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Journal Name

ARTICLE

Water Soluble EDTA-linked Chitosan as a Zwitterionic Flocculant for pH Sensitive Removal of Cu(II) ion

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A linear and non-crosslinked polymer, EDTA-linked chitosan (ED-ch), was synthesized by *N*-acylation of chitosan with EDTA (ethylenediaminetetraacetic acid) monoanhydride under from acidic to slightly basic aqueous conditions. The degree of substitution (D.S.) of EDTA residues in the product was changeable by the stoichiometry of the acylation reagent, and the structure was confirmed by elemental analysis, FT-IR, ^{1}H and ^{13}C NMR spectroscopy. The newly introduced functional group in ED-ch provided properties as a strong chelating reagent and an amphoteric polyelectrolyte. It was found that ED-ch had good water solubility in both acidic and basic region and precipitated in the narrow pH region. Applicability of ED-ch to removal of heavy metal ions by flocculation was proven by a model experiment using 10 ppm Cu(II) solution. Simple procedure, mixing ED-ch with D.S. 41% and 70%, and subsequent pH adjustment (4.5 and 2.5) and centrifugal separation of the flocs formed, was shown to remove almost complete Cu(II) from aqueous solution, respectively. The residual Cu(II) concentration after these flocculation/separation process with ED-ch reached 0.03 ppm.

Introduction

Flocculation/coagulation is a process that fine divided or dispersed particles are aggregated together to form large particles of such a size so as to cause their sedimentation and cause clarification by filtration. Thus it is an efficient and cost-effective technology for the removal of suspended insoluble organic and inorganic substances from industrial and domestic wastewater. Polyaluminum chloride (PAC) and polyferic sulfate (PFS) are commonly used coagulants that form cationic species in water, and adsorb anionic colloids, resulting in aggregation of particles (coagulation).[1] Since these aggregates are small and fragile, polymeric flocculants like polyacrylamide (PAM) and polyamine are used in combination with the coagulant to enhance the formation of larger flocs bridging the destabilized particles and to facilitate sedimentation.[2,3]

In addition to synthetic flocculants, natural polyelectrolytes or modified biopolymers recently received much attention because they are as environmentally friendly, biodegradable, and less toxic.[4-6] Chitosan, a linear polysaccharide of β-(1,4)-linked 2-amino-2-deoxy-D-glucopyranose, is a semi-synthetic polysaccharide made from such abundant and renewable biomass as shells of crabs, shrimps, and insects. Since chitosan is dissolved in aqueous acidic solutions and possesses polycationic property, chitosan and its grafted polymers have been investigated as flocculants to reduce turbidity

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in wastewater. As an example, the ability was revealed by removal of clay mineral such as kaolinite from water.[4,7]

Among a large number of environmental pollutants, heavy metals are highly toxic or carcinogenic, and tend to readily accumulate into living organisms through food-chain. Various methods are being used to remove heavy metal ions, *e.g.*, chemical precipitation,[8] ion exchange,[9] adsorption,[10] and membrane separation.[11] Flocculation of heavy metal ions, however, is generally inefficient, because it is difficult to accumulate the dissolving metal ions into large flocs. Therefore, flocculation has to be carried out in combination with another purification process. For example, conventional flocculants like PAM[12,13] and carboxyethyl chitosan[14,15] was used after chemical precipitation to form colloids of the heavy metal hydroxide. These combined methods result in enhanced usage of harmful reagents and running expense of wastewater treatment.

Because chitosan has coordination ability with transition metals,[16] chitosan based materials have been extensively investigated to remove heavy metal ions, *e.g.*, beads,[10] membranes,[17] and chitosan immobilized onto glass beads.[18] Furthermore, their chemical modification made it possible to enhance the chelation ability with heavy metal ions.[19-21] Recently, a strong chelating agent, ethylenediaminetetraacetic acid (EDTA), was introduced into chitosan skeleton to give a crosslinked and water insoluble material. This solid-material showed remarkable adsorption ability with dissolving ions of copper,[22] nickel,[23] and also rare earths.[24]

Under these situations, our interest focused on design and synthesis of a chitosan based flocculant that has an ability to remove heavy metal ions. The success of EDTA-chitosan adsorbent prompted us to investigate on synthesis and utilization of water soluble EDTA-linked chitosan (ED-ch). ED-ch has chelation site of heavy

metal ions together with both anionic carboxyl group and cationic amino group in one polymer molecule. These functional groups are expected to provide an ability to flocculate dissolving heavy metal ions. Here, we would like to describe the synthesis of ED-ch through *N*-acylation of amino groups of chitosan using EDTA monoanhydride and its applicability for heavy metal ion removal examined by use of Cu(II) ion. Since the World Health Organization (WHO) recommended that heavy metal ions in drinking water must be reduced to several mg/l (*e.g.*, copper: less than 2 mg/l),[25] we planed the removal experiments were conducted at ppm level.

Similar concept of flocculants with pendant ligands have been reported by several research groups. Sulfur-containing functional groups such as thiourea, xanthate, and dithiocarbamate, which are known as moderately strong ligands for transition metal ions,[26] were introduced into synthetic polymers[27-29] and polysaccharide based graft copolymers.[30,31] Their effectiveness was shown by removing Pd(II), Au(III), Cu(II), and Pb(II).

