Electronic supplementary information

Synthesis of a chitosan-based functional biopolymer with both catalytic and binding groups for protein and DNA hydrolysis

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1. **Determining the copper ion content of CCMS**

Materials and Methods

Inductively coupled plasma atomic emission spectrometry (ICP-AES) was performed by using IRIS Intrepid IIXSP to determine the content of copper ions in Cu(II)-cyclen-modified cross-linked chitosan microspheres (CCMS). The microspheres were completely digested by 1.0 M HNO_3 overnight before loaded on ICP-AES platform. The parameters were set as follows: RF power: 1150W; nebulizer flow: 26.0 PSI; auxiliary gas: 1.0 LPM.

Results

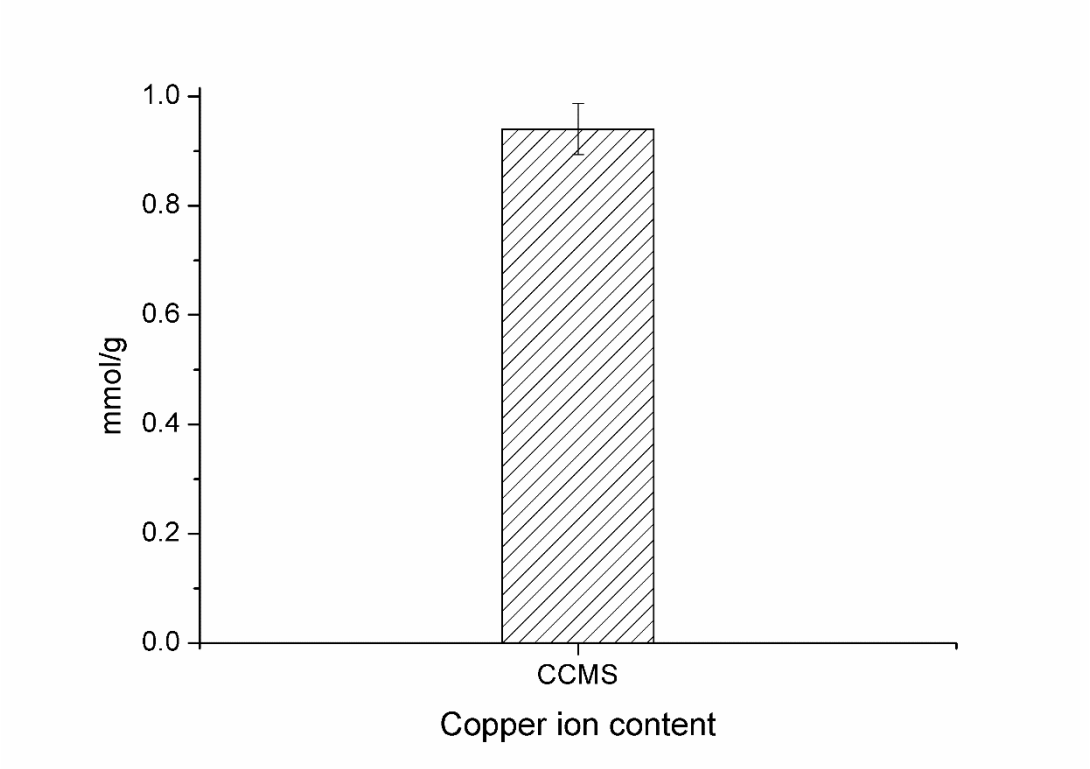


Fig. S1 Copper ion content of CCMS determined by ICP-AES.

As shown in Fig. S1, the copper ion content of CCMS was determined as 0.94 mmol/g. By calculating the number of the sugar unit, which was 0.54 mmol/g, the ratio of Cu^{2+}/sugar unit was determined as *ca.* 2:1.

2. **Elemental analysis of pure chitosan, CMS, A-CMS and CA-CMS**

Materials and Methods

Chitosan (degree of deacetylation 92.2%, MW: 5.5×10^5 Da), cross-linked chitosan microspheres (CMS), activated cross-linked chitosan microspheres (A-CMS) and cyclen-tethered activated cross-linked chitosan microspheres (CA-CMS) were subjected to elemental analysis. Elemental Analyzer (Vario EL III, Elemenatar Co. Ltd.) was used to determine the contents of carbon, nitrogen and hydrogen elements. Each sample was tested three times and the average values were presented as the results.

