Electronic Supplementary Material (ESI) for Analytical Methods

Supporting information

Materials and chemicals

Triblock copolymer poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) Pluronic P123 (EO20PO70EO20, Mav = 5800 g/mol) was purchased from Aldrich Chemical Inc. Aluminum iso-propoxide, polyvinylpyrrolidone (PVP, Mw = 30000) was purchased from Aldrich. 2, 2-Azobisisobutyronitrile (AIBN) (Acros) was recrystallized in methanol before use. Styrene (St) and ethanol were purchased from Shanghai Chem. Corp. Trifluoroaceticacid (TFA), β-casein, ammonium bicarbonate (NH_4HCO_3), bovine serum albumin; trypsin (from bovine pancreas). 3-(trihydroxysilyl)propyl methylphosphateand 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma Chemical (St. Louis, MO). Acetonitrile was purchased from Merck (Darmstadt, Germany). All aqueous solutions were prepared using Milli-Q water by Milli-Q system (Millipore, Bedford, MA). All other chemicals and reagents were of the highest grade commercially available.

Preparation of polystyrene microspheres

Monodisperse polystyrene (donated as PS) microspheres with a mean size of ~1.2 μm were prepared through a dispersion polymerization approach.[1] For a typical preparation, 16 g of styrene, 2.0 g of PVP, 0.3 g of 2,2-azobisiso-butyronitrile (AIBN) were dissolved in a mixture of ethanol (80 mL) and H_2O (7.6 mL). The obtained solution was then added into a 250 mL four-neck round bottom flask equipped with a mechanical stirrer, a refluxing condenser, and a nitrogen inlet. After sealing in a nitrogen atmosphere, the reactor was submerged in a water bath and the polymerization was carried out with a stirring speed of 100 rpm at 70 °C for 24 h. The resulting polymer microspheres were repeatedly washed with an ethanol_water mixture (1: 1

volume ratio) by centrifugation and then redispersed in water to obtain a colloidal solution. Finally, an aqueous dispersion (40 g) of PS microspheres (5 wt % solid content) was let to stand for a week to form a closely packed colloidal crystal at the bottom of the vessel by gravity sedimentation. After careful removal of the supernatant liquid and further drying at 25 ^{o}C for 48 h, the as-made colloidal crystals were produced and could be directly used as the hard template for hierarchically ordered macro/mesoporous alumina.

Preparation of hierarchically ordered macro/mesoporous alumina

The hierarchically ordered macro/mesoporous alumina (denotes as HOMMA) were prepared through a dual-templating synthesis approach with 3D ordered PS colloidal crystals as a hard template and amphiphilic triblock copolymer Pluronic P123 as a soft template, and alumina iso-propoxide as the alumina source. First, the precursor sol was prepared by mixing 1 g of Pluronic P123, 1.5 mL of concentrated nitric acid, 20 mL of ethanol and 2.04 g of alumina iso-propoxide for 20 h under magnetic stirring at room temperature. Second, the colloidal crystal monoliths were impregnated with the above solution to introduce the P123-alumina composites into the interstitial voids. Finally, the HOMMA materials were obtained by calcination of the impregnated colloidal crystals.

Characterization and measurements

Scanning electron microscopy (SEM) images were obtained on a Philips XL30 electron microscope (Netherlands) operating at 20 kV. Transmission electron microscopy (TEM) images were taken with a JEOL2011 microscope (Japan) operating at 200 kV. Wide-angle X-ray diffraction (WAXRD) patterns were recorded on a Bruker D4 X-ray diffractometer (Germany) with Ni-filtered Cu KR radiation (40 kV, 40 mA). The Brunauer-Emmett-Teller (BET) method

was utilized to calculate the specific surface areas (SBET) using adsorption data in a relative pressure range from 0.18 to 0.35. By using the Barrett-Joyner-Halenda (BJH) model, the pore volumes and pore size distributions were derived from the desorption branches of the isotherms, and the total pore volumes (*V*t) were estimated from the adsorbed amount at a relative pressure P/P_0 of 0.992.

