Asymmetric mesoporous silica nanoparticles as potent and safe immunoadjuvants provoke high immune responses

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Supplementary Information

Material and Methods:

Chemicals: Cetyltrimethylammonium chloride (CTAC) solution (25 wt% in H_2O); triethanolamine (TEA); chlorobenzene and tetraethylorthosilicate (TEOS, >98%), 3-aminopropyl triethoxysilane (APTES), rhodamine isothiocyanate (RITC), ammonia aqueous solution (28 wt %) and ethanol were purchased from Sigma-Aldrich. All chemicals were used as received without further purification.

Preparation of MSN

Dendritic nanoparticles were synthesised according to literature report with slight modification.[1] To 100 mL round bottom flask, 0.18 g of TEA, 24 mL of CTAC (25 wt%) solution and 36 mL of water were added and stirred slowly at 60 °C for 1 h. After 1 h, 16 mL of cyclohexane and 4 mL of TEOS was slowly added from sides of the flask and left the reaction under slow stirring for 24 h. The particles were collected after 24 h by centrifugation and washed for several times with ethanol. The surfactant was removed by calcination at 650 °C for 6 h. The calcined product was labelled as MSN.

Preparation of HTMSN

For the synthesis of HTMSN, 60 mg of uncalcined MSN were dispersed in 6 mL of milliQ water and 0.035 g TEA by sonication for 15 min. Then, 4 mL of CTAC (25 wt%) solution was added into the solution and kept at 60 °C at a stirring speed of 400 rpm for 1 h. After stirring for 1 h, 8.75 mL of chlorobenzene and a 1.25 mL of TEOS was added into the above solution. The reaction was continued at 60 °C for 12 h. Particles were collected by centrifugation at 15,000 rpm, washed with water and ethanol twice and dried at 50 °C overnight. The surfactant was removed by calcination at 650 °C for 6 h. The calcined product was named as HTMSN.

Characterizations

TEM images were taken using a JEOL 1010 microscope operated at 100 kV. For observing the nanoparticles under TEM, particles were dispersed in ethanol, and few drops were then dropped on copper grid. DLS and ζ potential measurements were carried out at 298 K using a Zetasizer Nano-ZS from Malvern Instruments. The samples were dispersed in deionized water by ultrasonication before measurement. Nitrogen sorption isotherms of the samples

were obtained at 77 K using a Micromeritcs Tristar II system. Before the measurements, the samples were degassed at 180 °C overnight on a vacuum line. The total pore volume was calculated from the amount adsorbed at a maximum relative pressure (P/P0) of 0.99. The Barrett-Joyner-Halenda (BJH) method was used to calculate the pore size of samples from the adsorption branches of the isotherms. The Brunauer-EmmettTeller (BET) method was utilized to calculate the specific surface areas. Fourier transform infrared (FTIR) spectra were measured on a Thermo Nicolet Nexus 6700 FTIR spectrometer equipped with a Diamond ATR (attenuated total reflection) Crystal. For each spectrum, 128 scans were collected at resolution of 4 cm^{-1} over the range 400–4000 cm^{-1}.

Amino modification on MSN and HTMSN

Amino silane was grafted onto the surface of MSN, and HTMSN to create positive charge particles. 200 mg of particles was suspended in 30 mL of toulene, and sonicated for 10 min, and then 0.19 mL APTES was added and the mixture was refluxed at 110 °C for 20 h. The product were collected by centrifugation and washed twice with ethanol and water and dried at 50 °C for overnight. The obtained samples were labelled as MSN-A and HTMSN-A.

Grafting of Rhodamine B isothiocyanate (RITC) on MSN-A and HTMSN-A

50 mg of amino modified particles were suspended in 20 mL of ethanol with sonication for 10 min. Afterwards, 5 mg of RITC was added and stirred the suspension for overnight in dark. RITC tagged particles were collected by centrifugation followed by several times washing with ethanol to remove the free RITC. The RITC tagged particles were labelled as RITC-MSN-A and RITC-HTMSN-A.

Loading of J8

Loading of J8 into MSN-A and HTMSN-A was done by mixing 2 mg/mL of J8 with 1mg/mL of particles and incubating at 4 °C for 2h. Following 2h of incubation, particles were collected using centrifugation and the loading amount was measured from the supernatant using high performance liquid chromatography (HPLC).