Results

Table S1 Results of elemental analysis of pure Chitosan, CMS, A-CMS and CA-CCMS

	N (%)	C (%)	H (%)	C/N
pure chitosan	7.972	44.260	6.950	5.551
CMS	4.523	48.152	6.795	10.642
A-CMS	4.147	49.426	7.017	11.918
CA-CCMS	4.829	48.210	7.642	9.983

The results of elemental analysis were shown in Table S1. After crosslinking with glutaraldehyde，the C/N value of CMS increased comparing to that of pure chitosan, indicating the crosslinking reagent was introduced onto chitosan molecules. The C/N value of A-CMS raised after the activation by epichlorohydrin (from 10.642 to 11.918)，suggesting that the microspheres were activated and the epoxy group was present in A-CMS. After the modification of A-CMS by cyclen, the C/N value of CA-CCMS increased comparing to that of A-CMS, indicating the existence of cyclen moiety. Based on the data of elemental content of A-CMS and CA-CCMS, the ratio of cyclen/sugar unit was estimated as *ca.* 1:3.

3. **Measuring crosslinking degree of the cross-linked chitosan microspheres**

Materials and Methods

Crosslinking Degree of CMS (cross-linked chitosan microsphere) was determined as the following steps. First, certain amount of the microspheres was kept in drying oven for 12 h until the mass of the microsphere reached a constant value. Then 0.25 g (W_1) dried microspheres was soaked in 2% (v/v) acetic acid solution for 24 h, followed by incubated at 105°C for 4 h. The microspheres were weighed and designated as W_2 (0.218 g). The Crosslinking Degree value (ξ):

$$\xi(\%) = \frac{W_1 - W_2}{W_1} \times 100\%$$

Results

The Crosslinking Degree value of CMS was determined as 12.67%, indicating that not all the sugar monomers reacted with the crosslinking reagent (glutaraldehyde). The intact $–NH_2$ and –OH groups on the monomers can react with epichlorohydrin which serves as a “linker” to tether cyclen moiety on the microspheres.

4. **Determining the nitrogen content of the protein solutions**

Materials and Methods

Horse heart myoglobin (Mb, purity >90%), Bovine serum albumin (BSA, purity >98%), Ovalbumin (Ova, purity >90%) were purchased from Sigma-Aldrich Co. Ltd. (Beijing, China). Collagen (Col, purity >70%) was obtained from Bio Basic Co. Ltd. The automatic Kjeldahl determination apparatus was provided by Hanon (Shanghai, China). Tris-base and analytical grade HCL were purchased from Shanggong Biotech. Co. Ltd (Shanghai, China). All other reagents were of analytical reagent grade. All solutions were prepared using Milli-Q purified water.

Aliquot of 1.0 mL initial solution (containing 1.0 mg/L protein substrate) was added to 9.0 mL H_2SO_4 containing 0.25g $CuSO_4$, the resulting mixture was heated on digestion cube for 30 min. After the digestion, the mixture was cool down to room temperature followed by loaded on automatic determination device. The hydrolysate solution was obtained by centrifuging the Eppendorf tube with CCMS catalyst, and then collected the supernatant liquid for automatic analysis.

