A proton-responsive ensemble using mesocellular foam supports capped with N, O-carboxymethyl chitosan for controlled release of bioactive proteins

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Electronic Supporting Information (ESI)

Experimental part:

Materials:

Triblock copolymer Pluronic P123, glycidoxypropyltrimethoxysilane (GPTMS), 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) and bovine serum albumin (BSA) were all purchased from Sigma-Aldrich (CA, America). Bone morphogenetic protein-2 (BMP-2, E. coli-derived) was purchased from Shanghai Rebone Biomaterials Co. Ltd. (Shanghai, China). Hydrochloric acid, Isopropyl alcohol and monochloroacetic acid were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), tetraethyl orthosilicate (TEOS), ammonium fluoride (NH_4F) and 1,3,5-trimethylbenzene (TMB) were from Shanghai Lingfeng Chemical Reagent Co. Ltd. (Shanghai, China). Chitosan with average molecular weight of 3.0×10^4 g·mol^{-1}, 90% deacetylation was purchased from Shanghai Kabo Reagent Co. Ltd. (Shanghai, China). DAPI was purchased from Beyotime Biotech Co. Ltd. (Jiangsu, China). p-nitrophenylphosphate (PNPPNa) was purchased from Sangon (Shanghai, China). All cell-culture related reagents were purchased from Gibco (Grand Island, NY).

Methods:

Synthesis of N, O-carboxymethyl chitosan (NOCC)

Chitosan powder (5 g) was dispersed in 50 mL of isopropyl alcohol and the mixed solution was rapidly stirred in a 250-mL round-bottom flask at 25 ^{o}C. Afterward, 5 ×

2.5 mL of aqueous NaOH solution (10 M) was added to the stirred solution over a period of 30 min. The alkaline mixed solution was continued stirring for another 30 min. In the next moment, 30 g of monochloroacetic acid was added, in 5 equal portions, at 2 min intervals. Subsequently, the reaction system was heated to 60 ^{o}C and stirred at this temperature for 3 hours. After that, the reaction mixture was centrifuged at 8000 rpm for 10 min and the solid was washed with methanol twice. Then the collected solid (NOCC) was freeze dried and stored at a dryer until used.

Synthesis of mesocellular foam (MCF)

Mesocellular foam was synthesized as per a procedure described in a literature with slight modifications.[1] 20 mL of concentrated hydrochloric acid (37%) and 130 mL of deionized water were added into a 500 mL beaker and then 8 g of Pluronic P123 was dissolved into the acidic solution. 8 g of TMB was added and the mixture was vigorous stirring at 38 ^{o}C for 2 hours. Afterwards, 18 mL of tetraethoxysilane (TEOS) was dropwise added into the stirred solution for 8 min. The resulting solution was transferred to a tetrafluoroethylene autoclave and aged at 40 ^{o}C for 24 h. Subsequently, 92 mg of NH_4F was added and the mixed solution was aged at 100 ^{o}C for another 24 h. The solution was centrifuged at 8000 rpm for 10 min to obtain the preparative precipitate. Then the solid was washed with ethanol and water twice and dried. The prepared white powder was grind and then calcined in air at 900 ^{o}C for 5 h.

Synthesis of N, O-carboxymethyl chitosan modified mesocellular foam (MCF-NOCC)

The synthetic step to produce the N, O-carboxymethyl chitosan modified mesocellular foam is showed as followed. 200 mg of NOCC was dissolved into 20 mL of water under intensively stirring to obtain a sticky solution and then 1 mL aliquot of 20% glycidoxypropyltrimethoxysilane (GPTMS) in ethanol solution was added (referred to as 1:10 GPTMS/NOCC in the research). The mixture was stored in a sealed plastic bottle over night at room temperature. 800 mg of MCF was added into the bottle and then strongly stirred at room temperature for another 12 h. The mixture was centrifuged and the solid was washed with water. The prepared MCF-NOCC was freeze dried and stored at a dryer until used.

Characterization

The FTIR spectrum of chitosan, NOCC, MCF and MCF-NOCC was measured by ATR on Nicolet 380 (Thermo, USA). The morphologies and microstructures of MCF and MCF-NOCC were measured with TEM and SEM. TEM and high-resolution TEM (HRTEM) were performed on a transmission electron microscope (JEM-2100, Japan) with a field emission gun operating at 200 KV. A scanning electron microscope (SEM, S4800, Japan) was applied to evaluate the surface morphology of our materials at 15 KV. The surface analysis was performed by N_2 adsorption isotherms in a Micromeritics ASAP2010 sorptometer (Micromeritics, USA) at 77 K. Before the sorption measurement, the MCF samples were outgassed for 6 h at 200 ^{o}C, while the MCF-NOCC samples were outgassed for 12 h at 80 ^{o}C. The surface areas were obtained by the BET method and the pore size distributions were calculated by the BJH method. Thermogravimetric analysis (TG) was measurement with an integrated thermal analyzer STA409PC (Netzsch, Germany). The zeta potential of our materials was studied with a particle size analyzer Malvern Nano HT Zetasizer (Malvern, UK). The release of protein was carried out with a UV/Vis spectrometer (Sunnyvale, CA) using the BCA Assay Kits (Beyotime, China).

