# Supporting information

# Nanoengineered hollow mesoporous silica nanoparticles for the delivery of antimicrobial protein into biofilm

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**Materials and Methods:**

**1. Synthesis of rod shaped iron oxide nanoparticles as core**

Briefly, 1.62 g $FeCl_3$ and 0.012 g $NaH_2PO_4$ were dissolved in milli-Q water and the final volume was topped up to 500 mL. Then the solution was transferred to autoclave under hydrothermal treatment at 100 °C for 2 days. The products were washed with water and ethanol, then dried in air.

**2. Synthesis of $Fe_2O_3$@MSN-LP.**

Briefly, 160 mg spindle shaped iron oxide nanoparticles were well dispersed in solution containing 4 mL CTAC, 6 mL $H_2O$ and 0.04 g TEA with ultrasonic treatment. The mixture was stirred and the temperature was raised to 60 °C. Then 9.1 mL chlorobenzene and 0.9 mL TEOS was added and stirred with a speed of around 450 rpm for another 12 h at 60 °C. The products were collected by centrifugation at 4,700 rpm for 10 min and washed with water and ethanol.

**3. Synthesis of $Fe_2O_3$@MSN-SP.**

A thin layer of $SiO_2$ (~13 nm) was coated on the outside of spindle shaped iron oxide nanoparticles. Hundreds (100) mg spindle shaped ion oxide nanoparticles were well dispersed in solution containing 200 mL isopropyl alcohol, 40 mL $H_2O$ and 6 mL ammonia solution (28%-30%). Then 0.1 mL TEOS was added and stirred at room temperature for 6 h. The products were collected by centrifugation at 4,700 rpm for 10 min and washed with water and ethanol.

**4. Synthesis of HMSN-LP/SP by removing the $Fe_2O_3$ core.**

For removal of the ion oxide core, a certain amount of $Fe_2O_3$@MSN-LP or $Fe_2O_3$@MSN-SP was dispersed in a mixture solution containing 40 mL acetone and 4 mL HCl (32%) and stirred at 80

°C for 8 h. This process was repeated 2~4 times until all ion oxide cores were removed. The products were washed with water and ethanol and dried at room temperature.

**5. Lysozyme loading assay**

For lysozyme loading, hollow silica nanoparticles (HMSN-LP, HMSN-SP) were dispersed in phosphate buffer solution (PBS, 10 mM, pH 7.4) by ultrasonication with concentration of 2 mg/mL, and then were mixed with lysozyme in PBS solution (4 mg/mL). After rotating at room temperature for 24 h, the mixtures were centrifuged at 15,000 rpm for 5 min. The supernatants were collected and the residual lysozyme content was measured by using a UV-vis spectrophotometer (UV-2450, Shimadzu) at a wavelength of 285 nm. The lysozyme loading amount can be calculated based on the original and residual protein concentrations and volumes. All experiments were performed in triplicates.

**6. Lysozyme release study**

The lysozyme release profile was studied under a fixed initial concentration of lysozyme (270 μg/mL). Varied amount of lysozyme loaded silica hollow spheres (calculated based on their loading capacities) were suspended in 2 mL of PBS and were shaken in an incubator at 200 rpm under 37 °C. The supernatants were collected at different time points and were replaced with 2 mL fresh PBS. The amount of released lysozyme was quantified by a UV-vis spectrophotometer at wavelength of 285 nm.

**7. Formation of *E. coli* biofilm**

In pre-culture, *E.coli* was inoculated in 10 mL LB (Luria-Bertani) broth (pH=7.0) and incubated at 37°C overnight. Afterwards, for main-culture, the old culture medium was added to 30 ml fresh LB broth and subsequently incubated at 37 °C for 16 h. The grown bacteria were then harvested by centrifugation and rinsed by fresh PBS. A concentration of $1 \times 10^5$ CFU/mL *E.coli* suspension was prepared for biofilm formation.

Bacteria were allowed to form biofilm statically at 37°C for 4 h. By the end of incubation, fresh PBS was added to replace the old culture medium to terminate metabolic activity inside biofilm.