Results

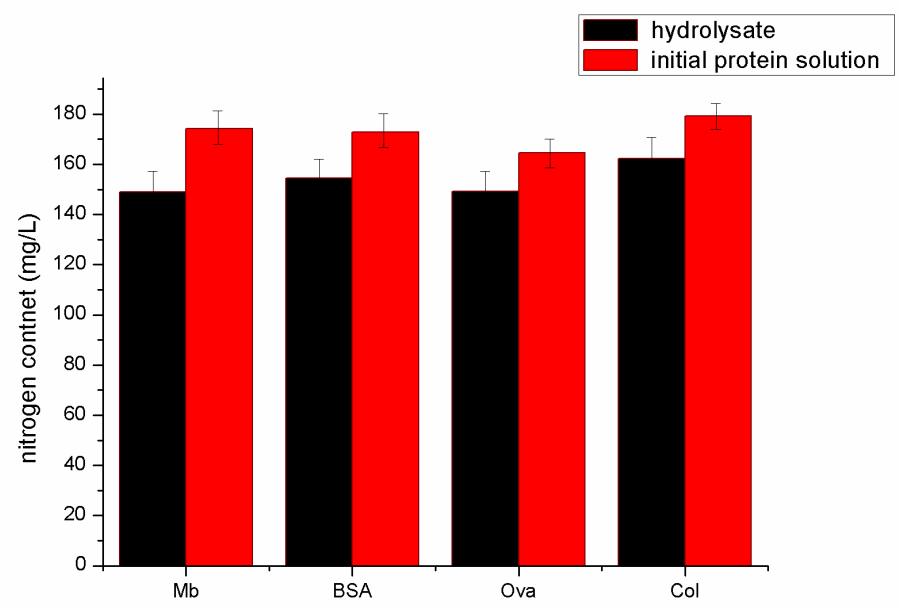


Fig. S2 Results of nitrogen content of initial protein solution and product solution.

It is demonstrated that 80% protein nitrogen was recovered from the product solution, indicating that the disappearance of the electrophoretic bands of the protein substrates was due to the proteolytic activity of CCMS rather than the adsorption onto the microspheres. The nitrogen content of initial protein solution was higher than that of the product solution. The possible explanation is that some of the product peptides were tethered onto the surface of the CCMS through the imine bond formed by the pendant aldehyde group and the Lys-amino group of the protein substrate.

5. Analyzing the free terminal amino in reaction supernatant by Ninhydrin reaction

Materials and Methods

Ninhydrin and $SnCl_2 \cdot 2H_2O$ was purchased from Bio Basic Inc. (Beijing, China). Other reagents of analytical reagent grade were obtained from local commercial source, and used without further purification. All solutions were prepared using Milli-Q purified water. The ninhydrin reagent used in the experiment was prepared as follow. Ninhydrin of 2.0 g was dissolved in 50 mL purified water containing 80 mg of $SnCl_2 \cdot 2H_2O$ followed by stirring for 2 h. The resulting mixture was kept in dark overnight and diluted to volume of 100 mL.

Ninhydrin reagent of 0.5 mL was added to 1.0 mL reaction supernatant (separated from the insoluble catalyst by centrifugation), followed by addition of 0.5 mL of phosphate buffer (pH=8.0). The resulting mixture was subjected to boiling water bath for 15 min, and cooled down to room temperature before diluted to the fixed volume of 25 mL. The absorbance of the resulting solution was measured at 570 nm.

