Electronic Supplementary Material (ESI) for RSC Advances

Mesoporous Silica Nanoparticles Enhance the Cytotoxicity of Curcumin

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1. Experimental

Chemicals:

Cetyl trimethylammonium bromide (CTAB), Tetraethoxy orthosilicate (TEOS), Sodium lauryl sulphate (SLS) and Curcumin was purchased from Sigma-Aldrich. Reagent grade sodium hydroxide (NaOH) was received from ChemSupply. Methanol AR was purchased from RCI labscan. Human skin cancer SCC-25 cell line was a kind gift of Prof. Nicholas Saunders from the Princess Alexandra Hospital, Brisbane. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) media was purchased from Sigma. Fetal calf serum was purchased from Moregate biotech. All primary IgG antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Depending on the primary antibody, the secondary antibody used was either horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Clarity Western ECL Substrate reagent was purchased from Bio-Rad (Australia).

Characterization:

X-ray diffractograms (XRD) were recorded on a Rigaku Miniflex X-ray diffractometer with Fe-filtered Co radiation. Transmission electron microscopy (TEM) images were obtained with a JEOL 1010 operated at 100 kV. Nitrogen physisorption measurements were carried out at -196°C by using a Micromeritics Tristar II 3020 system. MCM-41 and MCM-41-CUR samples were degassed respectively at 200 °C and 100 °C overnight on a vacuum line. The pore-size distribution was measured from the desorption branch of the isotherm using BJH model followed by gaussian fitting. Fourier transform infrared (FTIR) spectra were recorded on ThermoNicolet Nexus 6700 FTIR spectrometer equipped with Diamond ATR (attenuated total reflection) Crystal. For each spectrum, 128 scans and 4 cm^{-1} resolution was applied over the range of 400–4000cm^{-1}. Thermogravimetric and Differential Scanning Calorimetry (DSC)

measurements were performed by a Setaram TG92 instrument with a heating rate of 5 °C/min in air flow. Curcumin concentration was determined using UV-VIS spectrophometer (Shimadzu UV-2450). Dynamic light scattering (DLS) studies were carried out on a Malvern NanoZS zetasizer at 25°C in water.

Experimental Section:

MCM-41 was synthesized with slight modifications to the method reported by Yang et al.[1] 1.0 g of CTAB was added to 480 g of deionized water and kept under stirring until clear solution was obtained. To this solution, 3.5 ml of 2M NaOH was added and temperature was raised to 80 °C. Then, 6.7 ml of TEOS was added slowly to this solution as the silica source and kept under stirring for additional 2 h. The resultant product was obtained by filtration and dried at room temperature. The dried product was calcined at 550 °C for 5 h to remove the surfactant template. The calcined product was termed as MCM-41.

Curcumin loading and in vitro release measurements:

Curcumin loading was performed using rotary evaporation technique. 160 mg of MCM-41 was placed in rotary evaporation flask followed by addition of 40 mg of curcumin to it. 10 ml of methanol was added to the flask, sonicated for 2 mins using a bath sonicator and then attached to the rotary evaporator. Methanol was evaporated slowly from the suspension under vacuum and at a water bath temperature of 50 °C with circulating water maintained at 5 °C to obtain curcumin loaded sample which was designated as MCM-41-CUR.

The in vitro release of curcumin from MCM-41-CUR was evaluated using dialysis bag technique.[2] MCM-41-CUR equivalent to 1 mg of curcumin was weighed and suspended in 1 ml of 0.5 % SLS. This suspension was then placed in dialysis bag (Sigma Aldrich) with 10 kDa molecular weight cutoff and was immersed into 9 ml of 0.5 % SLS at 37°C with continuous stirring. At predetermined time intervals, 1 mL of the samples were withdrawn and immediately replaced with an equal volume of dissolution medium to keep the volume constant. Pure curcumin was studied along with MCM-41-CUR to compare the in vitro drug release profile by weighing 1 mg of pure curcumin and suspending it in 0.5 % SLS similar to that of MCM-41-CUR . These samples were then properly diluted and analyzed for curcumin content at 432 nm using UV-VIS spectrophotometer.

