Antimicrobial Activities and Metal Chelation Ability of New Water-Soluble Chitosan Derivatives Having N-carboxymethyl Groups

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Abstract

In this article, novel aminoamine grafted chitosans containing hyperbranched-N-carboxymethyl groups are synthesized, characterized and tested for their potential usage. The synthesis is achieved by, using a commercially available chitosan with known chemical conversions. Two successive steps, Michael addition of amino groups to methyl acrylates followed by amidation with ethylenediamine, are repeated to generate hyperbranched chitosans. Subsequent connection of N-carboxymethyl groups to the synthesized hyperbranched chitosans led to the modified chitosans containing hyperbranched-N-carboxymethyl groups. These novel modified chitosans are characterized by FTIR-UATR (Fourier transform infrared-universal attenuated total reflectance) and 13C CP/MAS (13C Cross polarization/magic angle spinning). The modified chitosans show significantly improved water solubility which is a highly desirable property for some applications, compared to the original chitosan. The antimicrobial activity test showed that the modified chitosans display higher efficiency for antimicrobial activity against Staphylococcus aureus ATCC 29213, Micrococcus luteus ATCC 10240 and Shewanella putrefaciens ATCC 8071 compared to the original chitosan. ICP-MS (inductively coupled plasma-mass spectrometer) analyses are used to confirm that these modified chitosans have superior affinity to chelate heavy metals, compared to the unmodified starting chitosan. These modified chitosans are considered high potential utilities for human life and environmental concern.

Keywords: Antimicrobial activity, Chitosan, Hyperbranched polymer, Metal chelation

Introduction

Chitosan is a natural polymer obtained by deacetylation of chitin, the second most abundant polysaccharide exists in nature. Chitosan is the main component found in exoskeleton of crustaceans such as crab and shrimp. It was also found in the cell walls of many fungi and yeast, and in the cuticles of insects (Song et al., 2018). Chitosan consists of repeating units of anhydro-N-acetyl-D-glucosamine and anhydro-D-glucosamine; the latter has higher proportion as shown in Figure 1.

Chitosan draw a lot of attentions of many research groups because of its unique properties, especially its non-toxicity, biocompatibility, and biodegradability (Sashiwa et al., 2003; Xing et al., 2005). Due to chitosan ability to chelate with metals using its free amine and primary and secondary hydroxyl groups, one potential application of chitosan is in water treatment (Ahmad et al., 2015; Qu et al., 2008). In addition, chitosan and its derivatives have been found to have antimicrobial activity (Goy et al., 2016; Mourya et al., 2008). However, the natural chitosan is only soluble in organic acids like acetic or formic acids which make its utilization quite limited. Fortunately, due to the presence of free active amino groups in chitosan, chemical modifications seem to be approachable means to overcome the solubility issue (Sashiwa et al., 2004; Baumann et al., 2001).

Dendrimers have attracted considerable attention due to their highly branched characteristics that

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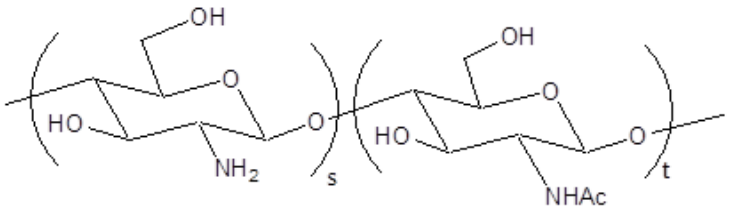


Figure1. Chitosan (s > t)

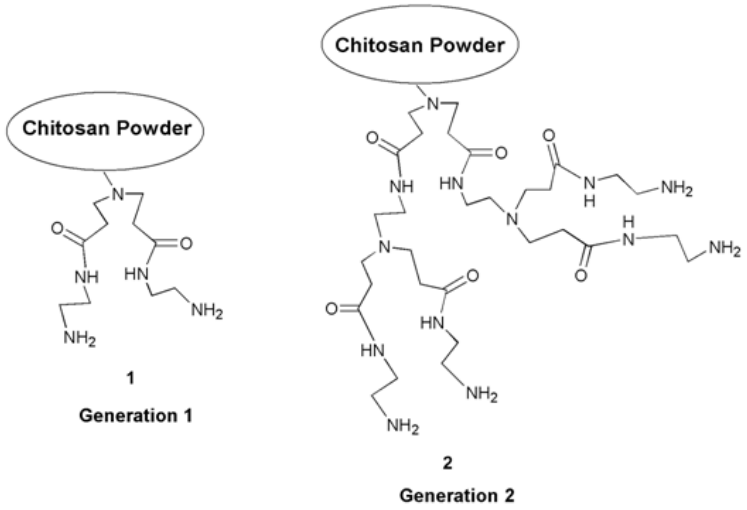


Figure2. Polyamidoamine dendrimer grafted chitosan powder.

can be modified to change their physical and electronic properties (Fréchet, 2003). As such, one could potentially modify chitosan with dendrimers to alter both dendrimer and chitosan properties. Chitosan-dendrimer hybrids were found to possess better water-soluble properties than the unmodified chitosan (Chanthateyanonth et al., 2010; Sashiwa et al., 2002). Polyamidoamine dendrimer can be successfully grafted on chitosan powder (Tsubokawa et al., 2000). (Figure 2)

In this work, we report an uncomplicated method for synthesizing aminoamine grafted chitosan containing hyperbranched-N-carboxymethyl groups with improved water solubility (Figure 3). The antimicrobial activity of these new modified chitosan and metal chelation ability were investigated, compared to the unmodified chitosan.

