Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information for

Effect of surface properties in protein corona development on mesoporous silica nanoparticles

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1. Methods

All reagents were provided by Sigma-Aldrich. N-hydroxysuccinimide esters of methoxy group-terminated polyethylene glycol molecules with FW = 333.33 (PEG_3) or FW = 1214.39 (PEG_{24}) were purchased from Iris Biotech.

Synthesis of 50 nm MSN and surface modification

A standard method with some modifications was used for the synthesis of unmodified MSN (MSN-OH).[1] Briefly, 1.00 g of hexadecyltrimethylammonium bromide (CTAB) was dissolved in 500 mL of NaOH 14 mM at 80 ºC with strong stirring. Then, 5.0 mL of tetraethyl orthosilicate (TEOS) was dropped slowly. The obtained gel was 1:0.12:0.31:1245 SiO_2/CTAB/NaOH/H_2O. After 2 h the resulting mixture was cooled in ice, filtered off and washed with water and methanol. The solid was dried at 100 ºC overnight and heat-treated at 540 ºC for 6 h in air.

Amine derivatized MSN (MSN-NH_2) were prepared by surface functionalization of MSN-OH with 3-aminopropyltriethoxysilane (APTES). 500 mg of particles was dried at 350 ºC and vacuum (8 torr) for 3 h. Subsequently, 20 mL of anhydrous toluene was added and the mixture was heated to reflux. Then, 975 μL of APTES (4.2 mmol) was added and the mixture was stirred for 3 h. The obtained product was filtered off, washed with toluene and methanol and freeze-dried (-55 ºC, 16 h).

To obtain MSN with a coating of carboxylic moieties attached to surface (MSN-COOH), 500 mg of particles MSN-OH was dried at 350 ºC and vacuum (8 torr) for 3 h. Afterwards, 5 mL of anhydrous toluene was added and the mixture was heated to reflux. Then, 40 μL of (3-cyanopropyl)-trichlorosilane (0.25 mmol) was added and the mixture was stirred for 3 h. The obtained product was filtered off, washed with toluene and methanol and dried at room temperature and vacuum for 16 h. This solid was dispersed in 75 mL of sulfuric acid (60%) and heated at 150 ºC for 3 h in a reflux system. Then, the acid suspension was diluted with 100 mL of distilled water, filtered off, washed with toluene and methanol and freeze-dried (-55 ºC, 16 h).

For preparation of PEG-derivatized nanoparticles 200 mg of MSN-NH_2 was suspended in 20 mL of anhydrous dichloromethane. Then, 250 μL of diisopropyl amine were injected under nitrogen atmosphere. Afterwards, 150 mg of PEG_3 or 500 mg PEG_{24} was added. The reaction was stirred overnight at room temperature. Afterwards, the solvent was removed under reduced pressure and the nanoparticles were suspended in 100 mL of ethanol by stirring. Later, the suspension was

filtrated off and washed with ethanol (300 mL). Finally, the material was freeze-dried (-55 ºC, 16 h).

Nanoparticle characterization

Nanoparticle morphology and size were studied by transmission electron microscopy (TEM) in a JEOL JEM 2100F microscope operating at 200 kV. Samples were dispersed in methylchloride (methylene chloride?) and transferred to carbon coated copper grids. Nitrogen gas adsorption isotherms were measured in a Micromeritics Flowsorb apparatus. Surface area calculations were carried out using the BET method, whereas pore size distribution was calculated according to the Kruk-Jaroniec-Sayari (KJS) estimation.[2] Particle size and Z-Potential measurements were conducted by diffuse light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Dried materials were re-suspended in deionized water at a concentration of 5 μg/mL and measurements were performed at 25 ºC. The mean hydrodynamic diameter was determined by cumulant analysis. Surface coverage of organic groups (R-NH_2, R-COOH, R-PEG_3-OCH3 and R-PEG_{24}-OCH_3) was calculated from carbon elemental analysis determination (FISONS, EA 1108 CHNS-O). The organic content on the different samples before and after protein adsorption was quantified by thermogravimetric analysis in a Mettler-Toledo TGA/SDTA 851_e apparatus.