Experimental

Materials

Low molecular weight chitosan (deacetylation degree of over 98%, average molecular weight of 1.56×10^4 g/mol) was obtained from Dainichi-seika Color and Chemicals Mtg. Co., Ltd. (Tokyo, Japan). High molecular weight chitosan (deacetylation degree of 95%, average molecular weight of $5-10 \times 10^5$ g/mol) was purchased from Hokkaido Soda Co., Ltd. (Hokkaido, Japan). Ethylenediaminetetraacetic acid (EDTA) was obtained from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). $Cu(NO_3)_2 \cdot 3H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$, $Pb(NO_3)_2 \cdot 4H_2O$, and $K_2Cr_2O_7$ of analytical grade and other regents of preparative grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and all chemicals were used without further purification. Water was purified by treatment with ion-exchanger followed by reverse osmosis with a Millipore Millex-3 apparatus.

Apparatus

Fourier transform infrared spectra (FT-IR) were recorded on a FT-210 spectrometer (Horiba Ltd., Japan) using a potassium bromide pellet at a resolution of 2 cm^{-1}. The samples were scanned from 4000 to 500 cm^{-1} using an average of 16 scans. ^{1}H and ^{13}C NMR spectra were recorded on a JNM-ECA600 spectrometer (Jeol Ltd., Japan) at 600.17 and 150.91 MHz and an ASX-300 spectrometer (Bruker BioSpin GmbH, Germany) at 300.13 MHz, respectively. Elemental analysis was performed by a MICRO CORDER JM-10 (J-science Co., Ltd., Japan). Turbidity was measured with a V-560 UV-visible spectrometer (Jasco Co., Japan), using a quart cell with an optical path length of 1 cm at 600 nm. Zeta potential was conducted on a Delsa Nano HC particle analyzer (Beckman Coulter Inc., USA) with a laser light with wavelength of 658 nm at 25 °C, using the same Beckman apparatus with the standard flow cell. The zeta-potential value was calculated using Smoluchowski equation. Concentration of Cu(II) in aqueous solution was measured by A-2000 atomic absorption spectrometer (Hitachi Ltd., Japan) which

equipped with a flame atomizer (air/acetylene) and a hollow cathode lamp of Cu (wavelength of 324.7 nm).

Synthesis of EDTA monoanhydride 3

This compound was prepared by a modified procedure reported by Capretta *et al.*.[32] Acetic anhydride (48 ml) was added to a suspension of EDTA tetraacid (**1**: 36.0 g, 120 mmol) in pyridine (62 ml) at room temperature under N_2 atmosphere. The mixture was stirred at 65 °C for 24 h and cooled. The resulting precipitates were filtered, washed successively with acetic anhydride and diethyl ether under N_2 stream, and dried under high vacuum to give EDTA dianhydride (**2**: 30.6 g, 97% yield) as white solid: ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.67 (s, 4H, N(CH_2)$_2$N), 3.70 (s, 8H, O=CCH_2N). The dianhydride **2** (30.0 g, 117 mmol) was dissolved in dry DMF (200 ml) at 80 °C under N_2 atmosphere. Water (2.1 ml, 117 mmol) was carefully added dropwise into the solution, and stirred at the same temperature for 3 h. Precipitate was formed during the reaction, and the turbid mixture was cooled, filtered, and washed successively with dry DMF and diethyl ether under N_2 stream. The white solid obtained was dried under high vacuum, giving the monoanhydride **3** (24.4 g, 75% yield): ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.59 (t, 2H, J 5.8 Hz, CH_2NCH$_2$C=O (carboxylate)), 2.78 (t, 2H, J 5.6 Hz, CH_2NCH$_2$C=O (anhydride)), 3.43 (s, 4H, NCH_2C=O (carboxylate)), 3.73 (s, 4H NCH_2C=O (anhydride)).

Synthesis of water soluble ED-ch 4

Low molecular weight chitosan (0.25 g, 1.56 mmol of D-glucosamine unit) was dissolved in 2% aqueous acetic acid-methanol (100 ml, 1:1(v/v)). The monoanhydride **3** (0.21 g, 0.78 mmol, 0.5 molar equivalents to the glucosamine unit) was added to the solution, and then the mixture was stirred vigorously at room temperature. After stirring for 24 h, pH of the reaction mixture was adjust to 8 by dropwise addition of 2% aqueous NaHCO$_3$, and further **3** (0.21 g, 0.78 mmol) was added to the mixture. The mixture was stirred at room temperature for 12 h, and precipitated by acidification of the mixture to pH 2-3 with 2 mol/l HCl. The resulting precipitate was separated by filtration, washed with methanol, and then dissolved in 2% aqueous NaHCO$_3$. The solution was subjected to ultrafiltration using a membrane (Advantec UK-10; Toyo Roshi Co., Ltd., Japan) with molecular weight cutoff 10,000 against deionized water, and lyophilized to give low molecular weight ED-ch (Na form) **4a** (0.37 g, D.S. 51%) as white hygroscopic amorphous powder. ^{1}H NMR (D$_2$O/NaOD, 80 °C, 600 MHz): δ 4.64 (s, H-1 of substituted), 4.45 (s, H-1 of unsubstituted), 3.91-3.31 (m, H3-6), 3.29 (s, NHCOCH_2N), 3.11 (s, NCH_2COONa), 2.66 (s, H-2 overlap with N(CH_2)$_2$N), 2.04 (s, NHCOCH_3). ^{13}C NMR (D$_2$O/NaOD, rt, 150 MHz): δ 182.5 and 182.1 (*C*OONa), 177.5 (NH*C*OCH$_2$), 61.6 (CO*C*H$_2$N), 55.0 (N(CH$_2$)$_2$N), 105.4, 103.9, 81.1, 77.8, 74.9, 63.1, 61.0, 59.4, and 58.1 (pyranose). FT-IR (KBr, cm^{-1}): ν 3422 (N-H and O-H str), 2959, 2888 (C-H str), 1653 (C=O amide str), 1636 (C-O carboxylate asym), 1592 (N-H amide bend), 1405 (C-O carboxylate sym), 1324 (C-N amide str), 1151, 914 (β-(1,4) glycoside bridge), 1071, 1035 (C-O-C str). Elemental analysis: calculate for (C$_6$H$_{11}$NO$_4$)$_{0.49}$(C$_{16}$H$_{22}$N$_3$O$_{11}$Na$_3$)$_{0.51}$: C 39.84, H 5.00, N 8.46%; found: C 40.37, H 5.53, N 8.57%.