Biological experiments:

Cell culture

Cell culture reagents were purchased from GIBCO Invitrogen Corporation/Life Technologies Life Sciences unless otherwise specified. Cell lines used including macrophage cell line RAW264.7(ATCC) and cervical cancer cell line HEK 293 were purchased from ATCC (American Type Culture Collection). Cells were maintained as monolayer cultures at 37 °C and 5 % CO_2 in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % feta; bovine serum (FBA) and 1% penicillin-streptomycine. The final concentration is 100 u/mL for penicillin, 100 u/mL for streptomycin. Fetal bovine serum, paraformaldehyde, antifade fluorescent mounting medium with 4'-6-diamidino-2-phenylindole (DAPI) and MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; thiazolyl blue) were purchased from Sigma-Aldrich.

MTT assay

Both RAW264.7, HEK 293 cells were seeded in a 96 well plate at a density of 5×10^3 cells per well and incubated for 24 h. To evaluate the cytotoxicity of different pure nanoparticles and CFA, different concentrations are prepared in culture medium supplemented with 10 % FBS and 1 % penicillin-streptomycin, and incubated for 24 h and 48 h at 37 °C. After 24 h and 48 h incubation 20 µL of 5mg/ml of MTT reagent was added and incubated for 4h. Cell viability was measured by adding DMSO and reading the absorbance at 540 nm using a synergy HT Microplate Reader.

Microscopy

For fluorescent imaging, 1×10^5 RAW264.7 cells were seeded in 6 well plate on cover slip one day before the assay. 20 µg/mL of particles were added to wells containing serum free medium, and incubated at 37 °C for 4 h. Afterwards, cells were washed twice with PBS and fixed with 1 mL of 4 % PFA/PBS at RT for 30 min. Next, 200 µL of Alexa Fluor 488 (5 µL of stock + 200 µL of 1% BSA solution) was added and incubated for 30 min at RT. Following washing twice with PBS, coverslips were collected and placed on glass slide containing 200 µL of anti-fade fluorescent mounting medium containing DAPI, and the cells were viewed under confocal laser scanning microscopy (CLSM).

Detection of cellular uptake

To quantitatively compare cellular uptake between MSN-A and HTMSN-A, 2×10^5 RAW264.7 cells were seeded in 6 well plates one day before the addition of particles. Nanoparticles (5 µg/mL) were incubated with cells under serum free medium for 4 h. Afterwards, cells were washed twice with PBS and harvested using cell scraper. After centrifugation, cell lysis buffer (Cell Signaling Technology) was added to lyse the cells. The supernatant was removed by centrifugation at 13,000 rpm for 5 min, and left the tubes for drying at 60 °C overnight. Aqueous NaOH solution (1 M) was added to allow dissolution of silica and solutions were measured by inductively coupled plasma atomic emission spectroscopy (ICPOES) using Vista-PRO instrument (Varian Inc, Australia) for silica amount.

Animal experiment

All protocols were approved by the The University of Queensland, and were carried out in accordance with the institutional guidelines for animal experimentation. Four to six weeks old female B10.BR mice were used for immunization. Mice (n=5/ group) were injected subcutaneously at the tail base on day 0 with 50 µg of MSN-A+J8/HTMSN-A+J8 and 30 µg of J8 in a total volume of 50 µL of sterile PBS. Mice received three further boosts at days 14, 21, and 28 with the same working concentration. Positive control received 30 µg of J8 emulsified in a total volume of 50 µL of CFA subcutaneously at the tail base. Negative controls were given 50 µL of sterile PBS. Another group received 30 µg of J8 in a total volume of 50 µL.

Collection of blood

Blood was collected from the tail artery of each mouse 1 day prior to each injection and 1 week after the last immunizations. The blood was left to clot at 37 °C for 1 h and then centrifuged for 10 min at 3000 rpm to remove clots. Sera were then stored at -20 °C.

