeting.

Engineering of nanoparticle (NP) physicochemical properties is of great importance for the successful development of nanocarrier-based drug delivery^{1,2}. Size³, shape³⁻⁵, charge⁶, chemical composition and surface chemistry^{7,8} of a nanocarrier, and the interplay among these⁹⁻¹¹, are well-investigated parameters that influence immune evasion, blood circulation, tumor extravasation and penetration, and cell internalization through passive- and/or active-targeting¹². The mechanical property of NPs (normally presented as stiffness or Young's modulus) is another physical attribute that has recently gained attention due to its roles at the nano-bio interface¹³⁻¹⁷. This is inspired by the sophistication exhibited by some biological entities, such as red blood cells^{18,19}, murine leukemia virus²⁰ and human immunodeficiency virus²¹, that can regulate their stiffness when performing complex biological functions. Thus, understanding the mechanical properties of NPs and their effects on passive- and/or active-targeting is required to rationally design optimal drug-delivery nanocarriers.

A considerable effort has recently been dedicated to fabricate stiffness-engineered NPs for drug delivery. To this end, an arsenal of nanomedicine platforms has been synthesized with NPs of varied stiffness, including liposomes²², hybrid polymer-lipid nanoparticles^{23,24} and hydrogel nanoparticles²⁵⁻²⁹. Modulation of their stiffness was achieved through the selection of phospholipid types (liposomes), the number of lipid layers (hybrid nanoparticles), and the degree of cross-linking (hydrogel nanoparticles). Nevertheless, there has to date been no consensus about the impact of stiffness especially in cancer targeted drug delivery due to the variation in the NPs properties and the tumor types and stages studied¹³. This limitation arises from the difficulty in engineering NP stiffness while keeping other physicochemical parameters constant. Moreover, the produced NPs exhibited a narrow range of stiffness which in turn resulted in contradictory results between studies. For example, Zhang et al.²⁶ investigated the effect of NPs'

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3 stiffness on their circulation and biodistribution using poly(carboxybetaine) nanogels (diameter =
4 120 nm) having Young's moduli ranging from 180 to 1350 kPa. Sun et al.²³ studied the role of
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6 NPs' stiffness in controlling their cellular uptake efficiency using polymeric-core lipid-shell NPs
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8 (diameter = 40 nm) with two different Young's moduli of 0.76 GPa and 1.2 GPa.
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13 Herein, we report an elegant strategy for synthesizing silica nanocapsules having a wide range of
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15 stiffness from very soft (0.04 N/m) to very stiff (14 N/m), with the computed Young's moduli
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17 ranging from 704 kPa to 9.7 GPa. The soft and stiff nanocapsules were modified with stealth
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19 polymer poly(ethylene glycol) (PEG) and folate-PEG to investigate the effect of stiffness on
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21 their passive and active tumor-targeting, while their size, shape and charge remain identical.
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23 Nanocapsules were tested using three biological models with increasing complexity: monolayer
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25 cell cultures, 3D tumor spheroids, and tumor xenograft in mice. Results of this study will provide
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27 a fundamental understanding on the effects of NP stiffness on cellular interactions, tumor
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29 penetration and *in vivo* tumor targeting, and will deliver a new strategy for designing high-
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31 efficiency drug delivery nanocarriers.
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RESULTS & DISCUSSION

Synthesis of silica nanocapsules with controllable stiffness

Silica nanocapsules with controllable stiffness were synthesized based on a nanoemulsion and bio-silicification dual-templating technology (Fig. 1a) using a dual-functional peptide SurSi (Ac-MKQLAHS VSRLEHA RKKRKKRKKRKKGGGY-CONH₂).³⁰ This peptide was designed to have both surface activity (Sur module, MKQLAHS VSRLEHA) and bio-silicification activity (Si model, RKKRKKRKKRKKGGGY). To make silica nanocapsules, nanoemulsion templates were prepared by sonicating the mixture of Miglyol 812 oil and the peptide SurSi aqueous solution. SurSi absorbs onto the oil–water interface by virtue of its surface activity, thus stabilizing the nanosized oil droplets. Then adding a silica precursor into the nanoemulsion initiates bio-silicification, allowing the formation of a solid silica shell on the droplet surface, with the shell thickness controlled *inter alia* by reaction time. To generate soft and stiff silica nanocapsules, two silica precursors with different chemical structures, triethoxyvinylsilane (TEVS) and tetraethylorthosilicate (TEOS) were used, based on the knowledge that the incorporation of organic moieties in silica frameworks by using organosilanes that can tune the mechanical property of silica.³⁰⁻³³ All the four ethoxy groups (–OC₂H₅) in TEOS are able to hydrolyze and form siloxane bonds (≡Si–O–Si≡) to build a compact and stiff structure, while TEVS comprises three ethoxy groups and a non-hydrolyzable vinyl group (–C=C) that reduces the cross-linking density of the silica shell, hence diminishing its stiffness. Moreover, the shell thicknesses of nanocapsules can be controlled by varying the reaction time to further regulate their mechanical properties.³¹

Both the stiff and soft nanocapsules prepared using our biomimetic dual-templating approach show a core–shell structure and a diameter of approximately 150 nm (Fig. 1b) under TEM. Their