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ED-ch **4b** was synthesized by similar reaction with high molecular weight chitosan (2.00 g, 12.5 mmol) and **3** (1.71 g, 6.25 mmol) for 1 day and subsequent with **3** (1.71 g, 6.25 mmol) for 12 h and purified by ultrafiltration through a membrane with molecular weight cutoff 20,000, giving high molecular weight ED-ch (Na form) **4b** (2.94 g, D.S. 41%) as white amorphous; elemental analysis: calculate for $(C_6H_{11}NO_4)_{0.54}(C_8H_{13}NO_3)_{0.05}(C_{16}H_{22}N_3O_{11}Na_3)_{0.41}$ ·0.5H$_2$O: C 39.29, H 5.37, N 8.18%; found: C 39.36, H 5.37, N 8.20%.

ED-ch **4c** was synthesized by similar reaction with high molecular weight chitosan (2.00 g, 12.5 mmol) and **3** (5.15 g, 18.8 mmol) for 1 day and subsequent with **3** (5.15 g, 18.8 mmol) for 12 h. Similar purification gave high molecular weight ED-ch (Na form) **4c** (4.37 g, D.S. 70%) as white amorphous; Elemental analysis: calculate for $(C_6H_{11}NO_4)_{0.25}(C_8H_{13}NO_3)_{0.05}(C_{16}H_{22}N_3O_{11}Na_3)_{0.70}$·0.5H$_2$O: C 38.34, H 4.86, N 8.19%; found: C 38.82, H 5.20, N 8.29%.

Solubility of ED-ch 4

The solubility was evaluated by turbidity measurement based on the method reported by Kubota *et al.*.[33] Fine powder of ED-ch (Na form) **4a-c** (5 mg) was mixed with deionized water (5 ml), and shook vigorously for 1 h. The optical transmittance of each sample was recorded on UV-visible spectrometer at 600 nm. The pH dependence on the solubility of ED-ch **4b, 4c**, and chitosan was also estimated from the optical transmittance in a similar way. Each sample was prepared by dissolving **4b, 4c**, or chitosan (20 mg) in 0.1 mol/l HCl (20 ml), and adjusted the pH by addition of small amount of 3 mol/l HCl or 3 mol/l NaOH solutions. The zeta potential value of each sample was also measured at different pH.

Removal of Cu(II) from aqueous solution

ED-ch **4b** or **4c** (1.00 g) was dissolved in deionized water (50 ml) to prepare 2wt% ED-ch solution. A Cu(II) solution (1000 ppm) prepared from Cu(NO$_3$)$_2$ and 0.1 mol/l HNO$_3$ was diluted with deionized water to give a Cu(II) stock solution (10.5 mg/l). One ml of the ED-ch solution was added to 19 ml of the Cu (II) stock solution in a 50 ml centrifuge tube, and pH of the mixture was adjusted in the range 1.0 to 6.0 by the addition of small amounts of 3 mol/l HNO$_3$ or 3 mol/l CH$_3$COONa. The initial concentration of ED-ch and Cu(II) in each sample was 1 g/l and 10 mg/l, respectively. The mixture was shaken at 120 rpm at room temperature for 24 h. The resulting precipitate was settled by centrifugation at 4,000 rpm for 15 min and the supernatant was collected immediately. Part of the supernatant was filtrated by ultrafiltration tube (vivaspin 6; Sartorius Stedim Biotech GmbH, Germany) with molecular weight cutoff 10,000 to rid ED-ch, and then the filtrate was taken. The residual Cu(II) concentration in the supernatant and the filtrate was determined by atomic absorption spectrometer.

The removal of Cu(II) with ED-ch was calculated according to the following equation:

$$\text{Removal (\%)} = \frac{C_0 - C_{sup}}{C_0} \times 100$$

where C_0 is the initial Cu(II) concentration and C_{sup} is the residual Cu(II) concentration of the supernatant. The experiments were per-
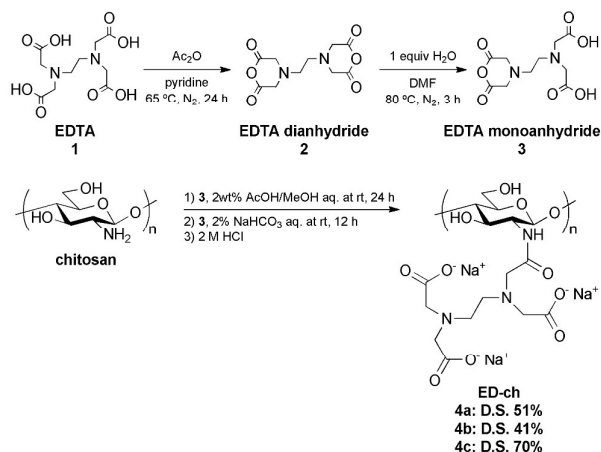
Scheme 1 Preparation of EDTA monanhydride **3** and water soluble ED-ch **4**.

formed three times. For comparison, the same experiments were also performed using 2wt% chitosan solution in aqueous acetic acid, which was prepared by dissolving in 1wt% acetic acid aqueous, instead of ED-ch solution.