Protein adsorption

Protein adsorption was achieved by incubating MSNs (1 mg) in 10% FBS/DMEM (1 mL) for 1 h at room temperature. Subsequently, the nanoparticles were isolated through centrifugation (14,800 rpm, 5 min) and the supernatant was discarded. The nanoparticles were then resuspended in PBS (1 mL) and sonicated. This process was repeated three times in order to remove as much unbound protein as possible.

SDS-PAGE

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on the proteins isolated after serum incubation. Removal of the hard corona was achieved by sonicating nanoparticles in Laemmli buffer (63 mM Tris-HCl, pH 6.8, 40 mM DTT, 0.01% (w/v) bromophenol blue, 10% glycerol, 2% (w/v) SDS). Following, nanoparticle suspensions were boiled for 5-10 min in a hot water bath. Nanoparticles were then removed from the suspension through centrifugation (14,800 rpm, 5 min) and the supernatant was saved for SDS-PAGE analysis. Protein separation was then performed on a Bio-Rad Mini-PROTEAN electrophoresis

system (120 V, 1.5 h). The gels were then stained for 2 h using GelCode blue stain reagent (Thermo Scientific), followed by de-staining overnight in deionized water.

Protein quantification by bicinchoninic acid (BCA) test

The hard corona was removed by sonicating the nanoparticles in extraction buffer (63 mM Tris-HCl, pH 6.8, 40 mM DTT, 10% glycerol, 2% (w/v) SDS). Nanoparticle suspensions were boiled for 5-10 min in a hot water bath; the nanoparticles were then removed from the suspension by centrifugation (14,800 rpm, 10 min). The supernatant containing the digested peptide (10 µL) was added to BCA reagent (200 µL taken from a solution of 4 mL 4% BCA, pH 8.5 and 80 mL of 4% $CuSO_4 \cdot 5H_2O$) and the mixture was incubated at 37 ºC for 30 min. Next, absorbance at 562 nm was measured in a Nanodrop ND spectrophotometer. Calibrates were prepared with a protein standard solution (QuantiPro^{TM} BCA Assay Kit, Sigma-Aldrich). Total protein content quantified in the hard corona of MSN-OH, MSN-NH_2 and MSN-COOH materials is presented in Table S1.

Protein digestion for mass spectrometry analysis

After removal of the final PBS wash, nanoparticles containing the hard corona were suspended in digestion buffer (36 µL, 50 mM ammonium bicarbonate, 0.25 mM urea, 4% acetonitrile) and reducing buffer (6 µL, 100 mM dithiothreitol in H_2O). These nanoparticle suspensions were allowed to incubate for 1 h at 70^{o}C. After 1 h, alkylation buffer (15 µL, 100 mM iodoacetamide) was added and the samples were incubated at room temperature for 20 min in a dark place. Protein digestion was achieved by adding trypsin (15 µL, 40 ng/µL) and incubating overnight at 37^{o}C. The following morning, formic acid (15 µL, 10%) was added to stop the digestion process. Prior to submitting the samples for proteomics analysis, the nanoparticles were centrifuged out of solution and the supernatant was submitted for analysis.

Proteomics analysis method

The digested peptide sample was desalted using a ZipTip C_{18} (P10, Millipore Corporation, Billerica, MA) according the manufacturer's protocol, and then dried in a SpeedVac. The dried peptide samples were dissolved in 20 µl 0.1% formic acid and 2% acetonitrile, and 5 µl were loaded onto a fused silica microcapillary LC column (12 cm x 100 µm inner diameter) packed with C18 reversed-phase resin (5 µm particle size; 20 nm pore size; Magic C_{18}AQ, Michrom Bioresources Inc.). Peptides were separated by applying a gradient of 3-60% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min for 45 min. Nanospray ESI was used to introduce peptides into a liner ion trap (LTQ)-Orbitrap mass spectrometer (Thermo Fisher Scientific) via a nanospray ionization source. Mass spectrometry data was acquired in a data-dependent

acquisition mode, in which an Orbitrap survey scan from m/z 400-2000 (resolution: 30,000 FWHM at m/z 400) was paralleled by 10 LTQ MS/MS scans of the most abundant ions. After an LC-MS run was completed and spectra were obtained, the spectra were searched against the IPI Bovine protein sequence databases (V 3.85) using Proteome Discoverer software (version 1.4; Thermo Electron, San Jose, CA). The search parameters permitted a 20 ppm precursor MS tolerance and a 1.0 Da MS/MS tolerance. Oxidation of methionine (M) and carboxymethylation of cysteines (C) were allowed as variable modifications. Up to two missed tryptic cleavages of peptides were considered. The cutoffs for SEQUEST assignments were: cross-correlation (Xcorr) scores greater than 1.9, 2.5, and 3.0 for peptide charge states of +1, +2, and +3, respectively; and a delta-correlation (ΔCn) score > 0.1. Then, all .srf files for each sample were inputted into Scaffold (version Scaffold_4.0.5, Proteome Software Inc., Portland, OR) for the calculations of total spectrum counts.