In vitro proteins loading and release

Two model proteins, bovine serum albumin (BSA) and bone morphogenetic protein-2 (BMP-2) were used to study proteins loading into the carriers and subsequent protein release behavior. As we know, the loading capacity of protein/drug depends greatly on the concentration of protein/drug. The aim of the experiment designed in this study is to compare the loading capacity and release characteristics of two kinds of proteins with different properties. Considering that the BMP-2 is very expensive, we kept the protein concentration same and fixed at a relatively low concentration of 5 mg/mL. Take BSA loading and release as an example. In the experiments, 2 beakers of 400 mg of MCF was soaked in 10 mL of deionized water and stirred, then 4 mL of 5 mg/mL BSA aqueous solution was added into the mixture for a time period of 3 to 4 h. one beaker of the mixture of MCF and BSA was centrifuged and then collected (MCF-BSA sample) while the solution in the other beaker was subsequently mixed with 10 mL NOCC/GPTMS solution (100 mg of NOCC was dissolved into 10 mL of water under intensively stirring to obtain a sticky solution and then 0.1 mL GPTMS was added, and then stored in a sealed plastic bottle over night). After being continuously stirred for another 12 h, the suspensions were centrifuged (MCF-BSA-NOCC). The precipitated

BSA-loading materials were washed with water once and the washing fluid was mixed with the supernatant (V_m), then detected the concentration of mixture (c_m). Quantification of drug loading was performed using the following equation:

Drug loading (%) = (Total protein amount - $c_m \times V_m$) / Total protein amount × 100%

To investigate the *in vitro* release of BSA from carriers, the nanocarriers were separately put into a 1.5 mL of centrifuge tube which containing 1 mL PBS solution (pH = 6.0 or 7.4). The protein release was performed for 72 h. At each time point, the released solution was centrifuged and 0.2 mL of the release medium was collected. Then 0.2 mL of fresh PBS was added into the release system immediately. A corrected method[2] was used to actual amount of BSA release from the carriers. The BSA concentration was measured with BCA Assay Kits. The test procedure of loading and release of BMP-2 is as the same with the loading and release of BSA, just change the loading concentration of BMP-2 into 0.5 mg/mL.

Conformation of the released proteins

The second structure of protein was always detecting with the Far-UV circular dichroism (CD) spectrum.[3] In our experiments, we used a spectropolarimeter (Model J-715, Japan) to measure the Far-UV CD spectra (190 - 260 nm) for studying the changes of the secondary structure and conformation of BSA and BMP-2. The free BSA and BMP-2, the proteins released from MCF and MCF-NOCC at pH 6.0 were analyzed at 25 ^{o}C in a 1.0 mm path length quartz cell with a time constant of 0.25 s and a scan rate of 100 nm/min. The concentration of proteins for test was 2 μg/mL in each group and the bandwidth was 1.0 nm and 5 scans were accumulated.

Cell culture

BMSCs were flushed out of the femora and tibias of rats with DMEM-LG (Gibco, CA) by a 10-mL syringe. The cells were seeded in 10-cm cell culture dish containing MEME-LG supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37 ^{o}C, and the medium was changed every 3 days. When the cells reached 80% confluency in the flasks, they were trypsinized with 0.25% trypsin/0.03% ethylene diamine tetraacetic acid (EDTA) and expanded into a plate as passage 1. All assays were performed on second passage cells after enlarged cultivation.

Cytotoxicity assay

The cytotoxicity of MCF and MCF-NOCC was evaluated with MTT assays.[4] The BMSCs were plated into 24-well plates at a density of 1.0×10^4 cells/well in 1.0 mL culture medium and incubated for 24 h. Subsequently, the culture medium was removed and the cells were washed with PBS twice, then each well was added with 1.0 mL medium with different samples at different concentrations of 100 and 200 μg/mL. The MTT assay was performed following the standard protocol after incubating cells for 1, 3 and 5 days.