Results and discussion

Preparation of water soluble ED-ch 4

Since EDTA linked chitosan so far reported was synthesized by using EDTA dianhydride **2** in aqueous acetic acid-methanol,[22,34] the product was insoluble due to crosslinking reactions occurred between chitosan chains. In order to prevent the crosslinking reaction and to obtain the corresponding water soluble material, we examined *N*-acylation by use of EDTA monoanhydride **3** instead of **2**. As shown in Scheme 1, **3** was prepared by a two-step reaction including dehydration of free EDTA **1** and selective hydrolysis.[32] *N*-Acylation of chitosan of both low and high molecular weight was carried out according to a slightly modified procedure developed by Satoh *et al.*,[35] under continually homogenous conditions in aqueous medium where *O*-acylation of chitosan would be restricted by hydrolysis of **3**. A solution of chitosan in aqueous acetic acid-methanol was treated with **3**, and then the reaction was continued under slightly basic media of pH 8. Precipitate obtained by acidification of the reaction mixture with aqueous HCl was collected by filtration. The carboxyl groups of EDTA residue were converted to sodium carboxylate groups by dissolving the product in aqueous NaHCO$_3$, and subsequently the solution was subjected to ultrafiltration to remove unreacted agents. Finally, lyophilization was performed to give ED-ch as white cotton-like hygroscopic amorphous. Reaction with equimolar amount of **3** provided ED-ch with D.S. 40-50% (**4a** and **4b**), and use of **3** fold excess reagent gave ED-ch with D.S. approximately 70% (**4c**) as summarized in Table 1.

Table 1 Preparation and properties of ED-ch **4**.

| Sample | Mw of chitosan (g/mol) | Molar ratio of the starting materials (**3** : chitosan NH₂) | D.S. (%) | | Water solubility[c] |
			Elemental analysis[a]	^{1}H NMR[b]	
4a	1.56×10^4	1 : 1	51	57	Soluble
4b	$5-10 \times 10^5$	1 : 1	41	39	Soluble
4c	$5-10 \times 10^5$	3 : 1	70	73	Soluble

[a] Calculation was based on the C/N ratio (see Experimental).
[b] Calculated from integral of H-1 protons.
[c] Sample (5 mg) was dissolved in H₂O (5 ml). Transmittance at 600 nm of the sample 95% higher than deionized water referred as "soluble".

Characterization of water soluble ED-ch

The structure of the product was characterized by FT-IR, ^{1}H and ^{13}C NMR spectroscopy using mainly low molecular weight **4a**. Fig. 1 shows the FT-IR spectra of chitosan and **4a**. In the spectrum of chitosan (Fig. 1 (a)), broad absorption at 3435 cm^{-1} corresponds to N-H and O-H stretching vibration, and absorption band between 2922 and 2880 cm^{-1} are attributable to the C-H stretching vibration. Moreover, the characteristic absorption bands were observed at 1653, 1597, and 1324 cm^{-1} due to the C=O stretching vibration (amide I), N-H bending vibration (amide II), and C-N stretching vibration (amide III) from the secondary amide, respectively.[36] The absorption bands at 1421 and 1384 cm^{-1} relate to O-H plane deformation of primary alcohol and C-H symmetric bending vibration in -CHOH-. The absorption bands of the β-(1,4) glycoside bridge at 1152 and 897 cm^{-1}, and C-O-C stretching vibration in the glucopyranose at 1091 and 1034 cm^{-1} were observed. Compared with the original chitosan, FT-IR spectrum of **4a** reveals several new absorption bands as shown in Fig. 1 (b). Absorption appeared at 1636 and 1405 cm^{-1} corresponds to C-O asymmetrical and symmetrical

stretching vibration of the carboxylate group, respectively,[24,37] and intensity of the amide I and II bands at 1653 and 1592 cm^{-1} increased. Furthermore, absorption band of ester (C=O stretch) was not observed at 1720-1770 cm^{-1},[38,39] suggesting highly selective formation of the amino group by the reaction with EDTA monoanhydride **3** under the aqueous conditions.

