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Ethylenediamine-Modified Amyloid Fibrils of Hen Lysozyme with Stronger Adsorption Capacity as Rapid Nano-biosorbents for Removal of Chromium(VI) Ions

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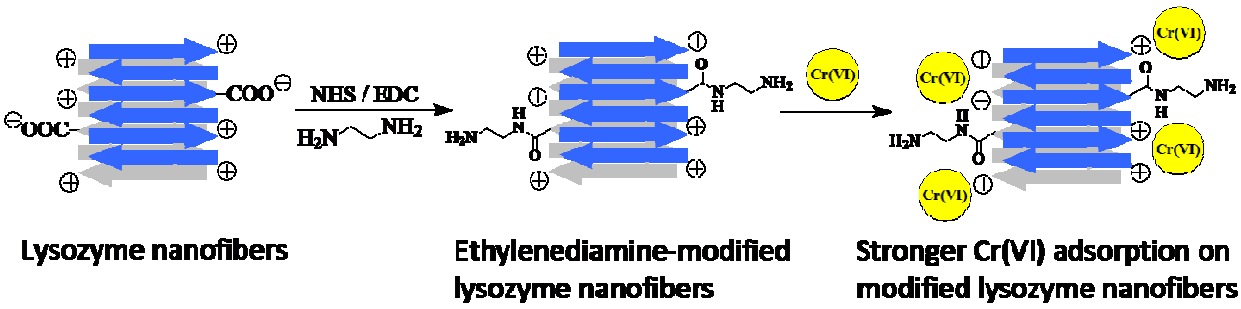

Amine-fortified amyloid fibrils of hen lysozyme as rapid nano-biosorbents with stronger adsorption capacity for removing toxic Cr(VI) ions:

Reducing the number of $-COO^-$ groups on lysozyme nanofibers through $-COO^-$/ethylenediamine conjugations enables the nanofibers to have higher net positive charges and offer a rapid and stronger charge-based adsorbing function towards Cr(VI).

Abstract

We report the development of ethylenediamine-modified amyloid fibrils (nanofibers) of hen lysozyme as rapid nano-biosorbents for removing toxic chromium(VI) ions in water. Ethylenediamine was covalently conjugated with the $–COO^-$ groups on positively charged lysozyme nanofibers through the formation of amide bonds using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as activating agents. Mass spectrometric results indicate that about 42% of lysozyme molecules in nanofibers are conjugated with ethylenediamine. The resulting ethylenediamine-modified lysozyme nanofibers, which have higher net positive charges as a result of the reduction in the number of $–COO^-$ groups through ethylenediamine conjugations, can adsorb Cr(VI) (existing as negatively charged chromate) rapidly in water and have stronger Cr(VI) adsorption capacity in acidic, neutral and alkaline media (pH 3.0–11.0) compared to unmodified lysozyme nanofibers. Results of the Langmuir isotherm model reveal that the adsorption sites on modified lysozyme nanofibers have higher affinity for Cr(VI) with respect to those on unmodified lysozyme nanofibers. Ethylenediamine-modified lysozyme nanofibers can maintain their Cr(VI) removal efficiency (~60%) after undergoing a series of desorption steps using NaCl as the desorbing agent. Ethylenediamine/$–COO^-$ conjugations can increase the adsorption capacity of lysozyme nanofibers for Cr(VI) in industrial wastewater (q_e = 0.68 mg g^{-1}) and river water (q_e = 1.90 mg g^{-1}) compared to unmodified lysozyme nanofibers (q_e = 0.40 and 1.44 mg g^{-1} for industrial wastewater and river water, respectively).

Introduction

Chromium(VI) pollution has attracted increasing worldwide attention because it can cause toxic, carcinogenic and mutagenic effects to humans and animals,[1] inhibitions of plant growth and changes in plant morphology.[2] Cr(VI) ions are usually produced by industrial activities (e.g. leather tanning, steel finishing and chrome plating) and can cause serious environmental pollution problems when they are disposed of as industrial wastes without proper treatments.[2] Previous environmental studies on Cr(VI) pollution around industrial tanning sites have shown that the natural water in the affected areas usually contained high Cr(VI) levels.[3, 4] Cr(VI) contamination in natural water and drinking water has been a major health concern because this toxic metal can bring about a serious health risk to the public. As such, the World Health Organization (WHO) and the European Commission of EU have set a maximum limit for total chromium [including less toxic Cr(III) and more toxic Cr(VI)] at 50 ppb level in drinking water,[5, 6] while the US Environmental Protection Agency (USEPA) has established a maximum contaminant level of 100 ppb for total chromium (based on the assumption that more toxic Cr(VI) constitutes the total Cr).[7] Given the serious health consequences caused by Cr(VI) to the public, extensive efforts have been made to develop different approaches to removing Cr(VI) in natural water/wastewater.

Chemical precipitation of Cr(VI) consists of a two-stage process involving the reduction of Cr(VI) to Cr(III) and precipitation,[8] resulting in the production of a large amount of sludge. Separation methods (e.g. membrane filtration, ion exchange and reverse osmosis), which usually require high pressure conditions and high operation costs, have been developed for removing Cr(VI) in water.[9] Adsorption methods provide an alternative way for efficient removal of Cr(VI) in water, and various bio- and synthetic materials have been developed as adsorbents for this application. Synthetic nanomaterials (e.g. polyaniline nanowires,[10] magnetic carbon fabrics[11] and porous ZnO nanoplates[12]), which usually need rather complicated synthetic methods and non-green/harsh conditions, have demonstrated their potential use as adsorbents in the removal of Cr(VI) ions. The use of industrial/agricultural wastes (e.g. sawdust,[13] fly ash,[14] clay,[15] Jatropha oil cake,[16] carrot waste,[17] and groundnut husk[18]) as well as natural materials/biomasses (e.g. activated carbon,[19] bacteria,[20] fungal,[21] chitosan,[22] and cellulose[23]) as adsorbents for Cr(VI) has also been studied in detail. While such materials have the advantage of low cost, their Cr(VI)-

removal processes usually require long adsorption time. Given the increasing need of removing toxic Cr(VI) in natural water for protecting public health (especially for developing countries with thriving industrial activities), it is highly desirable to further explore new materials with rapid Cr(VI)-adsorbing functions that can be prepared under green and mild aqueous conditions.

Amyloid fibrils represent a promising class of protein nanofibers in the development of nano-biosorbents for removal of Cr(VI) ions. Amyloid fibrils are the hallmark of amyloid diseases, which are human disorders involving the aggregation of proteins or peptide fragments into insoluble nanofibers (consisting of a common structural characteristic of cross-β structure) through the formation of intermolecular hydrogen bonds between the peptide units along individual polypeptide main chains under physiologically relevant conditions.[24] It is generally believed that amyloid fibril formation is a generic property of proteins and peptides (including those not related to amyloid diseases, such as hen lysozyme), because all proteins and peptides contain main chains that provide peptide units for the formation of intermolecular bonds during self-aggregations into fibrils (i.e. nanofibers).[24] Amyloid fibrils possess a number of advantageous properties that render themselves useful in the development of tailor-made nano-biomaterials for specific applications: (1) most amyloid fibrils can be easily prepared in aqueous solution under green and mild conditions in just one step; for example, amyloid fibrils of hen lysozyme can be rapidly produced in one step (~4–6 h) by incubation in phosphate buffer (pH 6.3) at 50°C with guanidine HCl as the chemical denaturant;[25] (2) amyloid fibrils, in general, have high structural stability and can maintain their robust structures in acidic and alkaline media;[26] and (3) amyloid fibrils usually carry charged amino acids, which are suitable for surface modifications (e.g. enzyme immobilizations and molecule couplings) into functional nanomaterials.[27, 28]

One of the useful functions of amyloid fibrils is that these protein nanofibers can act as rapid adsorbents towards ions and charged molecules. Our previous studies on colorimetric nanosensor, fluorescent biosensor and nano-biosorbent developments have shown that unmodified amyloid fibrils of hen lysozyme carry both net positive charges and hydrophobic regions along their structure, and these special structural properties confer rapid and efficient adsorbing functions onto lysozyme nanofibers.[26, 29, 30] In particular, the net positive charges of

unmodified lysozyme nanofibers play a very important role in the binding to charged molecules[26] and ions[30]. Thus, enhancing the net positive charges of unmodified lysozyme nanofibers is a crucial and effective way to further strengthen their adsorbing functions towards ionic species in various important applications (e.g. removal of chemical pollutants). Given that lysozyme nanofibers contain $–COO^-$ groups (e.g. Asp, Glu and the amino acid at the C-terminus), we reasoned that the charge-based adsorbing function of positively charged lysozyme nanofibers can be largely strengthened by reducing the number of $–COO^-$ groups along their fibrillar structure through the covalent conjugation of $–COO^-$ with an amine-containing molecule to form an amide. In this study, we demonstrate an effective approach to enhancing the net positive charges of lysozyme nanofibers by reducing the negative charges from their $–COO^-$ groups through the activation of $–COO^-$ with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)[31, 32] and subsequent conjugation with ethylenediamine (Scheme 1). Ethylenediamine was chosen because it not only neutralizes the negative charge of $–COO^-$, but also provides an additional "potential adsorption site" through the protonation of $–NH_2$ under aqueous conditions (Scheme 1). This strategic chemical modification not only increases the affinity of the adsorption sites (e.g. positively charged Lys and Arg) on lysozyme nanofibers for Cr(VI) ions (existing as negatively charged chromate), but also enables lysozyme nanofibers to adsorb Cr(VI) ions rapidly with stronger Cr(VI) adsorption capacity (compared to the unmodified form). More importantly, this study demonstrates a general strategy for enhancing the net charges of amyloid fibrils through the rational chemical modification of their oppositely charged amino acids to strengthen their charge-based adsorbing functions for different applications.

