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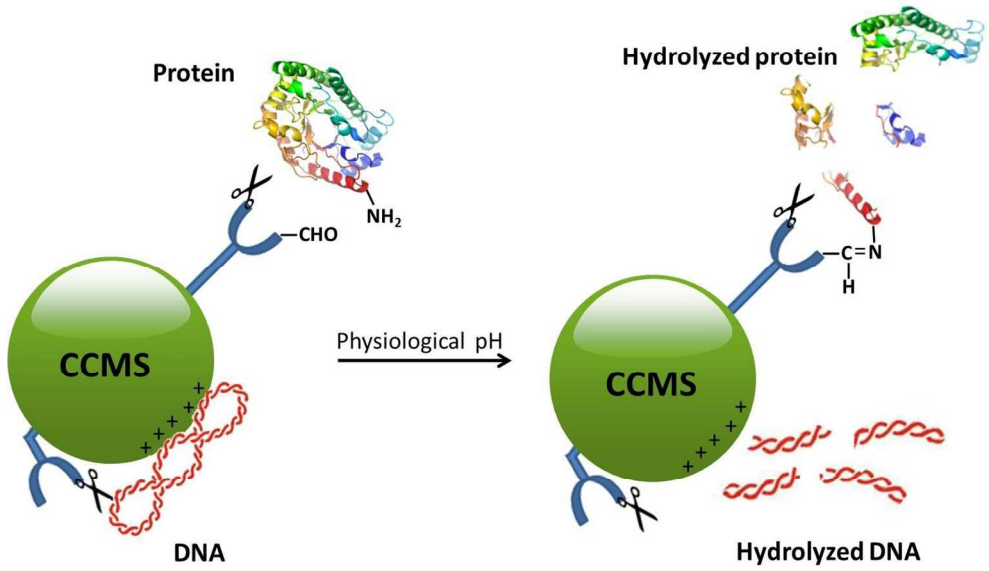

Protein and DNA hydrolysis by chitosan-based biopolymer with catalytic and binding groups
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Synthesis of a chitosan-based functional biopolymer with both catalytic and binding groups for protein and DNA hydrolysis

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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A novel chitosan-based biopolymer (CCMS) containing both catalytic and binding groups for protein and DNA hydrolysis was designed and prepared in this study. The catalytic center, consisting of 1, 4, 7, 10-tetraazacyclododecane (cyclen) coordinated with Cu(II), was constructed on the chitosan microspheres prepared by reverse-phase suspension crosslinking. A pendant aldehyde was introduced to the biopolymer as the binding group in view of its ability to form imine bonds with the amino groups of exposed on the surface of the protein substrate. FTIR analysis showed that the two functional groups were introduced to the biopolymer, which hydrolyzed all protein and DNA substrates tested in the study. The unique microenvironment on the surface of CCMS, imparted by the polysaccharide backbone, was conducive to peptide and phosphodiester bond cleavage. In addition, CCMS featured a desirable stability and can be reused for four times with minimal loss of proteolytic and DNA cleavage activity.

Introduction

Designing artificial proteinase and nucleases is of great attraction because their potential applications in biotechnology and food industry are promising.[1] However, the processing of constructing those synthetic enzymes is challenging due to the extreme stability of peptide bond and phosphodiester bond under physiological conditions.[2, 3] To build up artificial enzymes, several research groups employed heterocyclic chemicals coupled with metal for their structural similarity with the "catalytic center" found in natural enzymes.[4-6] It has been reported that heterocyclic compounds conjugating with Zr^{2+}, Co^{2+}, Cu^{2+}, Pb^{2+}, and Pt^{2+} are capable of cleaving the peptide or phosphodiester bonds in either hydrolytic or oxidative way.[7-10] The cleavage of protein and plasmid DNA can occur under physiological conditions, and the efficiency is comparable to that of natural enzymes. Those complexes are designed to be soluble, while missing the consideration that the catalytic efficiency might be seriously impaired by the strong polarity of solvent molecules. To solve this problem, some of researchers tried to embed the complexes into the insoluble polymers such as polystyrene or polysilica particles, which provided desirable hydrophobic microenvironment where the catalytic process would not be affected.[11]

The insoluble matrix promoted the catalytic efficiency and stability, as well as facilitated the separation of the synthetic enzyme and products. However, synthesis of the polymers materials is complicated, and these polymers are harmful to the environment because usually they take millions of years to be fully decomposed.[12] Therefore, it is necessary to find a degradable material to displace the synthetic polymers. As a natural alkali polysaccharide with great abundance, chitosan (CS) has been widely used, thanks to its attractive biocompatibility, biodegradability, low-toxicity and antimicrobial activity.[13] Moreover, CS is amenable to chemical modifications due to the presence of functional groups as hydroxyl, acetamido and amine. The CS-based microsphere, edible film, and drug delivery carrier have been prepared, indicating that CS is suitable for versatile application. Chemical and physical properties of the CS-based polymers can be manipulated by tethering functional groups such as

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† Electronic Supplementary Information (ESI) available: Nitrogen content of the protein solutions after CCMS hydrolysis; effect of shaking speed on the rate of protein and DNA hydrolysis; hydrolysing protein substrate using reduced-CCMS without pendant aldehyde group. See DOI: 10.1039/b000000x/

cyclodextrin, azacrown ether, and polyamine on the polymer backbone.[14-16] Since nature employs polypeptide as skeleton of enzymes, we are enlightened that CS and its derivatives might be used to build up scaffold of artificial enzymes. The CS-based artificial enzyme was designed to hydrolyze protein and DNA, in addition to its great biodegradability imparted by the natural polysaccharides.

In this work, we attempt to design a novel synthetic enzyme that combines metal complex with CS-based biopolymer. To better mimic the catalytic features of natural enzymes, complexes composed of heterocyclic compounds and metal ions are introduced onto the skeleton of the polysaccharides, where hydrolyzing reaction could take place with minimal interference caused by the strong polarity of the solvent. Meanwhile, the biopolymer was embedded with aldehyde group, which was able to form an imine complex to anchor the protein substrates.[17] By this design, efficiency of hydrolysis might be enhanced for increased affinity between catalyst and substrate. Experiment results demonstrated that the synthetic enzyme was of remarkable catalytic capability for both peptide and phosphodiester bond hydrolysis. This novel artificial enzyme may find its applications in biotechnological, pharmaceutical and biomedical areas, as a biocompatible and biodegradable material.

Experimental

Materials and Reagents

Chitosan (degree of deacetylation 92.2%, MW: 5.5×10^5 Da) was purchased from Li Zhong Biotech (Qingdao, China) and used without further purification. Cyclen (1, 4, 7, 10-tetraazacyclododecane) was provided by Kai Sai Chemical (Shanghai, China). 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. SDS-PAGE molecular-weight markers (7.8-97.4 kDa) and other electrophoresis reagents were purchased from Sigma Chemical Co. Ltd. Pageruler protein ladder (10-170 kDa) was provided by Thermo Fisher Scientific (Shanghai, China). Electrophoresis grade agarose was purchased from Takara Biotechnology (Dalian, China). Coomassie brilliant blue (CBB) and Ethidium bromide (EB) were obtained from Tiangen Biolab (Beijing, China). 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.