In vitro cytotoxicity assay:

SCC-25 cell line was propagated in a monolayer to sub-confluency at 37°C in 75 cm^2 flasks containing 10 ml of DMEM:F12 media, supplemented with heat inactivated 10% fetal calf

serum (FCS), 1% penicillin, 1% glutamine and 1% streptomycin and hydrocortisone (0.4µg/ml) in a fully-humidified incubator containing 5% CO_2 and 95% air.

The sensitivity of human skin cancer SCC-25 cells to the curcumin nano-formulation was determined by the MTT colorimetric assay. Cells (1×10^4 per well) were seeded in a flat-bottomed 96-well plate and incubated at 37°C and in 5% CO_2. Cells were exposed to silica nanoparticles alone or loaded with curcumin at the concentrations of 15, 30, and 45µg/ml in a dose and time dependent manner for 24, 48 and 72 h. Cells were then treated with MTT reagent (10µl/well volume from 5mg/ml solution in PBS) for 4 h at 37°C. Then DMSO (100µl) was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 570 nm in a microplate reader and percentage of residual cell viability was determined. All experiments were performed in triplicate.

Protein expression and Western blotting studies:

Briefly, 3×10^5/mL cells in 6-well plates were treated with MCM-41 and MCM-41-CUR for 48 h. The cells were then washed twice in PBS and lysed in 20 mM Tris (pH 7.5) containing 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM sodium vanadate, 1 µg of leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Following sonication, cell extracts were centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentrations were measured using a BCA assay. Protein samples (30 µg), diluted with SDS sample buffer, were electrophoresed on a 10% polyacrylamide gel and electro-blotted on a polyvinylidene difuoride (PVDF) membrane for immunoblot analysis. After blocking in 5% non-fat dry milk, the membrane were probed overnight at 4 °C with primary antibodies and immunoreactivity were detected using anti-mouse or anti-rabbit IgG conjugated peroxidase, then visualized using the Clarity™ Western ECL Substrate kit and the ChemiDoc MP system (Bio-Rad, Australia) according to the manufacturer's instructions.

Silica ICP-OES study:

This study was carried out to measure the silica nanoparticles endocytosis performance in human skin cancer SCC-25 cells (5×10^4 cells per well of 6 wells plate) from both MCM-41 and MCM-41-CUR. The SCC-25 cells were treated similar condition as mentioned in MTT assay and cells from each treatment were washed twice with cold phosphate-buffered saline (PBS, pH 7.4). The cells from both groups were collected separately after 8h following trypsinization and then washed twice with PBS and centrifuged at 5000 rpm for 10 min. Cell lysate were prepared in 200µl ice-cold (4°C) 1x cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-

glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) following sonication. Cell lysates were then centrifuged for 10 minutes at 14,000 rpm at 4°C and pellet fractions were collected and dissolved in 200µl of 1M NaOH solution. The collected samples (n=3) were then diluted in PBS and sent to the Agricultural Biotechnology Center at University of Queensland for ICP analysis of Si and expressed in milligram per litre unit.