Experimental

Materials and chemicals

Chromium(VI) solution (1000 ppm) was purchased from Fisher Scientific. 1,5-Diphenylcarbazide (DPC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), sodium chloride, sodium nitrate, guanidine hydrochloride, and hen egg white lysozyme were purchased from Sigma-Aldrich (St Louis, MO). Ethylenediamine was purchased from BDH chemicals Ltd (Poole, England). Potassium phosphate (monobasic) was bought from USB Corporation. All chemicals used in this study were of analytical grade. Milli-Q deionized water was used in this study.

Preparation of amyloid fibrils of hen lysozyme

Hen egg white lysozyme (200 mg) was prepared in 3.0 M guanidine hydrochloride solution containing 20 mM potassium phosphate buffer (pH 6.3), followed by heating at 50°C for 4 h.[25] White precipitates (lysozyme nanofibers) were formed and then collected by centrifugation at 14,000 rpm for 2 min. The lysozyme nanofibers were washed with deionized water for 10 cycles and stored at 4°C.

Chemical modification of lysozyme nanofibers with ethylenediamine

Amyloid fibrils of hen lysozyme (10 mg) were mixed with 3.5 mL of 200 mM ethylenediamine solution (pH 6.0), followed by the addition of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 108 mg) and N-hydroxysuccinimide (NHS, 80 mg); the mole ratio of –COOH from lysozyme molecules/EDC/NHS/ethylenediamine was 1:100:100:1000. The mixture was adjusted to pH 6.0 and then stirred for 3 h. The ethylenediamine-modified lysozyme nanofibers were collected by centrifugation at 14,000 rpm for 4 min, washed with deionized water for 5 cycles to remove excess EDC, NHS and ethylenediamine and then stored at 4°C.

Transmission electron microscopic studies

Ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers were characterized by transmission electron microscopy (TEM). Each sample (suspended in 10 μL deionized water) was placed on a formvar-coated carbon grid, followed by negative staining with

2% (w/v) phosphotungstic acid (pH 7.0) for 30 s. The grid was further washed with deionized water and then air-dried. The morphologies of the modified and unmodified lysozyme nanofibers were observed using a JEM-2010 transmission electron microscope (JEOL).

Mass spectrometric studies

Electrospray ionization mass spectrometric (ESI-MS) measurements were performed using an Applied Biosystem Qstar Pulsar quadrupole time-of-flight mass spectrometer. Ethylenediamine-modified lysozyme nanofibers (0.20 mg) were dissolved in DMSO (20 μL) and then mixed with 100 μL of acetonitrile and 100 μL of deionized water. The sample was injected into the standard ESI source at a flow rate of 5 μL min^{-1} for analysis. The mass spectrometer was operated in the positive ion mode, and MS data were acquired in the m/z range of 600–3000 for detection of multiply charged ions. The raw multiply charged mass spectra obtained were deconvoluted with the Transform program (MassLynx 4.1, Waters) after data format conversion with the Databridge program (MassLynx 4.1, Waters). For comparison, the same ESI-MS experiment was conducted on unmodified lysozyme nanofibers.

Determination of adsorption capacities

A Cr(VI) sample [initial concentration (C_i) = 3.0 mg L^{-1}, volume = 1.5 mL, pH 7.0] was first prepared. Ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) (1.5 mg) were added to the Cr(VI) solution, and the whole mixture was incubated for 15 min at 20°C. The mixture was then centrifuged at 14,000 rpm for 4 min to pellet down the lysozyme nanofibers, and the solution fraction collected. The concentration of Cr(VI) in solution was determined by the spectrophotometric method as follows. A 200 μL portion of the solution was mixed with 200 μL of 2.5 mg mL^{-1} DPC solution (in acetone) and 660 μL of 0.2 M sulfuric acid. The concentration of Cr(VI) remaining in the solution (C_e) (after adsorption) was determined by measuring the absorbance at 543 nm using a Varian Cary 4000 UV-Visible spectrophotometer and referring to a calibration curve constructed by Cr(VI) standards (final concentrations = 0.19, 0.38, 0.57 and 0.76 mg L^{-1}). The adsorption capacity of ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) for Cr(VI) was calculated using the following equation:

$$q_e = V(C_i - C_e) / M \quad (1)$$

where q_e is the adsorption capacity of lysozyme nanofibers (mg g^{-1}), V is the volume of Cr(VI) solution (L), C_i is the initial Cr(VI) concentration before adsorption (mg L^{-1}), C_e is the equilibrium Cr(VI) concentration after adsorption (mg L^{-1}), and M is the mass of lysozyme nanofibers (g).

Studies of pH effects

A series of Cr(VI) samples (3.0 mg L^{-1}, volume = 1.5 mL) was prepared at pH 3.0, 4.0, 5.0 , 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0). Ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) (1.5 mg) were added to each Cr(VI) solution. The mixture was incubated at 20°C for 15 min, and the solution fraction collected by centrifugation at 14,000 rpm (to separate the nanofibers). The concentration of remaining Cr(VI) was determined by the spectrophotometric method, and the adsorption capacity (q_e) of the modified (and unmodified) lysozyme nanofibers were calculated using [eqn (1), in the section of determination of adsorption capacities].

Adsorption kinetic studies

A Cr(VI) sample (3.0 mg L^{-1}) mixed with ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) (1.0 mg mL^{-1}) was incubated at 20°C and pH 7.0. A 400 μL portion of the mixture was collected at different time intervals (t = 0, 1, 4, 7, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min) and then centrifuged at 14,000 rpm for 4 min to pellet down the nanofibers. The concentration of remaining Cr(VI) at each time interval and the corresponding adsorption capacity (q_t) were determined by the spectrophotometric method and [eqn (1)] {with q_e and C_e replaced by q_t (the adsorption capacity at the time interval t) and C_t (the concentration of Cr(VI) at the time interval t after adsorption), respectively}, as described in the section of determination of adsorption capacities.

Dosage effect studies

The effects of varying the amount of ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) on Cr(VI) adsorption were studied as follows. A series of

Cr(VI) solution [C_i = 3.0 mg L^{-1}, volume (V) = 1.5 mL, pH 7.0] was prepared and added with different amounts of modified (and unmodified) lysozyme nanofibers (0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 mg). The mixtures were incubated for 15 min at 20°C. The lysozyme nanofibers in each mixture were then pelleted down by centrifugation at 14,000 rpm to collect the solution fraction. The concentration of Cr(VI) remaining in the solution (C_e) was determined by the spectrophotometric method (in the section of determination of adsorption capacities). The Cr(VI) removal efficiency was then determined by the following equation:

$$(Q_i - Q_e) / Q_i \times 100\% \quad (2)$$

where Q_i is the amount of Cr(VI) before adsorption (= VC_i, in g) and Q_e is the amount of Cr(VI) at equilibrium after adsorption (= VC_e, in g).

Studies of ionic strength effect

A series of Cr(VI) samples (3.0 mg L^{-1}, volume = 1.5 mL, pH 7.0) with different concentrations of sodium nitrate (and sodium chloride) (concentration = 0, 1, 5, 10, 50, 100, 250 and 500 mM) was prepared. Ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) (1.5 mg) were added to each solution for incubation for 15 min at 20°C. The solution fraction was separated from the lysozyme nanofibers by centrifugation at 14,000 rpm. The concentration of remaining Cr(VI) in the solution and the adsorption capacity (q_e) of modified (and unmodified) lysozyme nanofibers were determined by the spectrophotometric method and [eqn (1)], respectively (in the section of determination of adsorption capacities).