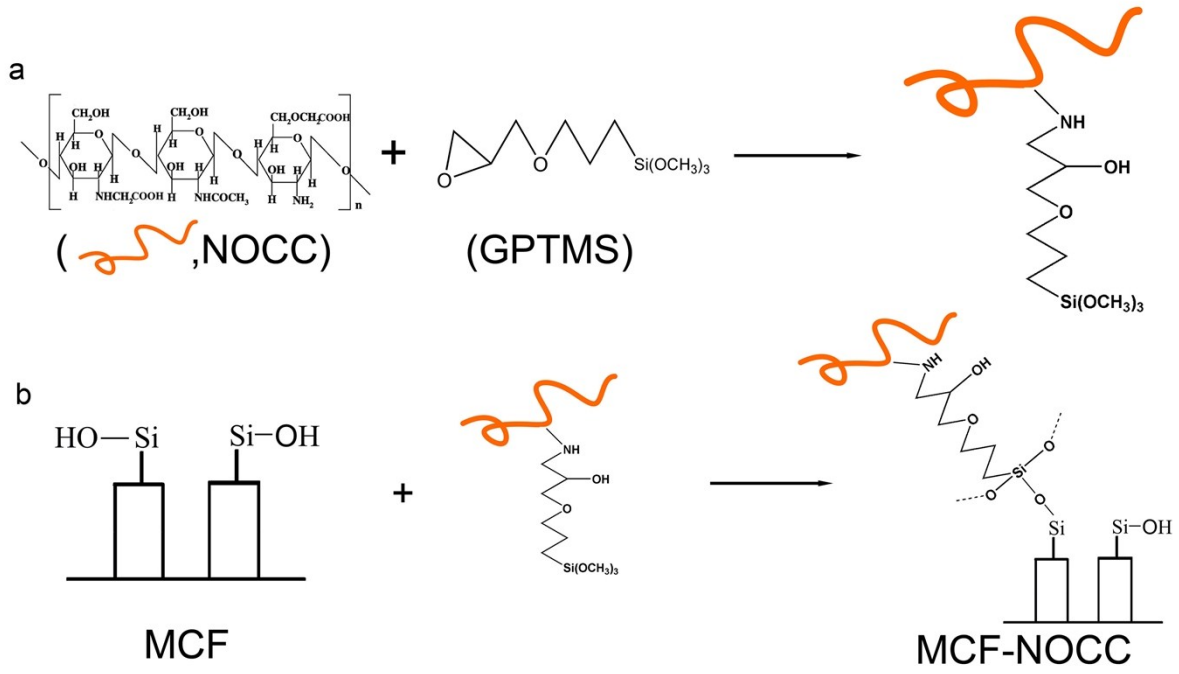
Determination of alkaline phosphatase (ALP) activity

BMSCs were cultured into a 24-well plate at 3.0×10^4 cells/well with DMEM-LG. After 24 h of cell adhesion, the DMEM was removed and then replaced with 1.0 mL DMEM-LG containing different concentrations of MCF-based materials. At day 4 and 7, the culture medium was removed. After that, about 200 μL of 1% Nonidet P-40 (NP-40) was added into each well and incubated for 60 min at room temperature. 50 μL of the obtained cell lysate was added to a 96-well plate, 50 μL of 2 mg/mL PNPP-Na substrate solution composed of 1 mmol/L $MgCl \cdot 6H_2O$ and 0.1 mol/L glycine was added. Then it was incubated at 37 ^oC for 60 min. The reaction was quenched by 100 μL of 0.1 N NaOH, and the ALP value was quantified at a wavelength of 405 nm using a microplate reader. ALP activity was expressed as 405 nm O.D. value per min per mg of total protein. Protein concentration was determined using the BCA Assay Kits, with bovine serum albumin as the standard.

The bioactivity of BMP-2 released from MCF and MCF-NOCC was detected by the ALP activity of BMSCs which cultured with BMP-2. In the protein released experiments, we have collected 0.2 mL released solution at each time point and only 0.02 mL released protein solution was used for measurement of protein concentration with BCA Assay Kits. The 0.18 mL remaining solutions collected at 13 time points were mixed homogeneously. A half of the mixed protein solution was sterilized with 0.22 μm sterilizing filters and diluted to the test concentration with a cell culture medium for bioactivity measurement.

BMSCs were cultured into a 24-well plate at 3.0×10^4 cells/well with DMEM-LG. After 24 h of cell adhesion, the culture medium were removed and then replaced with 1.0 mL DMEM-LG containing 1μg/mL BMP-2 (free BMP-2, BMP-2 released from

MCF, BMP-2 released from MCF-NOCC). At day 4 and 7, the culture medium was removed and the ALP activity was measured with the same method above. To determine the ALP activity histochemically, cells were fixed with 1% glutaraldehyde for 10 min on ice after 7 days cultivation. After that, they were washed with PBS, and incubated with 0.5 mL BCIP/NBT ALP Color Development Kit (Beyotime, Jiangsu, China) for 30 min at 37.0 ^{o}C. The stained cells were photographed with TE-2000U inverted light microscope (Nikon, Japan).