2. Results

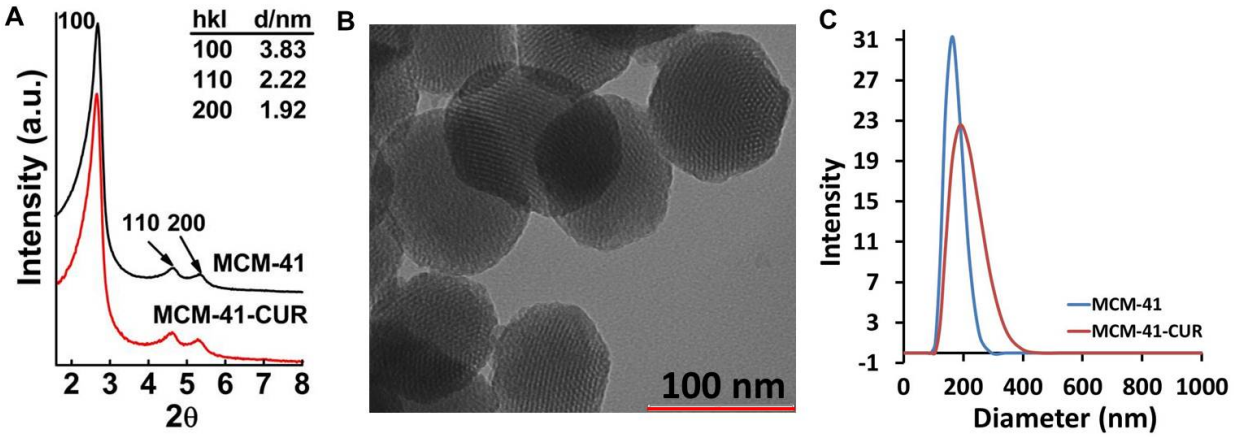

Fig S1. XRD patterns of MCM-41 and MCM-41-CUR (A), TEM image of MCM-41 (B) and DLS size measurements of MCM-41 and MCM-41-CUR (C).

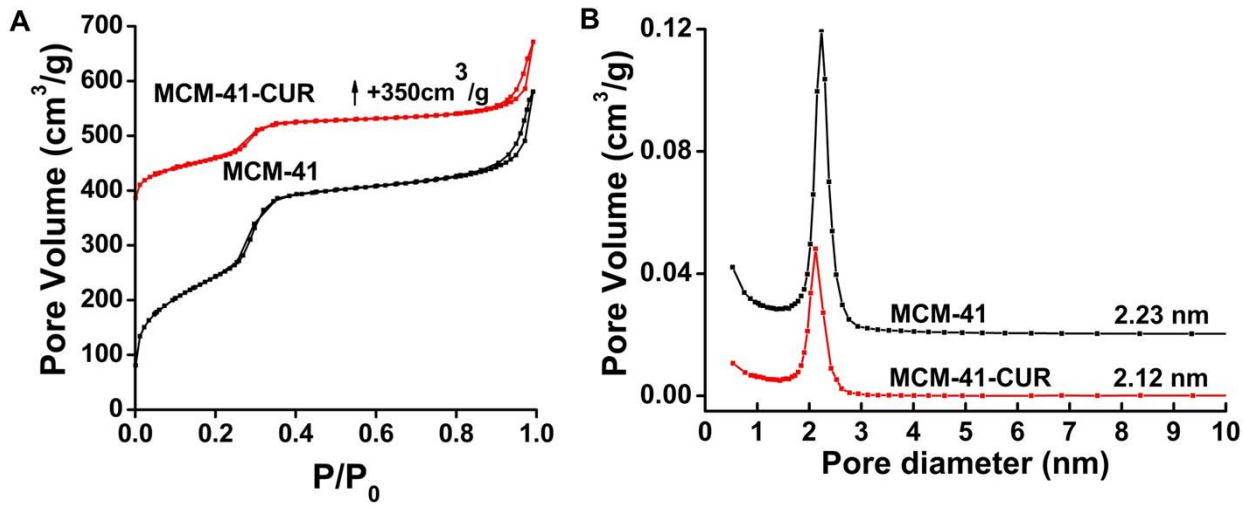

Fig S2. N_2 adsorption - desorption isotherms (A) and BJH pore size distribution plots (B) of MCM-41 and MCM-41-CUR.

