Supporting Information for

Decoupling the Effects of the Size, Wall Thickness and Porosity of Curcumin-loaded Chitosan Nanocapsules on their Anticancer Efficacy: Size is the Winner

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EXPERIMENTAL SECTION

The experimental aspect of this work in regards to synthesis of seven different type of chitosan nanocapsules is summarised in **Figure S1**.

Preparation of Solid Silica Particles

Based on a modified version of the Stöber method,[1] the solid core (SC) silica spheres were prepared in appropriate amounts of ethanol, deionized water (MilliQ) and ammonia solution (28%) as per **Table S1**, and the solutions were mixed well before heating to the required temperature. After the solution temperature had stabilized, 75 µL of tetraethoxysilane (TEOS) was added and the solution was mixed for 4 seconds. The solutions were then kept very still and held at their appropriate temperatures while the reaction took place over a 1 hour period.

Preparation of SC/MS Silica Templates

To grow the mesoporous shell (MS) of specified thickness and porosity on the solid cores, solutions containing mixtures of TEOS and n-octadecyltrimethoxysilane (TMS) were slowly added to the original solutions containing solid core silica particles over a period of 20 min at 25 °C under shaking conditions (**Table S2** for the TEOS and TMS volumes). Note that there are significant variations in the total volume of the TEOS/TMS solutions, as well as the ratios of TEOS:TMS that have been used. The solutions were then incubated under shaking at 25 °C for 1 hour, followed by centrifugation and washed three times with ethanol to obtain the precipitated powder. This powder was heated to 550 °C for 6 h to remove the porogen TMS through calcination, this resulted in the SC/MS silica particle templates.

Preparation of Chitosan Nanocapsules

Chitosan capsules were prepared by dispersing 5 mg SC/MS template particles in 450 µL of deionized water, followed by overnight incubation after adding 1 mL aqueous solution containing 4 mg low molecular weight chitosan (Sigma Aldrich – dissolved using 0.8% acetic acid) in pH 7.2 phosphate buffer saline (PBS). Following overnight incubation, the chitosan-infiltrated SC/MS particles were obtained by centrifugation, and redispersed in 800 µL PBS and 50 µL glutaric dialdehyde for 2 h to cross-link the chitosan within the mesoporous shell of SC/MS particles (**Figure S1**). After washing three times with deionized water, the SC/MS silica template was removed by hydrofluoric acid (2M) treatment in an ammonium hydroxide buffer (8M), followed by centrifugation and repetitive washing with deionized water (Warning: hydrofluoric acid is highly corrosive, and extreme care should be taken while handling hydrofluoric acid).

Nanomaterial Characterization

The obtained SC/MS templates and chitosan nanocapsules were characterized by electron microscopy (SEM and TEM). The average diameter of the SC/MS templates and chitosan nanocapsules were measured from the representative electron micrograph images using at least 100 particles of each type, and standard deviation was calculated. The surface area and pore diameter of the SC/MS templates were measured using the BET technique on a Micromeritics ASAP 2000 surface analyser.[2] The thickness of the capsule wall was determined from TEM images of ultramicrotomed sections of capsules embedded within the epoxy resin. The degree of chitosan loading within the mesoporous shell of SC/MS silica template was determined by thermogravimetric analysis (TGA) using Perkin Elmer Pyris 1 TGA instrument, wherein the change in weight was detected as the polymer burned off in air

between 110-600 ºC, with at a temperature ramping rate of 20 ºC/min and an air flow rate of 20 mL/min.

Loading and Quantification of Curcumin in Chitosan Nanocapsules

Chitosan nanocapsules (prepared from the equivalent of 5 mg of template material) were loaded with the lipophilic drug curcumin. This was achieved by initially dehydrating the capsules with ethanol through a quick ethanol wash, following which most of the ethanol was aspirated off before allowing the remainder ethanol to evaporate off at room temperature. The capsules were then dispersed in 1 mL of curcumin/oleic acid mixture (10 µg curcumin per mL of oleic acid), and the oil phase was allowed to infiltrate through the porous walls and fill the capsules over a period of 12 h on a rotary shaker, as has been previously demonstrated for the loading of lipophilic chemotherapeutic drugs doxorubicin and 5-fluorouracil into poly(methacrylic acid) capsules.[3] Following incubation, the curcumin-loaded chitosan nanocapsules were obtained by centrifugation and washed thrice with hexane to remove any non-infiltrated curcumin/oleic acid. After residual hexane was allowed to evaporate off, these curcumin-filled chitosan capsules were found to make stable dispersions on addition to water and biological buffers. It should be noted that the hexane washing step was found crucial to obtain capsules that could readily disperse in the aqueous media.