^{13}C NMR spectra of chitosan in DCl/D₂O and **4a** in NaOD/D₂O are shown Fig. 2. Six signals of chitosan (Fig. 2 (a)) at 98.3 (C1), 77.8 (C4), 75.6 (C5), 70.9 (C3), 61.1 (C6) and 56.8 ppm (C2) are attributable to the pyranose carbons of the 2-amino-2-deoxy-D-glucopyranose residue.[40] In the spectrum of **4a** (Fig. 2 (b)), the new signals were observed at 61.6 and 55.0 ppm, which were assigned to two kinds of methylene carbon in EDTA moiety.[41] The new signals at 182.5 and 182.1 ppm were assignable to carbonyl carbons of carboxylate groups in the EDTA moiety, and the signal at 177.5 ppm corresponds to carbonyl carbon of amide group. These results also supported the assigned structure of ED-ch **4**. Relatively sharp peaks observed at 171.1 and 173.9 ppm were probably due to low molecular weight contaminants such as CO₃[2-] in D₂O/NaOD.[42]
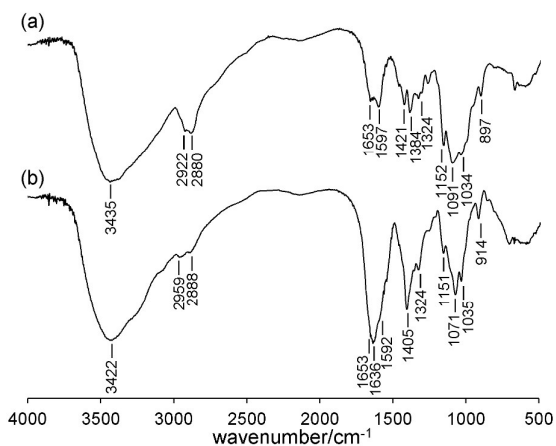

Fig. 1 FT-IR spectra of (a) chitosan and (b) ED-ch **4a** (D.S. 51%).

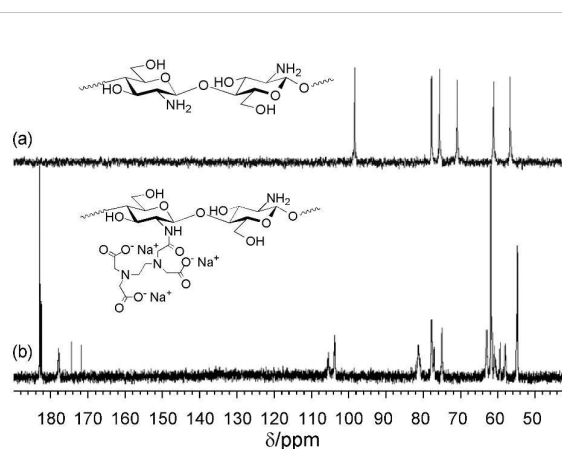**Fig. 2** ^{13}C NMR spectra of (a) chitosan in DCl/D₂O and (b) ED-ch **4a** (D.S. 51%) in NaOD/D₂O at rt.

^{1}H NMR spectra of ED-ch with various D.S. (**4a-c**) in NaOD/D$_2$O at 5 and 80 °C are shown Fig. 3. The spectra were measured at different temperatures in order to shift the large signal of HOD. The signals at 4.64 and 4.45 ppm were attributed to the anomeric protons of *N*-substituted (H-1') and un-*N*-substituted (H-1)[43] glucosamine residues, respectively. The signal at δ 3.91-3.31 ppm assigned to H-3-6 of glucosamine residues.[33] Generally, presence of *O*-acyl groups at O-3 or O-6 positions of glucosamine residue can be determined by strong downfield shift of the corresponding α-protons.[44] Observation of no signal at such lower magnetic field of δ 3.9-4.2 ppm in the spectrum (a) suggested the absence of the *O*-acyl groups. The signals at 3.29, 3.11, and 2.66 ppm correspond to protons of three kinds of methylene groups of EDTA residue. The peak at 2.66 ppm was an overlap signal of H-2 of chitosan and H-b of EDTA residue.

The degree of substitution (D.S.) of the EDTA residue in the products was calculated from the area ratio between the anomeric protons of substituted (H-1') and unsubstituted (H-1) glucosamine residues in ^{1}H NMR spectrum.

$$D.S.\,(\%) \,=\, \left(\frac{H-1'}{H-1'\,+\,H-1}\right)\,\times\,100$$

Moreover, D.S. was also calculated from C/N ratio of elemental analysis using the following equation:

$$C/N = \frac{73.26 + 120.1x}{14.01 + 28.02x}$$

where C/N is C/N ratio of elemental analysis and x is D.S.. The molar mass of carbon and nitrogen per mole of glucosamine unit are calculated to be 73.26 and 14.01, respectively. Those of EDTA
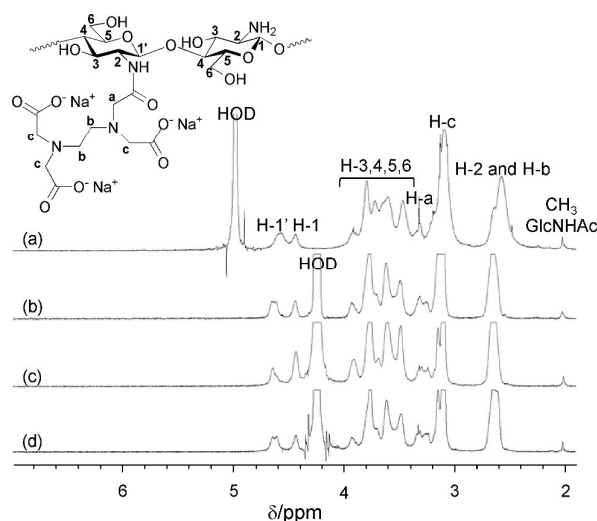