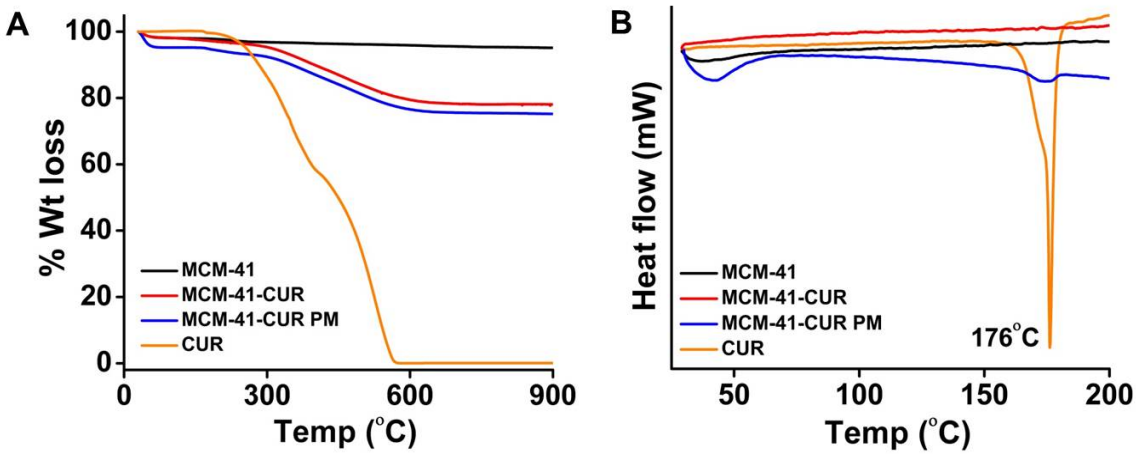

Fig S3. TGA (A) and DSC (B) curves of MCM-41, MCM-41-CUR, MCM-41-CUR PM and Curcumin.