Adsorption isotherm studies

A series of Cr(VI) samples with different initial Cr(VI) concentrations (C_i = 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mg L^{-1}, pH 7.0, volume = 1.5 mL) was prepared. Ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) (1.5 mg) were added to each Cr(VI) sample for adsorption at 20°C for 15 min. After adsorption, the mixture was centrifuged at 14,000 rpm to pellet down the lysozyme nanofibers. The equilibrium Cr(VI) concentration and the corresponding adsorption capacity (q_e) of the modified (and unmodified) lysozyme nanofibers were determined by the spectrophotometric method (in the section of determination of

adsorption capacities). The q_e and C_e values for the whole series of Cr(VI) samples were then fitted to the Langmuir isotherm model and the Freundlich isotherm model. The non-linear form of Langmuir isotherm model is shown below:

$$q_e = (q_{max} K_L C_e) / (1 + K_L C_e) \quad (3)$$

where q_e (mg g^{-1}) is the amount of adsorbate (Cr(VI)) adsorbed per gram of adsorbent (lysozyme nanofiber), q_{max} (mg g^{-1}) is the maximum adsorption capacity of the adsorbent, K_L (L mg^{-1}) is the Langmuir constant related to the affinity of the adsorption sites, and C_e (mg L^{-1}) is the concentration of adsorbate in solution at equilibrium after adsorption.[33-35]

The non-linear form of Freundlich isotherm model is shown below:

$$q_e = K_F C_e^{1/n} \quad (4)$$

where K_F is related to the adsorption capacity of the adsorbent (lysozyme nanofiber) ((mg g^{-1})(L $mg^{-1})^{1/n}$) and n reveals sorption favorability.[33-35]

Reusability studies

The ability of ethylenediamine-modified lysozyme nanofibers to adsorb Cr(VI) after undergoing desorptions was investigated as follows: (1) ethylenediamine-modified lysozyme nanofibers (1.5 mg) were first mixed with 1.5 mL of Cr(VI) solution [3.0 mg L^{-1}, pH 7.0] for adsorption for 10 min; (2) the Cr(VI)-adsorbed modified nanofibers were separated from the solution fraction by centrifugation at 14,000 rpm, followed by desorption with 1.5 mL of NaCl solution (500 mM) for 10 min; and (3) the desorbed modified nanofibers were then collected by centrifugation at 14,000 rpm and mixed with 1.5 mL of Cr(VI) solution [3.0 mg L^{-1}, pH 7.0] for adsorption for 10 min. The concentration of Cr(VI) in the solution after adsorption [step (3)] was determined by the spectrophotometric method (in the section of determination of adsorption capacities). The Cr(VI) removal efficiency of the modified lysozyme nanofibers [step (3)] was determined using [eqn (2)] (in the section of dosage effects of modified and unmodified lysozyme nanofibers). The adsorption/desorption cycle [step (1)–(3)] was performed for 4 cycles in total. The desorbing

effect of another desorbing agent [4.0 mL of 500 mM NaCl and then 4.0 mL of deionized water] was also studied with similar procedures.

Application studies

Raw water from the Dongjiang River and local industrial wastewater were used as real water samples in our application studies. To compare the overall ion concentrations in the river water and industrial wastewater samples, the conductance of each sample (50.0 mL, with gentle stirring) was measured using a RS Pro Conductivity Meter. The river water and industrial wastewater samples (1.5 mL) were each added with Cr(VI) (final concentration = 3.0 mg L^{-1}). Ethylenediamine-modified lysozyme nanofibers (and unmodified lysozyme nanofibers) (1.5 mg) were added to each sample, followed by adsorption at 20°C for 15 min. The solution fraction was then collected by centrifugation at 14,000 rpm, and the equilibrium Cr(VI) concentration was determined by the spectrophotometric method (in the section of determination of adsorption capacities). The Cr(VI) removal efficiency was then calculated using [eqn (2)] (in the section of dosage effects of modified and unmodified lysozyme nanofibers).

Data treatment

Data with an error bar appearing in figures were obtained by triplicate measurements.

Results and Discussion

Construction and characterization of ethylenediamine-modified lysozyme nanofibers

Hen lysozyme consists of 17 positively charged (Lys and Arg) and 9 negatively charged (Glu and Asp) amino acids and maintains positively charged under neutral and acidic conditions (pI = 9.3). Upon aggregation to form amyloid fibrils, hen lysozyme nanofibers will contain both positively and negatively charged amino acids, but maintain positively charged when pH<10.8.[30] A possible strategy for increasing the net positive charges of lysozyme nanofibers is to reduce the negative charges from their $–COO^-$ groups. To this end, the $–COO^-$ groups can be activated by NHS and EDC to form amine-reactive NHS esters, which are subsequently conjugated with ethylenediamine through the formation of amide bonds (Scheme 1). The use of NHS and EDC in the coupling of a carboxyl group with an amino group through the formation of an amide bond has been applied in proteins for specific purposes (e.g. immobilization on solid surfaces).[31, 32] Ethylenediamine conjugations not only neutralize the negative charges from the $–COO^-$ groups along lysozyme nanofibers, but also produce more $–NH_2$ groups as "potential adsorption sites" (through protonation in an aqueous medium); the pK_a values of the conjugate acid of ethylenediamine ($^+H_3NCH_2CH_2NH_3^+$) are 6.848 and 9.928.[36]

In this study, we conjugated the $–COO^-$ groups of lysozyme nanofibers with ethylenediamine using NHS and EDC as the activating agents. Briefly, lysozyme nanofibers were mixed with excess NHS, EDC and ethylenediamine (relative to the total number of mole of $–COO^-$ from lysozyme molecules), and the ethylenediamine-conjugation efficiency on the nanofibers was analyzed by electrospray ionization mass spectrometry (ESI-MS) after dissociating the modified nanofibers into monomeric lysozyme molecules with DMSO. For comparison, the same MS experiment was performed with unmodified lysozyme nanofibers. Fig. 1(a) shows the ESI mass spectrum of unmodified lysozyme nanofibers after dissociation by DMSO; the mass peak (M_1, m = 14304 Da) corresponds to the molecular mass of hen lysozyme (MW = 14304–14308 Da),[37] indicating that DMSO can dissociate solid lysozyme nanofibers into soluble lysozyme molecules. For the ESI-MS analysis of ethylenediamine-modified lysozyme nanofibers, in addition to the mass peak of unmodified lysozyme (M_2, m = 14306 Da), a new mass peak with an additional mass value of 42 Da (M_3, m = 14348 Da) also appears in the mass spectrum [Fig. 1(b)]. This new mass peak (M_3, m = 14348 Da) is consistent with the molecular

mass of ethylenediamine-modified lysozyme resulting from the conjugation of a carboxyl group in lysozyme (m = 14306 Da) with the $–NH_2$ of ethylenediamine (MW = 60.1 Da)[38] accompanied by the elimination of a H_2O water (MW = 18.0 Da).[38] This observation indicates that ethylenediamine can be covalently linked to the lysozyme molecules in nanofibers through the activation of NHS and EDC on $–COO^-$ groups. Analysis of the relative peak intensities of unmodified lysozyme (M_2, m = 14306 Da) and modified lysozyme (M_3, m = 14348 Da) revealed that 43% of lysozyme are modified with ethylenediamine [Fig. 1(b)]. Similar conjugation efficiency (41%) was also observed in the analysis of the relative peak intensities of the [unmodified lysozyme + H_2O] complex (M_{2W}, m = 14323 Da) and the [modified lysozyme + H_2O] complex (M_{3W}, m = 14364 Da) [Fig. 1(b)]. The formation of protein-water complexes is a possible phenomenon in electrospray ionization (ESI) processes, and this has also been observed with other proteins.[39] The MS findings reveal that part of the $–COO^-$ groups of lysozyme nanofibers are well exposed to the external aqueous environment for NHS/EDC activations and ethylenediamine conjugations.

The morphology of ethylenediamine-modified lysozyme nanofibers was analyzed by transmission electron microscopy (TEM) and compared with that of unmodified lysozyme nanofibers. In both cases, modified and unmodified lysozyme nanofibers show long fibrillar structures (Fig. 2). These is no significant difference in width between the modified and unmodified forms (width = 10–20 nm, Fig. 2), indicating that the overall morphology of lysozyme nanofibers remains similar before and after ethylenediamine conjugations.

Stronger adsorption capacity for Cr(VI)

Cr(VI) exists mainly as $HCrO_4^-$ and CrO_4^{2-} in aqueous solution under acidic and neutral/alkaline conditions, respectively.[40, 41] Cr(VI) can be probed by the Cr(VI)-specific colorimetric agent diphenylcarbazide (DPC) that can reduce Cr(VI) to Cr(III) to form a pink complex with its oxidized form (λ_{abs} = 543 nm).[42] To verify that ethylenediamine-modified lysozyme nanofibers can adsorb Cr(VI), we examined the absorbance of Cr(VI) solution at 543 nm with the aid of acidified DPC before and after adsorption by modified lysozyme nanofibers. As shown in Fig. 3, the light absorption peak at 543 nm shows lower absorbance after adsorption by modified lysozyme nanofibers compared to that before adsorption, indicating that

ethylenediamine-modified lysozyme nanofibers can adsorb Cr(VI) onto their structures. The effect of increasing the amount of ethylenediamine-modified lysozyme nanofibers on Cr(VI) adsorption was further investigated. The adsorption capacity (q_e) of ethylenediamine-modified lysozyme nanofibers for Cr(VI) increases when using more modified lysozyme nanofibers as adsorbents (0.2–1.0 mg mL^{-1}) and then reaches maximum afterwards (1.0–1.4 mg mL^{-1}) (Fig. 4). The Cr(VI) removal efficiency also increases with the concentration of modified lysozyme nanofibers (0.2–1.4 mg mL^{-1}) (Fig. S1 in the ESI). Similar adsorption experiments were conducted on unmodified lysozyme nanofibers; the adsorption capacity (q_e) of unmodified lysozyme nanofibers also increases with the concentration of nanofibers (0.2–1.4 mg mL^{-1}), but their q_e values are, in general, lower than those of ethylenediamine-modified lysozyme nanofibers under similar experimental conditions (Fig. 4). The Cr(VI) removal efficiency of unmodified lysozyme nanofibers also increases with their concentration (0.2–1.4 mg mL^{-1}), but is lower than that of modified lysozyme nanofibers (Fig. S1 in the ESI). These observations indicate that the ability of lysozyme nanofibers to adsorb Cr(VI) can be enhanced through the conjugation of their $–COO^-$ groups with ethylenediamine.