2. TEM micrographs of MSNs

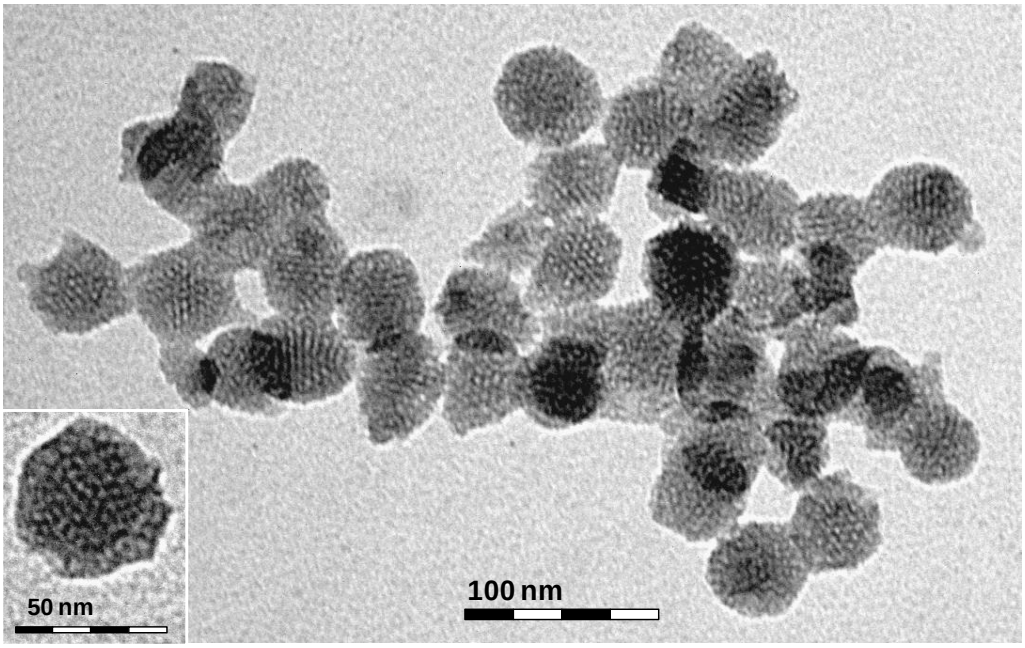

Figure S1. TEM micrograph of as-synthesized MSN-OH material. The inset shows the internal hexagonal order of a single 50 nm nanoparticle.