Construction of the artificial enzyme

Preparation of cross-linked chitosan microspheres

The cross-linked chitosan microspheres (CMS) tethering pendant aldehyde group were prepared by using reverse-phase suspension crosslinking. First, chitosan powder (10 g) was dissolved in 200 mL of 10% acetic acid solution to form chitosan solution with high viscosity. Then 200 mL of liquid paraffin, 2.5 mL of Span-80 and 40 mL of 0.2% polyethylene glycol (pore-forming reagent) were added to the solution orderly. The resulting mixture was stirred with the stirring speed of 180 r/min for 40 min. After that, 20 mL of formaldehyde (pre-crosslinking reagent) was continuously introduced to the mixture and reacted at 50 ℃ for 30 min. Then the pH of the mixture was adjusted to 9.0 by using 0.1 M NaOH solution, followed by the addition of 20 mL glutaraldehyde (crosslinking reagent) and 3 h reaction under 60 ℃ with the stirring speed of 300 r/min to form chitosan microspheres. Finally, CMS was washed by petroleum ether, absolute ethyl alcohol, deionized water respectively, and stored in DMF (N, N-Dimethylformamide) for the future use.

Activation of the CMS by epoxy reagent

To activate the microspheres for sequel modification, epichlorohydrin was used as the activation reagent. CMS (10 g) was added into a 35 mL of 1, 4-dioxane solution containing certain amount of NaOH that guaranteed pH value of the solution was maintained at 10. Then 5 mL of epichlorohydrin was introduced to the solution mixture dropwise, and the resulting mixture was kept at 60 ℃ for 3 h in a water bath shaker. After the reaction, the activated CMS was washed thoroughly with absolute ethyl alcohol and kept at 4 ℃.

Modification of the activated CMS by heterocyclic compound and metal ions

The activated CMS (20 g) was suspended in 200 mL of DMF, containing 2.7 g (1.5×10^{-2} mol) of cyclen. The suspension was kept at 50 ℃ for 4 h with magnetic stir. Then certain amount $CuCl_2$ was introduced to the system to guarantee that the final concentration of Cu^{2+} was 0.4 M. The resulting suspension was incubated at 45 ℃ for 8 h in a water bath shaker. Finally, the Cu(II)-cyclen-modified CMS (CCMS) were collected by filtration and flushed with distilled water and absolute ethyl alcohol repeatedly until no Cu^{2+} was detected in the eluent. The microspheres were stored in pure DMF for the future use.

Instrumental analysis

Surface morphology of CCMS

Microscopic observation of CCMS was performed on scanning electron microscope (SEM, JSM-6390LV, Jeol Co. Ltd.) and optical microscope (OM, SMZ745T, Nikon Co. Ltd.). For SEM observation, the beads were vacuum dried at room temperature, mounted on brass stubs and sputter-coated with gold in an argon atmosphere prior to examination under the SEM.

Particle diameter analysis

The mean diameter and particle size distribution of the microspheres were measured using a laser particle size analyzer (LPSA, Mastersizer 2000, Malvern Instruments Co. Ltd.). The CCMS particles were well dispersed in ultrapure Milli-Q water

before loaded on analysis platform. Average size of the beads was expressed as intensity-mean diameter.

XRD analysis

X-ray diffraction (XRD) was used to analyze the crystal structure of pure chitosan, CMS and CCMS. Analysis was performed using the X-ray diffractometer (D8 ADVANCE, Bruker AXS Inc.) with Cu anode, running at 40 kV and 30 mA, scanning from 5 °to 50 °at 2 °/min while the data was collecting.

FTIR

Fourier Transform Infrared Spectra (FTIR, Nexus 470, Nicolet Co. Ltd.) was used to characterize the chitosan-based polymers. All the samples were pulverised with IR grade KBr to form the table for IR analysis. IR signals were recorded from the accumulation of 32 scans in the 4000-500 cm^{-1} range at a resolution of two cm^{-1}. The spectral scan was repeated at least three times to minimize the background noise.

ICP-AES

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 parameters were set as follows: RF power: 1150W; nebulizer flow: 26.0 PSI; auxiliary gas: 1.0 LPM. All the tests of samples were performed in triplicate, from which standard deviation less than 5% was obtained.

Protein hydrolysis by CCMS

Mb, BSA, Ova, and Col were used as the substrates to check the proteolytic activity of CCMS. The typical reaction conditions for protein hydrolysis were as follows. Certain amount of CCMS was added to 1.5 mL of Tris-HCl buffer (100 mM, pH=7.8) containing a fixed concentration of the protein substrates. The reaction system was placed on an isothermal shaker with constant temperature and shaking speed. After a specific time interval, 20 μL of the solution was drawn from the reaction system, and subjected to SDS-PAGE analysis.

SDS-PAGE was conducted to investigate the hydrolysis efficiency. The intensity of protein bands in electrophoretic patterns, which represented the concentration of the corresponding substrate protein, was recorded and analyzed by Biorad gel imaging and processing system. The data were collected and processed to obtain the initial rate plots. Kjeldahl determination was employed to verify the fact that disappearance of the electrophoretic bands was attributed to the hydrolyzing ability of CCMS. To study the catalytic kinetics of the CCMS, the concentration of the catalyst was calculated by assuming that all the catalyst used in the experiments was dissolved in the solution.

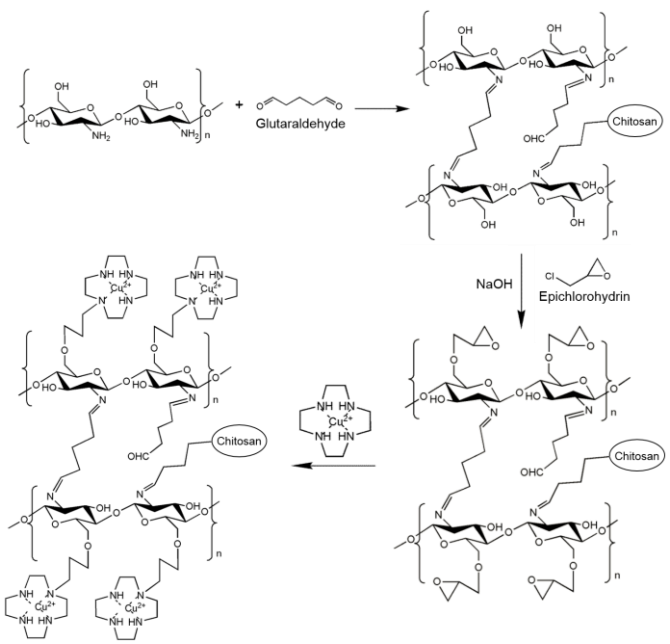
DNA Cleavage by CCMS

DNA cleavage experiments were carried out by using pUC-18 plasmid DNA as the substrate. Plasmid DNA (pUC-18, over 90-95% supercoiled) was stored in Tris-EDTA buffer and directly used for this study. The cleavage was carried out in 400 μL of NaH_2PO_4/Na_2HPO_4 buffer (200 mM, pH=7.2) with a certain amount of CCMS and pUC-18 with fixed concentration. The samples were then incubated at 37 ℃ with constant shaking speed. The reaction was terminated at a specific time interval by configuration that facilitated the separation of the insoluble catalysts from the cleavage DNA. The DNA was loaded on 8% PAGE gel, and the electrophoresis was carried out at 350 V for 90 min in TBE buffer followed by EB staining. Finally, bands were visualized under ultraviolet illumination at 302 nm and photographed by with a Biorad molecular imager. Estimation of the intensity of the DNA bands was performed by using Biorad gel processing system.