Fig. 3 ^{1}H NMR spectra of low and high molecular weight ED-ch (a) **4a** (D.S. 51%) at 5 °C, (b) **4a**, (c) **4b** (D.S. 41%), and (d) **4c** (D.S. 70%) at 80 °C in NaOD/D$_2$O.
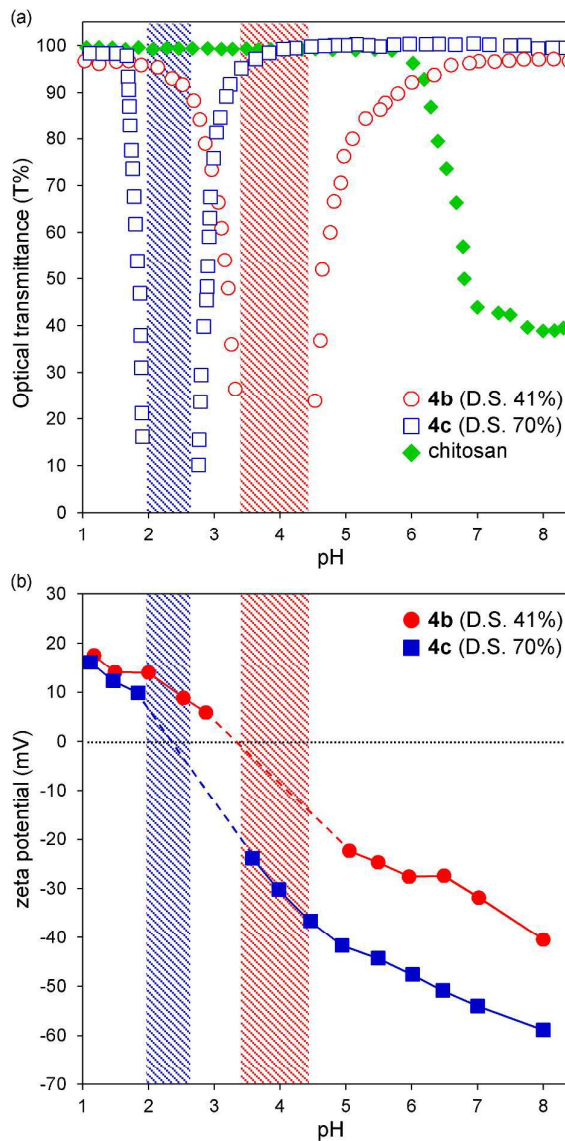

Fig. 4 (a) The optical transmittance (T%) and (b) the zeta potential of aqueous solutions of chitosan, ED-ch **4b** with D.S. 41%, and **4c** with D.S. 70% under various pH.

residue are 120.1 and 28.02, respectively. The results of calculation are shown in Table 1, and D.S. values obtained from ^{1}H NMR spectra are in good agreement with from C/N ratio of elemental analysis. D.S. of ED-ch **4** was able to change by the molar ratio of starting chitosan and EDTA monoanhydride used.

Solubility of ED-ch 4

Next our interest focused on pH dependence on solubility of ED-ch **4**, because behavior of the polymeric amphoteric compound in

aqueous media seemed to be important for application to flocculant. The pH dependence on solubility of 2 types of high molecular weight ED-ch with different D.S. **4b**, **4c**, and chitosan were investigated by turbidity measurement in aqueous solution in the pH range of 1.0-8.0. The sample was determined soluble when the transmittance was higher than 95%, compared to that of deionized water. As shown in Fig. 4 (a), the transmittance of chitosan solution was close to 100% between the pH region of 1.0-6.0, whereas it rapidly degreased at pH >6.0. The solubility of chitosan in acidic region is known to be caused by the protonation of amino groups.[45]

By contrast to chitosan, the EDTA derivatives **4b** and **4c** were found to be the soluble in the wide range of pH region, including both acidic and basic region. These results are explainable by consideration that ED-ch is amphoteric polyelectrolyte containing amino and carboxyl groups, which can be protonated or deprotonated depending on pH of the solution. In acidic solution, it should have unionized carboxyl groups (-COOH) but protonated amino groups (-NH_3^+), behaving as a cationic polyelectrolyte that causes intra- and inter-molecular electrostatic repulsive interaction. While the amino groups are deprotonated in basic solution, ED-ch can also act as an anionic polyelectrolyte due to the deprotonation of carboxyl group to carboxylate ions (-COO^-). The zeta potential was also corroborated these discussion (Fig. 4 (b)). The zeta potential of ED-ch **4b** and **4c** was positive value at acidic conditions, while it changed to negative value at neutral or basic conditions, *i.e.*, ED-ch had either positive or negative surface charge at the pH region we examined. These results suggested that ED-ch behaved as a cationic or an anionic polyelectrolyte when it was dissolved in aqueous solution.

