Electronic Supplementary Information

Inflammation-responsive nanocapsules for the dual-release of antibacterial drugs

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1. Materials and bacteria

Tetraethyl orthosilicate (TEOS, Acros Organics, 98%), chloroform ($CHCl_3$ Carlo Erba, 99%), dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (TPOAc, ACROS, 60%), sodium dodecyl sulfate (SDS, Acros, 99%), sodium oleate (SO, TCI, > 97%), hexadecane (HD, Acros Organics, 99%), phosphate buffered saline (PBS, Sigma Aldrich), amoxicillin trihydrate (AC, TCI, 98%), tetracycline hydrochloride (TC, TCI, 98%), calcium chloride ($CaCl_2$, Carlo Erba, 90%) hydrochloric acid (HCl, Carlo Erba, 37%), di(ethylene glycol) diacrylate (DEGDA, Sigma Aldrich, 75%), ammonium persulfate (APS, Carlo Erba, 98%), *N,N,N',N'*-tetramethyl ethylenediamine (TEMED, TCI, 98%), and acrylamide (AAM, Acros, 98.5%) were used as received. Deionized water was used through all the experiments. *Staphylococcus aureus* (DMST 8013) and *Escherichia coli* (DMST 4212) were purchased from the Department of Medical Sciences, Ministry of Public Health (Nonthaburi, Thailand). *Staphylococcus aureus* MRSA (ATCC 43300) and mouse fibroblast cells (L929) were purchased from American Type Culture Collection (ATCC, Virginia, America).

2. Hydrophobic ion pair with tetracycline

20 mL of a 2.2wt% aqueous solution of TC and 10 mL of a 2.2wt% aqueous solution of $CaCl_2$ were mixed and stirred for 15 min. 25 mL of a 2.4wt% aqueous solution of SO or SDS was then added and stirred for 3 h. The product was centrifuged and extracted three times with water to remove the non-complexed substances. Finally, the collected product was lyophilized for 3 days and stored at 10 °C in the dark for subsequent experiments (yield ~ 63%).

3. Entrapment of HIP complexes in silica nanocapsules (SiO_2NCs)

2 g TEOS and 125 mg HD were dissolved in 1 g $CHCl_3$ containing 0, 23.5 or 47 mg of drug complex (TC-O or TC-DS). Then, 30 mL of a 0.38wt% aqueous solution of TPOAc containing 0, 8.5, or 17 mg AC was added. The mixture was subsequently stirred at 540 rpm for 10 min and processed by ultrasonication (3 min, 50% amplitude in a pulsed regime with 3 s on and 1 s off, power output 66 W) under ice cooling. The resulting miniemulsions were stirred for 20 h at 25 °C and then further stirred for 6 h at 40 °C to obtain silica nanocapsules. For the determination of the encapsulation efficiency (EE), the dispersion of SiO_2NCs were centrifuged at 4 °C and 44,800 rcf for 45 min. Then, the contents of TC and AC in the

supernatants were measured by UV-vis spectroscopy at 360 and 225 nm, respectively (see Figure S7).

4. Release of drugs from SiO_2NCs

1 mL of the dispersions (total content of drugs = 500 μg) were placed in dialysis bags (MWCO = 3500 g·mol^{-1}) and immersed in 10 mL of PBS at pH 7.4 or 5.5 while shaking at 100 rpm at 37 °C. 10 mL of the release media were withdrawn at specific intervals (15, 30, 60, 180, 360, 540, 720, 1,440 and 2,880 min) and replaced by 10 mL of fresh media. The concentrations of TC and AC in the withdrawn samples were then measured by UV-vis spectrophotometry at 360 and 225 nm, respectively. The calibration curves are shown in Figure S7, Figure S8, and Figure S4. Release profiles were fitted with the Korsmeyer-Peppas model:[1]

$\log M_t/M_\infty = n\log t + \log k$. M_t and M_∞ are the cumulative amounts of drugs at time t and infinite time respectively. k is the rate constant of drug release and n is the diffusion exponent. A parameter value $n \leq 0.5$ corresponds to Fickian diffusion while $0.5 < n < 1$ indicates anomalous diffusion.

5. Zone of inhibition (ZOI)

The antibacterial potential was performed by the Kirby-Bauer disk diffusion method.[2] *Escherichia coli*, *Staphylococcus aureus* and MRSA were cultured on nutrient agar before the experiment and incubated at 37 ℃ for 18 to 24 h to activate the bacteria. Then, bacterial inocula were prepared by inoculating bacteria into sterile DI water. The concentrations of bacteria were adjusted to $1 \cdot 10^8$ CFU/mL by comparing their turbidity with McFarland No. 0.5. Afterwards, 100 μl of each bacteria dispersion was spread onto the surface of tryptic soy agar (TSA) (Merck, Germany). 20 μL of SiO_2NCs dispesions containing 10 μg drugs was added to a sensitivity disk, which was impregnated onto the surfaces of the TSA. The plates were then incubated at 37 °C for 24 h and the inhibition zones were measured.[3] The experiments were carried out in triplicate. For the investigation with MRSA, 20 μL of the release media of dialyzed SiO_2NCs_TC-O/AC dispersions at pH 7.4 or 5.5 at various time intervals (1, 3, 6, 9, 12, 24 h) was dropped on sensitivity disks, which were then permeated onto TSA.

6. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

MIC and MBC of SiO_2NCs were determined by the micro-dilution method according to a procedure of the Clinical and Laboratory Standards Institute (CLSI).[4] *Escherichia coli*, *Staphylococcus aureus,* and MRSA were cultured on nutrient agar and incubated at 37 ℃ for 18 to 24 h for activating the bacteria. Then, bacteria were inoculated into sterile DI water so that the final concentrations of the bacterial inocula were adjusted to $1{\cdot}10^6$ CFU/mL. The SiO_2NCs dispersions were diluted to several concentrations (500 to 2 μg/mL) with fresh tryptic soy broth (TSB). Then 100 μL of the diluted SiO_2NCs dispersions was added into a 96-well plate. Afterwards, 100 μL of bacterial inocula was mixed into each well and the plates were covered to prevent dehydration of bacteria and incubated at 37 ℃ for 24 h. Positive and negative controls were TSB with and without bacteria, respectively. The MIC value was set as the minimum concentration inhibiting bacteria growth.[5] For MBC, all wells showing no growth were dropped on nutrient agar and incubated at 37 ℃ for 24 h. The MBC value was set as the minimum concentration which could kill bacteria and showed no bacterial colony on agar. The experiments were carried out in triplicate.

7. *In vitro* cytotoxicity tests

Mouse fibroblast L929 cells were cultured at 37 ℃ in a 5% CO_2 humidified environment in MEM medium supplemented with 10vol% and 1vol% fetal bovine serum and penicillin-streptomycin, respectively. After 24-h of incubation, cells were washed with sterile PBS two times and the attached cells were eluted with 5% trypsin-ethylenediaminetetraacetic acid (EDTA) at 37 ℃. Then, eluted cells were centrifuged at 1500 rpm at 4 °C. Cells were resuspended in MEM (minimum essential medium) and the cell's concentrations were determined by the trypan blue exclusion method.

The cytotoxicity test was carried out following the ISO 10993-5 (2009) protocol.[6] The cytotoxic effects with normal cell line (L929) were tested with SiO_2NCs dispersion (7.8-4000 μg/ml). First, all samples were dispersed in MEM in an incubator shaker at 37 ℃ for 24 h. Then, extracted samples were withdrawn and tested against L929 cells. Before testing, L929 ($1.0{\cdot}10^4$) cells were added into 96 well plates and incubated for 24 h. Then, cells were washed with sterile PBS three times and treated with different concentrations of the extracted samples and incubated for 24 h. After incubation, the culture media were removed and 110

μl MTT solutions (2 mg/mL in serum free culture medium) were added to each well. Plates were incubated at 37 °C for 2 h and then MTT solutions were gently removed from the wells. 100 μl of isopropanol was added and mixed by swaying the plates. The absorbance of samples was measured at 570 nm using a microplate reader. Cell viability was calculated as the ratio between absorbance of cells treated with samples and absorbance of untreated cells.

8. Preparation of the hydrogels

AAM (28 mg, 0.4 mmol), DEGDA (2 mg, 9 μmol), and APS (40 mg, 0.18 mmol) were dissolved either in 0.2 mL water (0.2 mL) or SiO_2NCs dispersion. AAM (28 mg, 0.4 mmol), DEGDA (2 mg, 9 μmol), TEMED (40 mg, 0.34 mmol) were separately dissolved in 0.2 mL water (0.2 mL) or SiO_2NCs dispersion. The solutions were simultaneously injected in plastic circle molds (inner diameter = 1 cm). Formation of hydrogels was observed within 1 min.

9. Antibacterial activity of the hydrogels

Staphylococcus aureus MRSA were inoculated in 5 mL of TSB and incubated at 37 ℃ for 24 h to prepare a bacterial inoculum ($1 \cdot 10^8$ CFU/mL). Then, 10 μL of the inoculum was added into the new tubes containing 5 ml fresh TSB at pH 5.5 or 7.4. Afterwards, the gels were added into the inoculated tubes and incubated at 37 ℃ for 24 h. The incubated media (100 μL) was cultured on agar at 37 ℃ for 24 h and then the number of colonies on agar was counted.