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3 shell thickness increase from around 10 nm to 24 nm as the reaction time prolongs from 30 h to
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5 50 h (Fig. 1c). The stiff TEOS nanocapsules remain spherical irrespective of their shell
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7 thickness, whereas the soft TEVS nanocapsules deform significantly, especially for the softest
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9 TEVS nanocapsules produced with a reaction time of 30 h. The hydrodynamic diameters of the
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11 TEVS and TEOS nanocapsules are around 200 nm (reaction time of 30 h) and undergo a slight
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13 increase as the reaction time prolongs to 50 h (Fig. 1d). All DLS results reveal a polydispersity
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15 index (PDI) of less than 0.2, suggesting the formation of uniform nanocapsules (Fig. S1).
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17 Although silica is intrinsically negatively charged at neutral pH, the nanocapsules show positive
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19 zeta potentials of around +25 mV (Fig. 1e) likely due to the embedded peptide SurSi (highly
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21 positively charged at neutral pH) in the silica shell. Jakhmola et al.³⁴ reported a similar finding
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23 that silica prepared using the cationic poly-L-lysine also gave a zeta potential of +33 mV at
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25 neutral pH.
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32 Atomic force microscopy (AFM) was used to measure the mechanical properties of
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34 nanocapsules. Figure 2a displays the height profiles of our nanocapsules in air. Notably,
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36 compared with the stiff TEOS nanocapsules, the soft TEVS nanocapsules exhibited much
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38 smaller heights of around 18, 40, and 70 nm with the reaction times of 30, 40, and 50 h,
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40 respectively, due to their drastic flattening on the substrates during drying and imaging
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42 processes. The re-constructed 3D topographical images confirmed their disc-like structures (Fig.
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44 2b). The mechanical properties of nanoparticles are normally presented in Young's modulus and
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46 stiffness. Young's modulus (Pascals, Pa) is an intrinsic attribute of a material which defines the
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48 unidirectional relationship between stress and strain, and has been widely used in computational
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50 determination of deformability,³⁵ whereas stiffness (Newton per meter, N/m) is a heuristic
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52 measure that convolutes geometry with Young's modulus. The determination of Young's moduli
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of nanomaterials with a complex structure like ours, that is, core-shell structure, is very intricate, and requires separation of the fundamental stress-strain relationships in a given geometry. Therefore, new complex nanostructures that haven't been fundamentally characterized, such as the nanocapsules presented here, are more readily characterized by empirical and geometry-specific measures such as stiffness. Young's moduli can be computed afterwards as a supplement, which will allow a more straightforward comparison with other materials. To measure their stiffness, force-indentation curves (Fig. S2) of the nanocapsules were obtained, and the stiffness equals to the slopes of the linear region of the curves (Fig. 2c). Based on this method, direct measurement of the TEVS nanocapsules with reaction times of 30 and 40 h gives higher stiffness than that of 50 h, which is counter-intuitive as the former two have the same material but thinner shells. Considering that TEVS nanocapsules with reaction times of 30 and 40 h are highly deformable and very easy to flatten on the substrate in air, the measured stiffness is likely affected by the hard substrates. Therefore, the real stiffness of these extremely soft nanocapsules could not be determined in air. The TEVS nanocapsules produced with a reaction time of 50 h with a shell thickness of 24 nm only showed a stiffness of 0.4 N/m. In contrast, the stiffnesses of TEOS nanocapsules increase from 8.7 to 20.1 N/m as the shell thickness grows from 12.5 to 24.8 nm when the reaction time prolongs from 30 to 50 h. To further characterize the soft TEVS nanocapsules with a reaction time of 30 h, an AFM force mapping method in liquid phase was explored, wherein the nanocapsules flattened to a less extent, and the measured stiffness was 0.04 N/m (Fig. S3).

To study the impacts of nanocapsules' mechanical properties on their cellular interactions and *in vivo* tumor targeting, nanocapsules having distinct stiffness, namely TEVS and TEOS nanocapsules produced with a reaction time of 30 and 40 h, respectively (arrowed in Fig. 2c),

were utilized in the following experiments. They have very different stiffness of 0.04 N/m for the soft TEVS one, and 14 N/m for the very hard TEOS one. Considering the nanocapsule as a uniform material, the Young's moduli of the two nanocapsules were computed to be 704 kPa and 9.7 GPa (Fig. 2d) based on the Hertzian contact model.

Figure 1. Synthesis and characterization of silica nanocapsules. (a) Schematic illustration for the synthesis of silica nanocapsules having controllable mechanical property; (b) TEM

micrographs (scale bars = 200 nm), (c) shell thicknesses, (d) hydrodynamic diameters and (e) zeta potentials of the prepared silica nanocapsules. Values are means $\pm$ SD (n = 3).

Figure 2. Mechanical properties of the silica nanocapsules. (a) AFM height profiles of the nanocapsules (scale bars = 200 nm); (b) re-constructed 3D morphologies of TEVS nanocapsules;

(c) stiffness of the nanocapsules; (d) computed Young's moduli of the selected nanocapsules (TEVS-30 h and TEOS-40 h). Values are means $\pm$ SD (n = 10).