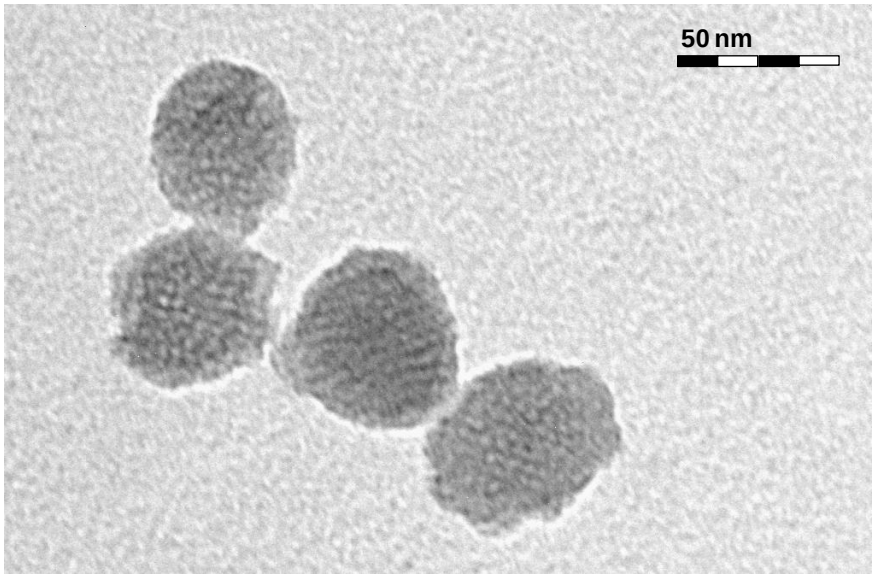

Figure S2. TEM micrograph of as-synthesized MSN-PEG_3-OCH_3 material. High mesoporous order and nanoparticle dispersion is observed even after functionalization.

3. Protein quantification by BCA test

Sample	TGA (wt% organic)[a]	BCA (% protein)[b]
MSN-OH	10.1	8.8
MSN-NH_2	14.4	13.2
MSN-COOH	2.3	3.2

[a] Protein adsorbed determined by TGA.

[b] Total protein content extracted as determined by BCA test.

4. Proteomics analysis of protein corona

Table S1. Combined spectral counts for all identified proteins.

Protein	Mw (kDa)	MSN-OH	MSN-NH_2	MSN-COOH	MSN-PEG3	MSN-PEG24
50 kDa protein	50	0	2	0	0	0
ACTA2 protein-like	26	16	0	3	0	0
Actin, alpha skeletal muscle	42	16	0	7	0	0
Actin, cytoplasmic 1	42	0	12	0	0	0
Alpha-1-antiproteinase	46	8	7	18	0	0
Alpha-1B-glycoprotein	54	0	0	0	0	0
Alpha-2-antiplasmin	55	0	7	0	0	0
Alpha-2-HS-glycoprotein	38	59	73	17	6	0
Alpha-2-macroglobulin	168	7	22	27	0	0
Antithrombin-III	52	0	3	0	0	0
Apolipoprotein A-I	30	42	10	30	0	0
Apolipoprotein A-I-like	24	22	0	12	0	0
Apolipoprotein A-II	11	28	41	17	0	0
Apolipoprotein A-IV	43	0	0	0	0	0
Apolipoprotein C-III	11	5	6	0	0	0
Apolipoprotein E	36	0	11	3	0	0
Apolipoprotein M	21	0	8	0	0	0
Beta actin	42	0	0	2	0	0
Beta-2-glycoprotein 1	38	0	0	6	0	0
CCP modules 3-12, with parts of CCP 2 and 13-like, partial	37	0	0	0	0	0
Clusterin	51	4	19	15	0	0
Coagulation factor X	55	0	12	0	0	0
Coagulation factor XII	67	0	0	0	0	0
Collectin-43	34	0	17	0	0	0
Complement C3 (Fragment)	187	4	61	2	0	0
Complement component 8, alpha polypeptide	66	0	0	2	0	0
Complement factor H	140	0	0	3	0	0
Complement factor H-like	106	0	0	0	0	0
Complement factor H-like	65	0	0	0	0	0
Complement factor H-like, partial	73	0	0	0	0	0
Complement factor H-related 1-like isoform 1	59	0	0	0	0	0
Complement factor I	69	0	0	9	0	0
ECM1 protein	58	0	3	3	0	0
Embryo-specific fibronectin 1 transcript variant	262	0	38	11	0	0
Factor XIIa inhibitor	52	0	5	0	0	0
Fetuin-B	43	3	2	0	0	0
Fibrinogen alpha chain	67	12	9	8	0	0
Gelsolin	81	0	0	16	0	0
Hemoglobin fetal subunit beta	16	12	10	33	0	0
Hemoglobin subunit alpha	15	0	13	4	0	0