Results and discussion

Synthesis of CCMS

Cu(II)-cyclen modified cross-linked chitosan microspheres (CCMS) was synthesized according to the following three steps. First, the cross-linked chitosan microsphere (CMS) was fabricated by using glutaraldehyde as a crosslinking reagent. The two aldehyde groups were able to form Schiff bases with C_2-NH_2 groups from different polysaccharide chains, a process that rendered the formation of the microspheres with insolubility and physical strength. However, in some cases, one of the aldehyde groups could not conduct Schiff base formation with C_2-NH_2 because of the steric hindrance caused by the molecule winding.[18] Therefore, pendant aldehyde group, which was able to form imine bond with amino group on protein substrate, was present in CMS. The crosslinking degree of CMS was determined as 12.67% (section 3, ESI), indicating that a fraction of sugar monomers of chitosan did not react with glutaraldehyde. The intact -NH_2 and -OH groups on the monomers can react with epichlorohydrin afterwards, which serves as a "linker" to tether cyclen moiety on the crosslinked microspheres.



Scheme 1 General synthesis procedure of CCMS

Epichlorohydrin bearing two functional groups, epoxide group and chloride, was used as the activation reagent here. C_6-OH of chitosan preferentially reacted with chloride than epoxy group. Since the amount of epichlorohydrin used in the experiment exceeded the stoichiometric proportion, some of the epoxy group tended to be unreacted in the activated CMS.[19] For its high activity, the rupturing of the epoxide ring can easily occur once electron-rich group, such as N atom of cyclen, was present.

The Cyclen-moiety was introduced onto CMS via the bond formation between -NH with the epoxy group. Reaction of -NH with epoxide took place in organic solvent, resulting in the attachment of cyclen to CMS. Elemental analysis of CMS and cyclen-modified CMS was performed to determine their C/N value (Table S1, ESI). After modification with cyclen, the C/N value decreased, suggesting the introduction of cyclen into the backbone of the microsphere. The ratio of cyclen /sugar unit was calculated as 1:3, signifying that one of three sugar units was tethered with a cyclen molecule. Cyclen has four nitrogen atoms with lone electron pair, which can form a tetradentate ligand with Cu(II) and other transition metal ions. Cu(II) coordinating with the azamacrocycle boasted a desirable stability for multi-ligand of N atoms, and served as the catalytic center for the artificial enzyme.[6, 20] The proposed synthetic strategy of CCMS was shown in Scheme 1.

Characteristics of CCMS

Surface detail and particle size of CCMS

Chitosan is the product of chitin deacetylation, is able to dissolve in acidic solution. After crosslinking, the chitosan-microsphere was found to be insoluble in both acidic and alkaline medium as well as in distilled water, and both the chemical property and physical strength were enhanced. It can be observed in Fig.1, CCMS were green, regular microspheres with spherical and smooth surface structure, a situation that favors the dispersion in water and the interaction with protein or DNA substrates.

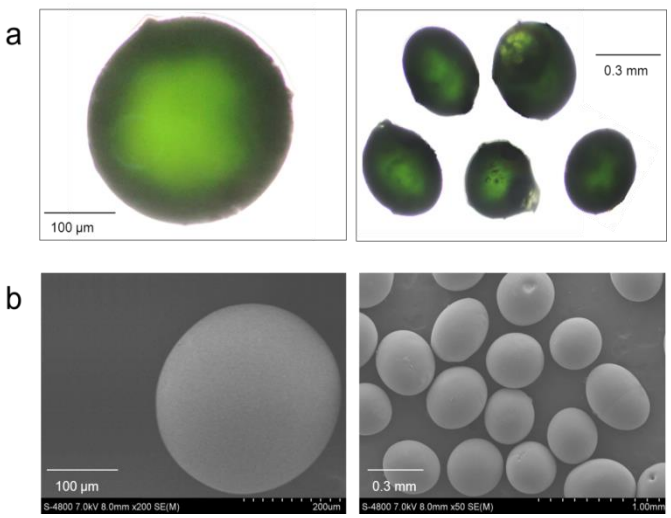


Fig. 1 Images of CCMS taken by optical microscope and SEM. (a) Optical microscope images of CCMS; (b) SEM images of CCMS.

Particle diameter analysis showed that the microspheres were homogenous in size, more than 90% of the microspheres were of the diameter distributing from 208.9 μm to 549.5 μm. The average diameter of CCMS was determined as 353.0 μm (Fig. 2). CCMS utilized copper ion to form the catalytic center. In this regard, the amount of copper ion chelated on CCMS is vital for its proteolytic activity. The stability of copper ion on CCMS was determined by inductively coupled plasma absorption-emission spectroscopy (ICP-AES). The microspheres were completely digested by 1.0 M HNO_3 overnight before loaded on ICP-AES platform, from which the content of copper ion on CCMS was obtained. After 24 hours' shaking, only 1.62% amount of ions dissociated off the matrix, indicating that CCMS boasted an appreciable capacity of stabilizing copper ions. The ratio of Cu(II)/sugar unit of chitosan was determined based on the copper content of CCMS (Fig. S1, ESI). The data showed that 0.94 mmol of Cu(II) chelated on 0.54 mmol of sugar unit of chitosan, indicating that approximately two Cu(II) ions coordinated with one chitosan monomer. The enhanced Cu(II)/sugar unit ratio can be attributed to the introduction of cyclen, which provided another coordination site for Cu(II) ion. The ratio of cyclen /sugar unit was obtained based on element content of microspheres before and after the modification cyclen (Table S1, ESI).

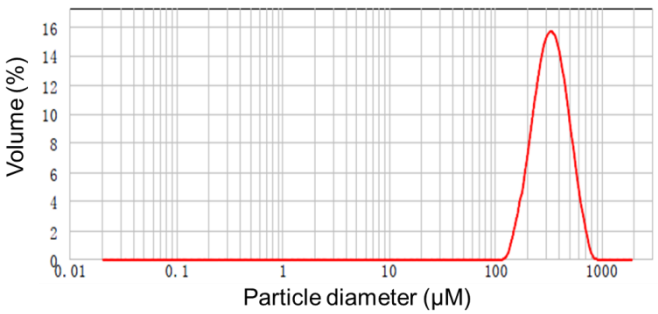


Fig. 2 Particle size distribution of CCMS

Crystallinity determined by XRD

The XRD analysis of pure chitosan, CMS and CCMS was shown is Fig. 3. In the spectra of pure chitosan, two typical crystalline diffraction peaks were observed at 10.9 ° and 21.4 ° respectively, demonstrating the presence of specific crystal structure (Fig. 3a). H-bond between hydroxyl and amine group was conducive to the formation of crystal lattice. However, in Fig. 3b, the typical diffraction peaks disappeared with the emergency of a weaker, amorphous one, suggesting that the formation of crosslinked products reduced the regularity of chitosan molecules.[21] The loss of crystallization ability in CMS can be explained by the fact that H-bond interaction was destroyed by the formation of a Schiff base between glutaraldehyde and amine group. In addition, glutaraldehyde crosslinking limited the mobility of the biopolymer molecules, which led to the decrease of the crystallization.[22] The XRD pattern of CCMS can be observed in Fig. 3c, which was similar with that of CMS, indicating that the basic structure of CCMS was not significantly changed by the attachment of Cu(II)-cyclen complex. The stable network structure demonstrated by XRD analysis, provided a great support for the complex to exert hydrolytic

ability, as well as shielded the interference caused by solvent molecules.