Results

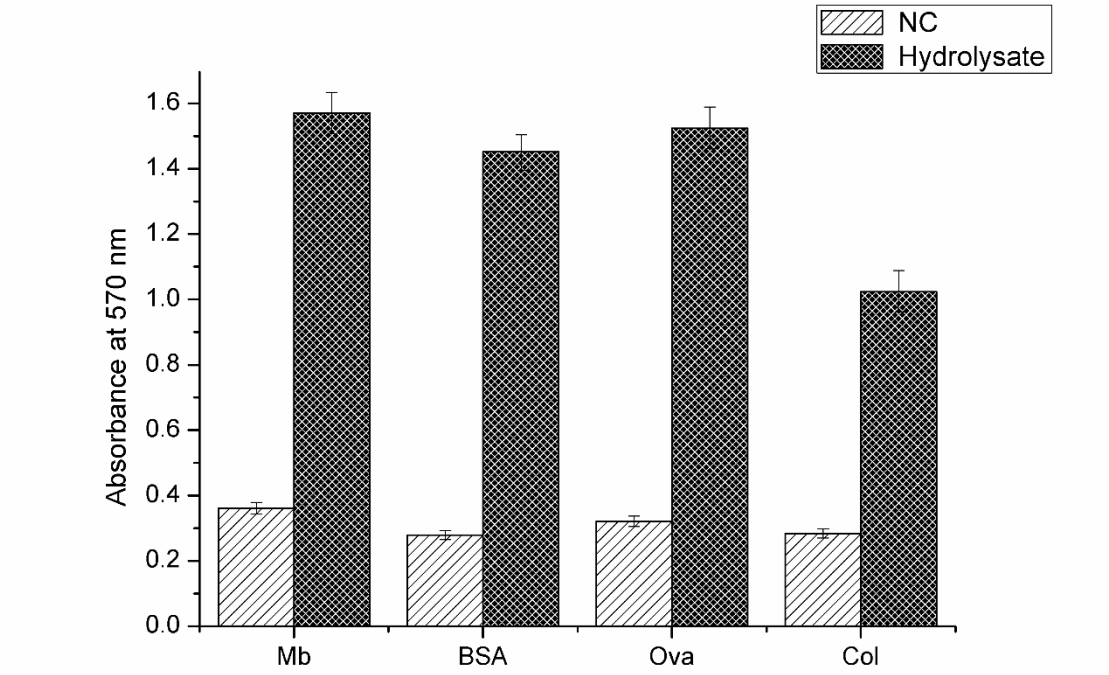


Fig. S3 Analysis of free terminal amino using Ninhydrin reaction. "NC" represented negative control reaction, which was carried out under the conditions described above expect for adding the CCMS catalyst. The duration of NC reactions and hydrolysis reaction were 4 h, 10 h, 8 h and 16 h for Mb, BSA, Ova and Col respectively.

As shown in Fig. S3, after incubated with CCMS, the amount of free terminal amino increased for all protein substrates examined, indicating that parent proteins were cleaved to generate nascent terminal amino.

6. **HPLC analysis of the BSA reaction supernatant with different incubate duration**

Materials and Methods

TSK-GEL3000 SW HPLC columns was purchased from TOSOH Inc. (Shanghai, China). Mobile phase A: 100% acetonitrile, Mobile phase B: 0.2% trifluoroacetic acid (TFA).

HPLC analysis was performed as follow: Opening the broken valve, using 0.2% TFA at flow rate gradually increasing from 1.0 ml/min to 1.0 ml/min. The velocity change every 30 s, stable at 5.0 ml/min for 1.0 min to remove the inorganic salt ion in the chromatograph instrument. Then 100% acetonitrile was used as the mobile phase under the condition described above. Finally, the flow rate was kept at 5.0 ml/min for 5 min, until no more bubbles appear, then shut down the broken valve followed by column loading. Using A, B each 50% mixed as mobile phase, keep the flow rate at 0.5 ml/min, smoothing the baseline for 20 min, sample is loaded by hand. The reaction tube was subjected to centrifugation to collect the supernatant as the sample.

Results

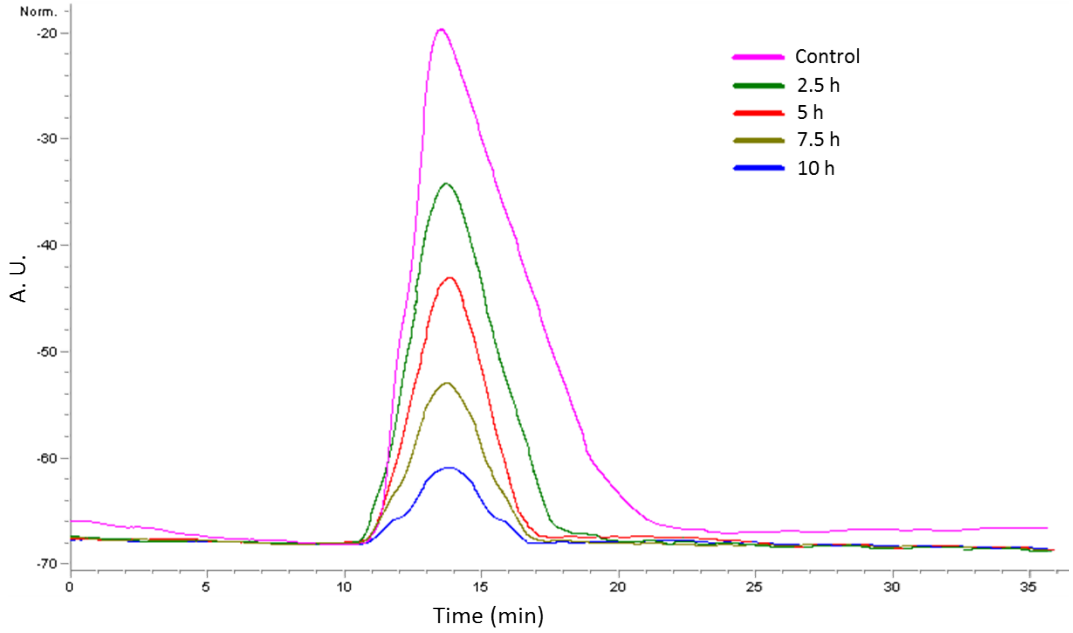


Fig. S4 Elution profiles of BSA hydrolysate supernatant from different reaction duration.