**8. Anti-biofilm effects of lysozyme-loaded nanoparticles using LIVE/DEAD staining method**

Three hundred (300) μL/well of various concentrations of lysozyme-loaded nanoparticles (with a lysozyme concentration of 800 μg/mL), free lysozyme and control (PBS) were added to 24-well plate containing grown biofilm. After 24 h of exposure, the nanoparticle suspension was removed and the remaining biofilm were gently rinsed by PBS.

The viability of biofilm was determined by Confocal Leaser Scanning Microscopy (CLSM, Nikon C2+ Confocal Microscope System, Japan). Specimens for CLSM were stained using LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Invitrogen, Thermo Fisher Scientific, Oregon, USA) which containing separate vials of the two component dyes (3.34 Mm SYTO 9 and 20 Mm propidium iodide in 1:1 mixture) in solution. The staining material was prepared fresh following the manufacturer's instruction, and then cultured with the biofilm in dark at 37°C for 30 min. Live

bacterium with intact membrane was demonstrated by green florescence at 506 nm, while dead bacterium with damaged membrane was demonstrated by red florescence at 560 nm.

**9. Anti-biofilm effect of lysozyme loaded nanoparticles using spread plates method**

Biofilm grown in 96 well plate was cultured with different concentrations of lysozyme-loaded nanoparticles, free lysozymes (200 µl/well with lysozyme concentration of 200, 400, 800 mg/mL) and controls (200 µl/well) at 37 °C for 24 h. The antibacterial agents were then gently replaced by fresh PBS (200 µl/well). After being mixed thoroughly, 100 µl bacterial suspension from each well was serially diluted with fresh PBS, evenly spread on LB agar plates, and incubated at 37 °C for 24 h. The number of colonies formed on each LB agar plate was counted. The colony forming unit was calculated by multiplying viable colonies with the dilution factor and expressed as CFU $mL^{-1}$.

**10. Penetration of HMSN-LP into *E. coli* biofilm using CLSM**

The biofilm on 24-well plate was cultured with labelled HMSN-LP (0.4 mg/mL, 300 µl/well) at 37°C for 24 and 48 h. The HMSN-LP was labelled with red fluorescence using RITC (rhodamine B isothiocyanate) according to reported protocol.[1] At designated time points, the nanoparticle solution was gently removed after the incubation and carefully rinsed by fresh PBS (300 µl/well) for 5 times. Then biofilm was fixed with 4% paraformaldehyde and the bacteria was stained with SYTO 9. The biofilm was observed using CLSM (Nikon C2+ Confocal Microscope System, Japan).

**11. Interaction of HMSN-LP and *E. coli* using CLSM and TEM**

To study the interaction of HMSN-LP and *E. coli* biofilms, after co-culture of the HMSN-LP with *E. coli* biofilm for 24 and 48 h. The biofilms were dispersed into single bacteria using very mild ultrasonic treatment. The relationship of nanoparticles and separated bacteria was observed using CLSM with high magnification lens (using above protocol) and transmission electron microscopy.

For TEM observation, samples were prepared by drop-coating method: droplet of HMSN-LP treated *E. coli* suspension was left on copper grids for 45 s, and the excess was removed with filter papers. Then *E. coli* was stained with uranyl acetate (2%) for 30 s and dried. TEM images were taken by Hitachi HT 7700 (Hitachi, Japan) with 80 kV."

**12. Cell toxicity study**

The cell toxicity of HMSN-LP was tested on NIH-3T3 (ATCC, American) cells by 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay. In brief, NIH-3T3 cells were firstly seeded in a 96 well plate at a density of 5000 cells per well. After incubation for 24 h, the culture medium was replaced by fresh medium containing HMSN-LP solutions at different concentrations. The cells were cultured for additional 24 hours, then the culture medium was replaced by MTT agent (final concentration of 0.5 mg/mL) and incubated at 37 °C for 4h. The

blue formazan crystals formed were dissolved by dimethyl sulfoxide. The cell viability was measured by reading the absorbance at 570 nm and samples were normalized to non-treated cells.