Immobilization of proteins/enzymes into HOMMA

For the immobilization test, 1.0 mg of HOMMA was added into 2 mL of protein/enzyme solution (1 mg/mL) in the ammonium bicarbonate buffer (25 mM, pH ~ 8.0). The mixture was stirred at 25 °C for different time to reach the adsorption maximum. The adsorbed amount was measured using a difference method with protein concentrations determined before and after adsorption by UV absorption at 280 nm on a V-550 UV/vis spectrophotometer.

Digestion and isolation of phospho species for MALDI-TOF MS analysis

For in-solution digestion, 1 mg of β-casein was dissolved in 1 mL of ammonium bicarbonate buffer (25 mM, pH ~ 8), digested for 16 h at 37 °C with an enzyme to protein ratio of 1:30 (w/w), then the digested β-casein was diluted to 20 ng/μL. For the digestion in the presence of HOMMA, 2 μL of 20 mg/mL HOMMA was directly added into 200 μL of β-casein in-solution digestion system (20 ng/μL or 2 ng/μL) and incubated at 37 °C for 30 min. For the biosample, 30 μL of bovine milk was diluted in 900 μL of NH_4HCO_3 aqueous solution at 25 mM. This solution was then centrifugated at 16 000 rpm for 15 min, and the supernatant was collected for tryptic digestion.

After the digestion, the mixture was centrifugated to separate the deposits and the deposits were washed by a solution of 0.1% TFA (50% ACN/water) 20 μL two times. Finally, 10 μL of

0.1% TFA (50% ACN/water) was added to the deposits, and the suspension (1 μL) was directly deposited on the MALDI plate. After evaporation of the solvent, 0.5 μL of DHB (10 mg/mL in 50% ACN/water, 1% H_3PO_4) was added and dried at room temperature. MALDI–TOF MS experiments were performed in positive ion mode on a 5800 Proteomics Analyzer (Applied Biosystems, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV.

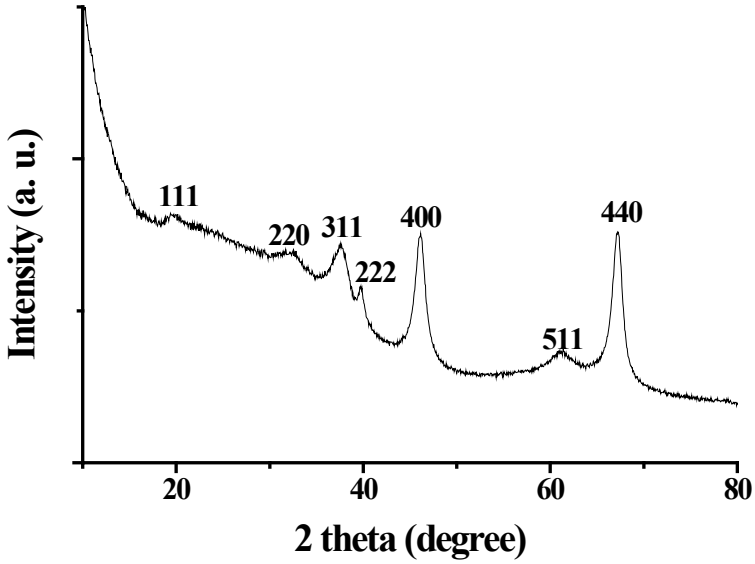

Figure S1. XRD patterns of HOMMA.