The results of HPLC analysis was shown in Fig. S4. The parent protein of BSA was digested gradually over time, which was consistent with the result of SDS-PAGE. No adsorption peak representing smaller peptides of cleaved proteins was observed, indicating that the cleaved intermediate proteins were subjected to further digestion preferentially that the parent protein molecules. The data revealed that the hydrolytic action of the artificial metalloprotease is of a unique fashion, which directly produced small peptides or amino acids without accumulation of intermediate protein fragments.

7. **Effect of shaking speed on the proteolytic activity of CCMS**

Materials and Methods

Horse heart myoglobin (Mb, purity >90%) was purchased from Sigma-Aldrich Co. Ltd. (Beijing, China) and used without further purification. Tris-base and analytical grade HCL were purchased from Sangong Biotech. Co. Ltd (Shanghai, China). Thermal stable shaking block was provided by Bioer Technology Co. Ltd. (Hangzhou, China). All other reagents were of analytical reagent grade. All solutions were prepared using Milli-Q purified water.

Mb was used as the substrate to study the effect of shaking speed. Tris-HCL buffer solution (pH=7.8) containing 6.25×10^{-2} mM Mb was incubated the insoluble catalyst (CCMS) with fixed concentration of 0.12 mM in 1.5 mL Eppendorf tube. The proteolytic reaction was carried out at 50°C for 4 h. At specific time interval, aliquot of 20 μL of product solution was pipetted and subjected to SDS-PAGE analysis. The speed of the isothermal shaker was set as 300, 450, 600, 750, 900, 1050, 1200 r/min respectively.

Results

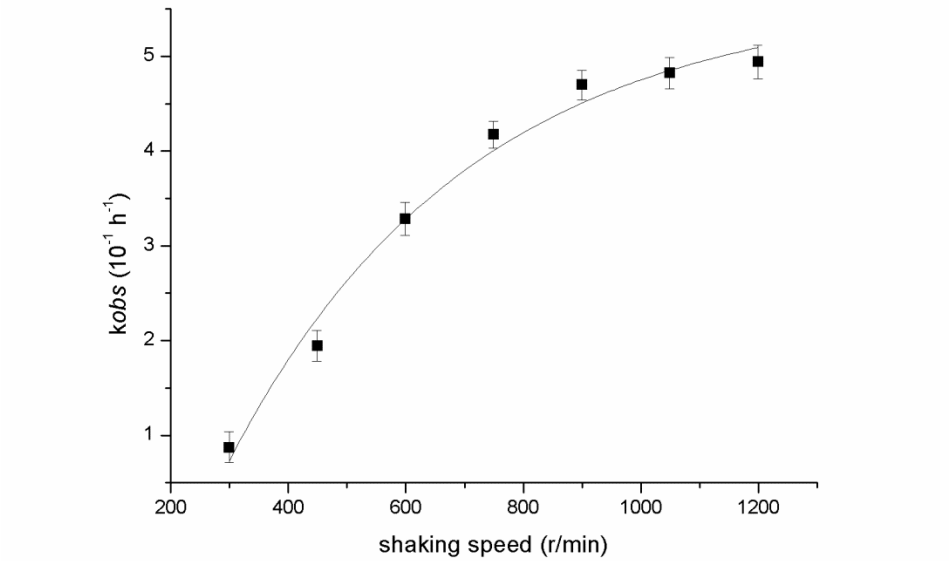


Fig. S5 Effect of shaking speed on the rate of protein cleavage (k*obs*). The apparent rate constant (kobs) for hydrolysis of Mb was estimated from the plot of $\ln[S]/[S_0]$ against time.