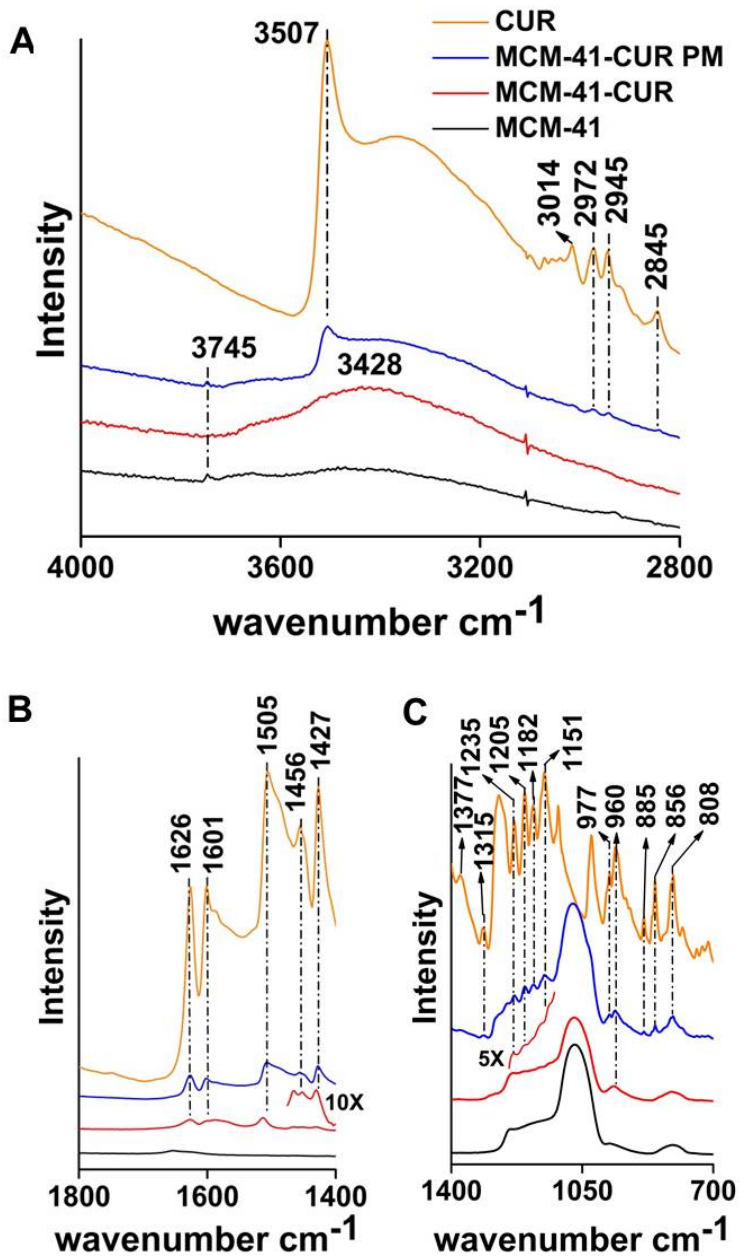

Fig S4. FTIR spectra of MCM-41, MCM-41-CUR, MCM-41-CUR PM and Curcumin.

FTIR spectra of CUR displays several peaks at 3507 cm^{-1} (free –OH group vibration), 3014 cm^{-1} (C-H stretching of aromatic ring), 2845 cm^{-1} (C-H stretching of methyl group), 1626 cm^{-1} (C=O stretching), 1505 cm^{-1} (C=O and C=C vibration), 1456 cm^{-1} (CH_2 bending) and 1377 cm^{-1} (CH_3 bending). A band of peaks is observed at 1000-1300 cm^{-1} in CUR which can be typically attributed to symmetric and asymmetric configurations of C-O-C chains. MCM-41-CUR reveals peak of both CUR and MCM-41. The band at 960 cm^{-1} (Figure S3C) is the characteristic peak for $\rho(CH_3)$, which overlaps with the band of silanols after the encapsulation of curcumin in pores of MCM-41.

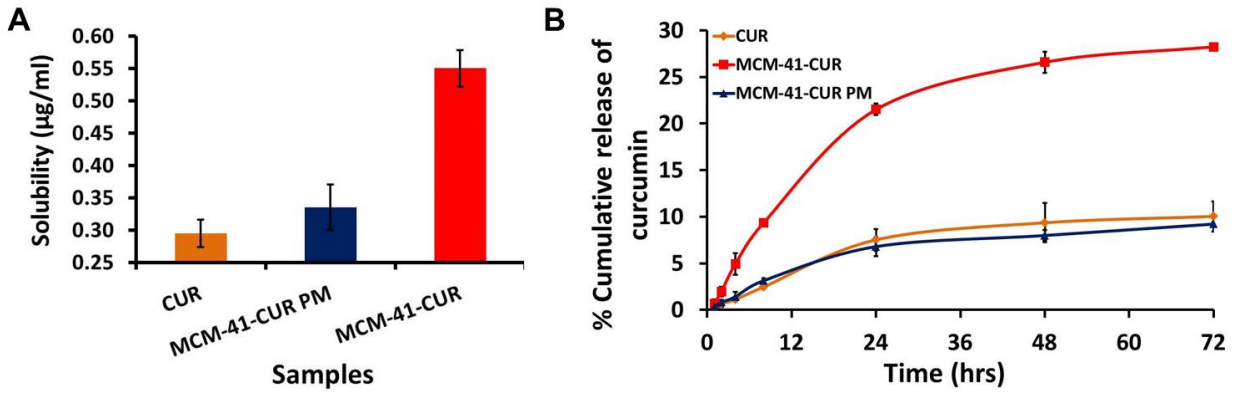

Fig.S5 Aqueous equilibrium solubility (A) and In vitro drug release (B) of Curcumin, MCM-41-CUR and MCM-41-CUR PM in 0.5% SLS.