We then studied the Cr(VI) adsorption of ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers at different pH values. Fig. 5 shows the adsorption capacities (q_e) of modified and unmodified lysozyme nanofibers over the pH range of 3.0–11.0. In both cases, Cr(VI) adsorption is weaker in acidic (pH 3.0–6.0) and alkaline (pH 8.0–11.0) media, but reaches maximum in the neutral medium (pH 7.0). Our previous study has shown that lysozyme nanofibers are positively charged when pH<10.8, but become negatively charged when pH>10.8.[30] At pH 7.0, Cr(VI) exists mainly as CrO_4^{2-} and interacts more strongly with positively charged lysozyme nanofibers through electrostatic attraction.[30] In acidic media, Cr(VI) exists mainly as $HCrO_4^-$ and therefore experiences weaker electrostatic attraction from positively charged lysozyme nanofibers.[30] In alkaline media, lysozyme nanofibers become less positively charged and hence exert weaker electrostatic attraction to Cr(VI) (existing mainly as CrO_4^{2-}).[30] The fact that the adsorption of Cr(VI) onto lysozyme nanofibers (modified and unmodified forms) is based on electrostatic interaction is further supported by the observations of decreasing adsorption capacities (q_e) of the modified and unmodified forms in the presence of increasing [NaCl] and [$NaNO_3$] (Fig. S2 and S3 in the ESI); such ions can neutralize the negative charge(s)

of $HCrO_4^-/CrO_4^{2-}$ as well as the positive charges of modified and unmodified lysozyme nanofibers and therefore weaken the electrostatic interaction between the nanofibers and Cr(VI).

It is interesting to note that ethylenediamine-modified lysozyme nanofibers, in general, have higher adsorption capacities (q_e) for Cr(VI) over the wide pH range (3.0–11.0) compared to unmodified lysozyme nanofibers, presumably due to the stronger electrostatic attraction of the modified form arising from the increase in net positive charge as a result of the reduction in negative charge caused by $–COO^-$/ethylenediamine conjugations and the possible increase in adsorption site ($–NH_3^+$) from attached ethylenediamine (Fig. 5). These factors are very likely to make ethylenediamine-modified lysozyme nanofibers more resistant than unmodified lysozyme nanofibers in the competitions with Cl^- and NO_3^- for Cr(VI), as revealed by the higher adsorption capacities (q_e) of modified lysozyme nanofibers within 100 mM Cl^- and NO_3^- (Fig. S2 and S3 in the ESI). These interesting observations highlight the fact that the net positive charges of lysozyme nanofibers can be enhanced to improve their adsorption capacity for Cr(VI) through rational chemical modifications.

Rapid Cr(VI) adsorption

We then studied the kinetics of Cr(VI) adsorption by ethylenediamine-modified lysozyme nanofibers. Briefly, Cr(VI) solution was collected at different time intervals, and the amount of Cr(VI) adsorbed by modified lysozyme nanofibers (q_t) was determined by the spectrophotometric method (described in Experimental). For comparison, a similar kinetic study was also conducted on unmodified lysozyme nanofibers. Fig. 6 shows the adsorption kinetic profiles of modified and unmodified lysozyme nanofibers with Cr(VI). Both modified and unmodified lysozyme nanofibers can adsorb Cr(VI) very rapidly; modified lysozyme nanofibers and unmodified lysozyme nanofibers reach their adsorption equilibrium at t = 1 min [q_t = 2.5 and 1.8 mg g^{-1} for the modified and unmodified forms (respectively), Fig. 6; t_{eqm} = 5 min if including the centrifugation time (4 min)]. This fast adsorbing function highlights the potential use of ethylenediamine-modified lysozyme nanofibers as rapid nano-biosorbents for immediate removal of Cr(VI) (e.g. accidental contamination of natural water), compared to other biomasses with relatively long adsorption equilibrium time (e.g. dead *Bacillus cereus*, inactivated fungal *Termitomyces clypeatus*, bio-chars from pyrolysis of *P. terebinthus* L. and alumina, acid-treated

macroalgae *Sargassum muticum*, beal fruit (*Aegle marmelos correa*) shell activated carbon, silver impregnation groundnut husk, sunflower stem, and autoclaved *Aspergillus flavus*, Table S1 in the ESI). The rapid adsorbing function of ethylenediamine-modified lysozyme nanofibers towards Cr(VI) ions can be attributed to their nano-sized structure carrying positive charges on their surface.

Adsorption isotherm studies

The adsorption properties of ethylenediamine-modified lysozyme nanofibers with Cr(VI) were studied with the Langmuir isotherm model and the Freundlich isotherm model. The adsorption capacity (q_e) at each equilibrium Cr(VI) concentration (C_e) was determined, and the data set of q_e against C_e was fitted to the Langmuir isotherm model [eqn (3)] and the Freundlich isotherm model [eqn (4)]. For comparison, similar experiments and data fittings were also performed with unmodified lysozyme nanofibers. The fittings of the two adsorption isotherms for ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers were shown in Fig. S4 and S5 in the ESI (respectively), and the results of the adsorption isotherm studies were shown in Table S2 in the ESI. The results indicate that both modified and unmodified lysozyme nanofibers follow the Langmuir isotherm model in Cr(VI) adsorption processes in which Cr(VI) ions bind to their adsorption sites in a monolayer.

It is interesting to compare the Langmuir constants K_L (related to the affinity of the adsorption sites on adsorbents for adsorbates) between ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers. The Langmuir constant of ethylenediamine-modified lysozyme nanofibers (K_L = 3.39 L mg^{-1}) is larger than that of unmodified lysozyme nanofibers (K_L = 0.86 L mg^{-1}), implying that the adsorption sites of modified lysozyme nanofibers have higher affinity for Cr(VI) compared to those of unmodified lysozyme nanofibers (Table S2 in the ESI). This interesting observation can be attributed to the increase in net positive charges of modified lysozyme nanofibers as a result of the reduction in their negative charges arising from $–COO^-$/ethylenediamine conjugations. Thus, the adsorption sites (e.g. positively charged Lys and Arg) on ethylenediamine-modified lysozyme nanofibers are likely to exert stronger electrostatic attraction towards Cr(VI), which exists as negatively charged chromate. This observation highlights the useful strategy of enhancing the net charges of

amyloid fibrils through rational chemical modifications in strengthening their charge-based adsorbing functions.

Reusability studies

We then investigated the ability of ethylenediamine-modified lysozyme nanofibers to adsorb Cr(VI) in water after undergoing a series of desorptions. Briefly, ethylenediamine-modified lysozyme nanofibers underwent adsorption with Cr(VI), desorption with 500 mM NaCl (1.5 mL) as the desorbing agent, and then adsorption with Cr(VI). Afterwards, the Cr(VI) removal efficiency of the modified lysozyme nanofibers was determined by the spectrophotometric method (see the experimental details for reusability studies). The initial Cr(VI) removal efficiency of modified lysozyme nanofibers (before the desorption step in cycle 1) is about 80%. After undergoing 4 cycles of desorption, the modified lysozyme nanofibers still have relatively constant Cr(VI) removal efficiency (64–60%) (Fig. 7). When using a larger volume of desorbing agent [500 mM NaCl (4.0 mL) and subsequently deionized H_2O (4.0 mL)], the Cr(VI) removal efficiency of modified lysozyme nanofibers maintains 67–52%, with a gradual decrease in Cr(VI) removal efficiency after undergoing each desorption cycle due to loss of the nanofibers (Fig. S6 in the ESI). In both desorption cases, there is a decrease in Cr(VI) removal efficiency after desorption in cycle 1 (from 80% to 64% with NaCl as the desorbing agent; from 80% to 67% with NaCl plus deionized H_2O as the desorbing agent), implying that part of the positively charged adsorption sites in the modified lysozyme nanofibers were strongly bound to Cl^- as a result of strong electrostatic interaction (and perhaps together with other interactions, such as hydrogen bonding). Nevertheless, the results indicate that ethylenediamine-modified lysozyme nanofibers can act as reusable nano-biosorbents for removing Cr(VI) ions using NaCl as the cost-effective desorbing agent.