Surface modification

To render their surface properties identical, the TEOS and TEVS nanocapsules were modified by PEGylation (MW = 5000 Da), and to explore the role of mechanical property in their *in vitro* and *in vivo* non-specific (passive) targeting and folic acid (FA)–folate receptor (FR) mediated specific (active) targeting, the nanocapsules were also functionalized with a targeting ligand FA. The introduction of PEG on the particles' surface can also create steric repulsion, thus enhancing their stability in physiological environments and extending their *in vivo* circulation time.³⁶ The conjugation of PEG and FA-PEG chains on nanocapsules increases their hydrodynamic diameters by about 15-20 nm (Table 1), while the TEM images show no significant difference between the pristine and surface-modified nanocapsules. Nanoparticle Tracking Analysis (NTA) technology was also used to characterize the size of modified nanocapsules (Fig. S4) and revealed a similar size as we observed using TEM. The PEG-modified nanocapsules display nearly neutral zeta potentials due to the charge-screening effect of PEG coatings, while the FA-PEG-modified nanocapsules show slightly lower zeta potentials. The successful conjugation of PEG chains on nanocapsules was also confirmed using Fourier transform infrared spectroscopy (FTIR) (Fig. S5). In addition, the surface modified nanocapsules displayed the same stiffness as the unmodified ones (Fig. S6).

The stability of unmodified and modified nanocapsules in physiological medium (DMEM completed with 10% FBS) was examined. The DLS results demonstrate a good stability of the naked TEVS and all surface-modified nanocapsules over 24 h, while the naked TEOS nanocapsules started aggregating after a 4-h incubation (Fig. S7). Therefore, a 4-h incubation time was adopted to conduct cellular uptake experiments. Moreover, the biocompatibility study

of our nanocapsules revealed a minimal cytotoxicity (24-h cell viability >85%) of both naked and modified nanocapsules (Fig. S8).

Table 1. Size, PDI and zeta potential of unmodified, PEG- and FA-PEG-modified silica nanocapsules. Values are means $\pm$ SD (n=3).

Silica Precursor	Triethoxyvinylsilane (TEVS)			Tetraethoxysilane (TEOS)		
Surface Modification	Unmodified	PEG	FA-PEG*	Unmodified	PEG	FA-PEG*
Z-Averaged Diameter [nm]	197 $\pm$ 4	213 $\pm$ 3	219 $\pm$ 3	220 $\pm$ 1	236 $\pm$ 2	235 $\pm$ 5
PDI	0.04 $\pm$ 0.02	0.10 $\pm$ 0.10	0.16 $\pm$ 0.04	0.11 $\pm$ 0.01	0.20 $\pm$ 0.03	0.11 $\pm$ 0.07
Diameter by TEM [nm]	142 $\pm$ 12	139 $\pm$ 9	144 $\pm$ 8	155 $\pm$ 8	156 $\pm$ 7	153 $\pm$ 5
Zeta Potential [mV]	+28.7 $\pm$ 0.7	+1.4 $\pm$ 0.3	-3.6 $\pm$ 0.6	+23.7 $\pm$ 1.1	+1.0 $\pm$ 0.2	-4.9 $\pm$ 1.1

*The coating of FA-PEG-modified nanocapsules consists of 10% FA-PEG and 90% PEG (in molar ratio).

The effects of nanocapsular stiffness on cellular uptake

In order to quantify the cellular uptake of nanocapsules, a fluorescent dye DiI was encapsulated in the nanocapsules using a pre-loading strategy. Briefly, DiI was dissolved in Miglyol 812 oil at a concentration of 500 μ g/mL. Then the DiI-loaded nanocapsules were synthesized following the standard procedure described previously. The encapsulation efficiency of DiI in the TEVS and TEOS nanocapsules was measured to be 92.2% and 93.4%, respectively (Fig. S9). Due to its lipophilicity, DiI showed minimal leakage from the oil-core silica-shell nanocapsules over 48 h in PBS at 37°C (data not shown).

Nanocapsule uptake by macrophages. Macrophages are a type of white blood cells involved in the clearance of cell debris, microbes, and foreign substances in the body. As the macrophage sequestration of NPs is a critical factor limiting their biological performance in clinical settings,³⁷ we first studied the uptake of unmodified and modified nanocapsules by RAW264.7 murine macrophages (Fig. 3a). The surface-modified nanocapsules (PEGylated) demonstrated a much lower (10 times less) uptake than the unmodified ones (naked), confirming the ‘stealth’ effect of PEG layers. The FA-functionalized nanocapsules didn’t show enhanced uptake due to the lack of FR on the RAW264.7 cell membranes. Moreover, for all three types of nanocapsules (unmodified, PEG- and FA-PEG-modified), the macrophage uptake of the stiff TEOS nanocapsules was always significantly higher than the soft TEVS nanocapsules ($p < 0.01$). This is in agreement with a host of previous findings in the literature that rigid NPs normally have a higher macrophage uptake than their soft counterparts.¹³ One possible reason is that soft NPs can undergo shape deformation during internalization, thus raising the energy barrier for the full wrapping of NPs by cell membranes.¹⁵ To prolong the blood circulation and enhance the EPR effect of NPs, their macrophage capture has to be minimized. In this respect, soft nanocapsules have more advantages than the stiff ones.

Nanocapsule uptake by tumor cells. The effects of nanocapsules’ stiffness on non-specific and folate receptor (FR)-mediated tumor cellular uptake were studied using MCF-7 and SKOV3 cells, respectively. The surface FR expression levels of the two cell lines were assessed (Fig. 3b). The MCF-7 cells showed no FR expression, whereas the SKOV3 cells displayed a great FR expression (9-fold higher) on the surface.