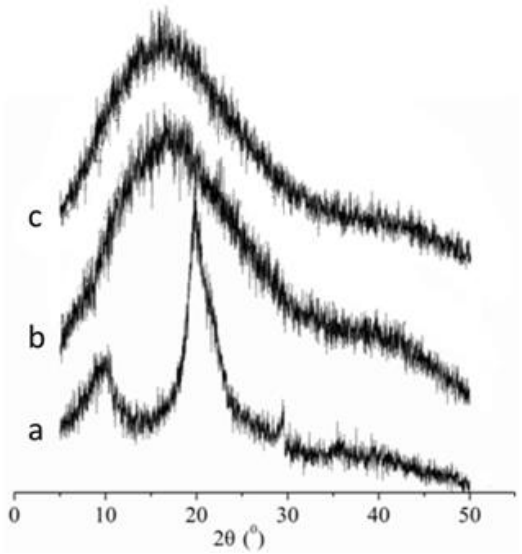


Fig. 3 XRD pattern of pure chitosan (a), CMS (b) and CCMS (c).

FTIR analysis

FTIR (Fourier transform infrared spectra) instrument is a useful tool to investigate composite of polymer material and presence of any functional groups. Here, FTIR was employed to unravel the fundamental structure information of pure chitosan, CMS and CCMS.

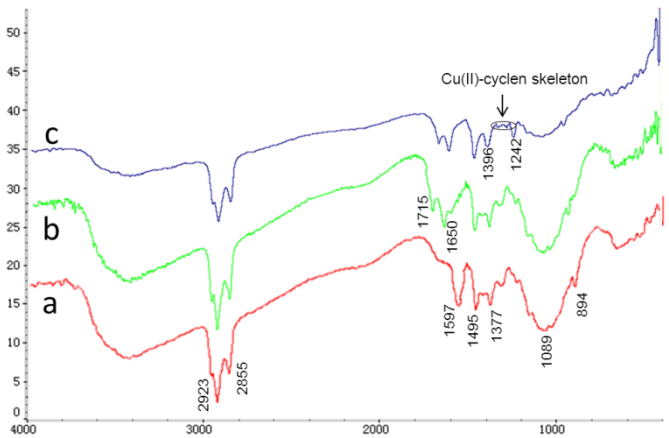


Fig. 4 FTIR spectra of pure chitosan (a), CMS (b) and CCMS (c).

As shown in Fig. 4a, the broad and strong band ranging from 3200 to 3600 cm^{-1} was attributed to the presence of -OH and -NH_2 groups for their stretching vibration. The adsorption peaks at 2923 and 2855 cm^{-1} can be assigned to asymmetric and symmetric stretching vibration of -CH_2 groups. The stretching and bending vibration of -CH_2 were found at 1459 and 1377 cm^{-1}. The peak located at 1597 was characteristic of amine deformation. The peak at 1089 cm^{-1} can be attributed to the C-O-C stretching. The characteristic absorption peak of β-D-pyran glycosidase was assigned to 894 cm^{-1}. For the IR pattern of CMS, most of the absorption peaks found in pure chitosan were retained, expect for two emerging peaks (Fig. 4b). One of them was located at 1650 cm^{-1}, which was corresponding to the C=N stretching vibration, suggesting the existence of Schiff base. The other one was found at 1715 cm^{-1}, can be attributed as the characteristic absorption peak of C=O stretching vibration, indicating that unreacted aldehyde group was present in CMS. The presence of aldehyde can be attributed to the following two aspects. First, the amount of glutaraldehyde used in crosslinking was excessive to the stoichiometric ratio; second, due to the steric hindrance caused by folding and winding of chitosan molecules, the accessibility to -NH_2 was reduced, resulting in the unreacted aldehyde group in CMS. For CCMS (Fig. 4c), the adsorption peak at 1396 cm^{-1} and 1242 cm^{-1} corresponded to the C-N and C-O-C stretching vibration, respectively, indicating that epichlorohydrin went through a ring-open reaction and formed a covalent bond with the N atom of cyclen.[23] The absorption band locating at 1374-1263 cm^{-1} represented stretching vibration of Cu(II)-cyclen skeleton.[24] The analytical data regarding CCMS indicated that the metal-azamacrocycle complex was successfully introduced the chitosan-based microspheres, a situation that was essential for hydrolysis reaction toward peptide and phosphodiester bond.

Proteolytic ability of CCMS

Electrophoresis analysis of proteins incubated with CCMS

Proteolytic ability of CCMS was examined by using myoglobin (Mb, M.V. 16 kDa), bovine serum albumin (BSA, M.V. 66 kDa), ovalbumin (Ova, M.V. 45 kDa) and collagen (Col, M.V. 40-300 kDa) as substrates. Then the hydrolysate was analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

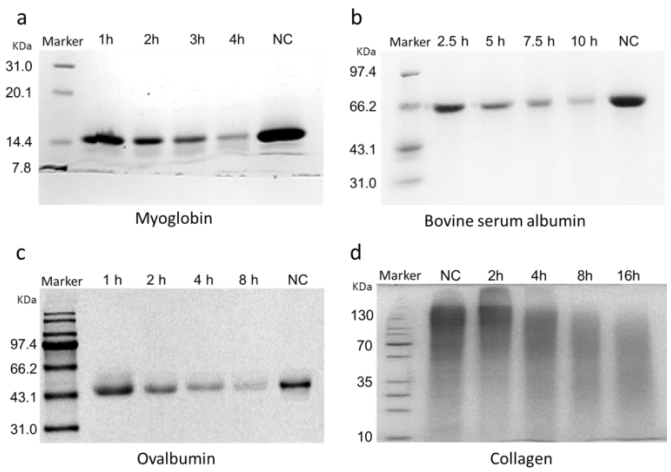


Fig. 5 SDS-PAGE analysis of protein substrates incubated with CCMS. (a)Mb; (b)BSA; (c)Ova; (d)Col. Conditions: concentration of protein substrates was 1.0 mg/mL, CCMS of 0.12 mM was added as the catalyst (catalyst concentration was calculated by assuming the insoluble microspheres were dissolved in buffer solution), Tris-HCl buffer (pH=7.8), 50 ℃ incubation, with shaking speed of 900 r/min. "NC" represented negative control reaction, which was carried out under the conditions described above expect for adding the CCMS catalyst. Duration of NC reactions were 4 h, 10 h, 8 h and 16 h for Mb, BSA, Ova and Col respectively.

The electrophoresis results of the protein incubated with CCMS were shown in Fig 5.The disappearance of the protein bands were observed in proportion to the incubation duration for all the proteins

tested. To confirm that the disappearance of the electrophoresis bands was not due to the adsorption on the chitosan microspheres, the total nitrogen content of the products solution (separated from the insoluble microspheres by filtration) was measured by Kjeldahl determination. The results showed that more than 80% of nitrogen of the original protein solution was obtained from the product solution (Fig. S2, ESI), indicating that the proteolysis was the primary cause for the fading of the bands. Ninhydrin reaction measuring free terminal amino was carried as well (Fig. S3, ESI), and the result confirmed that protein was cleaved for the increment of terminal amino after incubation with CCMS. Therefore, the chitosan polymer-based artificial proteinase was successfully established. The proteolytic activity of CCMS could be attributed to the following aspect. First, Cu(II)-cyclen complex provided the catalytic center using -OH group dissociating from H_2O to exert nucleophilic attack on peptide bond.[25] Second, when chitosan was crosslinked, certain cyclodextrin-like structures formed by self-folding of the chitosan chain, were fixed. These structures created a desirable hydrophobic microenvironment that can effectively block the interference of solvent molecules.[26, 27] Thus, the efficiency of catalytic reaction could be dramatically enhanced.