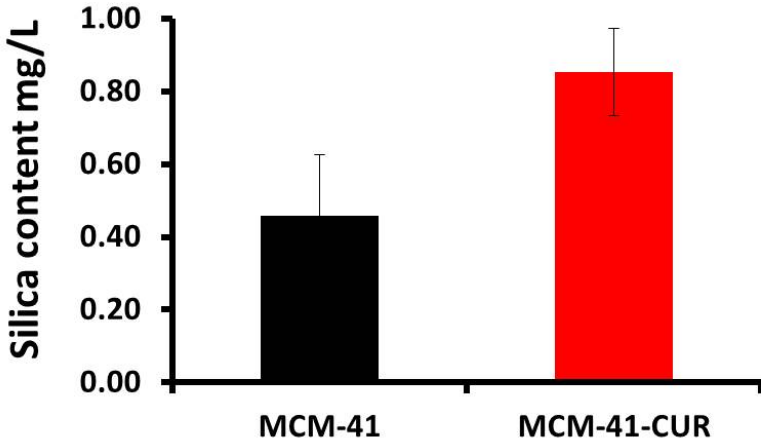

Fig.S6 Silica content analyzed by ICP-OES for MCM-41 and MCM-41-CUR endocytosed in SCC-25 cell line after 8h

Table S1. Physicochemical properties of calcined MCM-41 and curcumin loaded MCM-41

Sample	*a* (nm)	*P* (nm)	S_{BET} (m^2/g)	V_p (cm^3/g)	*Z.P.*
MCM-41	4.42	2.23	976	0.9	-21.03 ± 0.57
MCM-41-CUR	4.47	2.12	480	0.5	-20.53 ± 1.10

Note: *a*=cell dimension; S_{BET} = BET surface area; V_p = pore volume; *P* = pore size; Z.P. = Zeta potential.

Table S2. In vitro cytotoxicity of pure curcumin, MCM-41-CUR and MCM-41-CUR PM performed in SCC-25 cell line in triplicate after 24, 48 and 72h expressing % cell viability with standard deviation

Time	Sample	% Cell viability ± Standard deviation			
		CTL	15 ppm	30 ppm	45 ppm
24 h	CUR	100 ± 0.05	56.42 ± 0.03	50.96 ± 0.02	45.89 ± 0.04
	MCM-41-CUR	100 ± 0.06	52.13 ± 0.04	45.16 ± 0.01	37.17 ± 0.06
	MCM-41-CUR PM	100 ± 0.05	73.53 ± 0.01	75.94 ± 0.04	67.15 ± 0.08
	MCM-41	100 ± 0.05	97.41 ± 0.05	99.21 ± 0.01	97.37 ± 0.08
48 h	CUR	100 ± 0.06	58.41 ± 0.05	48.61 ± 0.03	43.11 ± 0.10
	MCM-41-CUR	100 ± 0.08	36.92 ± 0.03	20.09 ± 0.09	11.06 ± 0.07
	MCM-41-CUR PM	100 ± 0.14	79.33 ± 0.02	81.22 ± 0.02	76.04 ± 0.09
	MCM-41	100 ± 0.05	99.88 ± 0.02	96.26 ± 0.05	90.39 ± 0.05
72 h	CUR	100 ± 0.05	61.23 ± 0.05	51.88 ± 0.02	41.00 ± 0.03
	MCM-41-CUR	100 ± 0.08	15.94 ± 0.12	5.56 ± 0.01	2.66 ± 0.01
	MCM-41-CUR PM	100 ± 0.03	68.04 ± 0.02	62.33 ± 0.01	57.68 ± 0.04
	MCM-41	100 ± 0.12	96.73 ± 0.06	98.08 ± 0.04	96.42 ± 0.10

References

1. S. Yang, L. Z. Zhao, C. Z. Yu, X. F. Zhou, J. W. Tang, P. Yuan, D. Y. Chen and D. Y. Zhao, *J Am Chem Soc*, 2006, **128**, 10460-10466.
2. J. F. Shao, D. H. Zheng, Z. F. Jiang, H. E. Xu, Y. Hu, X. L. Li and X. W. Lu, *Acta Bioch Bioph Sin*, 2011, **43**, 267-274.