The high solubility of curcumin in absolute ethanol was exploited to determine the curcumin loading within different types of chitosan nanocapsules. This was performed by exposing the drug-infiltrated capsules three subsequent times to 1 mL each of absolute ethanol during precipitation-supernatant removal cycles. This resulted in the release of all the curcumin internalized within chitosan capsules to the 3 mL ethanolic supernatant, which was quantified by making standard curves of fixed amount of curcumin dispersed in ethanol using a Cary 50

Bio UV-Vis spectrophotometer at absorbance maxima of curcumin at 430 nm. All the experiments involving curcumin infiltration and release were conducted in triplicates to obtain statistically significant results and standard errors of means plotted.

Cellular Uptake and Cytotoxicity of Curcumin-loaded Chitosan Nanocapsules

P815 mouse mastocytoma cells (obtained from Manassas, VA, USA) were used to study both the uptake and the toxicity of the chitosan nanocapsules. The cells were cultured in RPMI-1640 media (Sigma) containing 10% FBS and supplemented with gentamycin, glucose, pyruvate, 2-mecaptoethanol and L-glutamine. The cells were maintained in a humidified incubator at 37 °C and 5% CO_2. For cell uptake studies, 10^5 P815 cells were exposed to 10^8 chitosan capsules for 24 h, followed by cell harvesting, washing three times with PBS , and seeding onto glass slides for imaging by confocal laser scanning microscopy (CLSM; Nikon A1 Confocal Microscope). As the glutaraldehyde cross-linking step during chitosan nanocapsules makes them highly fluorescent, fluorescent dye labeling was not required for CLSM studies.

To determine the efficiency of curcumin-infiltrated chitosan capsules on cell mortality of P815 mouse mastocytoma cells, 10^5 cells were exposed to different numbers (final concentration ranging from 10^5 to 10^8 capsules per treatment) of seven different types of capsules for 24 h in 96-well plates under appropriate growth conditions as explained in the previous section. This was followed by determination of cellular viability using a soluble tetrazolium-based colorimetric assay (Promega MTS CellTiter 96® aqueous kit) and measuring the absorbance at 490 nm using a microplate reader (Perkin Elmer, USA). Same experiments were performed on pristine chitosan capsules, which served as a control. Since pristine capsules did not show significant cytotoxicity, for brevity, only the mean cytotoxicity of seven different types of pristine chitosan capsules used in this study has been shown. The

cytotoxicity studies of curcumin dispersed in water was also performed for comparison. Each treatment was performed in quadruplicate and repeated three times, which means that the data presented here is an average of 12 experiments, with standard error of mean shown as error bars.

References

(1) Stöber, W.; Fink, A.; Bohn, E. *Journal of Colloid and Interface Science* **1968**, *26*, 62.

(2) Brunauer, S.; Emmett, P. H.; Teller, E. *Journal of American Chemical Society* **1938**, *60*, 309.

(3) Sivakumar, S.; Bansal, V.; Cortez, C.; Chong, S.-F.; Zelikin, A., N. ; Caruso, F. *Advanced Materials* **2009**, *21*, 1820.