10. Characterization techniques

Infrared spectra of dried HIP complexes were recorded by attenuated total reflection fourier transform infrared spectroscopy (ATR-FTIR, PerkinElmer Frontier FTIR, Universal-ATR) between 500 and 4000 cm^{-1} with a resolution of 4 cm^{-1}. The amount of TC in the HIP complex was measured by UV-Vis spectroscopy (PerkinElmer, Lambda 25) at 360 and 365 nm in PBS and DMSO, respectively. Average hydrodynamic diameters and size distribution of nanocapsules were measured at 25 °C by dynamic light scattering (DLS, Micromeritics, NanoPlus-3) at an angle of 90° and a laser wavelength of 660 nm with diluted dispersions (dilution factor = 20). The zeta potential of SiO_2NCs was determined at 25 °C by electrophoretic light scattering (ELS, Micromeritics, NanoPlus-3) with diluted dispersions (20 dilution factor). N_2 adsorption-desorption isotherm and pore size distributions were acquired

on a BET surface analyzer (MicrotracBEL, BELSORP-mini II). The measurements were performed under a nitrogen atmosphere and maintained at 63−77 K. Before measurements, the samples were calcinated at 400 °C for 5 h with a rate of 4 °C min^{-1} and degassed in vacuum at 100 °C for 24 h. The specific areas and mean pore diameters were determined by the standard BET equation at the relative pressure (p/p_0) of N_2 isotherms. Pore sizes distribution of the samples was calculated from the N_2 adsorption curve by the Barrett-Joyner-Halenda (BJH) method with the corrected Kelvin equation. The solid content of SiO_2NCs was measured by gravimetry after freeze-drying the dispersion for 5 days. Kinetic of SiO_2NCs reaction was studied by an AVANCE III HD console from 600 MHz Bruker and chemical shifts (δ) are expressed in ppm. The 600 μL of SiO_2NCs dispersion in D_2O was taken at 0.25, 1, 2, 3, 4, 5, 6, 20 h and measured by ^1H NMR spectroscopy. Isothermal titration calorimetry (ITC) experiments were carried out on Malvern, MicroCal PEAQ-ITC. Titrations of SiO_2NCs dispersions were operated by 19 injections of 2 μL of an AC aqueous solution (11 mM) into the calorimeter cell containing SiO_2NCs dispersion (3 g/L). Titration of TC in an $CaCl_2$ aqueous solution (0.226 μM) was operated by 19 injections of 2 μL of a sodium oleate aqueous solution (2.26 μM) into an isothermal titration calorimeter. The raw data from ITC were analyzed using MicroCal ITC analysis software version 1.0 with a 'one set of binding sites' model. TEM images were acquired on a transmission electron microscope (TEM, JEOL, JEM-ARM200F, Tokyo, Japan). Scanning electron microscopy (SEM) studies were done on a field emission microscope (SEM, JEOL, JSM-7610F, Tokyo, Japan) working at an accelerating voltage at 1.5 kV. Diluted SiO_2NCs dispersions were placed on silicon wafers and copper grids for SEM and TEM, respectively.

11. Supporting figures

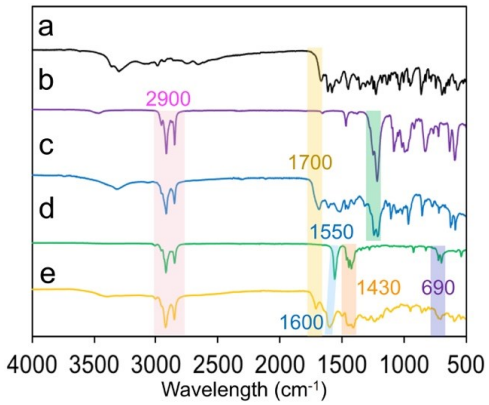


Figure S1. IR spectra of (a) TC, (b) DS, (c) TC-DS, (d) sodium oleate, and (e) TC-O.

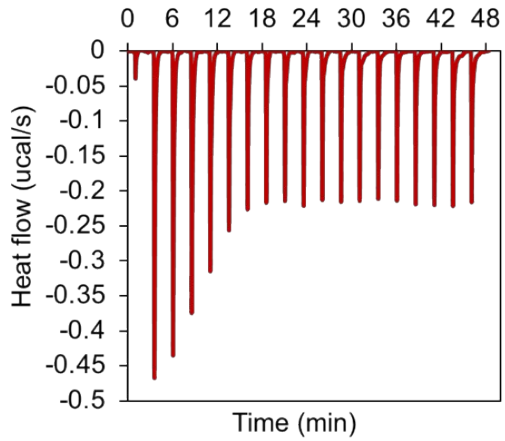


Figure S2. Heat flow for the titration of TC with sodium oleate measured by isothermal titration calorimetry.