Detection of antibody using ELISA

Detection of serum IgG antibodies against the J8 peptide was performed using an enzyme linked immunosorbent assay (ELISA) as previously reported. Briefly, ELISA plates were coated with J8 (10 mg/ mL) in carbonate coated buffer overnight at 4 °C and blocked with 150 µL/ well of 5% skim milk for 90 min at 37 °C. Serial dilutions of collected sera were prepared in 0.5% skim milk/ PBS-Tween-20 buffer. Absorbance values were read at 450 nm in a microplate reader after the addition of secondary antibody (peroxidase-conjugated antimouse IgG). The antibody titre was identified as the lowest dilution.

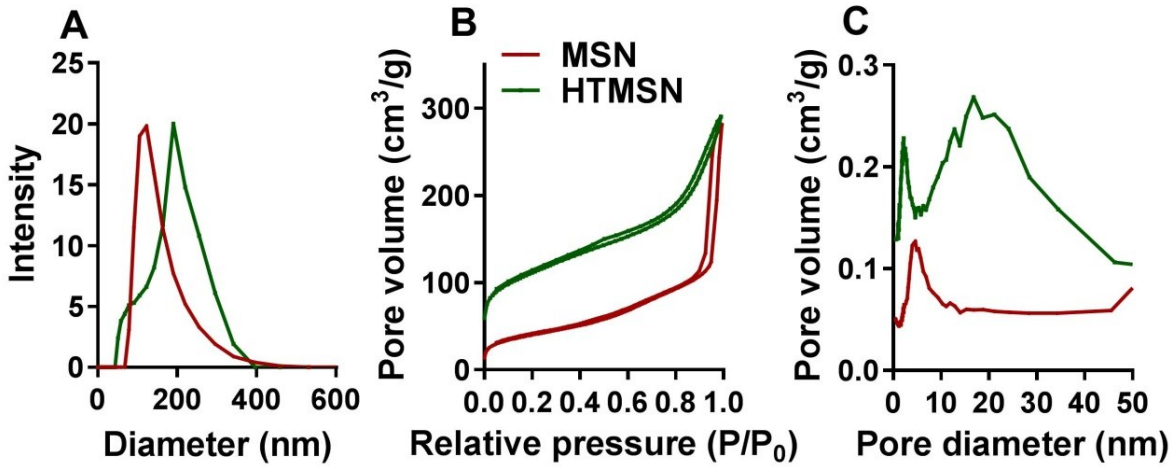

Figure S1. A) particle size, B) Nitrogen adsorption-desorption isotherms, and C) pore size distribution of MSN and HTMSN, respectively.

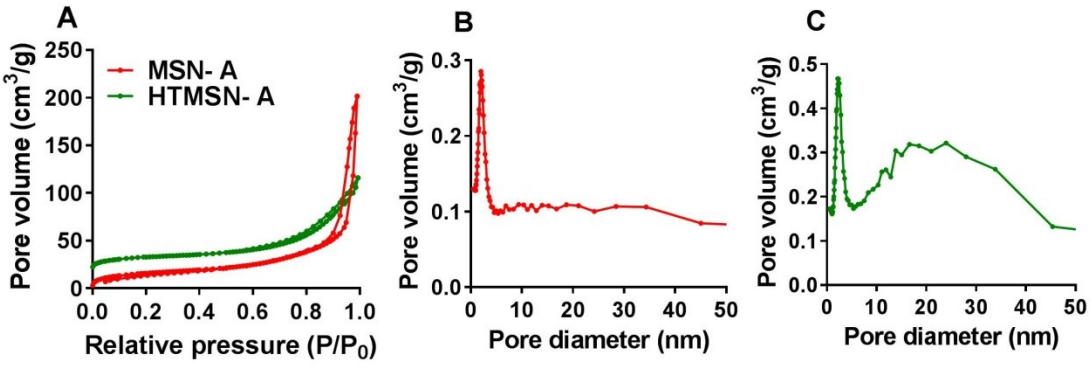

Figure S2. A) Nitrogen adsorption-desorption isotherms of MSN-A and HTMSN-A, and pore size distribution of B) MSN-A and C) HTMSN-A, respectively.