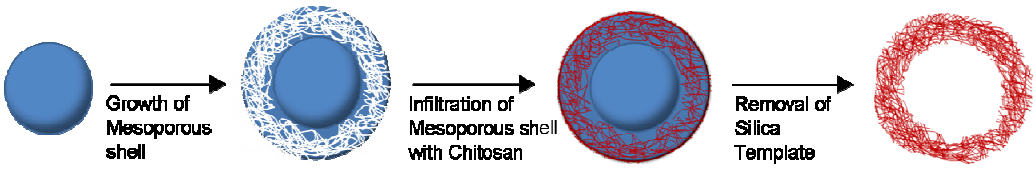

Figure S1-A. *A schematic representation of the steps to fabricate chitosan nanocapsules.*

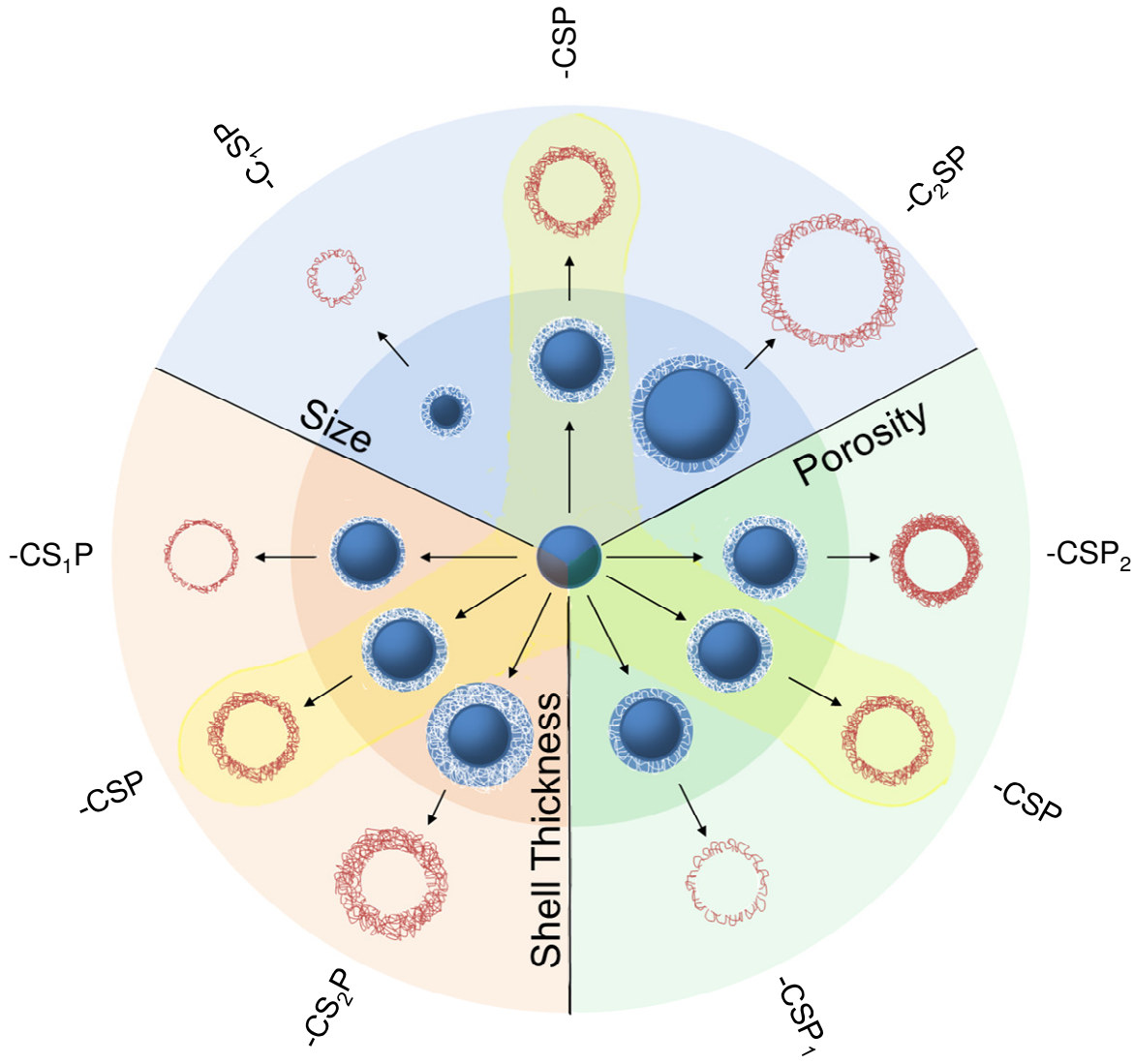

Figure S1-B. *Schematic representation of the solid core/ mesoporous shell (SC/MS) approach employed to fabricate seven different types of chitosan nanocapsules with different structural features, with one common type of SC/MS template in each category (highlighted by an arching triangle).*