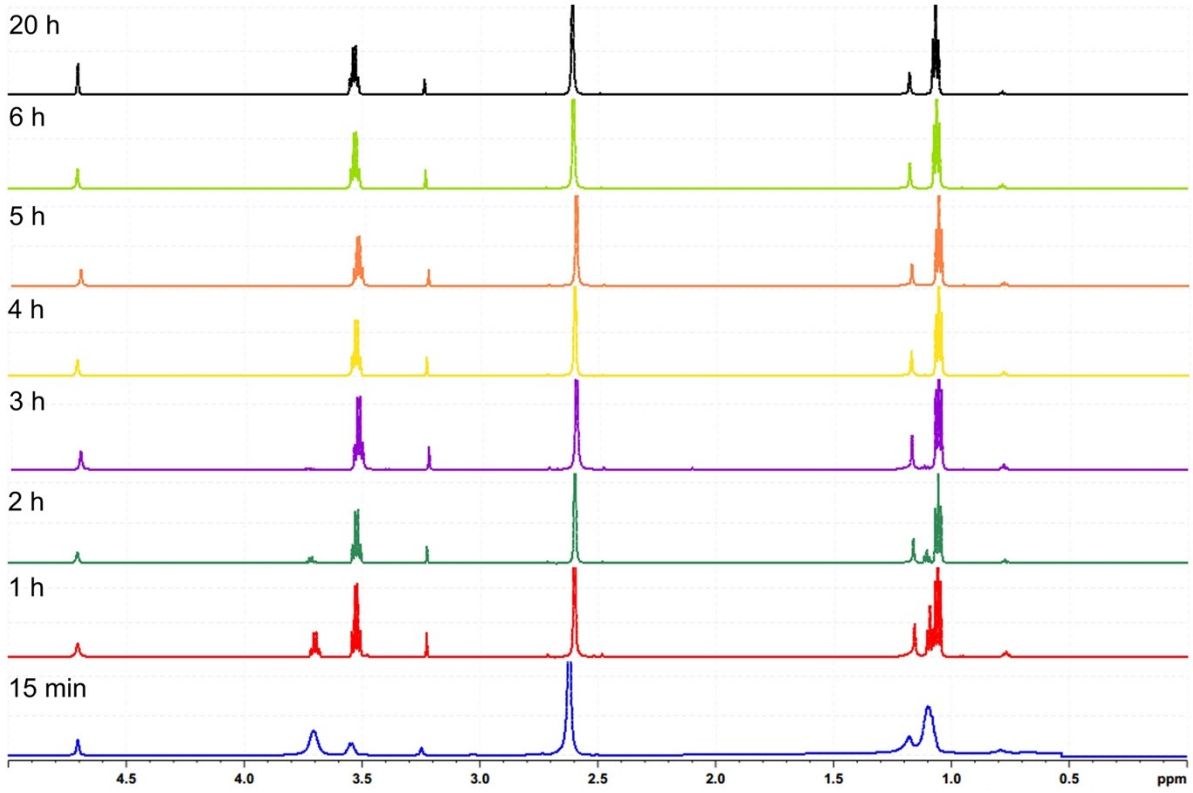


Figure S3. Temporal evolution of ^{1}H-NMR spectra showing the hydrolysis of TEOS and TPOAC measured in D_2O.

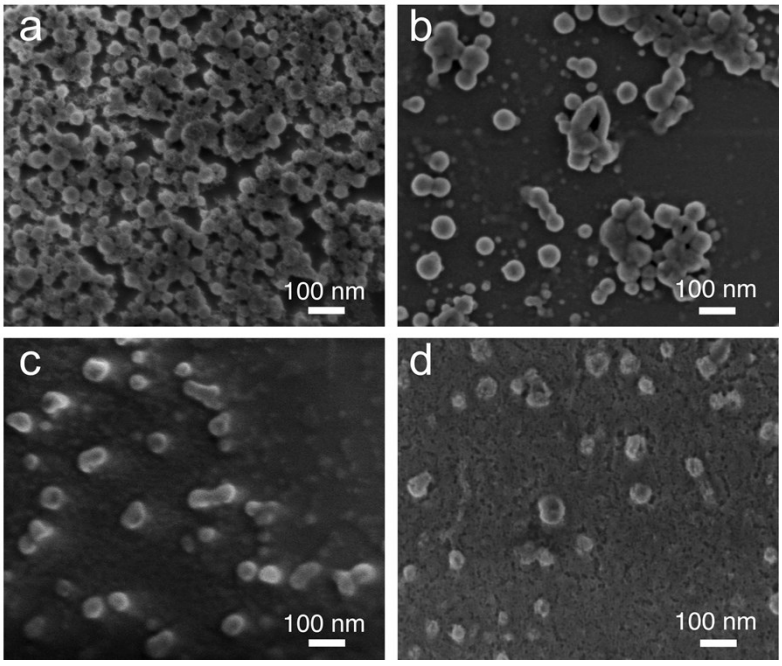


Figure S4. SEM micrographs of (a) SiO_2NCs, (b) SiO_2NCs_TC-O, (c) SiO_2NCs_AC, and (d) SiO_2NCs_TC-O/AC.