Scheme S1. NOCC was coated onto the surface of MCF via the cross-linking of GPTMS (glycidoxypropyltrimethoxysilane).

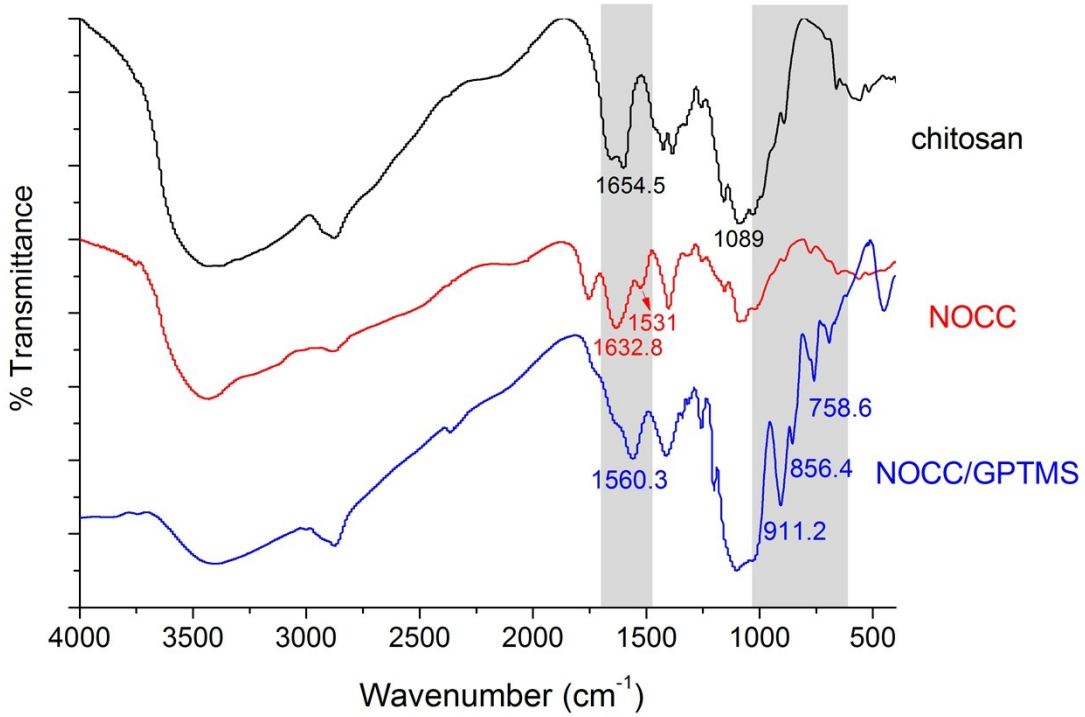


Fig. S1 FTIR spectra of chitosan, NOCC and NOCC/GPTMS. The peak centered at 1632.8 cm^{-1} was assigned to the characteristic stretching vibrations of the $–NH_2$ bond and carboxylic acid salt ($-COO^-$ stretch) in the main chain of NOCC, while the peak at 1531 cm^{-1} was assigned to the characteristic stretching vibrations of the -NH- bond. In the FTIR spectra of NOCC/GPTMS, a strong peak at 1560.3 cm^{-1} was corresponding to appearing large amount of the group of –NH–, which proved the successful introduction of epoxy and amine group. The peaks presented between 600 – 900 cm^{-1} was attributed to the stretching vibrations of C-Si bond, which was also proved that GPTMS was linked to the main chain of NOCC.