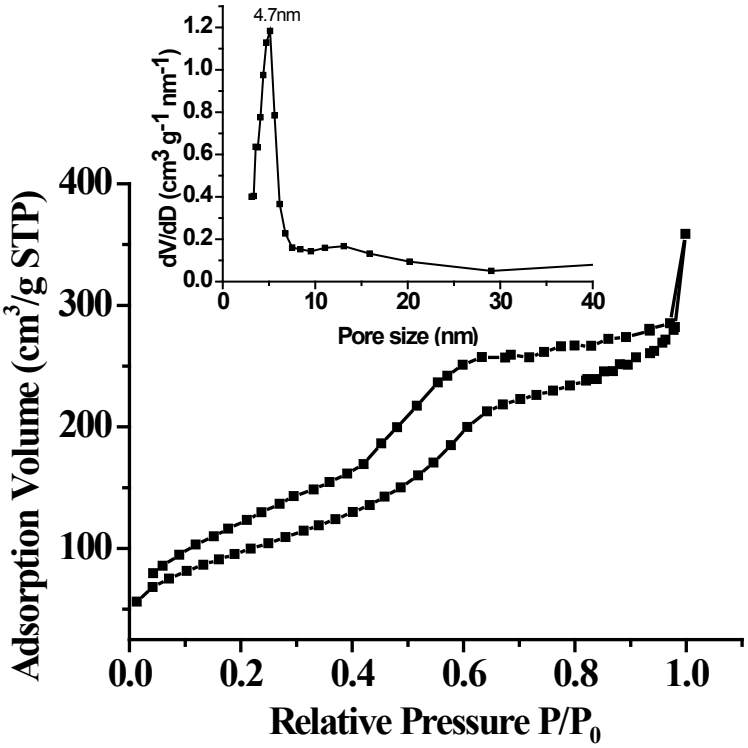

Figure S2. N_2 adsorption–desorption isotherms and pore size distribution (insert) of HOMMA.