Application studies

We then conducted a comparative study on the Cr(VI)-adsorbing functions of ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers in real samples. Briefly, samples of local industrial wastewater and river water were spiked with Cr(VI) (final concentration = 3.0 mg L^{-1}) and subsequently mixed with modified and unmodified lysozyme nanofibers. The adsorption capacities and removal efficiencies of both forms were then

determined. As shown in Fig. 8, ethylenediamine-modified lysozyme nanofibers have higher adsorption capacities (q_e) for Cr(VI) in both industrial wastewater and river water compared to unmodified lysozyme nanofibers; the q_e values for modified lysozyme nanofibers are 0.68 and 1.90 mg g^{-1} in the industrial wastewater and river water (respectively), whereas unmodified lysozyme nanofibers have lower q_e values in both cases (q_e = 0.40 and 1.44 mg g^{-1} for the industrial wastewater and river water, respectively). The observations that both modified and unmodified lysozyme nanofibers have lower adsorption capacities for Cr(VI) in the industrial wastewater compared to the river water are consistent with the fact that the industrial wastewater has much higher ion concentrations [conductance = 3013(±31) μS] relative to the river water [conductance = 106(±1) μS]. After modifying lysozyme nanofibers with ethylenediamine, the Cr(VI) removal efficiency increases from 12% to 22% and from 47% to 63% in the industrial wastewater and river water, respectively (Fig. S7 in the ESI). These results highlight the potential of amyloid fibrils to be further developed into stronger nano-biosorbents through rational chemical modifications.

Conclusions

We have successfully constructed ethylenediamine-modified amyloid fibrils of hen lysozyme with stronger adsorption capacity as rapid nano-biosorbents for removing Cr(VI) ions in water. The net charges on amyloid fibrils are very useful for rapid adsorption of oppositely charged ions/molecules.[26, 30] This study further underlines the "tunable nature" of the net charges of amyloid fibrils by adjusting the number of oppositely charged groups in amino acids through chemical modifications for enhancing their charge-based adsorbing functions; as demonstrated in this study, the net positive charges of lysozyme nanofibers can be enhanced by reducing the number of $-COO^-$ groups through chemical modifications with ethylenediamine, resulting in higher affinity of their adsorption sites for Cr(VI) and stronger Cr(VI) adsorption capacity. With their rapid charge-based adsorbing functions and nano-sized structures, amyloid fibrils can play an important role in the development of high-performance adsorbents for rapid and efficient removal of charged pollutants (e.g. heavy metal ions). In this regard, it would be interesting to explore the possibility of constructing hybrid biosorbents through the incorporation of amyloid fibrils (with rapid charge-based adsorbing functions) into other biomasses (with high adsorption capacities) and study their combined effects on pollutant adsorptions.

Acknowledgements

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Electronic supplementary information (ESI) available: ESI data of Cr(VI) removal efficiencies at different concentrations of modified and unmodified lysozyme nanofibers, studies of ionic strength effect, Langmuir and Freundlich isotherm studies, and Cr(VI) removal efficiencies of modified and unmodified lysozyme nanofibers in industrial wastewater and river water. See DOI: XXXXXX

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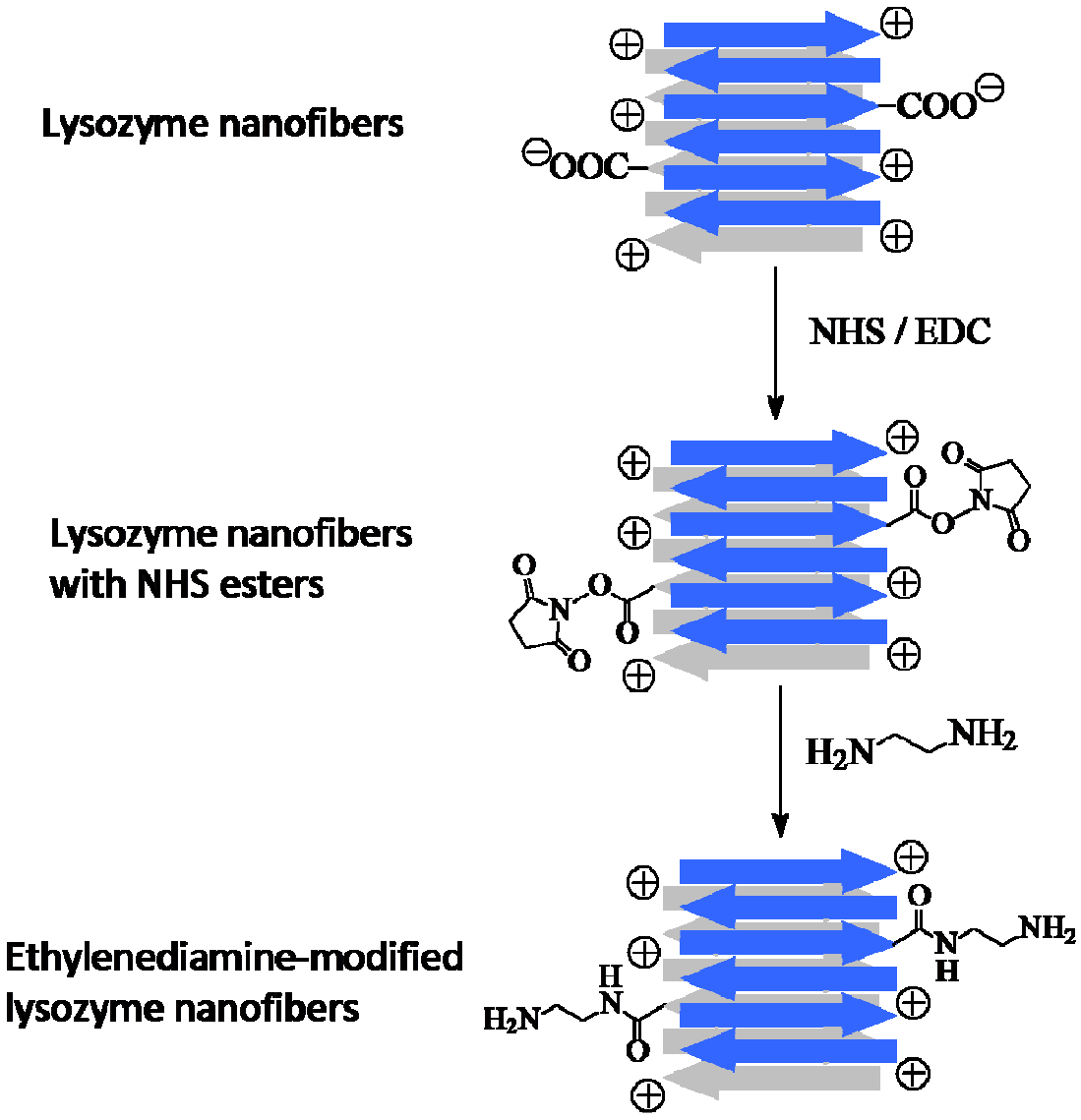
Scheme and Figures

Scheme 1. Chemical modification of amyloid fibrils of hen lysozyme with ethylenediamine.