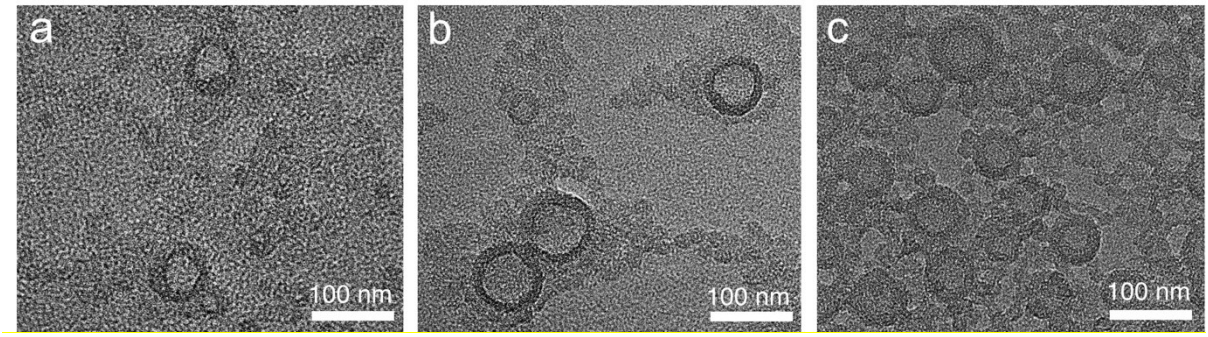


Figure S5. TEM micrographs of (a) SiO_2NCs, (b) SiO_2NCs_TC-O, and (c) SiO_2NCs_AC.

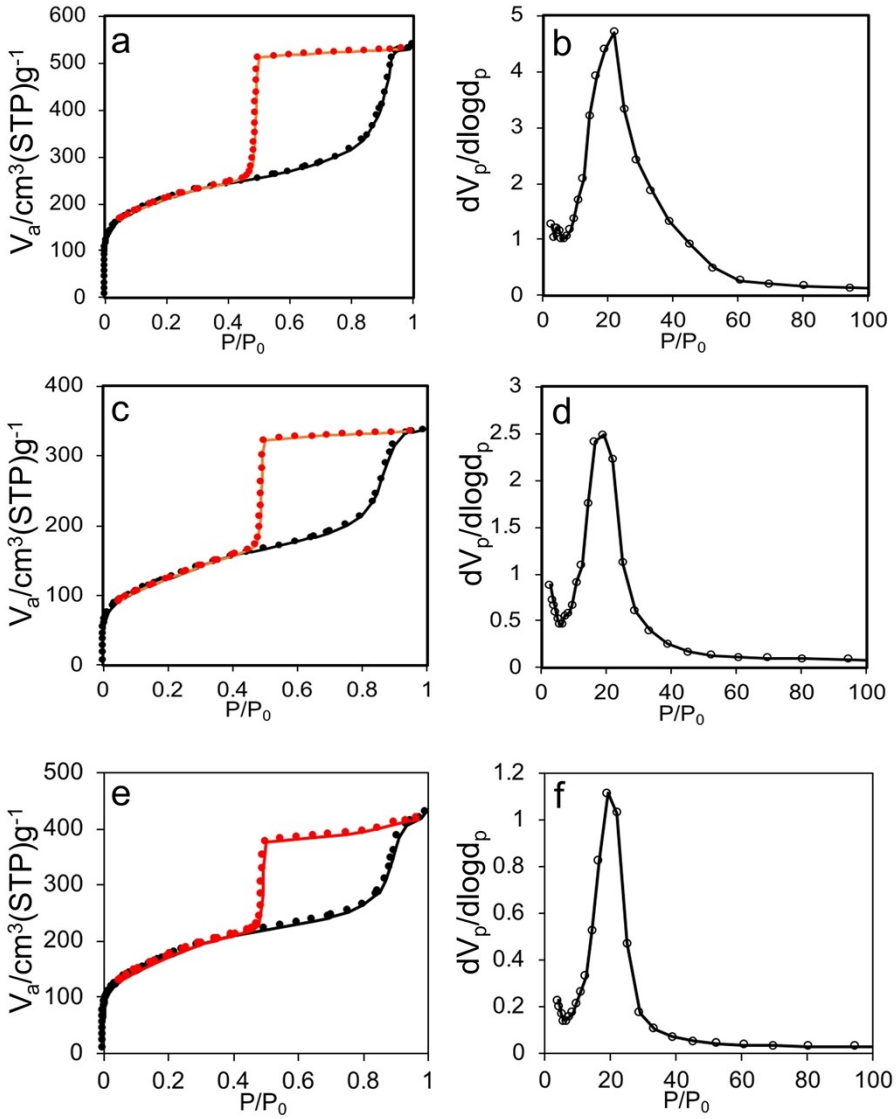


Figure S6. N_2 adsorption/desorption isotherms and pore size distribution of (a) SiO_2NCs, (b) SiO_2NCs_TC-O, and (c) SiO_2NCs_AC.