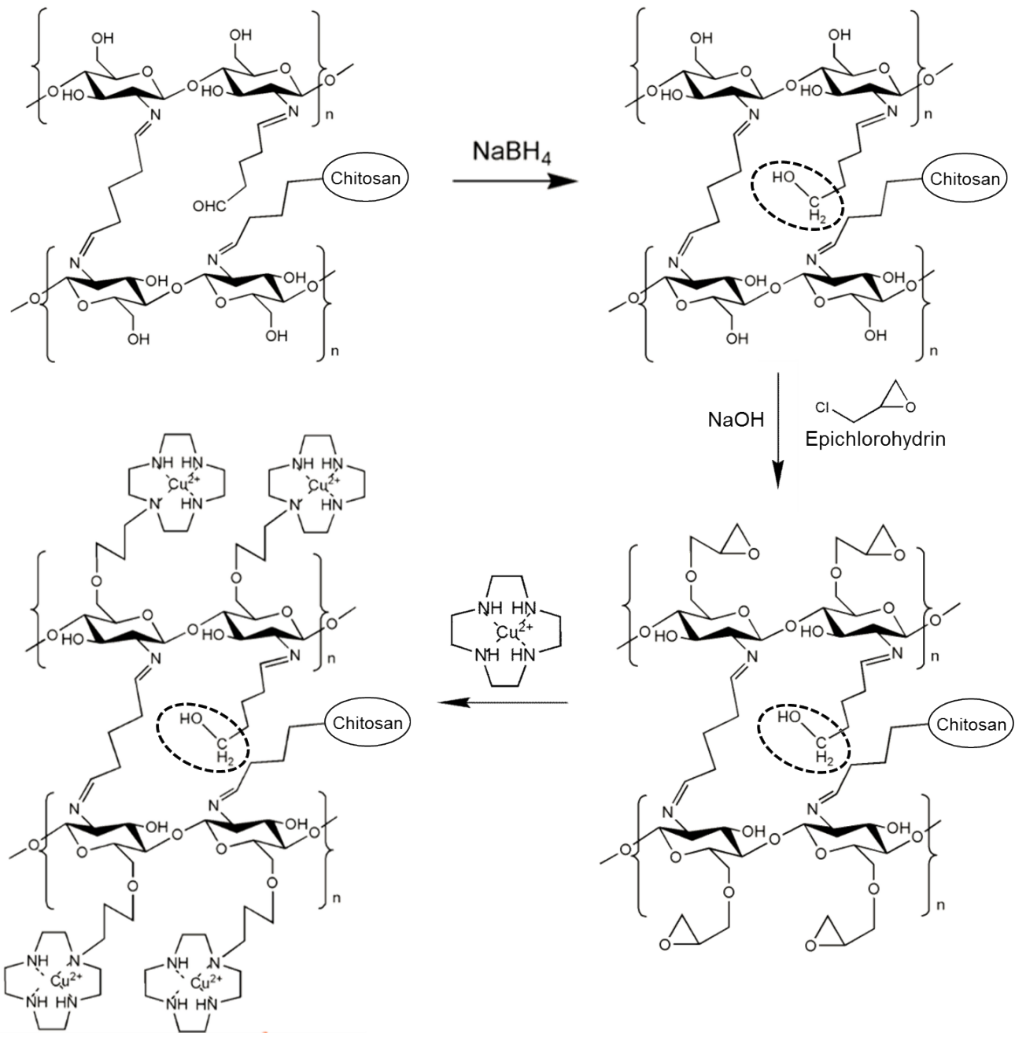
As shown in Fig. S5, the rate of protein cleavage is directly proportional to the shaking speed used in the specific reaction. Since bulky molecules like proteins tended to diffuse slowly to the reactive sites in CCMS particles, thus shaking speed that can accelerate the interaction of peptide and the reactive site might have a great influence on the rate of protein cleavage. The rate of protein cleavage reached a plateau when the shaking speed exceeded 900 r/min. Considering the balance between higher rate of hydrolysis and industrial cost of operating, the shaking speed was designated as 900 r/min for experiments in prospect.

8. **Hydrolyzing Mb using re-CCMS to confirm the function of the pendant aldehyde**

Materials and Methods

Horse heart myoglobin (Mb, purity >90%) was purchased from Sigma-Aldrich Co. Ltd. (Beijing, China) and used without further purification. Tris-base and analytical grade HCL were purchased from Sangong Biotech. Co. Ltd (Shanghai, China). $NaBH_4$ was provided by Solarbio Technology (Beijing, China). Thermo stable shaking block was obtained from Bioer Technology Co. Ltd. (Hangzhou, China). All other reagents were of analytical reagent grade. All solutions were prepared using Milli-Q purified water.