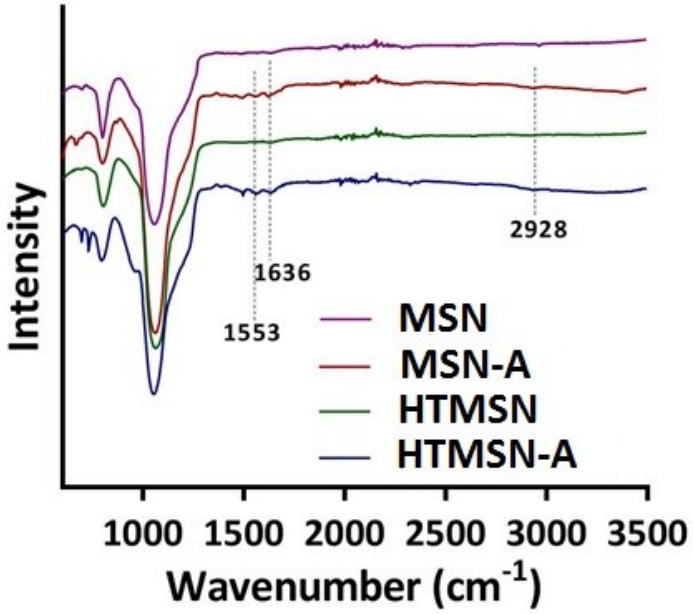

Figure S3. FTIR of MSN and HTMSN before and after amino modification.

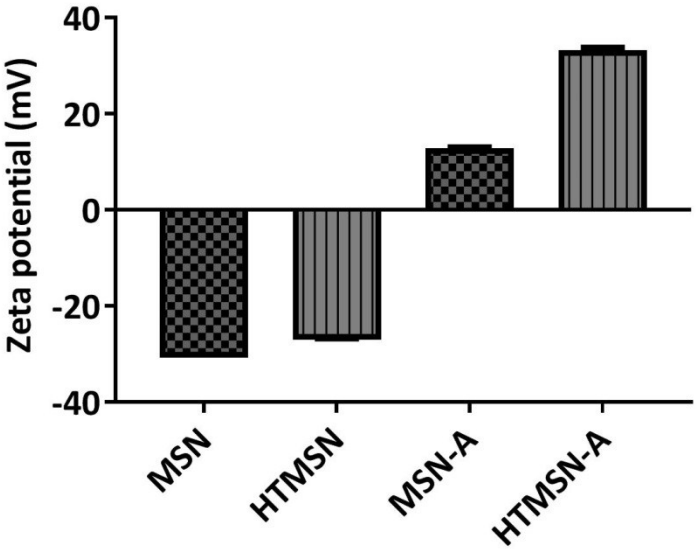

Figure S4. Zeta potential of MSN and HTMSN before and after amino modification.

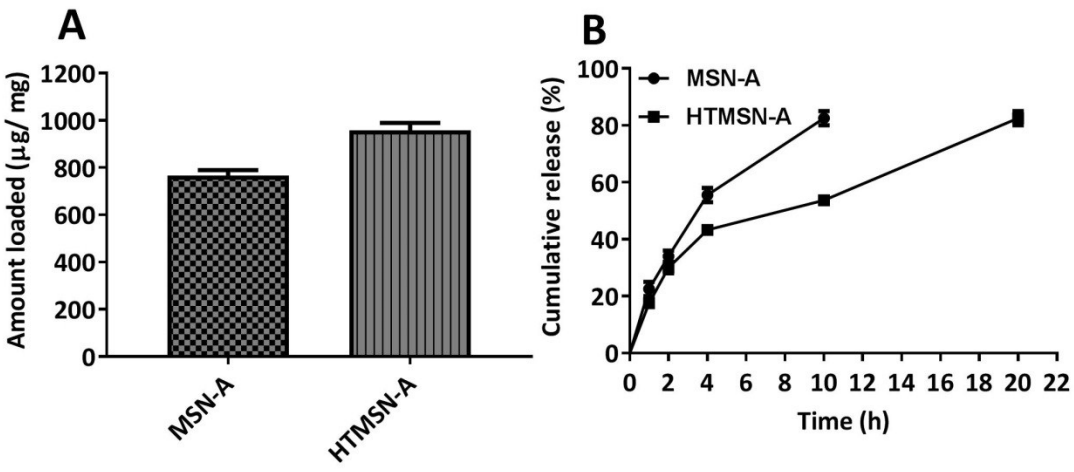

Figure S5. A) Loading, B) release of J8 from MSN-A and HTMSN-A.