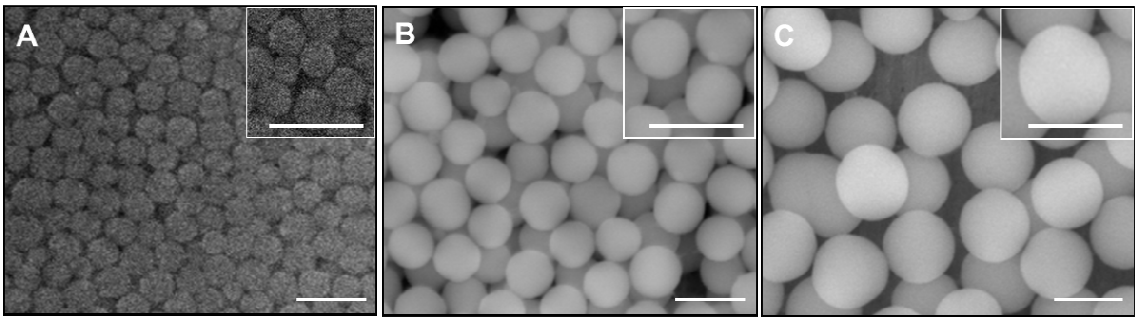

Figure S2. *SEM images of different sizes of solid silica cores, corresponding to (A) TempC$_1$, (B) TempC, and (C) TempC$_2$. These solid cores were then employed to fabricate the solid core/mesoporous shell (SC/MS) silica templates shown later in Figure S3. Scale bars correspond to 500 nm.*

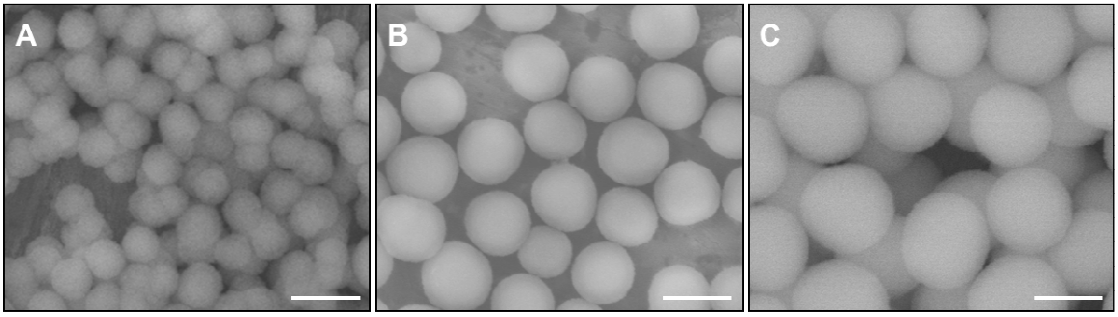

Figure S3. *SEM images of SC/MS silica template particles, corresponding to (A) TempC$_1$SP, (B) TempCSP and (C) TempC$_2$SP. These were fabricated using different sizes of solid silica cores shown in Figure S2. Scale bars correspond to 500 nm.*