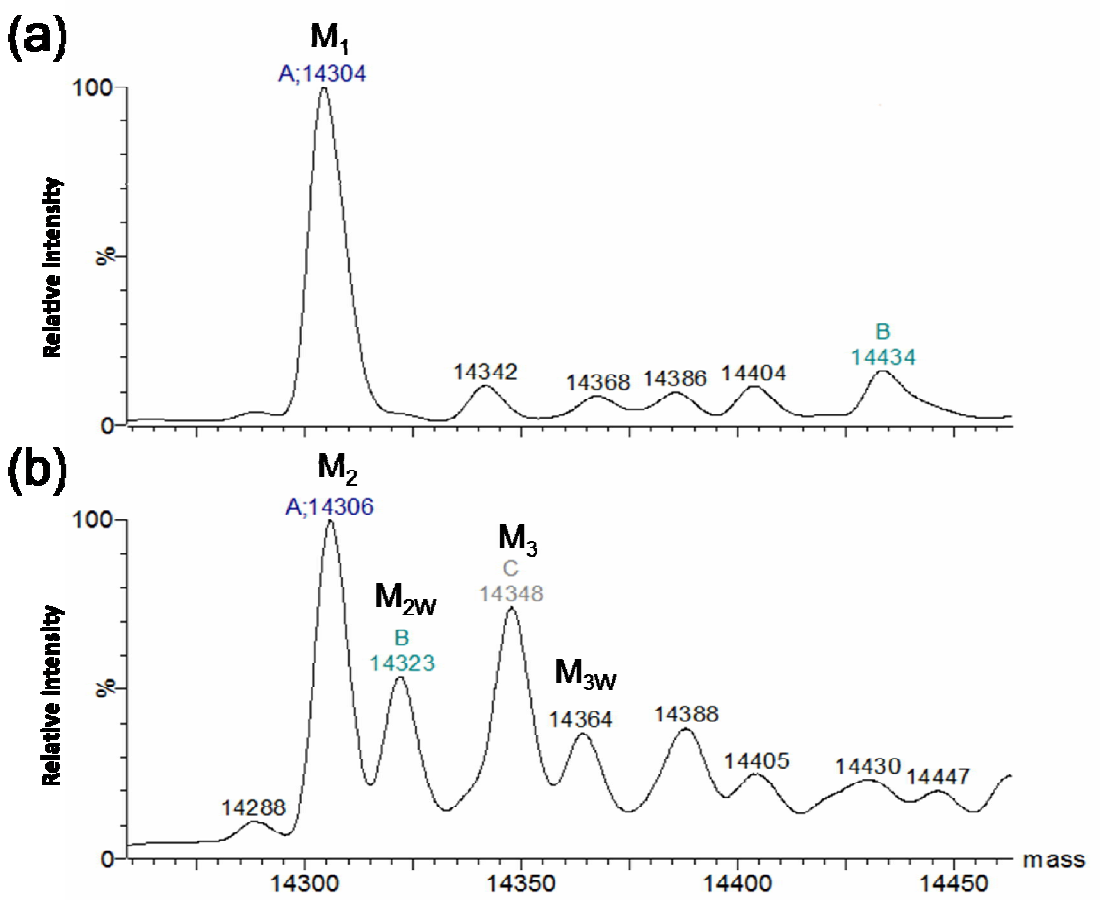


Fig. 1 ESI mass spectra of lysozyme molecules dissociated from unmodified lysozyme nanofibers and ethylenediamine-modified lysozyme nanofibers. **(a)** Mass spectrum of lysozyme molecules dissociated from unmodified lysozyme nanofibers; **(b)** mass spectrum of lysozyme molecules dissociated from ethylenediamine-modified lysozyme nanofibers. DMSO (20 μL) was used to dissociate the unmodified and modified lysozyme nanofibers (0.20 mg) into individual lysozyme molecules, followed by the addition of CH_3CN (100 μL) and deionized water (100 μL) to the lysozyme samples prior to ESI-MS analyses.

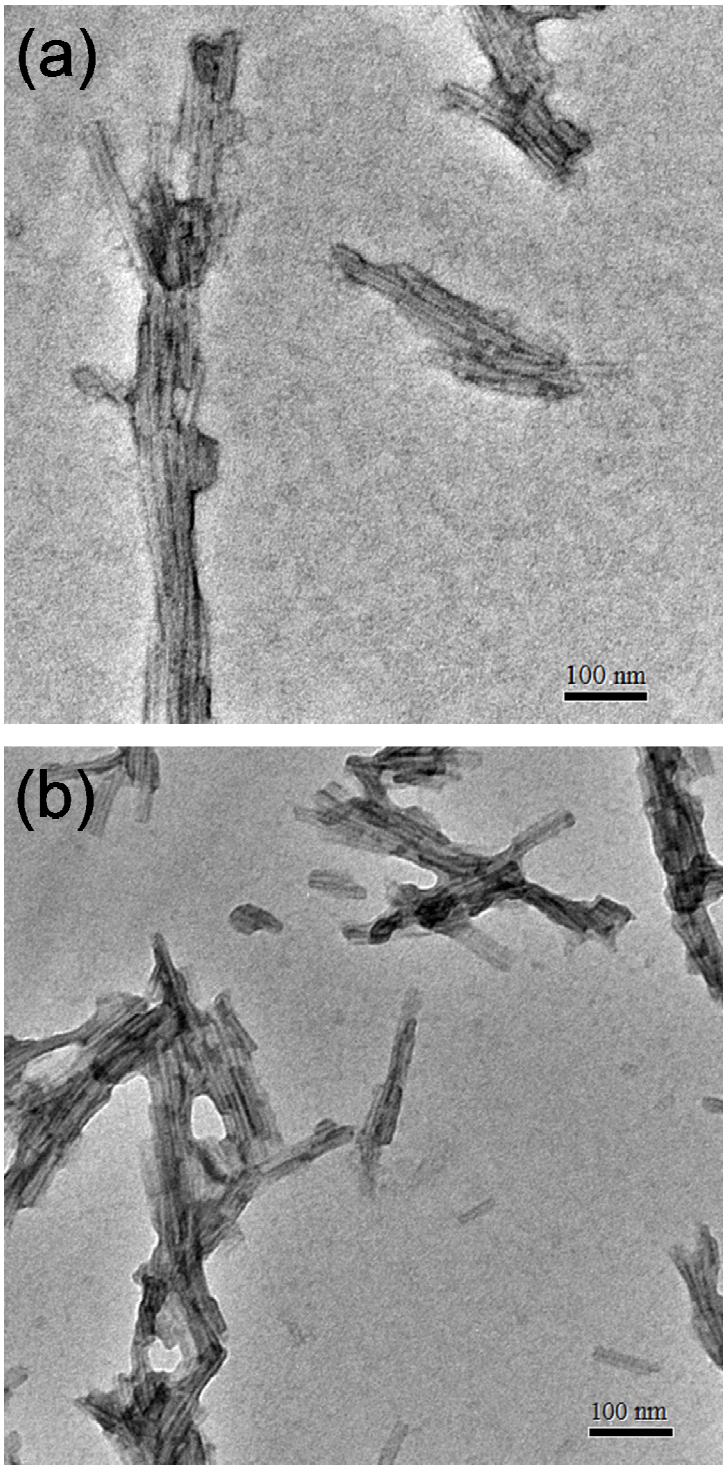


Fig. 2 Transmission electron microscopic image of unmodified lysozyme nanofibers and ethylenediamine-modified lysozyme nanofibers. **(a)** Unmodified lysozyme nanofibers; **(b)** ethylenediamine-modified lysozyme nanofibers. Scale = 100 nm.

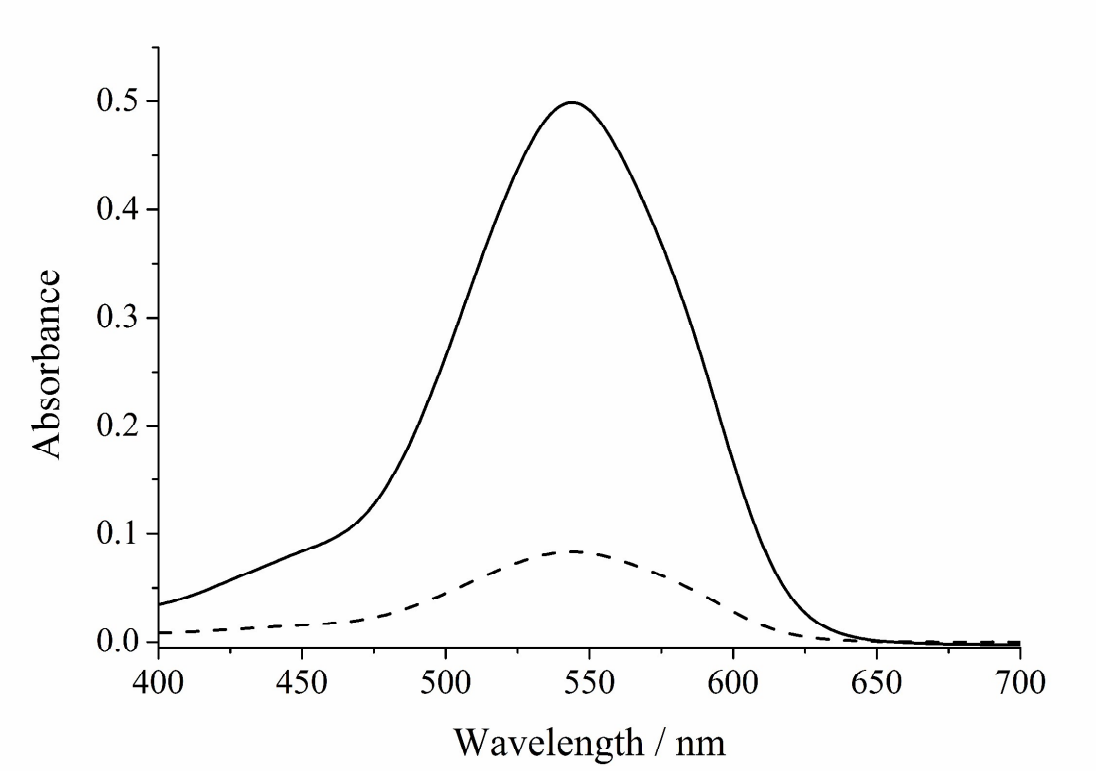


Fig. 3 UV-Visible absorption spectra of Cr(VI) solution with acidified diphenylcarbazide (DPC) before and after adsorption by ethylenediamine-modified lysozyme nanofibers. Upper curve (—): before adsorption by modified lysozyme nanofibers; lower curve (– –): after adsorption by modified lysozyme nanofibers. The concentrations of Cr(VI) and modified lysozyme nanofibers were 3.0 mg L^{-1} and 1.0 mg mL^{-1}, respectively (pH 7.0). The modified lysozyme nanofibers were removed from the Cr(VI) solution by centrifugation after adsorption.