The cross-linked chitosan microspheres (CMS) of 10 g were added to 1, 4-dioxane solution containing 0.06 M of $NaBH_4$. The resulting mixture was placed on a thermal stable incubator with stirring speed of 200 r/min and the reduction reaction was during for 3h. After the reaction, reduced-CMS was collected by filtration and subjected to the reaction steps as depicted for CCMS synthesis. The synthesis protocol of re-CCMS can be referred to Scheme S1.



Scheme S1 Synthesis procedure of reduced-CCMS

Results

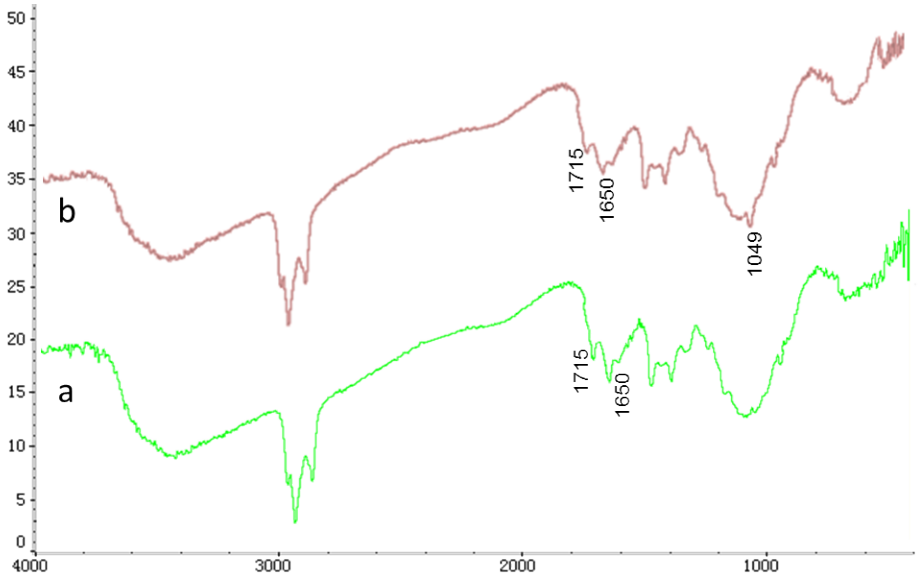


Fig. S6 FTIR spectra of (a) CMS and (b) reduced-CMS.

The results of FTIR analysis of CMS and reduced-CMS was shown in Fig. S3. The typical adsorption peaks of aldehyde group (-CHO) and Schiff base (-N=C) can be found in Fig. S6a. However, those two adsorption peaks became weaker in reduced-CMS, indicating that the pendant aldehyde group and Schiff based took part in the reduction reaction. As shown in Fig. S6b, emerging adsorption peak was found at 1049 cm^{-1}, which representing the stretching vibration of (C-O), suggesting CMS was reduced by $NaBH_4$, generating more hydroxyl groups.

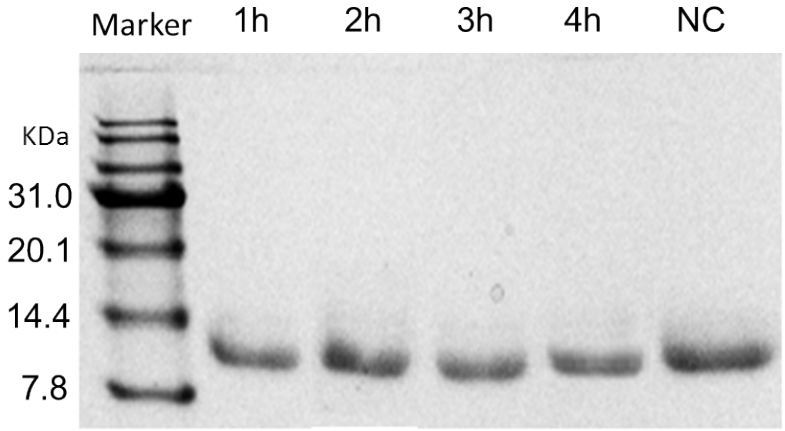


Fig. S7 Hydrolyzing Mb by the reduced-CCMS (re-CCMS). re-CCMS of 0.12 mM was added as the catalyst (the catalyst concentration was calculated by assuming the insoluble microspheres were dissolved in buffer solution), Tris-HCl buffer (pH=7.8), 50°C incubation, shanking speed at 900 r/min. "NC" represented negative control reaction, which was carried out under the conditions described above expect for adding the CCMS catalyst. The duration of NC reaction was assigned as 4 h.