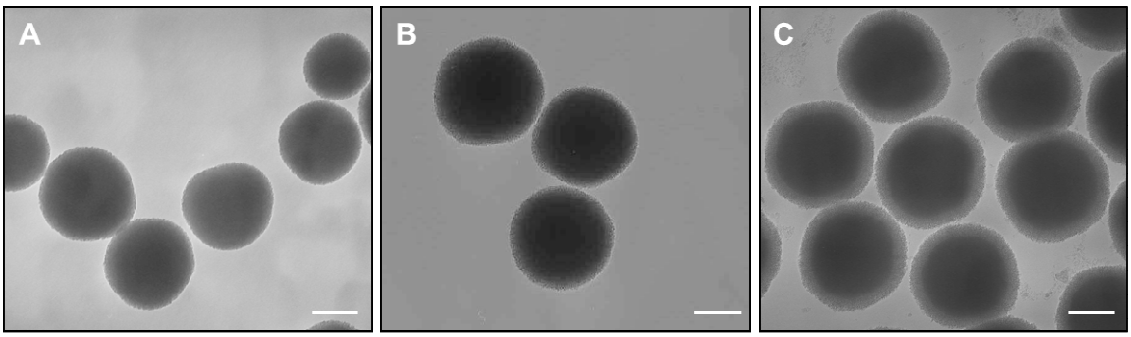

Figure S4. *TEM images of solid core/mesoporous shell (SC/MS) silica template particles, consisting of the same core diameter of 290 nm but different mesoporous shell thickness of (A) 31 nm, (B) 45 nm, and (C) 55 nm. These correspond to (A) TempCS$_1$P, (B) TempCSP and (C) TempCS$_2$P. Scale bars correspond to 200 nm.*

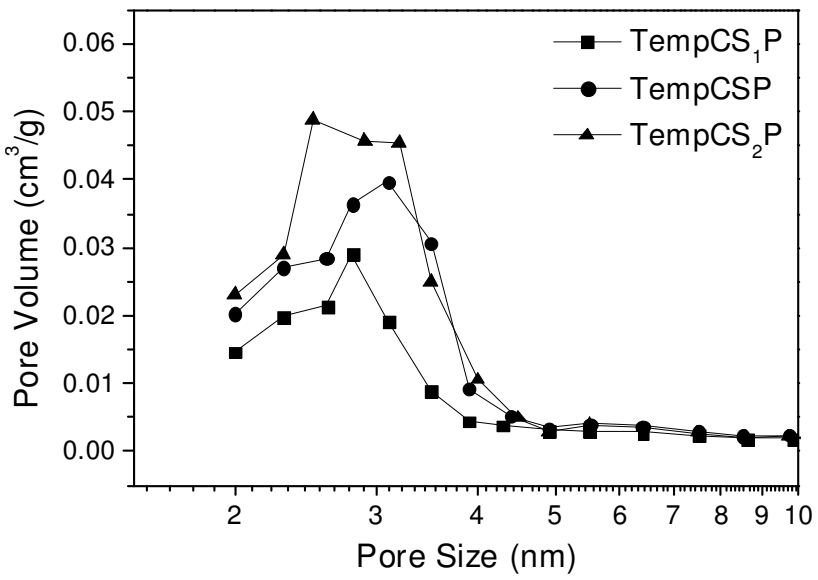

Figure S5. *BET-BJH pore distribution with regards to the templates of various shell thickness.*

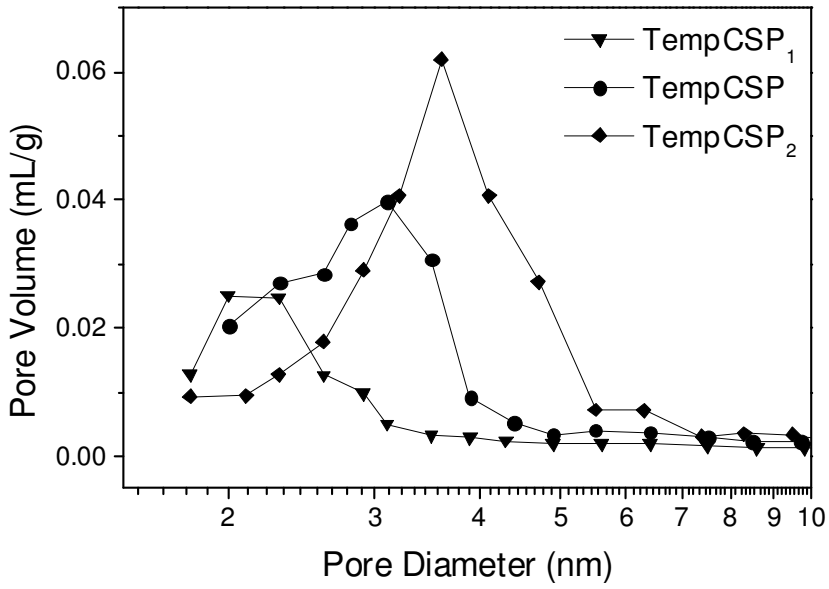

Figure S6. *BET-BJH pore distribution with regards to the templates of various porosities.*