Figures 3c and 3d demonstrate the uptake of surface modified nanocapsules by MCF-7 and SKOV3 cells, respectively. In contrast to the significantly enhanced cellular uptake of the soft

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3 SNCs by the RAW264.7 macrophages, the PEG-modified stiff and soft nanocapsules exhibited a
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5 similar level of uptake by both MCF-7 and SKOV3 cells, suggesting that mechanical properties
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7 may play a less important role in non-specific tumor cell uptake. Surface functionalization by FA
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9 did not enhance the uptake of nanocapsules by MCF-7 cells due to the lack of FR on the cell
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11 surface, but significantly increased their uptake by the FR-positive SKOV3 cells. A competitive
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13 uptake assay was conducted by incubating SKOV3 cells and FA-functionalized nanocapsules in
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15 a medium containing free FA (1 mg/mL). As a result, the enhanced uptake was reduced to a
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17 similar level as that of the PEG-modified nanocapsules, indicating the specificity of FR-mediated
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19 cellular uptake. Notably, the FA-PEG-modified stiff nanocapsules showed more than 2-fold
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21 higher uptake than the soft ones ($p < 0.01$), indicating a higher targeting efficacy of the stiff
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23 nanocapsules. This was also confirmed by observing the cellular uptake of the soft and stiff
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25 nanocapsules using confocal microscopy (Fig. 3e). FA-PEG-modified TEOS (stiff) nanocapsules
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27 displayed a clearly higher uptake than the TEVS (soft) ones at both 1 h and 4 h, whereas the
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29 PEG-modified nanocapsules show very limited uptake even at 4 h.
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37 The stiffness of nanocapsules exerts different effects on their non-specific and ligand–receptor
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39 mediated cellular interactions, indicating a coupled effect between the stiffness and the surface
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41 functionalization of nanocapsules, which might be a result of the different endocytic mechanisms
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43 involved. As pointed out by other researchers, the non-specific cellular uptake of NPs normally
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45 adopts a pinocytosis pathway,³⁸ while the FR-mediated internalization of NPs tends to utilize the
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47 clathrin-mediated endocytic pathway.³⁹ In the latter case, an important component of
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49 cytoskeleton, F-actin, is involved in the key processes of internalization such as the invagination
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51 of cell membrane and the pinch off of vesicles.^{40, 41} Moreover, it has been extensively reported
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53 that F-actin plays a key role in the sensing of extra-cellular environmental mechanical cues (e.g.,
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3 rigidity of substrate) by cells.⁴² Therefore, it is likely that in the FR-mediated uptake of
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5 nanocapsules, due to the involvement of F-actin and its cooperation with other related proteins
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7 such as dynamin and clathrin, cells become more sensitive to the variation in nanocapsules'
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9 stiffness, leading to their preference in taking up stiffer nanocapsules.
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13 Given this hypothesis, we further looked into the role played by F-actin in the cellular uptake of
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15 FA-PEG-modified nanocapsules. To do this, SKOV3 cells were pre-treated with different
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17 concentrations of cytochalasin D (CytD), an inhibitor of actin polymerization, before co-
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19 incubated with nanocapsules. Herein, due to the indispensable role of F-actin in the endocytosis,
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21 when the concentration of CytD increased, the cellular uptake of both stiff and soft nanocapsules
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23 dropped, the extent to which, however, is different between the stiff and soft nanocapsules (Fig.
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25 3f). When there was no CytD in the medium, the uptake of soft nanocapsules was 45% of the
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27 stiff ones, however, this ratio increased to 64% at a CytD concentration of 1 $\mu\text{g/mL}$. The
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29 alteration of actin filament structure upon CytD treatment is also demonstrated by the confocal
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31 micrographs (Fig. 3g). Compared to the cells without CytD treating, which display intracellular
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33 actin filaments, cells exposed to 1 $\mu\text{g/mL}$ of CytD show a de-activated F-actin state. Overall,
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35 these findings indicate that the FR-mediated uptake of stiff nanocapsules is more dependent on
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37 the F-actin activation of cells than the soft ones. To some extent, this represents how cell
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39 mechanics influences their interaction with stiff and soft NPs in ligand–receptor mediated
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41 manner: when the cells become ‘softer’, their ability of sensing NP stiffness tends to diminish.
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Figure 3. The effects of nanocapsular stiffness on cellular uptake. (a) Uptake by RAW264.7 murine macrophages, the inset is the uptake of surface-modified nanocapsules; (b) surface folate receptor expression of MCF-7 and SKOV3 cells; (c) uptake by MCF-7 cells; (d) uptake by SKOV3 cells. (e) Fluorescence microscopic images displaying nanocapsule uptake by SKOV3 cells (scale bars = 10 μ m). (f) Flow cytometry results and (g) fluorescence microscopic images

displaying the cellular uptake of FA-PEG-modified nanocapsules by CytD-treated SKOV3 cells. The blue (Hoechst) and green (AF647-phalloidin) fluorescence represent cell nuclei and F-actin, respectively, while the red fluorescence indicates nanocapsules labelled with DiI. Values are means $\pm$ SD (n = 3). * p <0.05, ** p <0.01, *** p <0.001 as determined by t -test.

The effects of nanocapsular stiffness on their interactions with 3D tumor spheroids

To further explore the biological fates of stiff and soft nanocapsules in clinical settings, their interactions with 3D tumor spheroids were investigated. Compared to 2D monolayer cells, tumor spheroids better mimic the real *in vivo* tumor conditions in terms of their spatial complexity and heterogeneity, so they are expected to give more relevant information about the effect of nanocapsules' mechanical property on their uptake and penetration in tumor tissues *in vivo*.