Protein	Mw (kDa)	MSN-OH	MSN-NH_2	MSN-COOH	MSN-PEG3	MSN-PEG24
Hemoglobin subunit beta	16	10	0	17	0	0
Histidine-rich glycoprotein	62	0	0	5	0	0
Histidine-rich GLYCOPROTEIN=FACTOR XIIIA substrate (Fragment)	24	0	3	0	0	0
Hyaluronan-binding protein 2	62	0	7	0	0	0
IGL@ protein	25	0	7	0	0	0
Inter-alpha-trypsin inhibitor heavy chain H1	101	0	13	0	0	0
Inter-alpha-trypsin inhibitor heavy chain H3	100	0	30	0	0	0
Inter-alpha-trypsin inhibitor heavy chain H4	102	12	0	44	0	0
Isoform HMW of Kininogen-1	69	0	0	0	0	0
Isoform HMW of Kininogen-2	69	0	20	0	0	0
Isoform LMW of Kininogen-1	48	0	23	0	0	0
Inter-alpha-trypsin inhibitor heavy chain H2	106	0	88	8	0	0
Periostin	93	0	6	0	0	0
Pigment epithelium-derived factor	46	0	0	3	0	0
Pregnancy-zone protein-like	159	0	0	2	0	0
Protein AMBP	39	0	46	0	0	0
Prothrombin (Fragment)	71	0	0	0	0	0
QSOX1 protein	63	12	0	2	0	0
Serum albumin	69	0	77	14	0	0
Similar to complement component 4A	193	0	64	8	0	0
Talin-1	270	0	8	2	0	0
Tenascin-X	447	0	0	0	0	0
Tetranectin	22	2	0	5	0	0
Thrombospondin-1	130	12	0	4	0	0
Uncharacterized protein	163	0	0	25	0	0
Uncharacterized protein	78	16	27	13	0	0
Uncharacterized protein	161	0	12	3	0	0
Uncharacterized protein	193	0	60	5	0	0
Uncharacterized protein	47	0	0	5	0	0
Uncharacterized protein	39	0	0	6	0	0
Uncharacterized protein	33	0	0	0	0	0
Uncharacterized protein	52	0	13	0	0	0
Uncharacterized protein	55	0	9	0	0	0
Uncharacterized protein	87	0	2	0	0	0
Uncharacterized protein	303	0	7	0	0	0
Uncharacterized protein	516	0	4	0	0	0
Uncharacterized protein	52	0	0	2	0	0
Uncharacterized protein (Fragment)	174	0	0	3	0	0
Uncharacterized protein (Fragment)	176	0	0	8	0	0
Uncharacterized protein (Fragment)	46	0	0	2	0	0
Vitamin D-binding protein	53	0	11	0	0	0
Vitamin K-dependent protein S	75	0	13	0	0	0
Vitronectin	54	0	13	10	3	0
von Willebrand factor	308	0	16	0	0	0