Template Type	Ethanol (µL)	Water (µL)	Ammonia (µL)	Temperature (°C)	Core Size (nm)
$TempC_1$	1000	135	100	18	170 ± 12
TempC	1000	135	113	25	290 ± 24
$TempC_3$	1000	135	133	30	490 ± 490

Table S1. *Experimental conditions for the synthesis of the solid cores of the SC/MS silica template particles.*

Template Type	TEOS (µL)	TMS (µL)	Shell Thickness (nm)
TempCSP	65	13	46 ± 7
$TempC_1SP$	125	25	49 ± 16
$TempC_2SP$	5	1	42 ± 13
$TempCS_1P$	25	5	31 ± 21
$TempCS_2P$	135	27	55 ± 12
$TempCSP_1$	70	7	42 ± 9
$TempCSP_2$	50	25	48 ± 14

Table S2. *Experimental conditions for the synthesis of the mesoporous shell of the SC/MS silica template particles.*

Template	Description	Core Size (nm)	MP Shell Thickness (nm)	Surface Area (m^2 g^{-1})	Pore Size in Shell (nm)	Pore Volume (mL g^{-1})
	Solid Cores					
$TempC_1$	Small diameter	170 ± 12	-	25.86	-	-
TempC	Medium Diameter	290 ± 24	-	14.37	-	-
$TempC_2$	Large Diameter	490 ± 14	-	8.50	-	-
	Solid Cores, Mesoporous Shell					
TempCSP	Medium Diameter, Medium Shell Thickness, Medium Pores Size	290 ± 24	46 ± 7	252.6	3.68	0.27
$TempC_1SP$	Small diameter, Medium Shell Thickness, Medium Pore size	170 ± 12	49 ± 16	300.0	5.12	0.44
$TempC_2SP$	Large Diameter, Medium Shell Thickness, Medium Pore Size	490 ± 14	42 ± 13	164.2	3.30	0.16
$TempCS_1P$	Medium Diameter, Small Shell Thickness, Medium Pores Size	290 ± 24	31 ± 21	144.1	3.83	0.16
$TempCS_2P$	Medium Diameter, Large Shell Thickness, Medium Pores Size	290 ± 24	55 ± 12	308.7	3.33	0.30
$TempCSP_1$	Medium Diameter, Medium Shell Thickness, Small Pores Size	290 ± 24	42 ± 9	184.0	2.90	0.13
$TempCSP_2$	Medium Diameter, Medium Shell Thickness, Large Pores Size	290 ± 24	48 ± 14	253.1	4.85	0.33

Table S3. *A tabular summary of the structural characteristics of the SC/MS silica template particles.*

Capsule Type	Description	Capsule Diameter (nm)	Shell Wall (nm)	Chitosan Loading (mg g-1)	Curcumin Loading (fg/capsule)
CapCSP	Medium Diameter, Medium Wall Thickness, Medium Porosity	270 ± 14	45 ± 4	76.35	2.155
$CapC_1SP$	Small Diameter, Medium Shell Thickness, Medium Porosity	220 ± 19	47 ±5	97.73	0.163
$CapC_2SP$	Large Diameter, Medium Wall Thickness, Medium Porosity	440 ± 37	40 ± 5	73.36	23.492
$CapCS_1P$	Medium Diameter, Thin Wall Thickness, Medium Porosity	235 ± 21	28 ± 4	65.84	0.748
$CapCS_2P$	Medium Diameter, Thick Wall Thickness, Medium Porosity	325 ± 12	56 ± 5	83.25	6.870
$CapCSP_1$	Medium Diameter, Medium Wall Thickness, High Porosity	269 ± 24	40 ± 3	47.11	0.851
$CapCSP_2$	Medium Diameter, Medium Wall Thickness, Low Porosity	283 ±26	42 ± 4	81.26	4.507

Table S4. *A tabular summary of the structural characteristics and loading capabilities of the chitosan nanocapsules.*