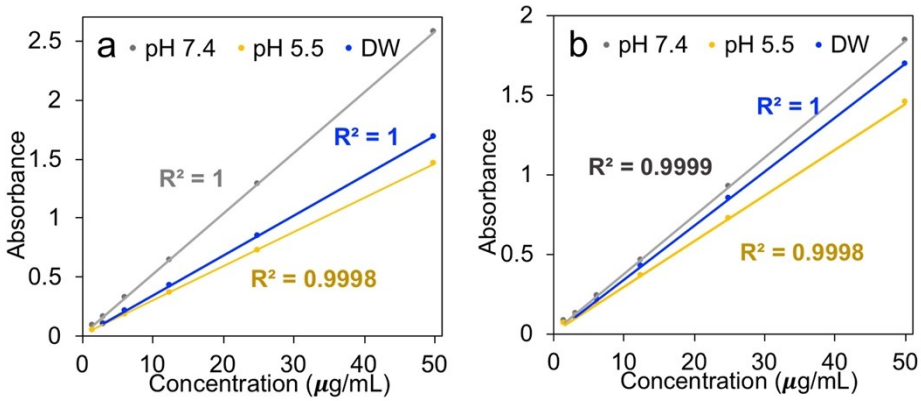


Figure S7. Calibration curves for UV-Vis spectroscopy measurements of (a) TC, (b) AC at pH 7.4, 5.5, and deionized water (DW). The absorbance of TC and AC was measured at 360 and 225 nm, respectively.

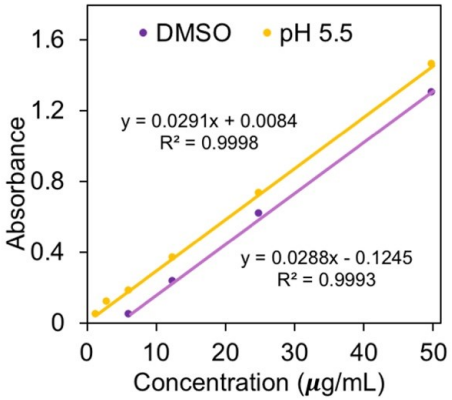


Figure S8. Calibration curves for UV-Vis spectroscopy measurements of TC at pH 5.5 (yellow) and DMSO (violet). The absorbance of TC was measured at 360 nm.

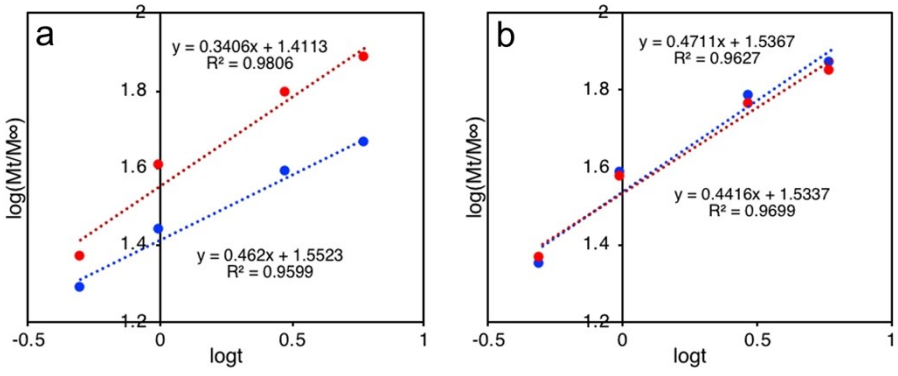


Figure S9. Logarithmic variation of tetracycline (blue) and amoxicillin (red) fraction released from SiO_2NCs_TC-O/AC between 0.5 and 6 h in PBS pH 7.4 (a) and 5.5 (b).