It was quite interesting that the transmittance of **4b** and **4c** decreased in the acidic ranges of pH 2.4-6.3 and 1.7-3.6, respectively. The solution became opaque in these pH regions due to formation of precipitates. Furthermore, the observed transmittance became unstable in the narrow pH regions of 3.3-4.5 (**4b**) and 1.9-2.7 (**4c**), which are shown by oblique lines in Fig. 4. Leaving these samples to stand for 10 min, we found the transmittance gradually increased to maximum of approximately 95%. These tendencies of **4b** could be observed visually as shown in Fig. 5. Precipitate formation of transparent solution of pH 7.0 (fig. 5 (a)) occurred by changing to pH 4.6, 4.0 (Fig. 5 (b), (c)). At pH 4.0, fine particles presented in the suspension (Fig. 5 (c)) gradually became larger aggregates and settled out in the clear after leaving for 10 min (Fig. 5 (d)). As shown in Fig. 4(b), the zeta potential of ED-ch **4b** and **4c** decreased with increasing pH. Although the potential could not measure when the sample contained precipitations, it changed from positive value to negative value at the boundary pH regions. The zeta potential of the precipitates can be presumed to be close to zero by interpolation of these data. These ranges of pH are affected by the effect of the isoelectric point (zeta potential = 0) at which equimolar of NH_3^+ and COO^- exists in the molecule. When the pH approaches the isoelectric point, the electrostatic attractive interaction between NH_3^+ and COO^- dominates the electrostatic repulsive interaction, which makes
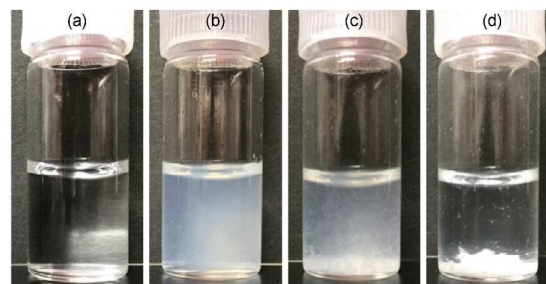

Fig. 5 The photograph of ED-ch **4b** (D.S. 41%) with 0.1wt% in aqueous solution at (a) pH 7.0, (b) pH 4.6, (c) pH 4.0 immediately after adjusting pH, and (d) pH 4.0 after leaving to stand for 10 min.

polymer chain aggregate[46] thus the solution exhibited low optical transmittance. These behaviors are agreed characteristic of most of amphoteric polyelectrolytes.[47-49] It is reasonable for ED-ch with higher D.S. **4c** to precipitate in the lower pH region because it has less amount of amino groups.

pH dependent removal of Cu(II) from aqueous solution

Having data of unique behavior of ED-ch with depending on pH in our hand, we undertook to perform preliminary removal experiment by use of Cu(II) as a model pollutant of heavy metal ion. Our interest focused on applicability of ED-ch to flocculation/precipitation process. Since Cu(II) forms hydroxide precipitation in the higher pH region over 6.3,[50] our removal experiments were conducted at pH ranging from 1.0 to 6.0 where effect of precipitation of the hydroxide may be negligible. The experiments were carried out in the initial concentrations of Cu(II) and a polymer ligand (two high molecular weight ED-ch **4b** (D.S. 41%), **4c** (D.S. 70%), and chitosan) 10 ppm and 1 g/l, respectively. The molar ratio of EDTA residue and Cu(II) for ED-ch **4b** with D.S. 41% and **4c** with D.S. 70% were 8.6 and 11.1, respectively. After contacting Cu(II) and the chitosan material for 24 h, a part of Cu(II) in the mixture thought to be chelated with the polymer ligand, which is presented either in precipitate or in solution. Therefore, the distribution of Cu(II) was first examined in order to obtain the conditions of flocculation removal of Cu(II). The experiment was performed by separation of the mixture by 2 different ways. Centrifugal separation of the mixture removed precipitate to give supernatant, which was then subjected to ultrafiltration to remove chitosan material. Subsequent measurement of the Cu(II) concentration by atomic absorption spectroscopy of the liquid fractions gave data for the calculation of amounts of Cu(II) in the precipitate as well as those in solution bound with the polymer ligand and non-chelated.
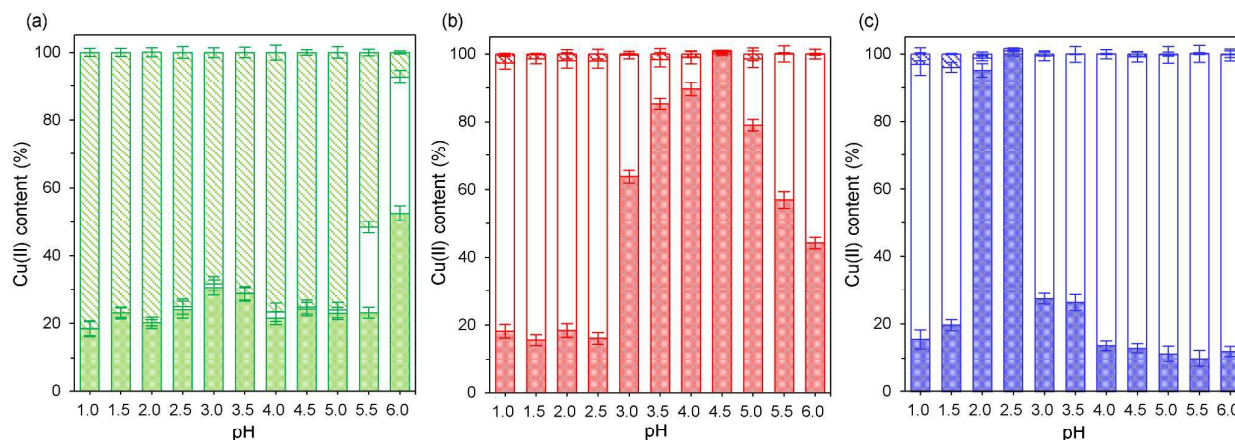
Fig. 6 The effect of pH on the distribution of Cu(II) contacting with (a) chitosan, ED-ch (b) **4b** with D.S. 41%, and (c) **4c** with D.S. 70%. Chelated Cu(II) with polymer ligand in precipitate (closed column), in solution (open column), and non-chelated Cu(II) (hatched column). Initial concentration of Cu(II) 10 mg/l, dosage of polymer ligand 1 g/l, contact time 24 h, shaking rate 120 rpm, rt.