**Characterization**

The SEM images were obtained using JEOL JSM 7800 field-emission scanning electron microscope operated at 0.8-1.5 kV using gentle bean mode without any coating. For FE-SEM measurements, the samples were prepared by dispersing the powder samples in ethanol, after which they were dropped to an aluminum foil piece and attached to conductive carbon film on SEM mount. Then the samples were dried in a vacuum oven at 60 °C for 12 hours and cleaned with evactron 25/45 De-contaminator RF plasma cleaning system.

The TEM images were obtained using JEOL 1010 operated at 100 kV. The ET experiment was performed in bright-field TEM mode on a FEI Tecnai F30 operated at 300 kV with a Gatan double tilt/rotation holder. All TEM images were recorded digitally at a given defocus in bright-field mode to show the mass–thickness contrast. The ET specimens were prepared by dispersion of the samples in ethanol by ultrasonication for 5 min, and then deposition directly onto a formvar film supported by a copper grid (3×1 mm slot, Electron Microscopy Science). Colloidal gold particles (10 nm) were deposited on both surfaces of the grid as fiducial markers for the subsequent image alignment procedures. The tomographic tilt series were carried out by tilting the specimen inside the microscope around a dual axis under the electron beam. TEM projected images were recorded over a tilt range of +65 ° to -65 ° at an even increment of 1.5 °. By using image processing software (IMOD) and reconstruction (IMOD and AMIRA) techniques, the fine structures of the synthesized products were obtained.

Dynamic light scattering (DLS) test of HMSN-LP was carried out using a Zetasizer Nano-ZS (Malvern Instruments) at room temperature. HMSN-LP was dispersed in milliQ water and LB culture medium placed in a cuvette for DLS measurement.

Nitrogen adsorption-desorption measurements were conducted at -196 °C with a Micromeritcs Tristar II system, before testing the samples were degassed at 100 °C overnight on a vacuum line. The total pore volume was calculated from the amount adsorbed at a maximum relative pressure ($P/P_0$) of 0.99. The Brunauer-Emmett-Teller (BET) method was utilized to calculate the specific surface areas. The Barrett-Joyner-Halenda (BJH) method was used to calculate the pore size of samples from the adsorption branches of the isotherms. The measurements were repeated for three times and an average of the values was calculated along with the corresponding standard deviation.

Circular dichroism spectra were recorded on a Jasco J-815 spectropolarimeter (Jasco Inc., Easton, MD) at 20 °C in a 1 mm quartz cell. Raw ellipticities were converted to molar ellipticities using following equation:

$$[\theta] = \frac{100\ \theta}{C * l}$$

where [θ] is molar ellipticity in deg $cm^2$ $dmol^{-1}$, θ is raw ellipticity in mdeg, C is the lysozyme concentration in mM and l is the path length in cm."