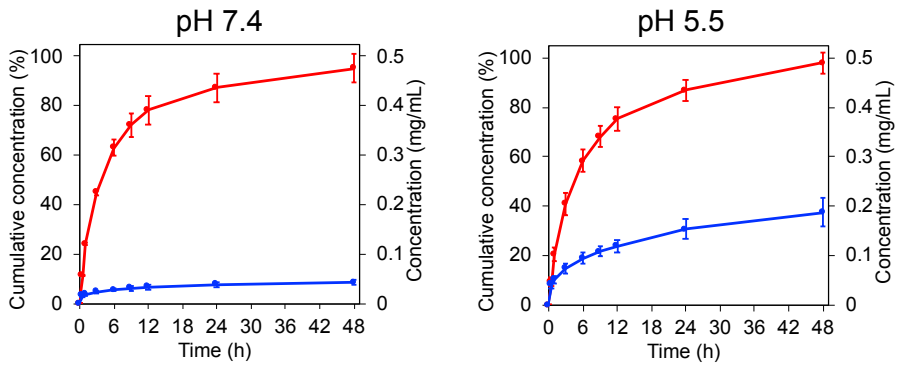


Figure S10. Release profile of TC (blue line) and AC (red line) from SiO_2NCs_TC-O at pH 7.4 and 5.5 (right) at 25 °C.

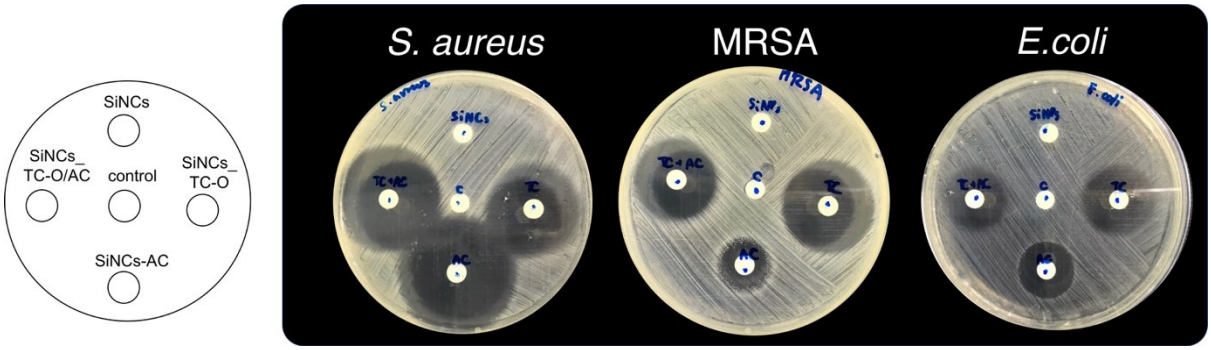


Figure S11. Zone of inhibition for SiO_2NCs against *S. aureus*, methicillin resistant *Staphylococcus aureus* (MRSA) and *E. coli.*

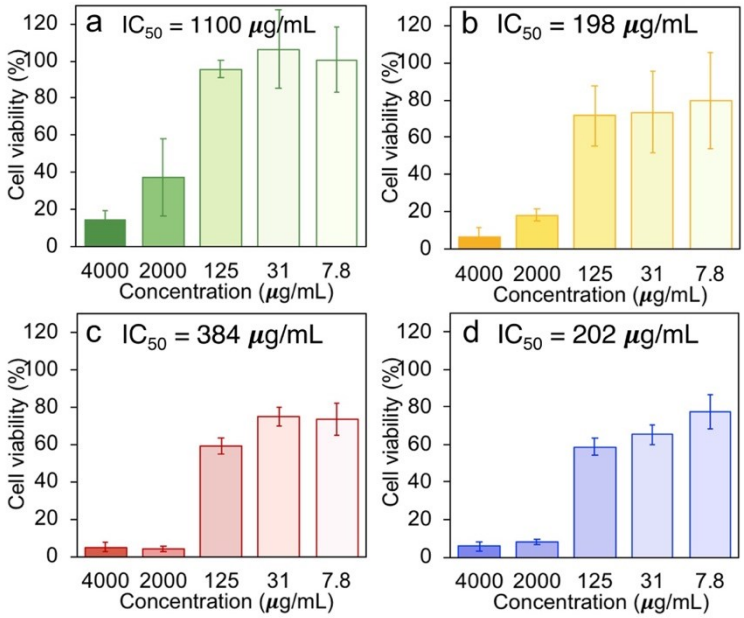
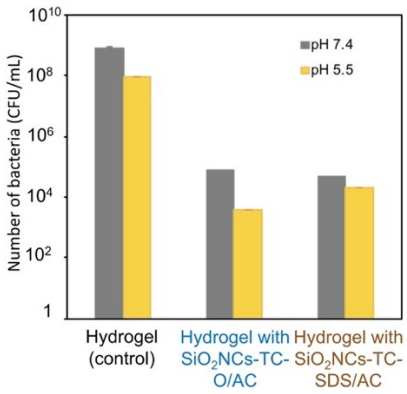


Figure S12. Cell viability of (a) SiO_2NCs, (b) SiO_2NCs_TC-O, (c) SiO_2NCs_AC, and (d) SiO_2NCs_TC-O/AC in fibroblast cells.