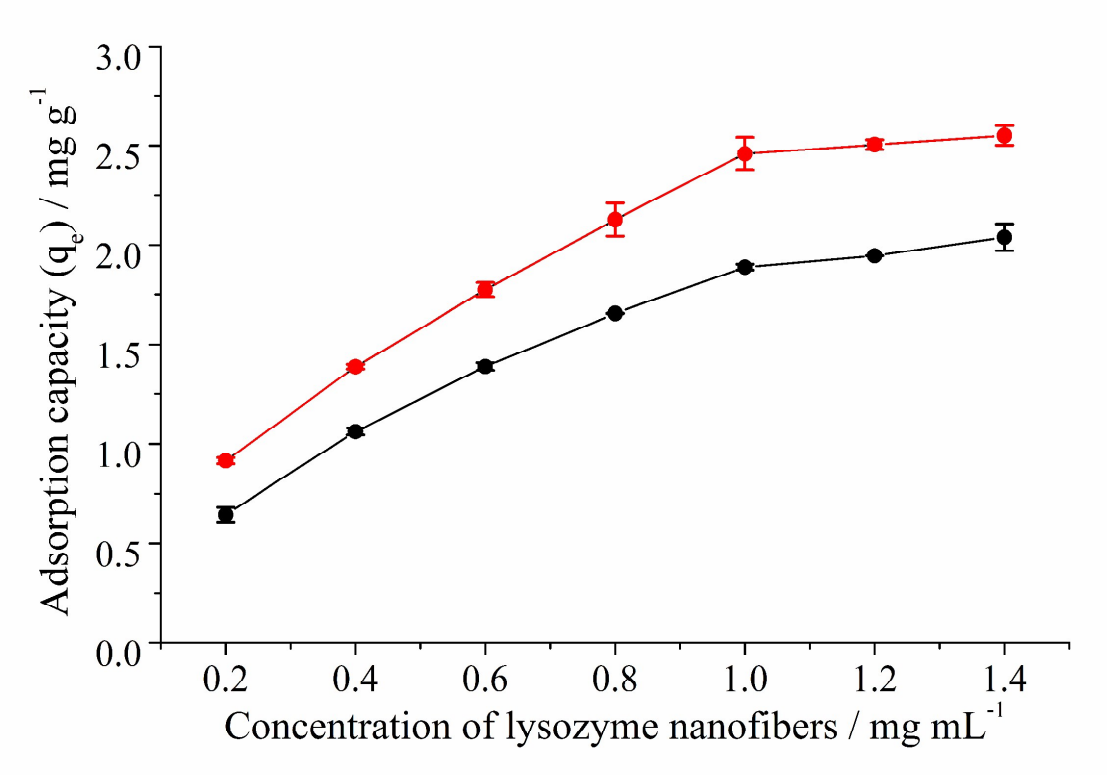


Fig. 4 Profiles of Cr(VI) adsorption capacities against different concentrations of ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers. Red curve: Cr(VI) adsorption by modified lysozyme nanofibers; black curve: Cr(VI) adsorption by unmodified lysozyme nanofibers. Initial Cr(VI) concentration = 3.0 mg L^{-1}; pH = 7.0. The concentration of Cr(VI) after adsorption was determined using acidified diphenylcarbazide (DPC) as the probing agent.

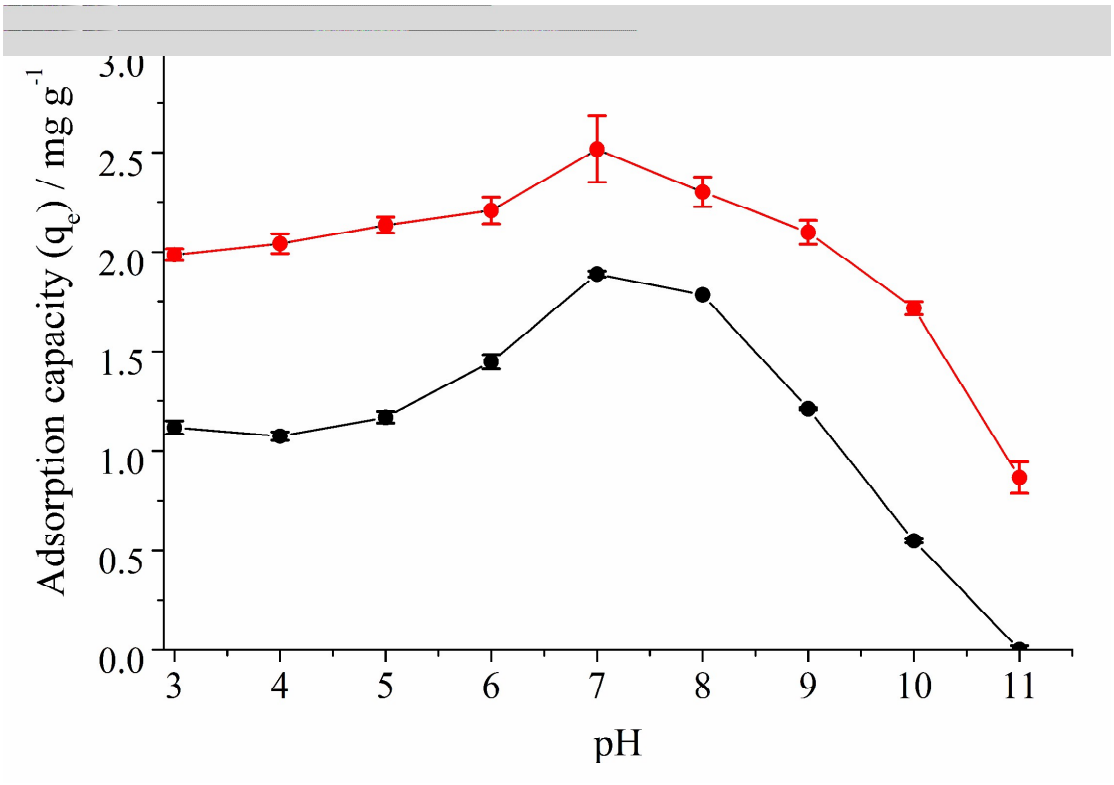


Fig. 5 Profiles of Cr(VI) adsorption capacities at different pH values for ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers. Red curve: Cr(VI) adsorption by modified lysozyme nanofibers; black curve: Cr(VI) adsorption by unmodified lysozyme nanofibers. The initial concentration of Cr(VI) was 3.0 mg L^{-1}. The concentration of Cr(VI) after adsorption was determined using acidified diphenylcarbazide (DPC) as the probing agent. Concentration of modified and unmodified lysozyme nanofibers = 1.0 mg mL^{-1}.