It was noticeable that the electrophoresis patterns of CCMS hydrolysate were different from enzyme cleavage, where small bands could be observed below the parent band. The phenomenon suggested that proteins or peptides with sizes large enough for detection by the electrophoretic method did not accumulate appreciably during the reaction. Since proteins with small sizes tend to diffuse much faster than large ones in the biopolymer matrix, the small intermediate proteins would be hydrolyzed preferentially, resulting in the smaller peptides that cannot be detected by electrophoresis.[28] HPLC analysis was performed characterize the BSA hydrolysate. The result was consistent with the analysis by SDS-PAGE, based on the fact that no adsorption peak designating smaller peptide or cleaved protein was observed, while the parent protein was digested over time (Fig. S4, ESI).

Kinetic behavior of the proteolytic activity

Kinetic studies on the rate of protein hydrolysis by CCMS were carried out by measuring the decrease in the electrophoretic band intensity corresponding to the specific protein substrate. Considering that the rate of protein hydrolysis might be affected by the shaking speeding applied during reaction, hydrolyzing protein substrate with varying shaking speeding was carried out. The results showed that, the cleavage rate reached a plateau at shaking speed more than 900 r/min (Fig. S5, ESI). Therefore, kinetic data for all protein substrates tested were collected under this speed.

The apparent rate constant (k*obs*) for hydrolysis of Mb was estimated from the plot of ln[S]/[S_0] against time as illustrated in Fig. 6, where "[S]" represents the protein concentration at designated time point, "[S_0]" stands for the initial concentration of the protein substrate. Since no bands smaller than the parent band was observed, k*obs* only reflected the kinetic behavior of hydrolyzing parent proteins and do not provide information on further digestion of cleaved products. For other protein substrates tested in this study, k*obs* were calculated by the same method.

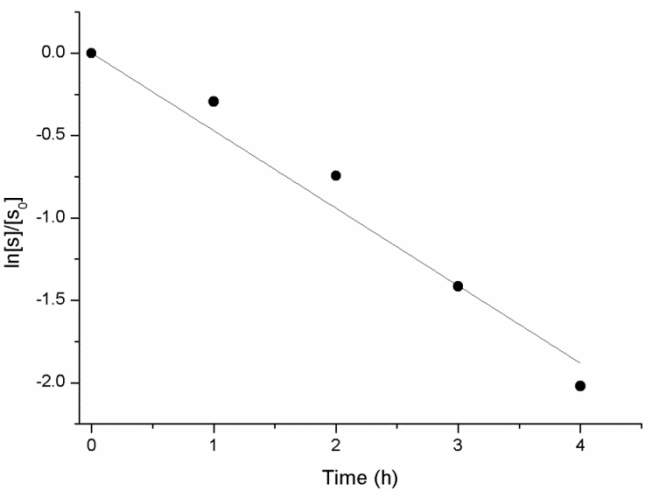


Fig. 6 Plot of ln[S]/[S_0] against time representing electrophoretic bands in Fig. 5a. The relative concentration of the protein substrate was measured by analyzing the density of the electrophoretic bands. The straight line corresponds to k*obs* of 4.7×10^{-1} h^{-1}.

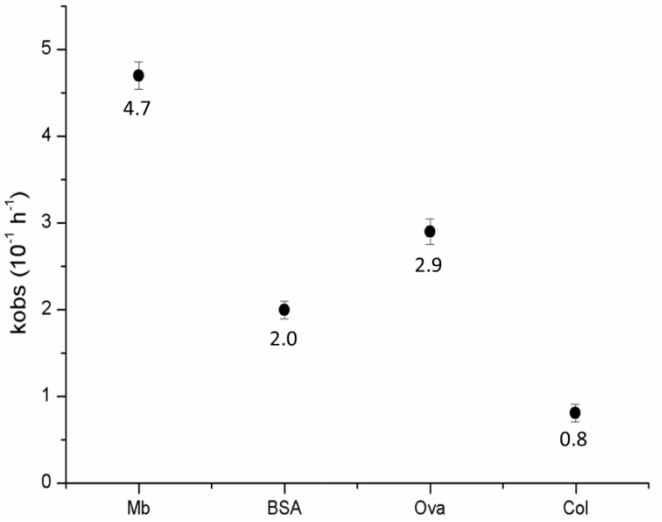


Fig. 7 Rate of protein hydrolysis by CCMS evaluated in terms of k*obs*.

As shown in Fig. 7, CCMS cleaved protein substrates with different efficiency. The fastest proteolytic reaction in this study was found when CCMS incubated with Mb. More than 90% of the protein molecules were digested during 4 h incubation. For BSA and Ova, CCMS delivered desirable proteolytic activity since more than 90% of the proteins were digested after 8 h and 10 h reaction respectively. However, for Col, the hydrolysis rate was low since 16 hours' shaking reaction merely hydrolyzed 70% of the protein substrate. The difference in hydrolysis rates can be attributed to the different molecular sizes. Since bulky molecules tend to diffuse slowly in the microspheres, their chance to interact with catalytic center is lower than small ones.[29] The fact that the rates of protein hydrolysis for all the substrates examined in this study were inversely proportional to their molecular weights confirmed the point. CCMS demonstrated a low efficiency when used to hydrolyze Col.

The unique spatial structure and amino acids composition of Col could be responsible for the phenomena. Collagen molecule is composed of three subunits protein chain intertwining with each other, the compact structure hinders its interaction with the reactive site of CCMS.[30] Furthermore, in collagen, Pro and Gly account for more than 40% of total amino acids composition.[31] These amino acids are not conducive to hydrolysis reaction for the lacking of -OH group on the side chain. Therefore, hydrolysis of Col was much difficult than other protein substrates tested in this experiment.

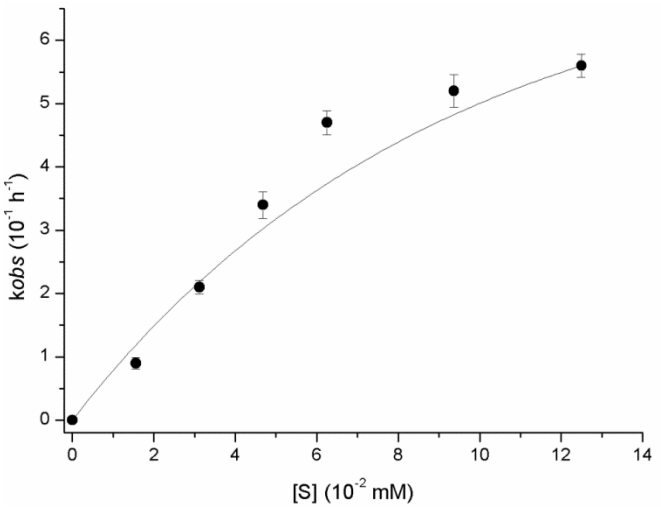


Fig. 8 Effect of initial Mb concentration on the rate of protein hydrolysis by CCMS. The parameters for CCMS hydrolyzing Mb were calculated from the data according to Lineweaver-Burk double-reciprocal method. Conditions: 0.12 mM of CCMS, Tris-HCl buffer (pH=7.8), incubated at 50 ℃ with shaking speed of 900 r/min.