SKOV3 tumor spheroids were cultured for 7 days to reach a diameter of around 600 μ m before they were co-cultured with the nanocapsules. The confocal microscopic images demonstrate a very limited uptake of PEG-modified nanocapsules by the tumor spheroids (Fig. 4a), while the uptake of FA-PEG-modified nanocapsules is much more as indicated by the stronger fluorescence signal. Flow cytometry results (Fig. 4b) of the nanocapsule uptake in tumor spheroids reveal a similar trend as in 2D monolayer cells, but the overall amount of uptake becomes less compared to the 2D monolayer cellular uptake, highlighting the diffusion barrier of the complex 3D structure. Specifically, the FA-PEG-modified stiff nanocapsules show a 1.6-time higher tumor spheroids uptake (p <0.05) than the soft ones, which is less pronounced than in 2D cells (2.2 times, p <0.01). To evaluate the penetration of FA-PEG-modified nanocapsules into tumor spheroids, their distribution in tumor spheroids at a depth of 150 μ m was analysed (Fig.

4c). For the stiff nanocapsules, a strong fluorescence signal appears at the periphery of tumor spheroids, which sharply attenuates along the direction towards the interior of the spheroids. In comparison, the soft nanocapsules show a deeper penetration, with their fluorescence distribution peaking (marked by blue arrows) at a distance around 50 μm from the surface of the spheroid. This discrepancy emphasizes the impact of nanocapsules' mechanical property on the interactions with biological models that are more complex than monolayer cells. As illustrated in Fig. 4d, the stiff nanocapsules might be unable to pass through the narrow intercellular spaces due to their poor deformability, leading to a high accumulation at the marginal area of the tumor spheroids. The soft nanocapsules, however, are likely to squeeze through the intercellular spaces and reach deeper inside the spheroids. Apart from this, the higher cellular uptake propensity of stiff nanocapsules might also hinder their diffusion into tumor spheroids as they are readily internalized once exposed to the outer-layer cells.

Figure 4. The interactions of stiff and soft nanocapsules with SKOV3 tumor spheroids. (a) Layer-by-layer and z-stack confocal microscopic images showing the uptake of nanocapsules by tumor spheroids (scale bars = 200 μm); (b) flow cytometry results of nanocapsule uptake by

tumor spheroids; (c) DiI fluorescence intensity distribution of FA-PEG-modified nanocapsules along the dashed line across the tumor spheroids at the depth of 150 μm ; (d) schematic illustration showing the penetration of stiff and soft nanocapsules in the tumor spheroids. Values are means $\pm$ SD ($n = 3$). $*p < 0.05$ as determined by t -test.

The effects of nanocapsular stiffness on *in vivo* tumor targeting

The *in vivo* passive and active tumor targeting of the stiff and soft nanocapsules was investigated using a SKOV3 xenograft mouse model. To do this, the nanocapsules were loaded with a near-infrared (NIR) dye, DiR, and intravenously injected into SKOV3 xenograft bearing Balb/c mice. Mice were then imaged at a series of time points over 72 h (Fig. 5a). It appears that for all types of nanocapsules, livers and spleens exhibit the strongest signal over the mice bodies, indicating that most of the injected nanocapsules are captured by these reticuloendothelial system (RES) rich organs. Moreover, the accumulation of soft nanocapsules in the tumor areas can be observed reaching the highest around 12-24 h after injection, then decreased. In comparison, no obvious tumor accumulation can be observed in the mice injected with the stiff nanocapsules. The organs and tumors of mice (one from each group) were collected and imaged at 24 h after injection. From the *ex vivo* images for the mice sacrificed at 24 h after injection (Fig. 5b), both the soft and stiff nanocapsules accumulated mainly at the livers and spleens. The signal of spleens was much higher than that of livers, probably due to the size of the nanocapsules (>150 nm). It has been reported that for intravenously injected NPs with a size larger than 100 nm, as their sizes increase, they tend to be captured and accumulate at the spleen, and their uptake by Kupffer cells in the liver decreases concurrently⁴³.

A semi-quantitative analysis based on the *in vivo* images was conducted to further understand the roles of liver and spleen in the clearance of nanocapsules. The integral fluorescence per unit mass of tumor ($IF_{\text{tumor}}/\text{g}$) was normalized to that of the liver ($IF_{\text{liver}}/\text{g}$) and spleen ($IF_{\text{spleen}}/\text{g}$) to obtain the tumor-to-liver (Fig. 5c) and tumor-to-spleen (Fig. 5d) ratios. Using these ratios rather than absolute values can reduce the inevitable errors from differences between mice, such as different tumor volume, different individual organ mass, and different mice weight, etc.⁴⁴ More importantly, the influence of livers and spleens on the nanocapsule's tumor targeting efficiency can be better understood.