5. Heat map identifying most abundant proteins

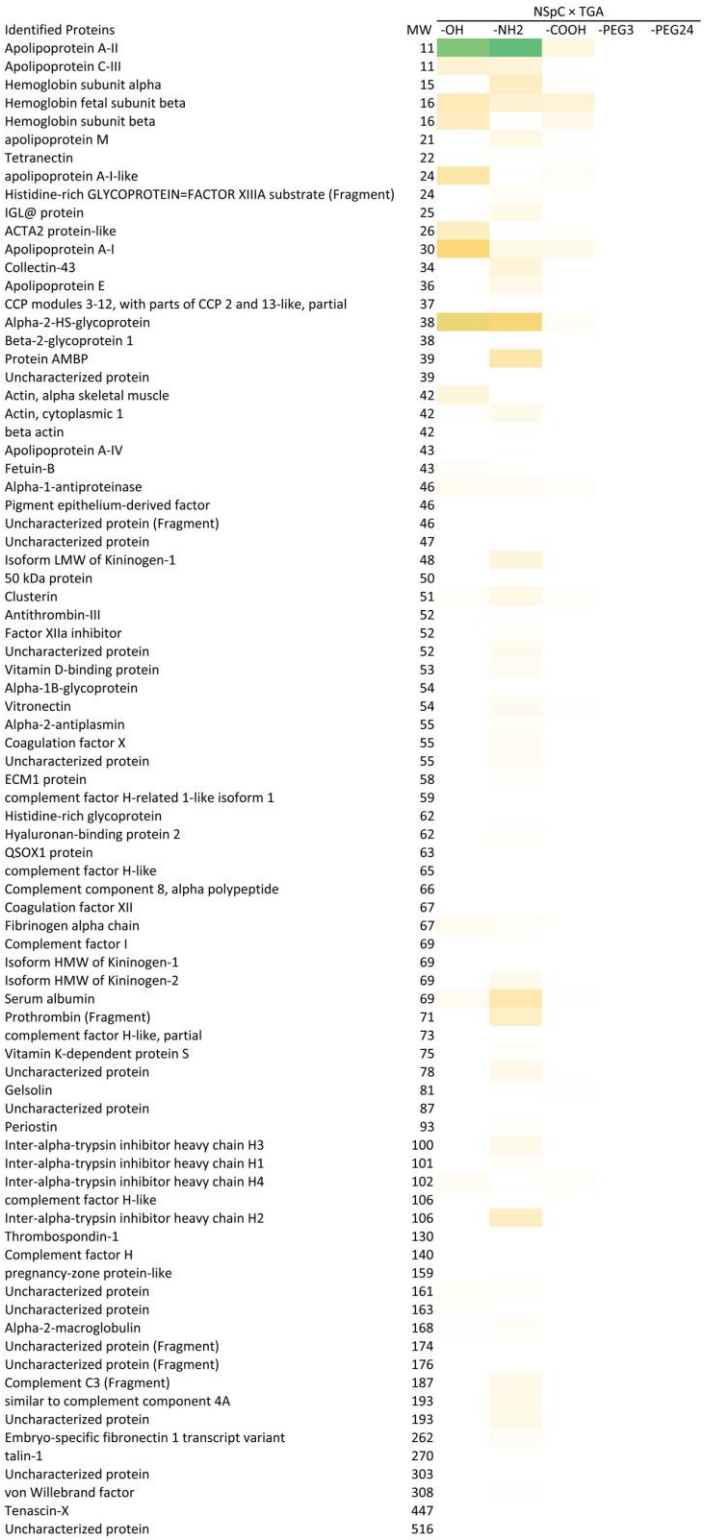

Figure S3. Heat map of all identified proteins, organized by increasing molecular weight. In this figure, color corresponds to NSpC × TGA. The highest value in is in green, the value at 50% is gold, the lowest value is in white, and all other values are colored on a linear scale between these values.

6. Histogram of relative protein composition based on molecular weight

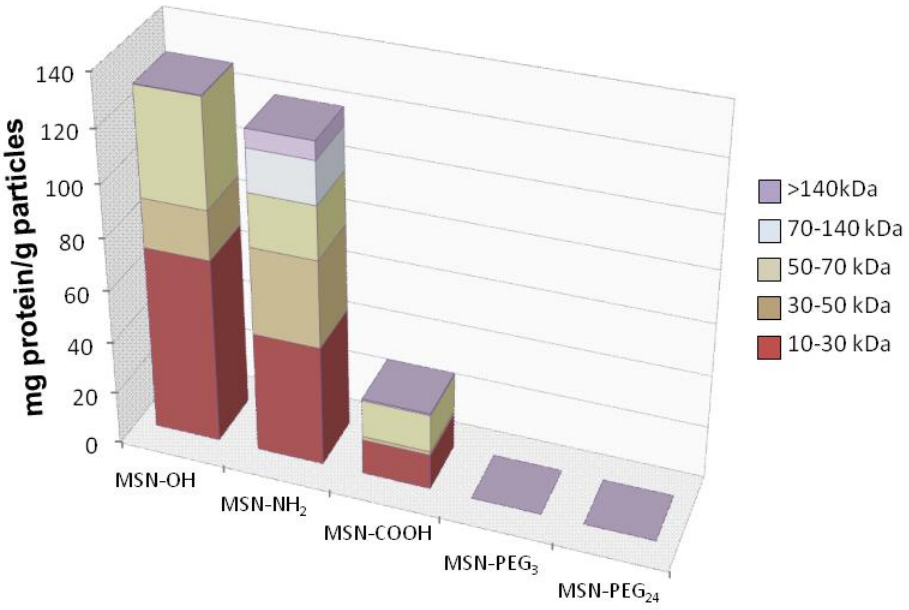

Figure S4. Protein composition was grouped according to molecular weight range depicted in histogram. As shown, low molecular weight proteins composed the highest weight percentage of the protein corona.

7. References

1. Q. Cai, Z.-S. Luo, W.-Q. Pang, Y.-W. Fan, X.-H. Chen and F.-Z. Cui, *Chem. Mater.*, 2001, **13**, 258-263.
2. M. Kruk, M. Jaroniec and A. Sayari, *Adsorption*, 2000, **6**, 47.