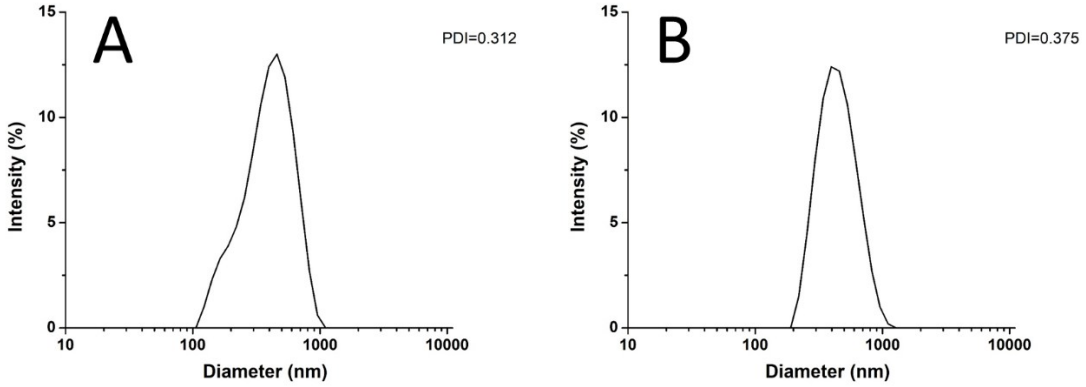


**Fig. S1** HMSN-LP size distribution curve in deionized water (A) and LB culture medium (B).

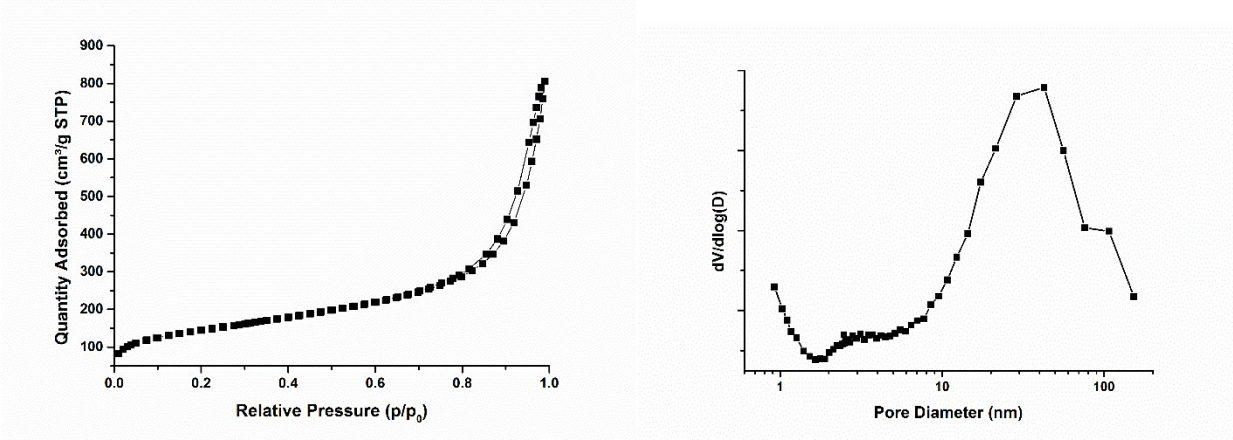


**Fig. S2** Typical $N_2$ sorption and pore size distribution plot of HMSN-LP. A pore size of around 30 nm could be calculated from adsorption branch by BJH method.