Surprisingly, the tumor-to-spleen ratios are nearly 10-fold smaller than the tumor-to-liver ratios, which means our silica nanocapsules are much more susceptible to the clearance by spleen than by liver, if do not consider their mass difference. There is no significant difference between the tumor-to-liver ratios of all the four groups except the last time point of 72 h with a higher ratio of the two soft nanocapsules compared to the two stiff groups. However, as for the tumor-to-spleen ratios, the two soft nanocapsule groups show much higher ratios than the two stiff groups, indicating that soft nanocapsules accumulates less at the spleens. Functionalizing the soft nanocapsules with the targeting ligand FA didn't significantly enhance their tumor accumulation, which corresponds well with the *in vitro* results. Comparing the two stiff nanocapsule groups, the FA-PEG-modified nanocapsules demonstrated a higher accumulation at 24 h for both the tumor-to-liver and tumor-to-spleen ratios. The distinct fates of the stiff and soft nanocapsules in spleens are likely due to their interactions with the splenic sinuses. The anatomical features of splenic sinuses are cavities in the splenic red pulp that are lined up by non-continuous endothelial cells.⁴³ The narrow slits between the endothelial cells allow the spleen to sieve the red blood cells in blood circulation. Healthy erythrocytes in splenic cords are able to squeeze across the slits of

splenic sinuses and return to the blood circulation, while aged and diseased erythrocytes that have lost their deformability are retained in the splenic red pulp and destroyed eventually. This mechanism has also been reported involved in the splenic sequestration of NPs, especially for those with a size larger than 200 nm.⁴³

In combination with previous findings, the stiff nanocapsules have greater specific receptor-mediated cellular uptake when directly exposed to cultured cells and tumor spheroids. However, they also show higher macrophage capture and splenic sequestration, which ultimately led to a compromised *in vivo* tumor targeting efficiency. In comparison, the soft nanocapsules show the ability of overcoming biological barriers such as intercellular spaces and the splenic sinusoid slits, thus displaying higher tumor accumulation in the mouse model. These findings may partially explain why most currently commercialized nanomedicines are based on soft materials (e.g., liposomes, albumin NPs and polymer micelles).

Figure 5. The effects of nanocapsular stiffness on *in vivo* tumor targeting. (a) *In vivo* images of SKOV3 tumor xenograft bearing mice at different time points after the injection of nanocapsules. The dashed circles indicate the tumor locations; (b) *ex vivo* images of the organs and tumors collected from mice at 24 h after nanocapsule injection; (c) semi-quantitative analysis showing the ratio of integral fluorescence per unit mass (IF/g) between tumor and liver, and (d) tumor and spleen. Values are means $\pm$ SD (n = 3). * $p < 0.05$ as determined by *t*-test.

CONCLUSION

In the current study, silica nanocapsules with very different stiffness were synthesized and modified with PEG and FA-PEG to reveal the effects of NPs' mechanical property on their biological performance, including non-specific and ligand–receptor mediated cellular uptake and *in vivo* passive/active tumor targeting. By using soft and stiff nanocapsules having stiffnesses of 0.04 and 14 N/m (calculated Young's moduli of 700 kPa and 9 GPa), respectively, we demonstrated the increased cellular uptake of the stiff nanocapsules by macrophages via non-specific pathways, and also by tumor cells through a receptor-mediated manner. In parallel, the stiffness of nanocapsules may also affect their ability of passing through the biological barriers. As a consequence, when exposed to 3D tumor spheroids, the stiff nanocapsules tend to accumulate at the marginal areas, whereas the soft ones are likely to pass through the intercellular spaces and penetrate deeper. In the tumor xenograft model, the soft nanocapsules display a higher tumor accumulation efficiency than the stiff ones, while the undesirably high splenic clearance proved to be the dominant obstacle of the tumor targeting of stiff nanocapsules. Overall, this work provides a proof of concept on how NPs' mechanical property modulate their

biological behaviors, especially when there is a coupled effect between the stiffness and targeting ligands. Given the complexity of *in vivo* conditions, the relevance of NPs' mechanical properties upon their drug delivery efficiencies should be completely evaluated in different models. Studies only in a cellular uptake level might lead to misleading conclusions.

In the future design of NP-based drug delivery systems, their mechanical properties ought to be carefully considered and manipulated to generate nanomedicines having both higher tumor targeting and selectivity to the receptors. For example, NPs that have a soft nature in the blood circulation but can be stiffened by certain stimuli when reaching the targeted area to boost their specific cellular association. Additionally, there might be an 'optimal' stiffness of NPs which can result in the highest therapeutic efficacy from the trade-off between a higher cellular association and a better tumor accumulation. These topics might stimulate further investigations and will be of great relevance for nanomedical engineering.

EXPERIMENTAL

Materials

Peptide SurSi was custom-synthesized by GenScript Corporation (Piscataway, NJ, USA) with a purity $\geq 95\%$. Miglyol 812 oil was purchased from Caesar & Loretz GmbH (Hilden, Germany) and passed through heat-activated silica gel (Sigma-Aldrich, Castle Hill, Australia) prior to use. Water having resistivity $>18.2 \text{ M}\Omega\cdot\text{cm}$ was obtained from a Milli-Q system (Millipore, Australia) equipped with a $0.22 \mu\text{m}$ filter. Methoxy poly(ethylene glycol) succinimidyl carbonate ester (mPEG-NHS, MW 5000) and folate poly(ethylene glycol) succinimidyl carbonate ester

(FA-PEG-NHS, MW 5000) were obtained from Nanocs (New York, NY, USA). DiI and DiR stain was purchased from Thermo Fisher Scientific (Scoresby, Australia). Mouse anti-human folate binding protein antibody was obtained from Abcam (Cambridge, UK). Alexa Fluor[®] 488 conjugate goat anti-mouse IgG secondary antibody was purchased from Invitrogen (Carlsbad, CA, USA). Other reagents and chemicals were of analytical grade purchased from Sigma-Aldrich and used as received unless otherwise stated.