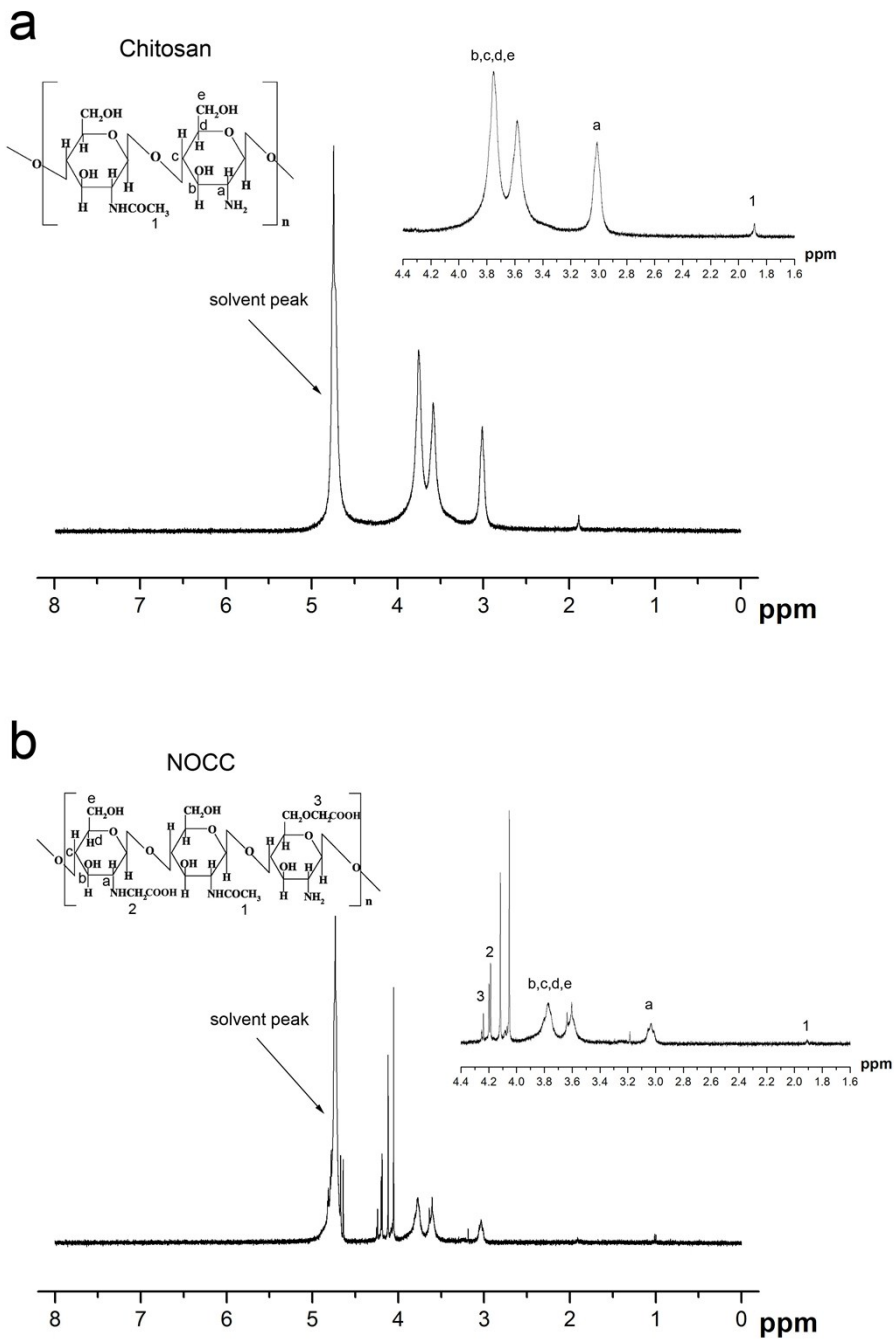


Fig. S2 ^{1}H NMR spectra of chitosan (a) and NOCC (b). In the NMR spectra of chitosan, there were four chemical shifts at 1.9, 3.0, 3.6 and 3.8 ppm, which were also appeared in the spectrum of NOCC. Meanwhile, as shown in the spectrum of NOCC, there appeared another two chemical shifts at 4.1 and 4.2 ppm corresponding to the ^{1}H of -CH_2COOH at the N-position at C_a and the O-position at C_e of NOCC, respectively.