The effect of initial concentration of Mb on k*obs* was shown in Fig. 8. With a fixed catalyst concentration, the hydrolytic rates fitted the first-order reaction kinetic at lower Mb concentration but exhibited a saturation trend at higher substrate concentration, suggesting that the catalytic behavior complied with Michaelis-Menten mechanism. Equations elucidating the mechanism of Mb hydrolysis were listed below.

$$CCMS + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CCMS\text{—}S \xrightarrow{k_{cat}} CCMS + P \quad (1)$$

$$k_{obs} = \frac{k_{cat}[CCMS]}{K_m + [CCMS]}, \quad K_m = \frac{k_{-1} + k_{cat}}{k_1} \quad (2)$$

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{[S]} \quad (3)$$

k_1, k_{-1} and k_{cat} represent rate constants for different phage. V_{max} is the maximum reaction rate, *K*m is Michaelis constant. K_m and V_{max} value of CCMS hydrolyzing Mb were calculated using Lineweaver-Burk double-reciprocal method based on equation (3) and the data shown in Fig. 8. The V_{max} was calculated as 70.4×10^{-1} h^{-1}, K_m as 8.3×10^{-5} mM. The data confirmed that the kinetic behavior of CCMS hydrolyzing Mb followed Michaelis-Menten mechanism as natural enzymes. The low K_m value suggested that affinity between CCMS and Mb molecules was high, a feature that promoted the interaction between the protein substrate and the reactive sites on CCMS. Comparing to the half-life of peptide bond at 25 ℃, pH 7.0, which is 500 years, CCMS speeds up the proteolytic reaction more than 1.5×10^5-fold without relying on the extreme pH where noticeably self-degradation would happen. [32, 33]

Many research teams reported the proteolytic activities of metal ion-heterocycle complexes, and the mechanism of peptide bond hydrolysis has been summarized by several review articles.[1, 34, 35] Generally, metal ions act as Lewis acid to mediate the cleavage of peptide bond, while heterocyclic compounds serve as ligands that stabilize and provided desirable reaction atmosphere by stabilizing the metal ions.[36] The mechanism of peptide bond hydrolysis by Cu(II)-cyclen can be proposed as follow: the Cu(II) center polarizes carbonyl group of the scissile peptide bond by binding at carbonyl oxygen, meanwhile facilitates Cu(II)-bound hydroxide ion acting as a potent nucleophile to promote the hydrolysis reaction.[37] However, the proteolytic activity of Cu(II)-cyclen complex itself is negligible.[25] A possible explanation is that the dimerization of two Cu(II)-cyclen complexes are formed in homogeneous system. Once the complexes are dimerized, it is hard for catalytic center to interact with protein substrates.[38] The purpose of applying the chitosan-based particles as the scaffold is to prevent the complexes from being dimerized by fixing Cu(II)-cyclen on the surface of CCMS. In addition, the unique microenvironment formed by folding of chitosan chains is also conducive to proteolytic reaction via lowering the polarity of the medium.

Effect of the pendant aldehyde group on proteolytic activity

We reported the first chitosan-based artificial metalloprotease in 2012, which was able to digest 90% of BSA more than 48 h.[39] However, the proteolytic activity of that synzyme towards other protein substrates was insignificant, suggesting its narrow substrate selectivity. In this study, we attempted to improve the selectivity by introducing pendant aldehyde group to the biopolymer. The pendant aldehyde group in CCMS was able to form an imine bond with the amino groups of Lys residues exposing on the surface of proteins, a process that could enhance the affinity of catalyst and substrate.[40] Since the imine bonds are readily hydrolyzed, the chitosan-based synzyme equipped with aldehyde groups may be able to reversibly form complexes with a variety of proteins, contributing to broad selectivity. To confirm the functional of the pendant group on CCMS, a control experiment was performed by using re-CCMS, whose aldehyde groups were reduced by $NaBH_4$. The detailed methods and results of the control experiment can be found in ESI. It was demonstrated by FTIR spectra that Schiff base and pendant aldehyde group were reduced to –NH– and –OH by $NaBH_4$. When re-CCMS was used to hydrolyze Mb, the proteolytic activity was found to be insignificant. Only 21.6% of the parent protein was digested for 4 hour's incubation, compared to the efficiency of Mb cleavage by CCMS, it was safe to conclude that the pendant aldehyde group is essential for the synzyme to perform with high efficiency and broad selectivity. The desirable properties of CCMS can be attributed to the fact that it has both catalytic group and binding group that can work synergic fashion to enhance hydrolysis

activity. By incorporating more catalytic elements to the reactive site with a better-defined structure, the chitosan-based artificial metalloprotease would become more effective and achieve higher reaction rates with controllable sequence specificity in prospect.

DNA cleavage activity of CCMS

Hydrolytic cleavage of plasmid DNA by CCMS

DNA is extremely stable under physiological conditions for its responsibility of carrying genetic information. The half-life of DNA toward spontaneous hydrolysis is 130,000 years at 25 ℃, pH=7.0.[41] Cleavage of DNA in oxidative pathway has been studied and some metal complexes reported with remarkable cleavage rate.[4, 42, 43] However, the oxidative cleavage protocols require the addition of external oxidative or reductive chemicals and thus limit their *in vitro* and *in vivo* applications. The progress of DNA cleavage in hydrolytic way is slow for the stability toward hydrolysis reaction. The stability is a result of the repulsion between negatively charged phosphodiester backbone and potential nucleophiles.[44] To minimize the repulsion force, positively charged insoluble support polymer could be introduced to construct artificial nucleases. Since chitosan is positively charged when dissolved in acidic medium, we envisaged that the unique property could be used to create a novel artificial nuclease. CCMS is a chitosan-based functional biopolymer with a catalytic center built on. It is conceivable that CCMS could cleavage DNA efficiently in a hydrolytic pathway. Herein, the DNA cleavage property of CCMS was investigated.

The experiment was carried out using supercoiled plasmid DNA (pUC-18) as substrate. Plasmid pUC-18, 2686 bp in length, is a covalently-closed-circular double-stranded DNA. Commonly, the plasmid exists in a supercoiled form in water solution. When natural nuclease or artificial nuclease cleaves the plasmid and leaves a nick on one of the DNA double strands, the supercoiled form is relaxed to become the nicked form. The nicked plasmid may be subjected to further cleavage, both strands are cut and results in linear form. The three forms of DNA showed different migration speed on gel electrophoresis, thus hydrolytic rate of pUC-18 can be determined by PAGE analysis. Fig. 9a showed the electrophoresis results of DNA cleavage catalyzed by CCMS. The supercoiled plasmid was converted to nicked and linear form gradually during 8 h incubation, indicating that CCMS was capable of cleaving DNA in a hydrolytic mechanism. It was proposed that the supercoiled DNA was transformed to nicked form in the first 6 h, then the nicked pUC-18 was further digested to linear form during 6 h to 8 h. Relative quantity of different forms of DNA was shown in Fig. 9b. The sum of nicked and linear plasmid was less than 100%, suggesting that some plasmid DNA was digested into small fragments that were not detectable under the electrophoresis conditions. Cu(II)-cyclen complex is one of the most effective synthetic catalysts discovered so far for DNA hydrolysis. Hydrolytic nature of the DNA cleavage by the Cu(II) complex has been proposed for the catalytic action of the Cu(II) complexes: the Cu(II) center binds the phosphate anion of the phosphodiester linkage to polarize the O=P bond, meanwhile it promotes the adjacent Cu(II)-bound hydroxide ion to exert intramolecular attack at the phosphorus atom of the bound substrate.[45] Finally, the phosphodiester bond is cleaved in the hydrolytic path. However, since the nucleophilic attack could take place at any phosphoester bond, two types of products with 5′-OH or 5′-P are released.[46]