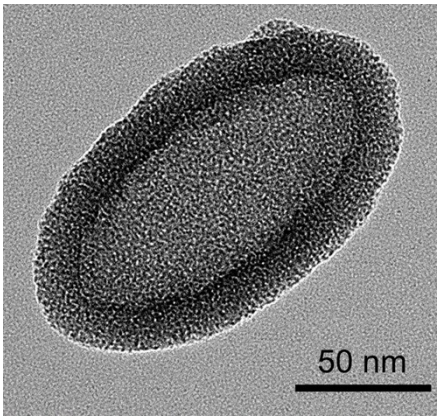


**Fig. S3** High resolution TEM image of HMSN-SP.

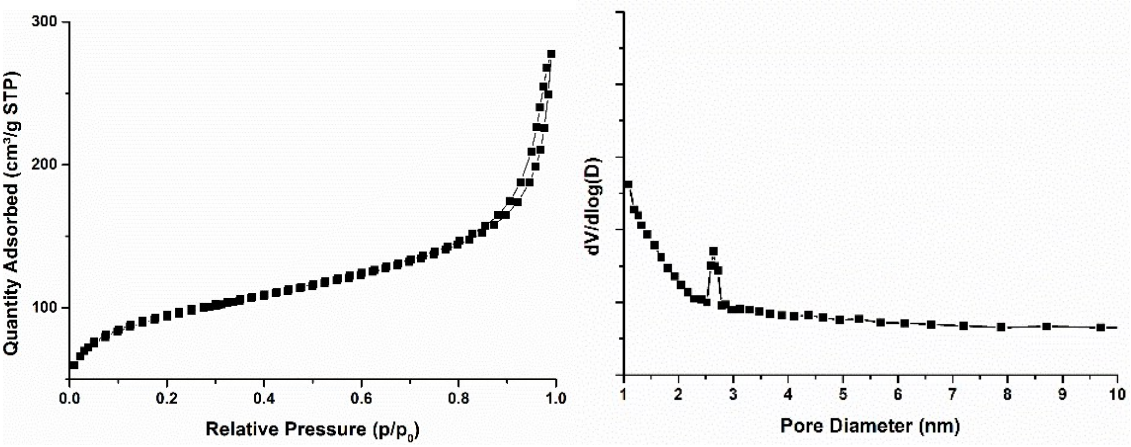


**Fig. S4** Typical $N_2$ sorption and pore size distribution plot of HMSN-SP.

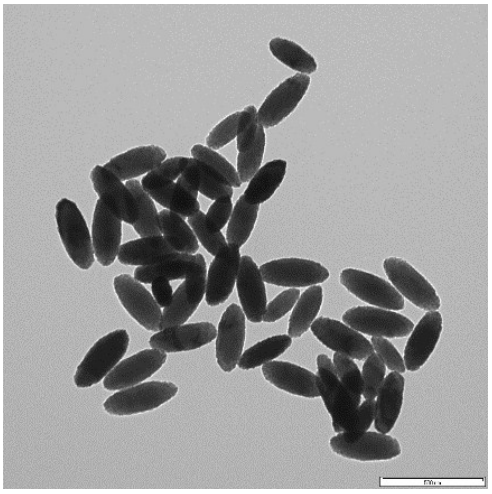

**Fig. S5** TEM image of spindle shaped $Fe_2O_3$ which was used as template.

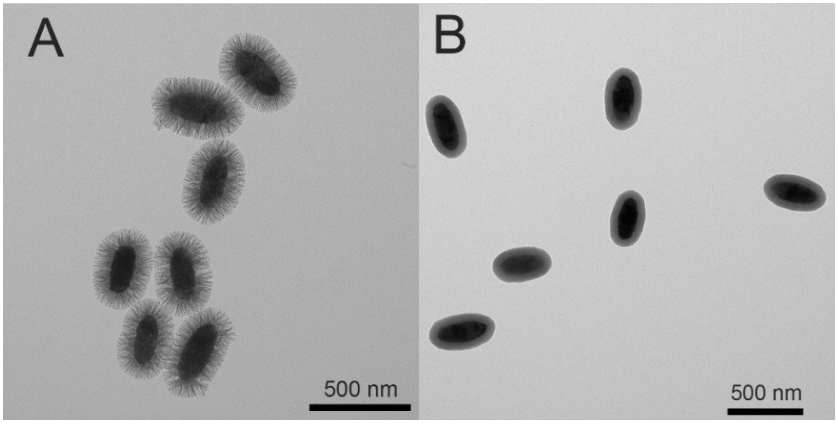


**Fig. S6** TEM images of MSN-LP (A) and MSN-SP (B) with $Fe_2O_3$ seed inside.

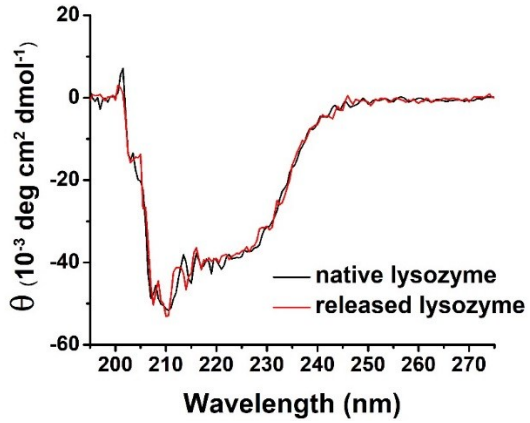


**Fig. S7** CD spectra of lysozyme released from HMSN-LP and native lysozyme in 20 mM PBS at pH 7.4.

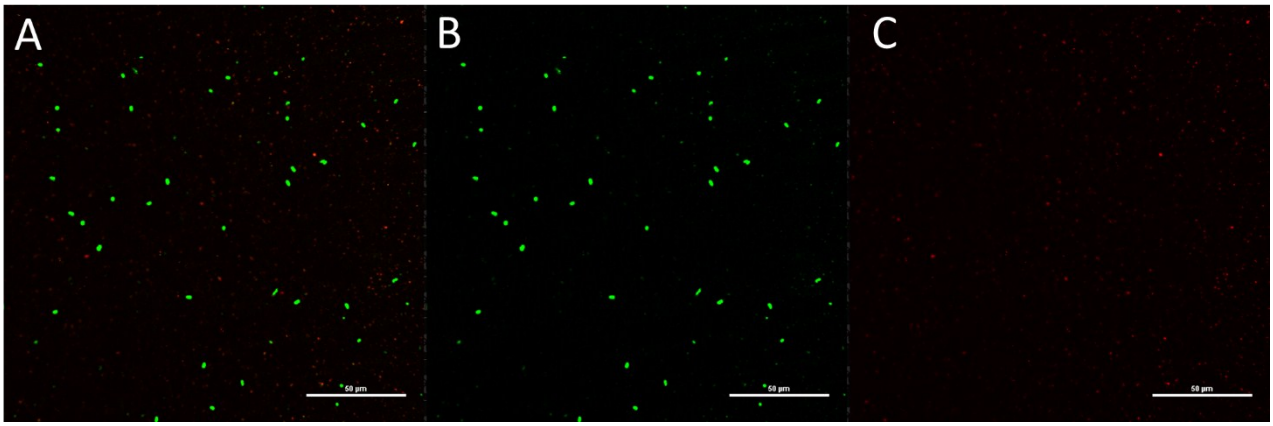