Experimental procedure

All chemicals, purchased from Fluka Chemical and Sigma-Aldrich companies were used as received. Low molecular weight chitosan was obtained from Fluka Chemical (degree of deacetylation = 72%, Mn = 150 kD) and Sigma-Aldrich (degree of deacetylation = > 85%, Mn = 50 kD) companies. Compounds 1 and 2 were prepared following the method described in the literature (Tsubokawa et al., 2000). All reactions were performed under a dry nitrogen atmosphere.

FTIR-UATR spectra were recorded on a Perkin Elmer System 2000FT-IR spectrometer. Samples for IR were examined using a universal attenuated total reflectance (UATR) with a diamond crystal. ^{13}C CP/MAS NMR spectra were recorded on a Bruker DPX-300 spectrometer. ICP-MS is a method for the determination of cadmium, nickel, or copper in samples. to cadmium(II) than to nickel(II) and copper(II).

Synthesis and characterization of generation one (G-1) (A, Mn = 150 kD), (B, Mn = 150 kD, further partial hydrolysis), (C, Mn = 50 kD), and (D, Mn = 50 kD, further partial hydrolysis) and generation two dendrimer (G-2) (E, Mn = 150 kD), (F, Mn = 150 kD, further partial hydrolysis), (G, Mn = 50 kD) and (H, Mn = 50 kD, further partial hydrolysis)

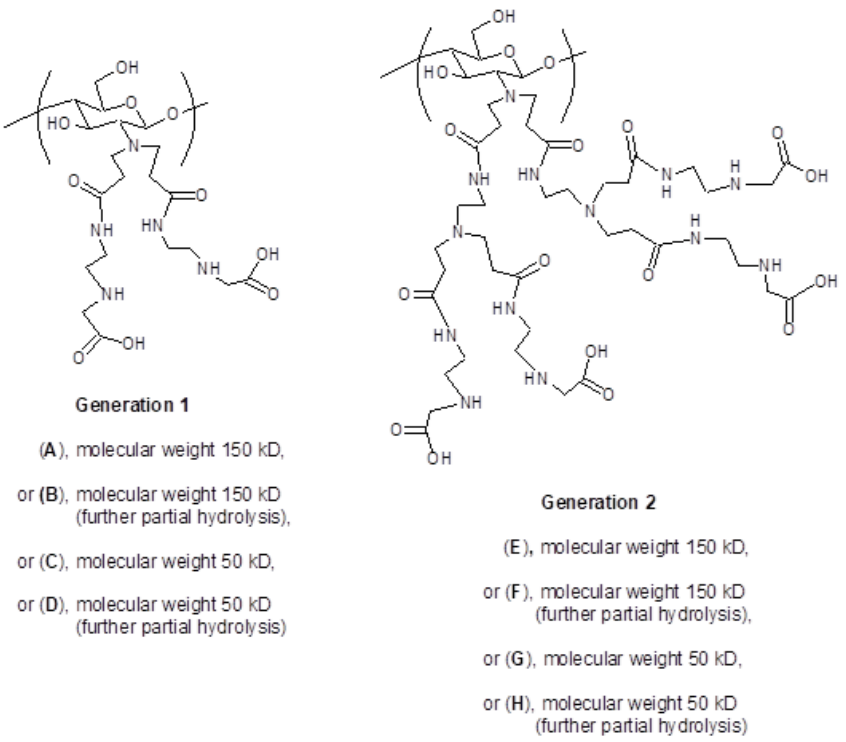


Figure 3. Chitosan derivatives containing hyperbranched-N-carboxymethyl groups.

A modified literature procedure was used (Colo et al., 2004). Either compound 1 or 2 (0.5 g) is dissolved in an aqueous 0.7% (v/v) acetic acid solution (50 mL). Any insoluble materials are filtered off before glyoxylic acid (10 mL) is added to the solution at room temperature. The mixture is then stirred further at that temperature for 2 hours before 1 M NaOH is added drop by drop until the pH value reaches 4.5. After that the mixture is stirred for an additional 1 hour. Then, sodium cyanoborohydride aqueous solution is added dropwise. The mixture is stirred further at room temperature for 1 hour after the addition is complete. Ethanol was slowly added to precipitate a pale- yellow powder, which was filtered, washed with ethanol several times, and dried to afford generation one dendrimer (G-1) or generation two dendrimer (G-2).