The electrophoresis analysis of Mb cleavage by re-CCMS was illustrated in Fig. S7. The apparent rate of Mb cleavage by re-CCMS was calculated from the plot of $\ln[S]/[S_0]$ against time, where "[S]" represents the protein concentration at designated time point, "$[S_0]$" stands for the initial concentration of the protein substrate. The k*obs* was 0.7×10^{-1} h^{-1} for re-CCMS hydrolyzing Mb. The rate was much slower than that of CCMS (4.7×10^{-1} h^{-1}), indicating that the presence of pendant aldehyde group is essential for the synzyme to perform with high efficiency.

9. **Effect of shaking speed on DNA cleavage activity of CCMS**

Materials and Methods

Plasmid pUC-18 was purchased from Thermo Fisher Scientific (Shanghai, China) and stored in Tris-EDTA buffer (10 mM, pH=7.6). All other chemicals were of analytical grade and purchased from local commercial sources. Milli-Q purified water (resistance of 18.2 M) was used throughout all experiments. Thermal stable shaking block was provided by Bioer Technology Co. Ltd. (Hangzhou, China). All other reagents were of analytical reagent grade. All solutions were prepared using Milli-Q purified water.

For plasmid cleavage, 0.12 mM of CCMS was added to 400 μL NaH_2PO_4/Na_2HPO_4 buffer (pH=7.2) containing 5.0 ng/μL plasmid DNA. The resulting mixture was incubated at 37°C with different shaking speed. Aliquot of 10 μL cleavage product was pipetted at specific time point and subjected to PAGE analysis. "NC" represented negative control reaction, where experiment was performed without adding the catalyst (CCMS). The speed of the isothermal shaker was set as 300, 450, 600, 750, 900, 1050, 1200 r/min respectively.

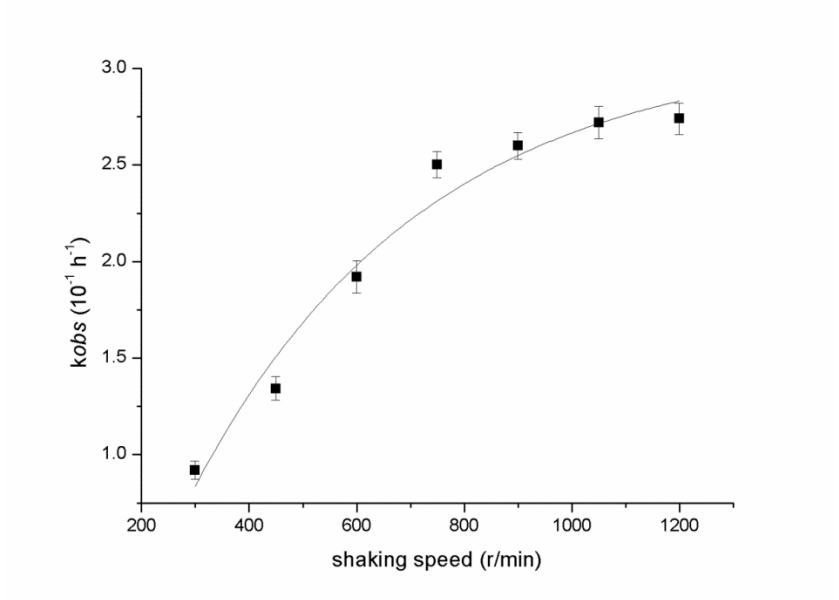


Fig. S8 Effect of shaking speed on the rate of DNA cleavage (k*obs*). The apparent rate constant (kobs) for hydrolysis of Mb was estimated from the plot of $\ln[S]/[S_0]$ against time.

As shown in Fig. S8, the rate of DNA cleavage is directly proportional to the shaking speed used in the specific reaction. Since bulky molecules as DNA tended to diffuse slowly to the reactive sites in CCMS particles, thus shaking speed that can accelerate the interaction of phosphodiester bond and the reactive site might have a great influence on the rate of protein cleavage. The rate of DNA cleavage reached a plateau when the shaking speed exceeded 750 r/min. Taking account of the balance between higher rate of hydrolysis and industrial cost of operating, the shaking speed was designated as 750 r/min for experiments in prospect.