Synthesis of silica nanocapsules

The silica nanocapsules were synthesized based on a nanoemulsion-templated and bio-silicification method reported recently by our group.³⁰ First, the nanoemulsion template was prepared using the peptide SurSi as a surfactant. Briefly, a solution of peptide SurSi (400 μ M) and ZnCl₂ (800 μ M) in HEPES buffer (25 mM, pH 7) was prepared. Then 2940 μ L of the peptide solution and 60 μ L of Miglyol 812 oil (2% v/v) was mixed and sonicated using a Sonifier 450 ultrasonicator (Branson Ultrasonics, Danbury, CT) to form nanoemulsion. The nanoemulsion was dialyzed in HEPES buffer (25 mM, pH 7.5) using a dialysis membrane having a cut-off molecular weight of 10 kDa (Thermo Fisher Scientific) for 20 h at 4 °C to remove the excess peptide molecules in the bulk solution. To make oil-core silica-shell nanocapsules, the dialyzed nanoemulsion was diluted by a factor of two. 3 mL of the diluted nanoemulsion was mixed with either triethoxyvinylsilane (TEVS, 80 mM) or tetraethoxysilane (TEOS, 40 mM). The reaction was conducted under magnetic stirring at room temperature for 30, 40 and 50 h to allow the formation of nanocapsules having varied shell thicknesses. The nanocapsule suspensions were then collected and dialyzed in HEPES buffer (25 mM, pH 7.5) at 4 °C for 20 h to stop the reaction and remove any excess chemicals.

Surface modification of silica nanocapsules

To make PEG-modified silica nanocapsules, 3-aminopropyltriethoxysilane was added into the as-prepared nanocapsule suspension at a final concentration of 4 mM and the suspension was stirred for 4 h at room temperature to allow amination of the nanocapsules. After this, mPEG-NHS was added into the suspension at a final concentration of 8 mM, the suspension was then kept stirred for 16 h at 4 °C. The modified nanocapsules were washed twice by centrifugation (14000 rpm, 10 min) and resuspending in PBS. To make FA-PEG-modified nanocapsules, all procedures were kept the same as described above except that the 8 mM mPEG-NHS was replaced by a mixture of 7.2 mM mPEG-NHS and 0.8 mM FA-PEG-NHS.

Characterization.

Dynamic light scattering (DLS). Hydrodynamic sizes and zeta potentials of the nanocapsules were determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at a scattering angle of 173° and a temperature of 25 °C.

Transmission electron microscopy (TEM). Nanocapsule morphologies were observed using a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan) operated at 100 kV. To prepare samples, 2 μ L of the nanocapsule suspensions were placed on Formvar-coated copper grids (ProSciTech, Townsville, Australia) and dried in air. The nanocapsule sizes and shell thicknesses were analyzed using iTEM software (version 3.2, Soft Imaging System GmbH, Münster, Germany).

Atomic force microscopy (AFM). The mechanical properties of silica nanocapsules were measured using a Cypher atomic force microscope (Asylum Research, Santa Barbara, CA,

USA). To prepare AFM specimens, the suspensions of nanocapsules were desalted by dialysis in Milli-Q water for 24 h. Then 20 μL of dialyzed nanocapsule suspensions were placed onto freshly cleaved mica surfaces and air-dried. Cantilevers with a nominal spring constant (k_N) of 4 N/m (Etalon, NT-MDT, Russia) were first calibrated on a clean glass surface and the deviation was within $\pm 10\%$. Imaging and localization of the nanocapsules were completed by scanning the mica surface in tapping mode in air. Force-indentation profiles were obtained by positioning the AFM probe above the centre of individual nanocapsules and pressing the nanocapsules at a velocity of 1 $\mu\text{m/s}$ and a load of 10 nN. Raw force-indentation data were processed using Igor Pro software (v. 6.37). Stiffness values of the nanocapsules were computed as the gradients of the linear part of the approaching force-indentation curves. For every sample, 10 different nanocapsules were analyzed to obtain mean stiffness.

Uptake of nanocapsules by 2D monolayer cells

Cell cultures. RAW264.7 (murine macrophage cells), MCF-7 (human breast adenocarcinoma cells) and SKOV3 cells (human ovarian adenocarcinoma cells) were obtained from American Type Culture Collection (ATCC), and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin (Gibco) at 37 °C in a humidified atmosphere with 5% CO_2 .

Assessment of folate receptor (FR) expression on MCF-7 and SKOV3 cell membranes. Surface FR expression was assessed by indirect immunostaining using a primary antibody and an Alexa Fluor[®] 488 conjugated secondary antibody. Briefly, MCF-7 and SKOV3 cells were collected, washed and resuspended to a density of 1×10^6 cells/mL in ice cold PBS containing 1% BSA