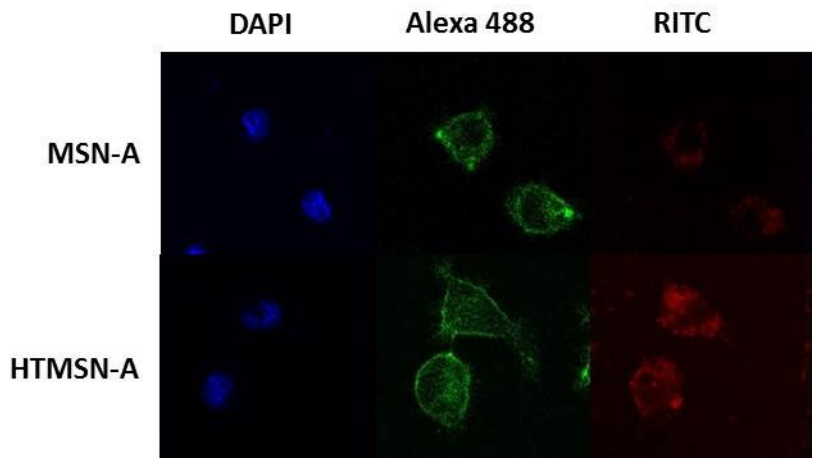

Figure S6. Confocal images of RAW 264.7 cells. Different color scheme in the images represent the following; blue: cell nucleus; Alexa Fluor® 488 phalloidin (green): cell cytoskeleton; and red: Rhodamine B isothiocyanate (RITC) labelled particles.

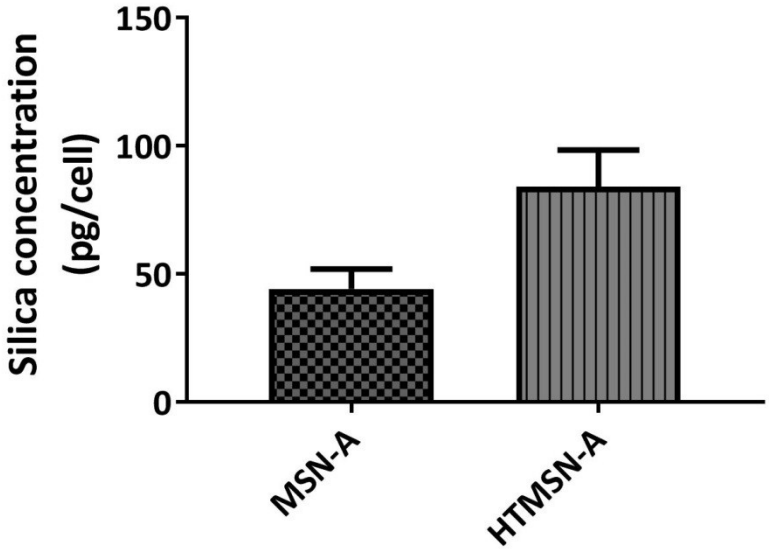

Figure S7. Cellular uptake performance of MSN-A and HTMSN-A measured by ICP-OES.

Table S1. Physical properties of MNS, MSN-A, HTMSN and HTMSN-A

Samples	S (m^2/g)	V (cm^3/g)	D (nm)	PDI
MSN	173	0.50	4.1	0.25
HTMSN	290	0.40	2.3, 16.8	0.30
MSN-A	100	0.31	1.72	
HTMSN-A	220	0.30	2, 15.3	

Note: *S* is BET surface area, *V* is total pore volume, *D* is the pore size.

Reference:

1. D. Shen, J. Yang, X. Li, L. Zhou, R. Zhang, W. Li, L. Chen, R. Wang, F. Zhang, D, Nano Lett, 2014, 14, 923-932.

Acknowledgements:

The authors acknowledge the financial support from the Australian Research Council and the Queensland Government. We also thank the Australian National Fabrication Facility and the

Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, Centre for Advanced Imaging for the technical assistance.