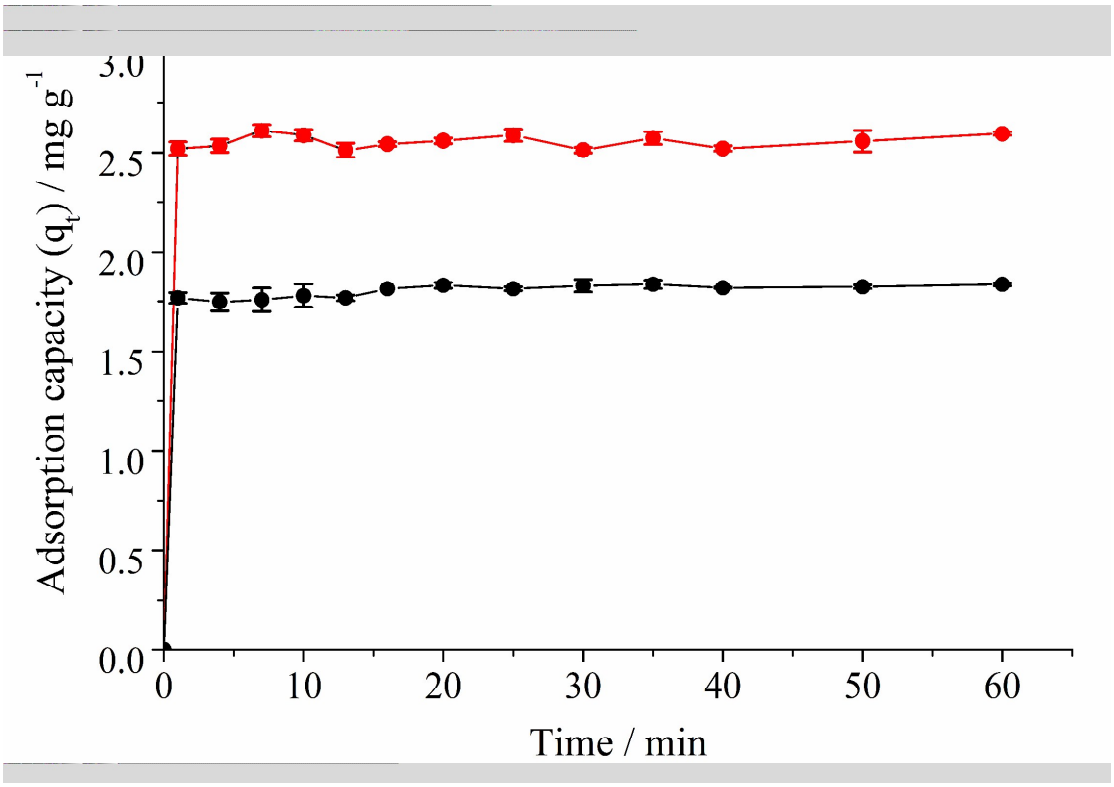


Fig. 6 Adsorption kinetic profiles of ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers with Cr(VI). Red curve: Cr(VI) adsorption by modified lysozyme nanofibers; black curve: Cr(VI) adsorption by unmodified lysozyme nanofibers. The concentrations of Cr(VI) and lysozyme nanofibers (modified and unmodified forms) were 3.0 mg L^{-1} and 1.0 mg mL^{-1} (respectively); pH = 7.0.

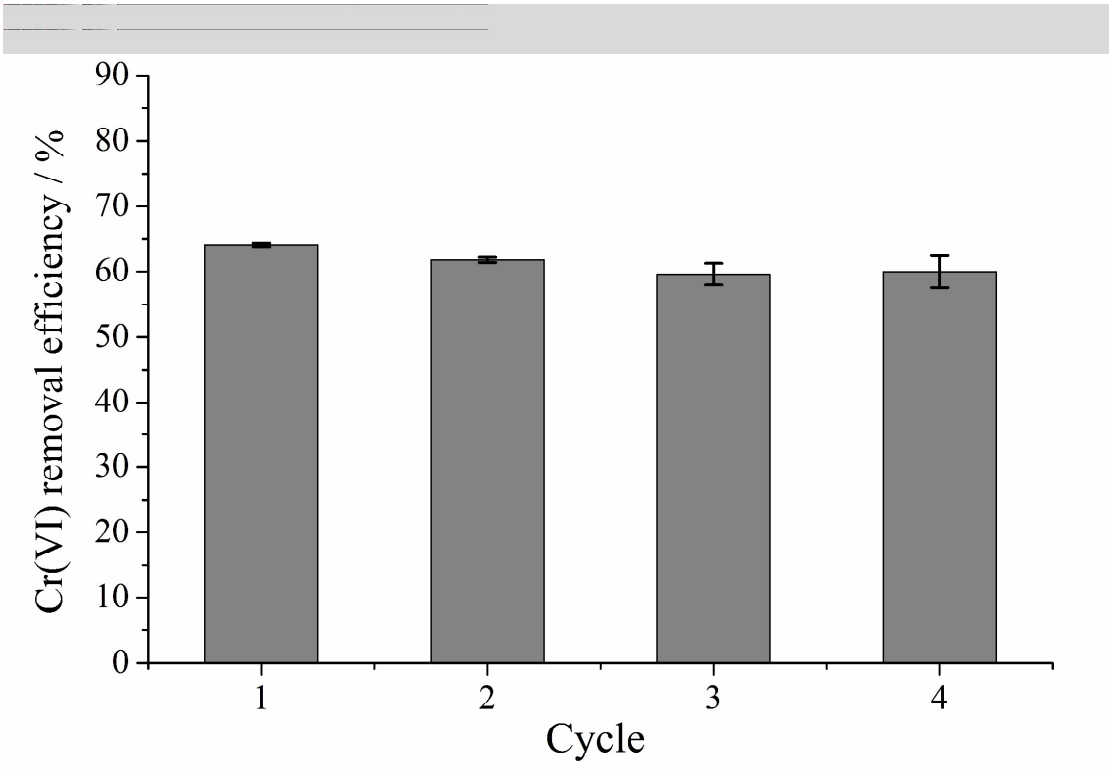


Fig. 7 Reusability study of ethylenediamine-modified lysozyme nanofibers in the removal of Cr(VI) ions. In each cycle, the modified lysozyme nanofibers underwent Cr(VI) adsorption and then Cr(VI) desorption using 500 mM NaCl as the desorbing agent. The Cr(VI) removal efficiency of the modified lysozyme nanofibers was then determined after adsorbing Cr(VI) ions. The concentrations of Cr(VI) and modified lysozyme nanofibers were 3.0 mg L^{-1} and 1.0 mg mL^{-1} (respectively); pH = 7.0.

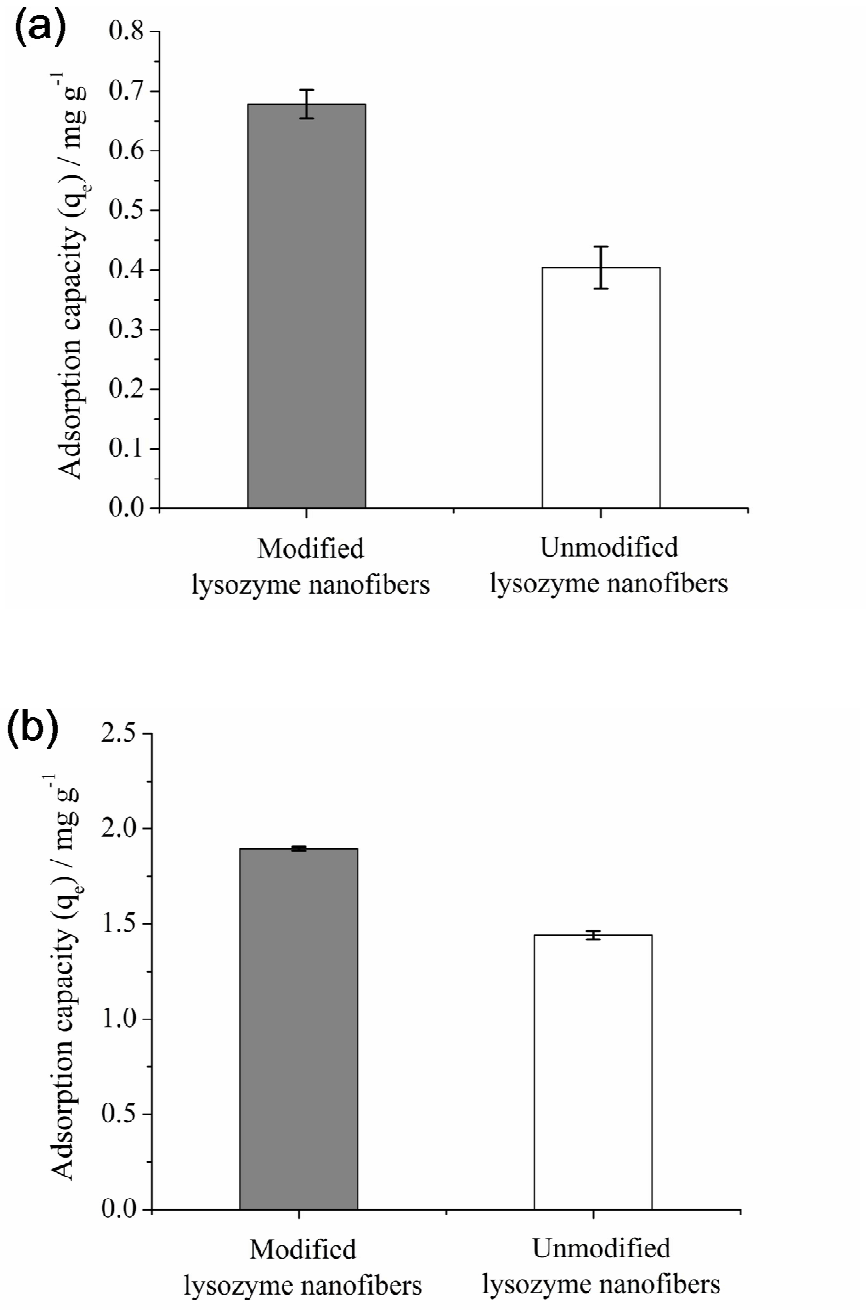


Fig. 8 Adsorption capacities of ethylenediamine-modified lysozyme nanofibers and unmodified lysozyme nanofibers for Cr(VI) in industrial wastewater and river water. **(a)** Cr(VI) adsorption capacities of modified and unmodified lysozyme nanofibers determined in industrial wastewater; **(b)** Cr(VI) adsorption capacities of modified and unmodified lysozyme nanofibers determined in river water. The concentrations of Cr(VI) and lysozyme nanofibers (modified and unmodified forms) were 3.0 mg L^{-1} and 1.0 mg mL^{-1}, respectively.