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3 and 0.5% sodium azide (buffer A). The cells were then co-incubated with mouse anti-human
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5 folate binding protein primary antibody (10 $\mu\text{g/mL}$) for 30 min. After this, the cells were washed
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7 three times and resuspended in buffer A and co-incubated with Alexa Fluor[®] 488 conjugate goat
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9 anti-mouse IgG secondary antibody for 30 min. Then the cells were washed three times,
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11 resuspended in 0.5 mL buffer A and their fluorescence intensities were measured using flow
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13 cytometry (Accuri C6 flow cytometer) (BD Biosciences, North Ryde, Australia). The excitation
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15 and emission wavelengths were 496 nm and 519 nm, respectively. Cells incubated with only
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17 secondary antibody were regarded as the control groups.
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22 ***Cellular uptake of silica nanocapsules.*** Cellular uptake of the nanocapsules by RAW264.7,
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24 MCF-7 and SKOV3 cells was studied using flow cytometry. To do this, cells were seeded in 24-
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26 well plates (2×10^5 cells per well) and cultured for 24 h. Cells were then co-incubated with DiI-
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28 loaded silica nanocapsules (at a final nanocapsule concentration of around 3×10^9 capsules/mL,
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30 see see Supplementary Fig. 4) for 4 h. The cells were then trypsinized and washed three times,
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32 and resuspended in 0.5 mL PBS before their fluorescence intensities were measured. Non-treated
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34 cells were regarded as the blank group. For competitive inhibition cellular uptake, cells were
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36 cultured in medium containing free FA (1 mg/mL) for 4 h, and were then co-cultured with both
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38 nanocapsules and free FA (1 mg/mL) for another 4 h.
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44 To study the nanocapsule uptake by Cytochalasin D (CytD) treated SKOV3 cells, the cells were
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46 seeded in 24-well plates (2×10^5 cells per well) and cultured for 8 h to allow attachment. The cells
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48 were then cultured in medium containing 0, 0.01, 0.1 and 1 $\mu\text{g/mL}$ of CytD for 16 h before they
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50 were co-cultured with the nanocapsules for 4 h. The fluorescence intensity of the cells were then
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52 measured. Cells treated with same concentration of CytD but without nanocapsules were
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54 regarded as the control groups.
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Confocal laser scanning microscopy (CLSM). SKOV3 cells at a density of 2×10^5 cells/well were seeded in 24-well plates containing coverslips (diameter of 12 mm) in every well and cultured for 24 h. Then the cells were co-incubated with DiI-loaded nanocapsules for 4 h. After this, the cells were washed with PBS for three times and fixed in cold 4% paraformaldehyde solution for 10 min. To stain the cytoskeleton, the cells were treated sequentially with 0.1% Triton X-100 solution for 5 min and 1% BSA containing PBS for 30 min, and were incubated with Alexa Fluor® 647 conjugated phalloidin for 30 min. This was followed by the nucleus staining using Hoechst 33342. Finally, the coverslips were mounted to glass slides using histology mounting medium and observed using CLSM (Zeiss 710, Jena, Germany).

Uptake of nanocapsules by SKOV3 tumor spheroids

To establish the tumour spheroid models, SKOV3 cells were seeded in 96-well plates (the wells were pre-coated with 50 μ L of 2% low melting point agarose) at a density of 5×10^3 cells/well. The cells were then centrifuged (1000 $\times$ g, 10 min) and cultured for 7 days to form tumor spheroids. The tumor spheroids were co-cultured with DiI-loaded nanocapsules (around 3×10^9 capsules/mL) for 4 h, and were washed 3 times with PBS and fixed in 4% paraformaldehyde before observed using CLSM. To quantitatively assess the nanocapsule uptake, after being co-cultured with nanocapsules, 10 tumour spheroids were gathered and incubated with 200 μ L Accumax solution for 30 min (with pipetting every 5 min) at 37 °C to dissociate the cells. The cells were then washed with PBS for 3 times and their fluorescence intensities were analyzed using flow cytometry.

***In vivo* tumor targeting of nanocapsules**

All animal experiments in this study adhered to the *Australian code for the care and use of animals for scientific purposes* and were approved by University of Queensland Animal Ethics Committee. To establish tumor xenograft models, 32 female Balb/C nude mice at the age of 7 weeks were randomly divided into 4 groups (8 mice per group) and subcutaneously injected with 1×10^7 of SKOV3 cells at their left armpit. When the tumor sizes reached around 0.5 cm in diameter, DiR-loaded nanocapsules were intravenously injected into mice through the tail vein at a dose of 100 μ L (with a DiR concentration of 10 μ L/mL). At time points of 2, 6, 12, 24, 48 and 72 h after injection, mice were imaged using an in vivo MS FX Pro instrument (Bruker Corporation, Billerica, MA). At 6, 12, 24 48 and 72 h after injection, one mouse from each group was euthanized, with their organs (hearts, livers, spleens, lungs and kidneys) and tumors collected, imaged and weighed. At the end of experiments, all mice was euthanized, and their organs and tumors were weighted for semi-quantitative analysis. Imaging processing was conducted using image-J software FIJI (v1.51 n).

Statistics

Statistics were computed with Microsoft Excel 2013. Standard unpaired *t*-test was used to test for statistical significance between groups, with *p* value ≥ 0.05 , < 0.05 , < 0.01 and < 0.001 denoted as N.S., *, **, and ***, respectively.

CONFLICTS OF INTEREST

The University of Queensland (UQ) filed a patent on the use of mineralizing peptides for making silica nanocapsules. A.P.J.M., C.-X.Z., and D.W. are named inventors on this patent and through

1
2
3 their employment with UQ hold an indirect interest in this intellectual property. The other author
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5 declares no conflict of interest.
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40 **SUPPORTING INFORMATION**

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43 Supporting information is available free of charge via the internet at <http://pubs.acs.org>.
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Graphical Table of Contents