Entry	pH	Colony count (10^3CFU/mL)
SiO_2NCs	7.4	860,720.00 ± 85310.62
SiO_2NCs_TC-O/AC		74.41 ± 1.68
SiO_2NCs_TC-DS/AC		45.47 ± 7.95
SiO_2NCs	5.5	89,668.00 ± 10439.14
SiO_2NCs_TC-O/AC		3.73 ± 0.18
SiO_2NCs_TC-DS/AC		20.50 ± 6.41

Figure S13. Colony counts of methicillin resistant *Staphylococcus aureus* bacteria after being treated with hydrogels with or without SiO_2 NCs loaded with drugs for 24 h and at pH 7.4 and 5.5.

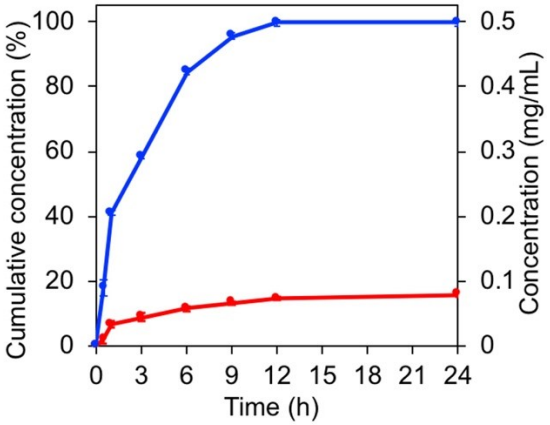


Figure S14. Release profiles of TC from TC-DS (red line) and TC-O (blue line) HIPs at pH 5.5.

12. Tables

Table S1. Binding constant (K_b), Gibbs free energy changes (ΔG), enthalpy change (ΔH), and entropy changes (ΔS) for the complexation of TC-O and absorption of AC on silica shell at 25 °C.

Entry	K_b [M^{-1}]	ΔG [kcal mol^{-1}]	ΔH [kcal mol^{-1}]	TΔS [kcal mol^{-1}]
TC-O	3.01×10^9	-12.9	-80.0	-67.1
SiO_2NPs_AC	3.03×10^9	-4.6	0.16	-3.6

Table S2. Evolution of the hydrodynamic diameter of SiO_2NCs_TC-O/AC during the sol-gel reaction in miniemulsion.

Time after sonication [h]							
0		1		2		20	
D_h [nm]	PDI	D_h [nm]	PDI	D_h [nm]	PDI	D_h [nm]	PDI
197	0.265	95	0.217	84	0.228	54	0.204

Table S3. Hydrodynamic diameters, zeta potential, encapsulation efficiency (EE), surface area, mean pore diameter, shell thickness, and solid content of SiO_2NCs encapsulating HIP complexes and hydrophilic drug.

Entry	Drug	D_h		ζ [mV]	Surface area [$m^2\ g^{-1}$]	Mean pore diameter [nm]	EE [%]	Shell thickness [nm]	Solid content [%]
		[nm]	PDI						
SiO_2NCs	-	54	0.208	+29	722	4.8	-	5	2.4
SiO_2NCs_TC-O	TC	91	0.201	+28	434	4.8	80	4	2.5
SiO_2NCs_AC	AC	77	0.200	+30	590	4.5	84	5	2.5
SiO_2NC_TC-O/AC	TC/AC	100	0.211	+40	647	4.3	79(TC)/66(AC)	7	2.6

Table S4. Hydrodynamic diameter of SiO_2NCs_TC-O/AC at various pH values and time intervals.

pH	Time [h]					
	24		36		48	
	D_h [nm]	PDI	D_h [nm]	PDI	D_h [nm]	PDI
7.4	98	0.260	102	0.235	100	0.226
5.5	100	0.266	100	0.251	98	0.249

Table S5. Calculated parameters (*n*, *k*) of the Korsmeyer-Peppas model fitting the release profile of AC and TC from SiO_2NCs_TC-O/AC) between 0.5 and 6 h.

Drug	pH	*n*	*k*	R^2
TC	7.4	0.34	25.78	0.9806
	5.5	0.47	34.41	0.9627
AC	7.4	0.46	35.67	0.9599
	5.5	0.47	34.17	0.9699

Table S6. Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and zone of inhibition (ZOI) of SiO_2NCs and SiO_2NCs containing drugs.

Samples	MIC [μg/ml]			MBC [μg/ml]			ZOI [mm]		
	S. aureus	MRSA	*E. coli*	*S. aureus*	MRSA	*E. coli*	*S. aureus*	MRSA	*E. coli*
SiO_2NCs	>250	>250	>250	>250	>250	>250	-	-	-
SiO_2NCs_TC-O	>250	0.98	2	>250	3.91	62.5	26	28	21
SiO_2NCs_AC	1	31.25	31.3	2	250.00	31.3	31	13	18
SiO_2NCs_TC-O/AC	2	1.95	7.8	2	15.63	62.5	33	24	19

13. References

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