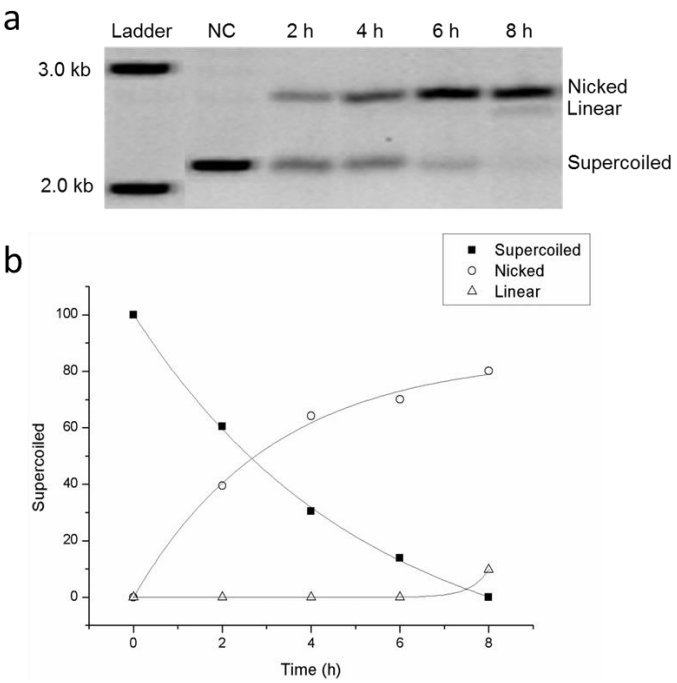


Fig. 9 Cleavage of plasmid DNA by CCMS. (a) PAGE analysis of supercoiled pUC-18 cleavage at different time span. For plasmid cleavage, 0.12 mM of CCMS was added to 400 μL NaH_2PO_4/Na_2HPO_4 buffer (pH=7.2) containing 5.0 ng/μL plasmid DNA. The resulting mixture was incubated at 37 ℃ with shaking speed of 750 r/min. Aliquot of 10 μL cleavage products was pipetted at a specific time point and subjected to PAGE analysis. "NC" represented negative control reaction, where experiment was performed without adding the catalyst (CCMS). (b) Relative quantity of different forms of plasmid in terms of %. Relative quantity of each band was obtained by comparing the band intensity to that of "NC" in Fig. 9a, which was designated as 100.

Kinect behavior of DNA cleavage by CCMS

As noticed, the shaking speed affected the rate of hydrolysis of DNA as well. The rate increased as the shaking speed was raised, reaching a plateau value at 750 r/min (see Fig. S8 in ESI). Thus, kinetic measurement was performed at this speed. The apparent rate constant (k*obs*) of CCMS hydrolyzing DNA was estimated from the plot illustrated in Fig. 10. The data were collected based on the band intensity of the supercoiled DNA. The k*obs* was determined as 2.5×10^{-1} h^{-1} for DNA hydrolysis, which was high than that of the Cu(II)-based complexes reported previously.[24, 47] The high cleavage efficiency could be attributed to the affinity between DNA substrate and the surface of CCMS. The chitosan matrix of CCMS was positively charged under the reaction conditions, therefore, negatively charged DNA can readily cling to the particle surface where the catalytic center was able to cleave the substrate DNA.[48] When single molecule DNA interacted with a CCMS particle, the gel-like microenvironment of CCMS surface may be conducive to hydrolysis.[49] Furthermore, one DNA molecule can cover a certain area of CCMS surface, where hundreds of Cu(II)-cyclen complexes can conduct hydrolytic reaction simultaneously.[50] As a result, higher

hydrolytic efficiency than the insoluble metal-heterocycle complexes was achieved.

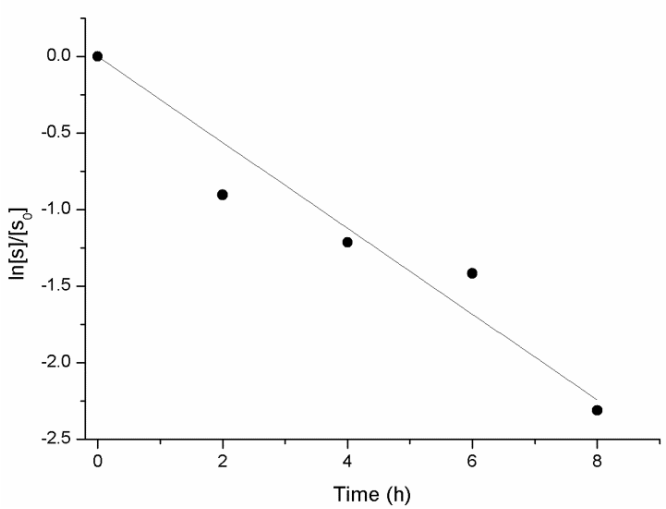


Fig. 10 Plot of ln[S]/[S_0] against time representing supercoiled DNA in Fig. 9a. The relative concentration of the supercoiled DNA was measured by analyzing the density of its electrophoretic bands. The straight line corresponded to k*obs* of 2.5×10^{-1} h^{-1} for CCMS hydrolyzing plasmid DNA.

Effect of catalyst amount on the rate of DNA cleavage

PAGE analysis of DNA cleavage by CCMS with increasing amount was shown in Fig. 11a. As the amount of the catalyst raised, the rate of DNA cleavage accelerated. For the reaction with 0.72 mM of CCMS, all three forms of plasmid DNA were practically digested for 3 hours' incubation. However, there was a possibility that the decrease in the band intensity (Fig. 11a) was due to adsorption of the DNA substrate onto the microspheres. This possibility was ruled out by testing absorbance of product solution (CCMS were separated by filtration) at 260 nm after the plasmid DNA completely disappeared. Substantial amount of the nucleotides was recovered in the product solution, because the absorbance at 260 nm of the product solution was greater than that of the plasmid DNA solution. Relative quantity of three forms of DNA can be found in Fig. 11b. The accumulation of nicked DNA was reversely proportional to the intensity of the supercoiled DNA, indicating that the supercoiled DNA was primarily transformed to the nicked DNA. However, the accumulation of linear DNA was modest, suggesting that the DNA was digested into small fragment in a much more efficient fashion. One of the reasonable explanations was that the completely relaxed linear DNA tended to cover a larger area of CCMS surface, a situation enables interaction with more hydrolytic Cu(II)-cyclen complexes, resulting in the fast hydrolyzation. Many transient metal based systems have been reported for the nature of cleavage DNA in hydrolytic path.[51] Among them, Scrimin's team represented a series of Zn(II)-mediated complexes as artificial metallonucleases. The fast DNA hydrolysis was realized by Zn(II)-based multivalent complex tethering on Au-nanoparticle.[52] More than 90% supercoiled DNA substrate was digested after 24 h incubation. However, for CCMS, 90% supercoiled DNA was digested within 3 h, demonstrating the higher hydrolytic efficiency.