Fig. 6 shows the distribution of Cu(II) after contacting with the three polymer ligands at various pH. Chitosan showed that, in acidic solution of pH below 5.0, most of Cu(II) was non-chelated with chitosan and presented in solution, and very small amount of Cu(II) was bound with chitosan (Fig. 6 (a)). The amount in precipitate was approximately 25% in this pH region. Most of the chitosan amino groups may be protonated, and resulted in poor coordination ability with Cu(II).[51] While chitosan exhibited an increased coordination ability toward Cu(II) and decreased solubility in upper pH region, and Cu(II) contents in both soluble chitosan and precipitated chitosan were increased. That of precipitate reached 52.6% at pH 6.0.

By contrast, the distribution profiles of ED-ch **4b** with D.S. 41% (Fig. 6 (b)) and **4c** with D.S. 70% (Fig. 6 (c)) were remarkably different. Amount of non-chelated Cu(II) in solution was very small (less than 5%) in the all pH region examined (pH 1.0-6.0). When Cu(II) was presented in the solution, formation of precipitation was also observed at the almost same pH as the solubility experiments, *i.e.*, **4b** and **4c** for pH around 4.0 and 2.5, respectively. The amount of Cu(II) in precipitate **4b** was increased as the increment of pH

from 3.0 to 4.5, reached maximum of 100% at pH 4.5, and subsequently decreased gradually with increasing pH. Similarly, that of precipitate **4c** reached maximum of 100% at pH 2.5. These data exhibit that ED-ch possessed high chelation ability both acidic and neutral aqueous media, and the Cu(II) captured polymer was precipitated by pH control.

The removal experiments of Cu(II) with **4b** and **4c** were carried out on the basis of above results. We observed visually formation of blue-colored flocculent precipitate at the optimum pH, and it was readily separated by centrifugation. The results are summarized in Table 2. The residual Cu(II) concentration treated with ED-ch **4b** (D.S. 41%) reached the lowest (0.03 mg/l) at pH 4.5, which means the dissolving Cu(II) was almost completely removed. In case of **4c** (D.S. 70%), the highest removal performance was attained at 2.5. Comparing with chitosan (Fig. 6 (a)), these results show excellent flocculation ability of ED-ch toward Cu(II). ED-ch can removed Cu(II) to much less than the permissible level[25] from aqueous solution at optimal pH. These optimal pH of ED-ch flocculation suggest that the electrostatic attraction between NH_3^+ and COO^- in ED-ch is

Table 2 The effect of pH on the residual concentration of Cu(II) after flocculation/precipitation process.[a]

pH	Initial Cu(II) concentration (mg/l)[b]	Residual Cu(II) concentration of supernatant (mg/l)[b]		
		4b	**4c**	chitosan
2.0	10.41	8.50	0.49	8.31
2.5	10.14	8.50	0.03	7.71
4.0	10.40	1.08	8.98	8.16
4.5	10.38	0.03	9.04	7.88
6.0	10.31	5.75	9.07	4.88

[a] Flocs formed by contacting ED-ch **4b** (D.S. 41%), **4c** (D.S. 70%), or chitosan were removed by centrifuge.
[b] Concentration of initial and residual Cu(II) were measured with atomic adsorption spectrometry.

reduced the repulsion of the polymer at around isoelectric point, which would cause a high efficient flocculation ability.

We also performed preliminary experiments with ED-ch **4b** to examine applicability to the removal for such toxic metal ions as Cd(II), Pb(II), and Cr(VI) in the same manner as described for Cu(II) using the each metal aqueous solution (10 mg/l) at pH 4.5. Although low efficiency removal (14.8%) was observed for Cr(VI) that exists oxyanion form in aqueous solution,[52] Cd(II) and Pb(II) were found to be removed effectively and the removal ratios were 88.0 and 93.5%, respectively. These results would be indicative of usefulness of ED-ch as a flocculation material which can get rid of harmful metal ions from wastewater.

Conclusions

In this study, a chitosan-based flocculant, ED-ch, for removal of heavy metal ions was successfully synthesized through *N*-acylation of chitosan with EDTA monoanhydride under aqueous acidic conditions followed by basic conditions. ED-ch was characterized for the flocculation performance against Cu(II) in terms of pH value. The newly introduced functional group in EDTA-linked chitosan (ED-ch) provided properties as a strong chelating reagent and an amphoteric polyelectrolyte. It was found that ED-ch had good water-solubility in both acidic and basic region and precipitated in narrow pH region. The flocculation performance was very sensitive to the pH of aqueous solution. ED-ch exhibited a high efficient removal toward Cu(II) with a high flocculation within the narrow pH range. Similar results were observed in the removal experiments against Cd(II) and Pb(II). The results demonstrated that ED-ch was superior to original chitosan for application as flocculant for removal of heavy metal ion. ED-ch is expected to be applied to the field of remediation and treatment of wastewater contained heavy metal ion. Further studies along this line, involving the selectivity in metal ion adsorption and precipitation mechanism, are now in progress.

Acknowledgements

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Water Soluble EDTA-linked Chitosan as a Zwitterionic Flocculant for pH Sensitive Removal of Cu(II) ion

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Water Soluble EDTA-linked Chitosan was synthesized and applied to remove Cu(II) utilizing its property of chelation and pH sensitive precipitation.