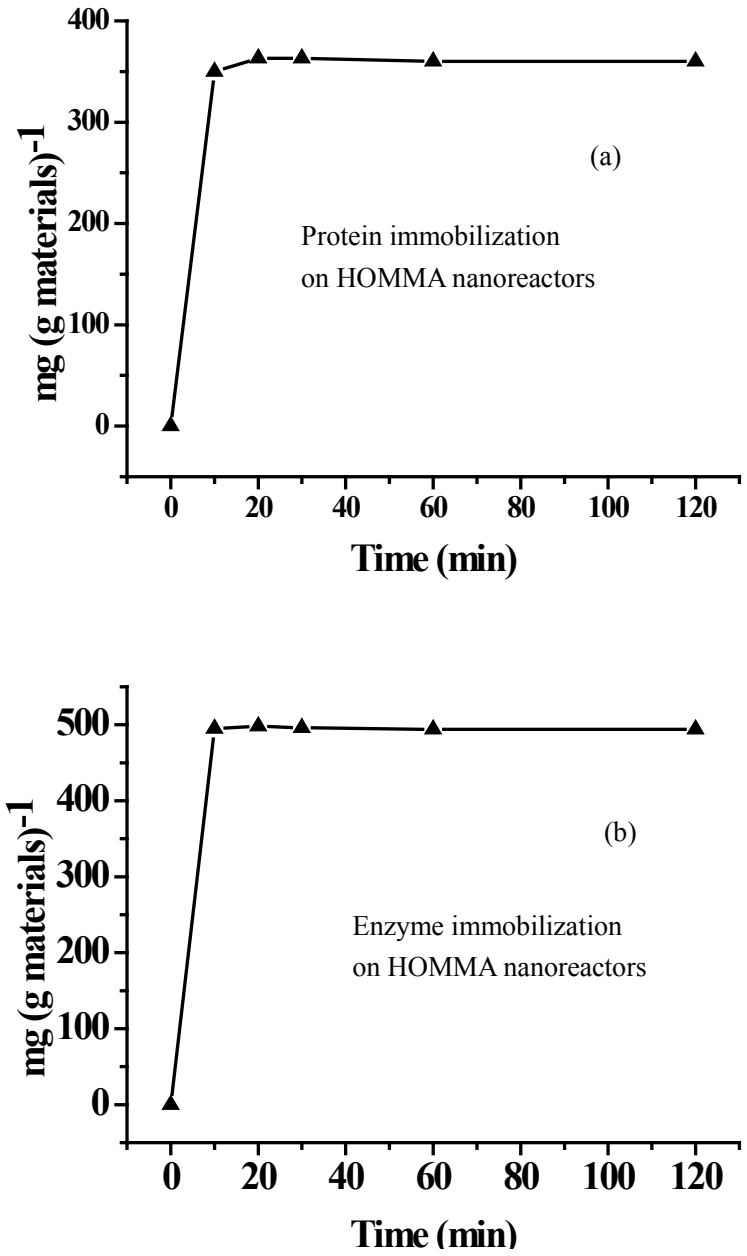

Figure S3. Adsorption of (a) proteins and (b) enzymes into HOMMA nanoreactors as a function of time.

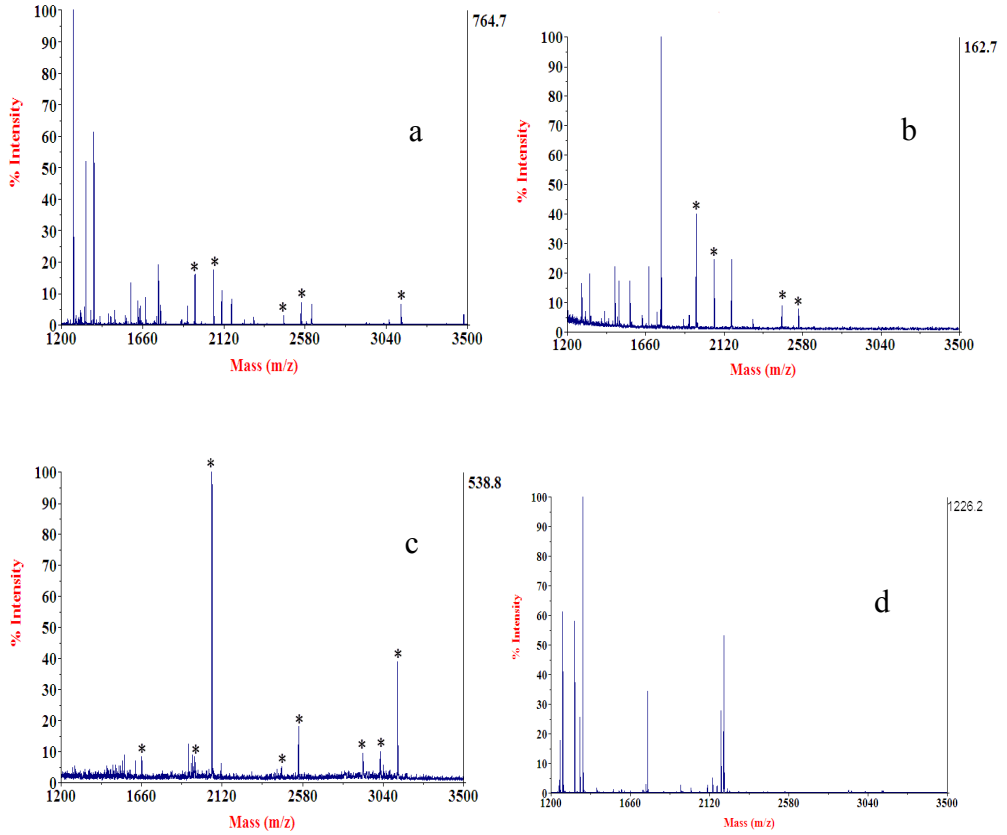

Figure S4. Mass spectra of proteolysis products for the supernatant of 30 min HOMMA-catalyzed β-casein (a) 20 ng/uL; (b) 2 ng/uL digestion; (c) MALDI mass spectrum of peptides for the fraction enriched by HOMMA from in-solution β-casein digestion; (d) MALDI mass spectrum of peptides for the supernatant of in-solution β-casein digestion with HOMMA enrichment, where the * indicates the phosphopeptides.

[1] C. Z. Li and J. H. He, *Langmuir* 2006, **22**, 2827.