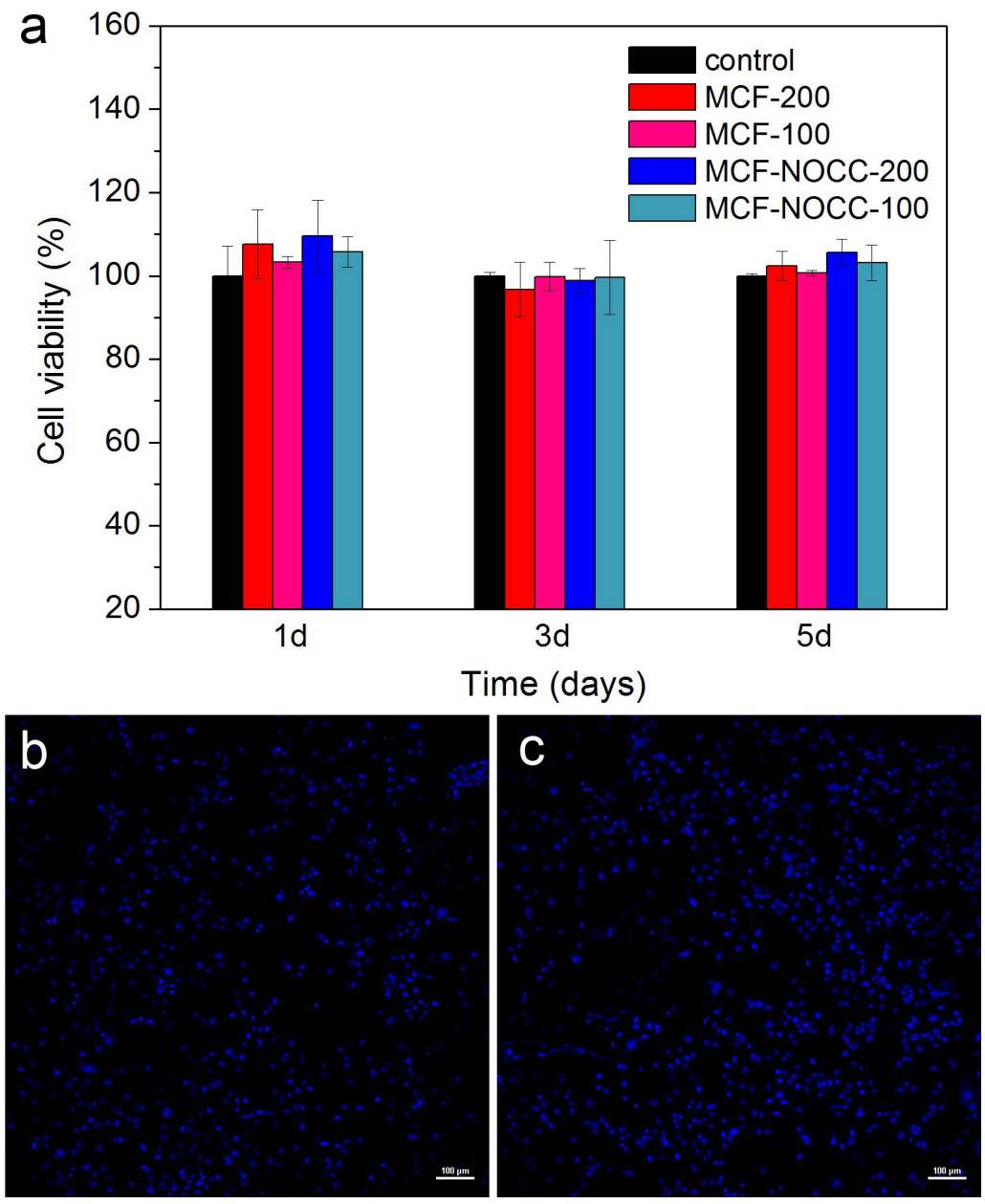


Fig. S3 (a) Cell viability of BMSCs cultured with MCF or MCF-NOCC (100 and 200 μg/mL) after 1, 3, 5 days determined by MTT assay. CLSM images of BMSCs cultured with MCF (b) and MCF-NOCC (c) after 5 days. Cells were stained by DAPI before photographed. Scale bar = 100 μm.