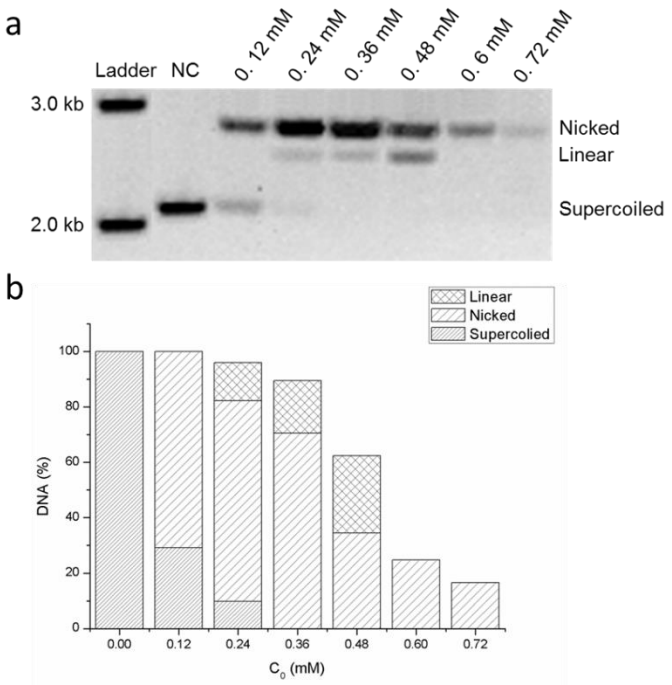


Fig. 11 Effect of initial amount of CCMS on hydrolysis of plasmid DNA. (a) PAGE analysis of plasmid DNA cleaved by various amount of CCMS. "NC" represented the negative control reaction. Reaction conditions: concentration of plasmid DNA 5.0 ng/μL, in 400 μL NaH_2PO_4/Na_2HPO_4 buffer (pH=7.2), 37 ℃, 750 r/min, the duration of reactions was 3 h. (b) Relative quantity of different forms of plasmid DNA. The data were calculated by comparing the band intensity to that of "NC" in Fig. 11a, which was designated as 100.

DNA cleavage using component of CCMS

A control experiment was carried out by using each component of CCMS, in order to confirm that the DNA cleavage activity was derived from the collaboration of each part. Cross linked chitosan microspheres (CMS) and Cu(II)-cyclen complex was added to DNA solution as the catalyst.

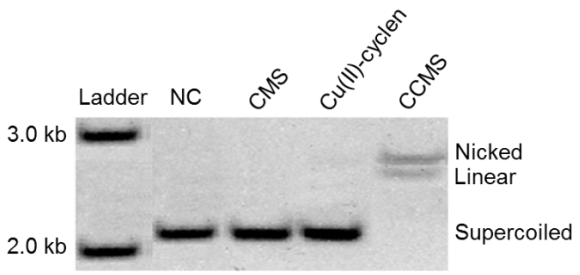


Fig. 12 Hydrolysis of plasmid DNA using components of CCMS. All the reactions were performed in 400 μL, NaH_2PO_4/Na_2HPO_4 buffer (pH=7.2) containing 5.0 ng/μL plasmid DNA, incubated at 60 ℃ for 10 h, with shaking speed of 750 r/min. After reaction, 10 μL of product solution was pipetted and subjected to PAGE analysis.

As shown in Fig. 12, both CMS and Cu(II)-cyclen failed to exhibit any hydrolytic activity, as compared to CCMS, which was able to convert all supercoiled DNA to nicked and linear form after 10 h incubation. The result demonstrated the synergistic fashion of CCMS for DNA hydrolysis. Construction of artificial metallonucleases based on Cu(II)-heterocycle were also reported by other research teams.[4, 26, 44] However, their hydrolytic efficiency

towards DNA substrate was lower than that of CCMS. One of the possible explanations is that the catalytic reaction was hindered by solvent molecules with srtong polarity. CMS used as an insoluble matrix can provide favorable microenvironment where Cu(II)-cyclen mediate nucleophilic attack toward phosphoester bond with least interference.

Reusability of CCMS

As an artificial enzyme, CCMS held the promise to be used as a heterogeneous catalyst. The insoluble chitosan matrix enabled the separation of the catalyst from the product as well as improved its mechanical strength. By recollection from the product solution and regeneration with water and ethanol, CCMS could act as a recyclable catalyst for protein and DNA hydrolysis.

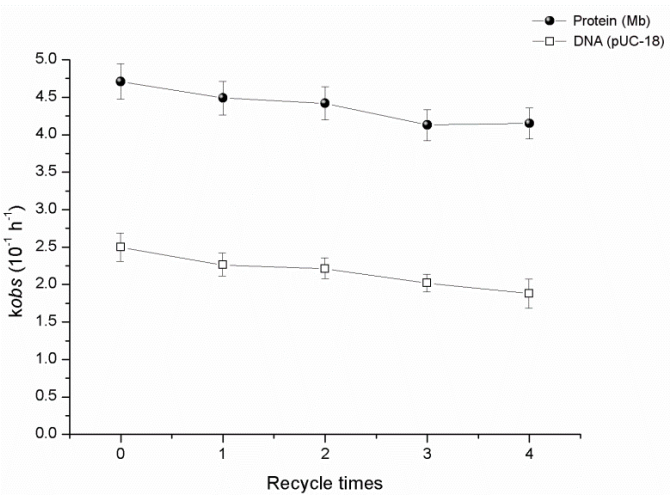


Fig. 13 Reusability of CCMS for protein and DNA hydrolysis. After each reaction, CCMS was recollected by centrifugation and washed with purified water and ethanol three times for regeneration. For protein hydrolysis, 6.25×10^{-2} mM of Mb was incubated with 0.12 mM of CCMS in 1.5 mL Tris-HCl buffer (pH=7.8). The proteolytic reaction was performed at 50 ℃ with shaking speed of 900 r/min. For DNA hydrolysis, 0.12 mM of CCMS was added to 400 μL NaH_2PO_4/Na_2HPO_4 buffer (pH=7.2) containing 50 ng/μL plasmid DNA. The resulting mixture was incubated at 37 ℃ with shaking speed of 750 r/min.

The reusability of CCMS was shown in Fig.13, evaluated in terms of k*obs*. The catalytic activity displayed a small decrease after each cycle. For protein hydrolysis, more than 88% activity was retained after being used for four times, for DNA, maintenance was 75%. The loss of catalytic activity could be mainly ascribed to the dissociation of Cu^{2+} off the catalytic center during regeneration. The high maintenance suggested that the deactivation or poison was not serious during the catalytic reaction and afterward recovery. The results indicated that CCMS were of great activity and stability for protein and DNA hydrolysis, a feature might enable its application in industrial area as an artificial proteinase or nuclease with high efficiency and low cost.[53]

Conclusions

In this work, we fabricated a novel insoluble artificial enzyme, dubbed CCMS, which can hydrolyze protein as well as DNA. FTIR analysis revealed that both catalytic group [Cu(II)-cyclen] and binding group (pendant aldehyde) were constructed onto the chitosan-based biopolymer support, which was degradable and friendly to the environment. CCMS hydrolyzed protein substrates under physiological pH at 50 ℃, featuring high efficiency and broad selectivity. The broad selectivity was attributed to the pendant aldehyde that can reversely form imine bond with protein molecule to enhance the substrate affinity. CCMS can also be used to hydrolyze DNA substrate under mild conditions. Kinetic studies revealed k*obs*=2.5×10^{-1} h^{-1} and the linear formed DNA was easily to be digested. To the best of our knowledge, this is the first chitosan-based biopolymer featuring high hydrolytic activity toward both protein and DNA. In addition, CCMS catalyst was stable and easy to be recovered, and it could be reused at least four times with tiny loss of catalytic activity. Further studies concerning the cleavage site on DNA and modification of chitosan using other functional chemicals are in progress.

Acknowledgements

We are grateful for the financial sponsored from Natural Science Foundation of China (NO. 31371731).

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