**Fig. S8** Confocal laser scanning microscope images of HMSN-LP and *E. coli* after ultrasonic treatment (A: combined image; B: *E. coli* (green) which was labelled with SYTO9; C: HMSN-LP (red) which was labelled with RITC).

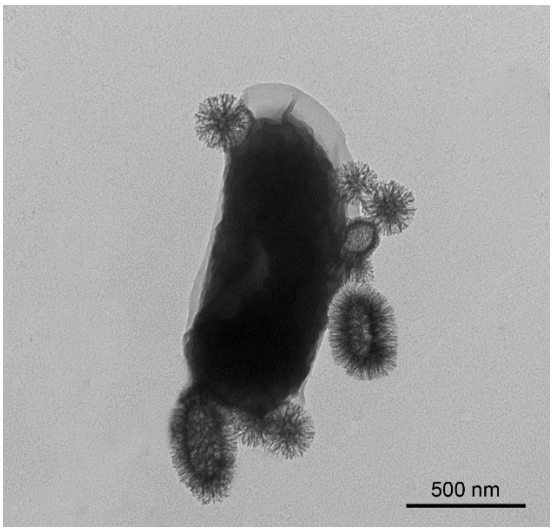


**Fig. S9** TEM image of HMSN-LP adhered outside of *E. coli* after ultrasonic treatment.

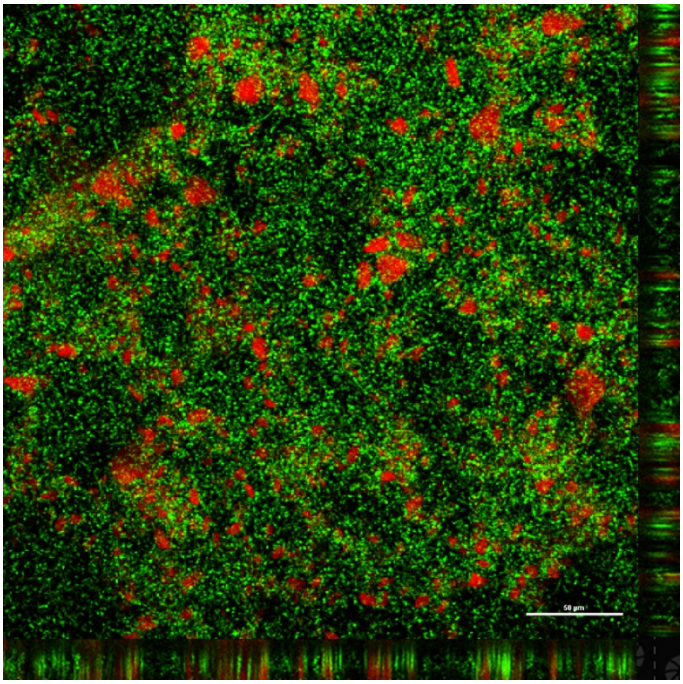

**Fig. S10** Confocal laser scanning microscope image showed the penetration of HMSN-LP into *E. coli* biofilm after co-culture for 48 h (scale bar=50 μm).