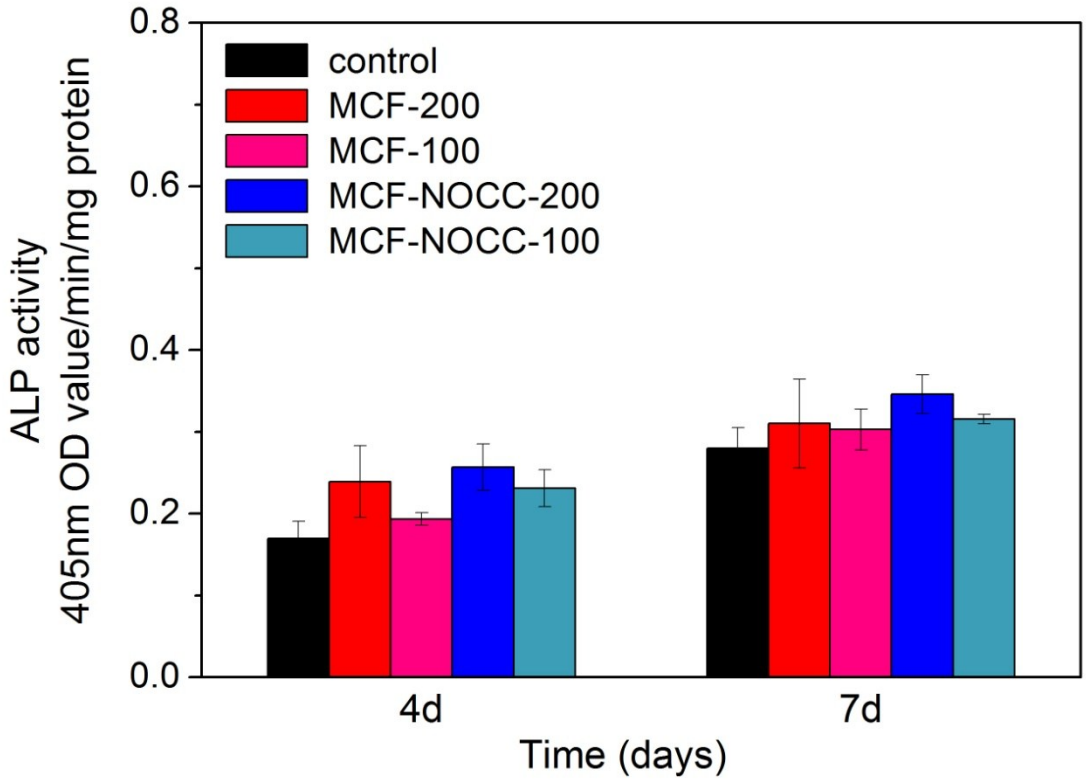


Fig. S4 ALP activity of BMSCs cultured with MCF or MCF-NOCC (100 and 200 μg/mL) after 4 and 7 days.

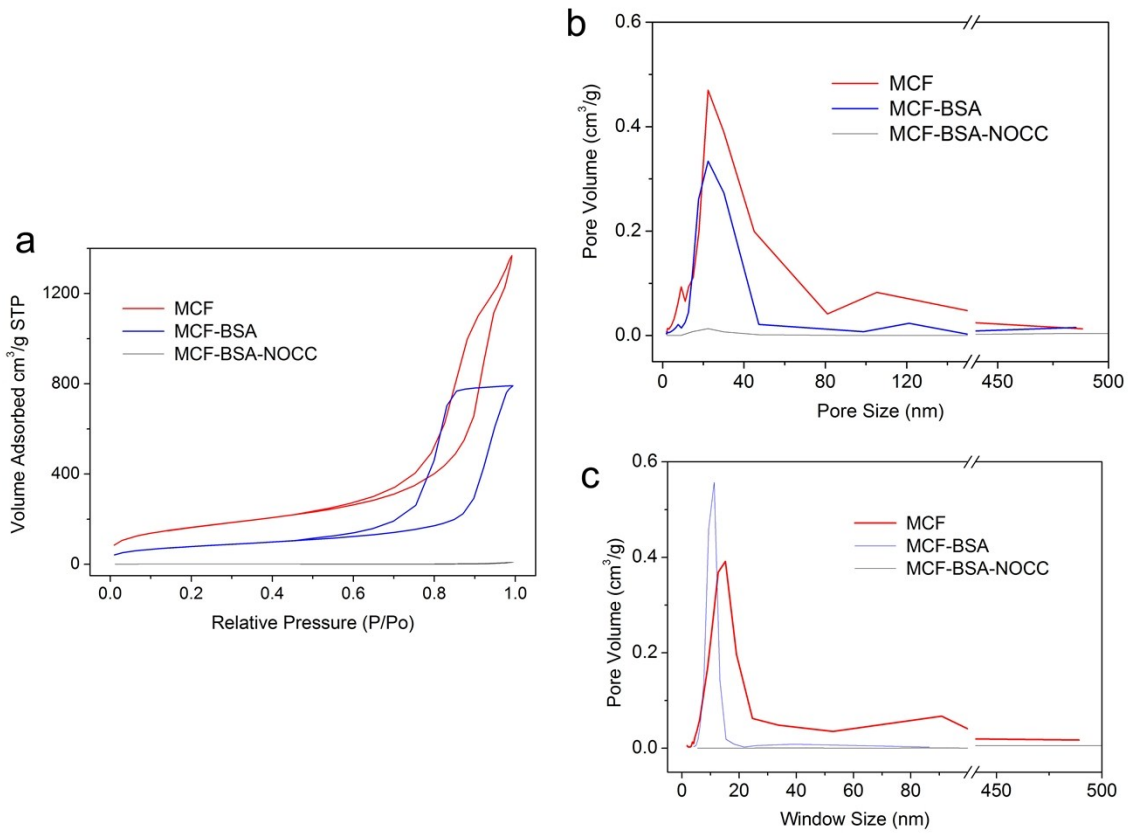


Fig. S5 (a) Nitrogen adsorption / desorption isotherms of prepared MCF, MCF-BSA and MCF-BSA-NOCC. (b) Pore size and (c) window size distribution of MCF, MCF-BSA and MCF-BSA-NOCC.