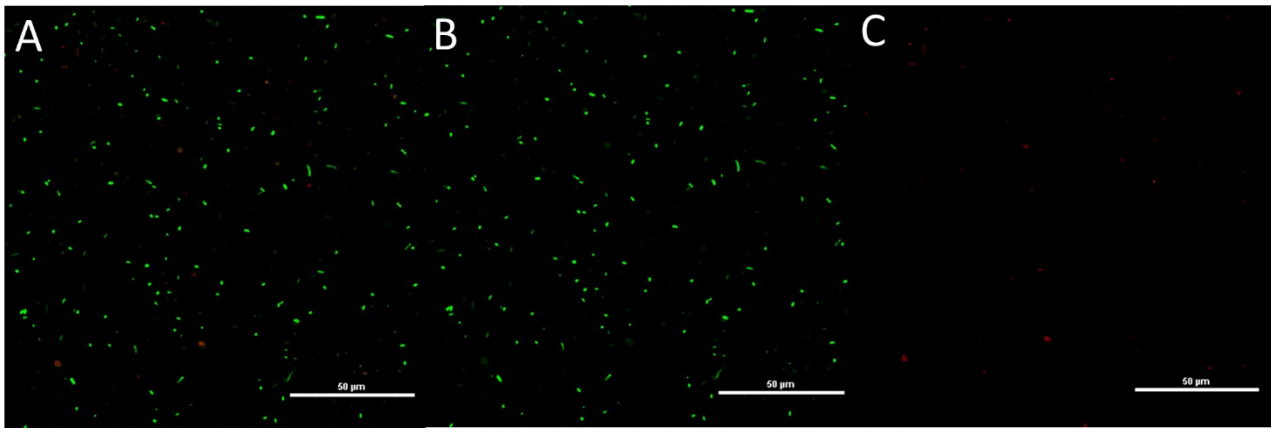


**Fig. S11** Confocal laser scanning microscope images of HMSN-LP and *E. coli* after ultrasonic treatment (A: combined image; B: *E. coli* (green) which was labelled with SYTO9; C: HMSN-LP (red) which was labelled with RITC).

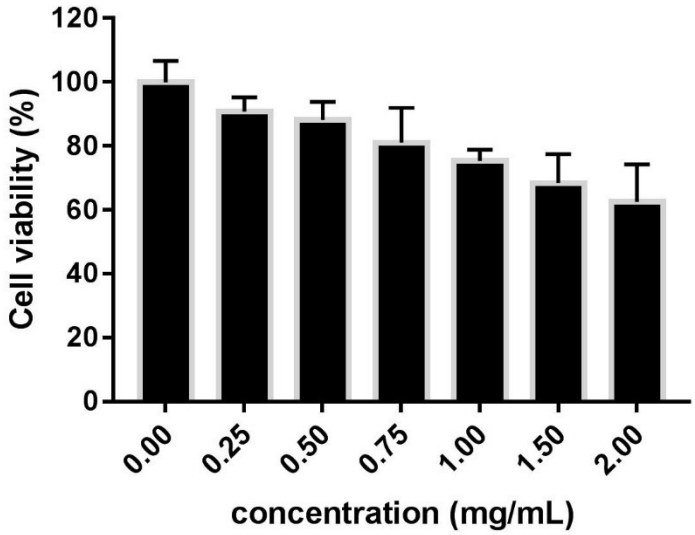


**Fig. S12** Cell toxicity of HMSN-LP towards NIH-3T3 cells.

**Table S1** Physical characterization of HMSN-LP and HMSN-SP

| Sample | Pore size (nm) | Total pore volume ($cm^3/g^{-1}$) | BET surface area ($m^2/g^{-1}$) |
|---|---|---|---|
| HMSN-LP | 40 | 1.32±0.05 | 500.5±0.95 |
| HMSN-SP | 3 | 0.49±0.08 | 313.40±1.49 |

## Reference

1 C. Xu, M. Yu, O. Noonan, J. Zhang, H. Song, H. Zhang, C. Lei, Y. Niu, X. Huang, Y. Yang and C. Yu, Small, 2015, 11, 5949-5955.