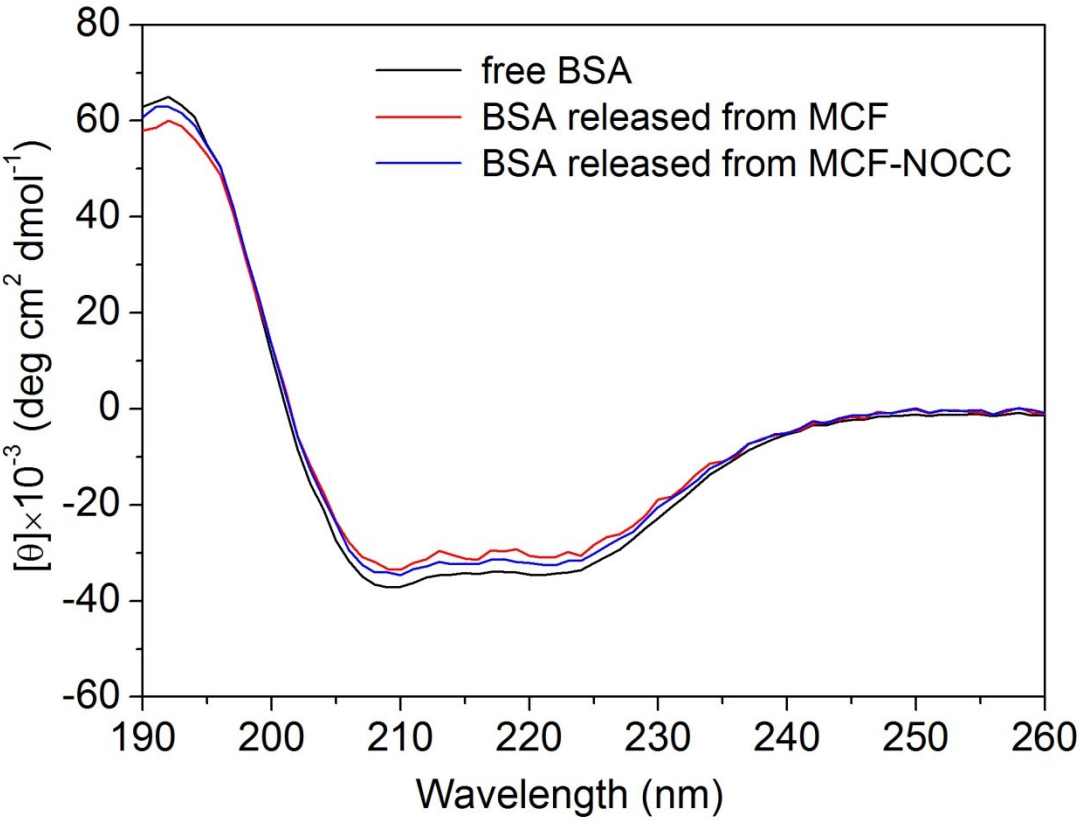


Fig. S6 Far-UV CD spectra of free BSA, BSA released from MCF and MCF-NOCC at pH 6.0. The concentration of BSA for test is 0.1 mg/mL.

Table S1. Nitrogen adsorption/desorption isotherms and ζ-potential measurement data of MCF and MCF-NOCC samples.

Sample	BET surface area (m^2/g)	Pore volume (cm^3/g)	Pore size (nm)	Window size (nm)	ζ-potentials (mV)
MCF	600	2.12	41.2	16.3	-27.3
MCF-NOCC	53.1	0.21	-	-	-6.4
MCF-BSA	278.2	1.23	25.6	10.5	
MCF-BSA-NOCC	3.27	0.016	-	-	

Table S2. Secondary Structure of BMP-2, BMP-2 released from MCF and MCF-NOCC in 0.5 mM PBS as determined by CD spectra [(a)].

Secondary structure compositions	Free BMP-2	BMP-2 released from MCF	BMP-2 released from MCF-NOCC
α-helix (%)	12.2	8.6	11.2
β-sheets (%)	24.2	25.5	26.8
β-turns (%)	17.8	24.8	18.3
Rndm. Coil	43.6	43.8	42.1
Changes of the folding structure (%)	-	12.1	5.6

[(a)] CDNN V2.1 software was used to evaluate the secondary structure using the "complex" spectra category and the 190–260 nm region of the spectra.

Table S3. Secondary Structure of BSA, BSA released from MCF and MCF-NOCC in 0.5 mM PBS as determined by CD spectra [(a)].

Secondary structure compositions	Free BSA	BSA released from MCF	BSA released from MCF-NOCC
α-helix (%)	91.9	91.5	90.7
β-sheets (%)	2.0	1.4	2.1
β-turns (%)	6.4	3.7	6.6
Rndm. Coil	1.7	3.7	2.0
Changes of the folding structure (%)	-	5.7	1.8

[(a)] CDNN V2.1 software was used to evaluate the secondary structure using the "complex" spectra category and the 190–260 nm region of the spectra.

References

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