Generation one dendrimer (G-1) (A) (0.6 g) IR (cm^{-1}) 3285, 2931, 1630, 1571, 1370, 1017; ^{13}C CP/MAS NMR δ (ppm) 174.9, 164.5, 104.9, 85.0, 75.2, 62.0, 41.7, 23.5.

Generation one dendrimer (G-1) (B) (0.6 g) IR (cm^{-1}) 3281, 2919, 1622, 1572, 1377, 1019; ^{13}C CP/MAS NMR δ (ppm) 175.5, 164.7, 105.4, 84.1, 75.6, 60.3, 39.4.

Generation one dendrimer (G-1) (C) (0.6 g) IR (cm^{-1}) 3279, 2875, 1624, 1570, 1376, 1024; ^{13}C CP/MAS NMR δ (ppm) 174.2, 164.7, 105.5, 84.0, 75.2, 61.5, 48.7, 38.2, 24.1.

Generation one dendrimer (G-1) (D) (0.6 g) IR (cm^{-1}) 3276, 2860, 1623, 1566, 1436, 1023; ^{13}C CP/MAS NMR δ (ppm) 175.1, 164.8, 105.6, 83.7, 75.3, 60.8, 47.9, 38.4.

Generation two dendrimer (G-2) (E) (0.6 g) IR (cm^{-1}) 3280, 2872, 1630, 1571, 1374, 1027; ^{13}C CP/MAS NMR δ (ppm) 174.9, 164.5, 104.9, 85.0, 75.2, 62.0, 41.7, 23.5.

Generation two dendrimer (G-2) (F) (0.6 g) IR (cm^{-1}) 3288, 2867, 1635, 1563, 1376, 1021; ^{13}C CP/MAS NMR δ (ppm) 174.3, 164.8, 105.6, 75.0, 61.6, 48.7, 38.7.

Generation two dendrimer (G-2) (G) (0.6 g) IR (cm^{-1}) 3276, 2870, 1623, 1566, 1436, 1023; ^{13}C CP/MAS NMR δ (ppm) 174.0, 164.5, 104.8, 84.6, 75.1, 62.4, 37.8, 23.6.

Generation two dendrimer (G-2) (H) (0.6 g) IR (cm^{-1}) 3280, 2872, 1622, 1575, 1373,1027; ^{13}C CP/MAS NMR δ (ppm) 174.6, 164.7, 104.5, 75.2, 60.7, 40.4.

General procedure for determination of water solubility

The procedure described in the literatures was used (Chanthateyanonth et al., 2010). The derivatives or starting chitosan (30 mg) were distributed in water

(10 mL) for 48 hours. Solutions of 0.1 M HCl and 0.1 M NaOH were used to adjust the pH of the suspensions. The water solubility was determined at pH 5, 6, 7, 8, and 9. The weight of the dry remaining undissolved derivatives and starting chitosan was determined. The weight of dissolved derivatives and dissolved starting chitosan was determined as well after evaporation of the aqueous solution under reduced pressure. To minimize the measurement error, at least four measurements were averaged for each sample.

Antimicrobial activity

The antimicrobial activity of the derivatives and starting chitosan was evaluated against Staphylococcus aureus (S. aureus) ATCC 29213, Micrococcus luteus (M. luteus) ATCC 10240 and Shewanella putrefaciens (S. putrefaciens) ATCC 8071, obtained from the Faculty of Medical Technology, Mahidol University, Thailand, using agar dilution method. S. aureus, a Gram-positive bacterium, is a powerful pathogen in human disease (Shelburne et al., 2011). M. luteus, a Gram-positive bacterium, can cause meningitis and endocarditis (Gupta et al., 2019). S. putrefaciens, a Gram-negative bacterium, can cause bacteremia and cellulitis (Vignier et al., 2013).

The tested compounds were individually mixed with Muller Hinton Broth (MHB), a medium containing beef infusion, casamino acids or peptone, and starch, to obtain a final volume of 2 mL. The microorganisms were cultured in MHB at 37 °C for 24 h and diluted with 0.9% normal saline solution to adjust the cell density of 10^8 CFU/mL compared with 0.5 McFarland. The microorganisms were further incubated at 37 °C for 24-48 h. For agar dilution, the solutions with defined numbers of bacterial cell are spotted directly onto the nutrient plates that have incorporated different antimicrobial and antibacterial agent concentrations. The following concentrations of chitosan derivatives were used 2500, 1250, and 625 µg/mL at pH 5.75 MHB (Muller Hinton Broth). All the plates were incubated at 37 °C for 24-48 hours.

The tested solution was transferred to the Muller Hinton Agar (MHA) by two-fold dilution to obtain the concentrations ranging of 625, 1250, and 2500 µg/mL. After incubation, the presence of bacterial colonies on the plates indicates the growth of the organism. The antimicrobial activity of tested compounds was evaluated by observing the growth of microorganisms on MHA at pH 5.75 and grading 4+ (100%), 3+ (75%), 2+ (50%), 1+ (25%), and no growth 0 (0%).

General procedure for adsorption of metals at pH 7

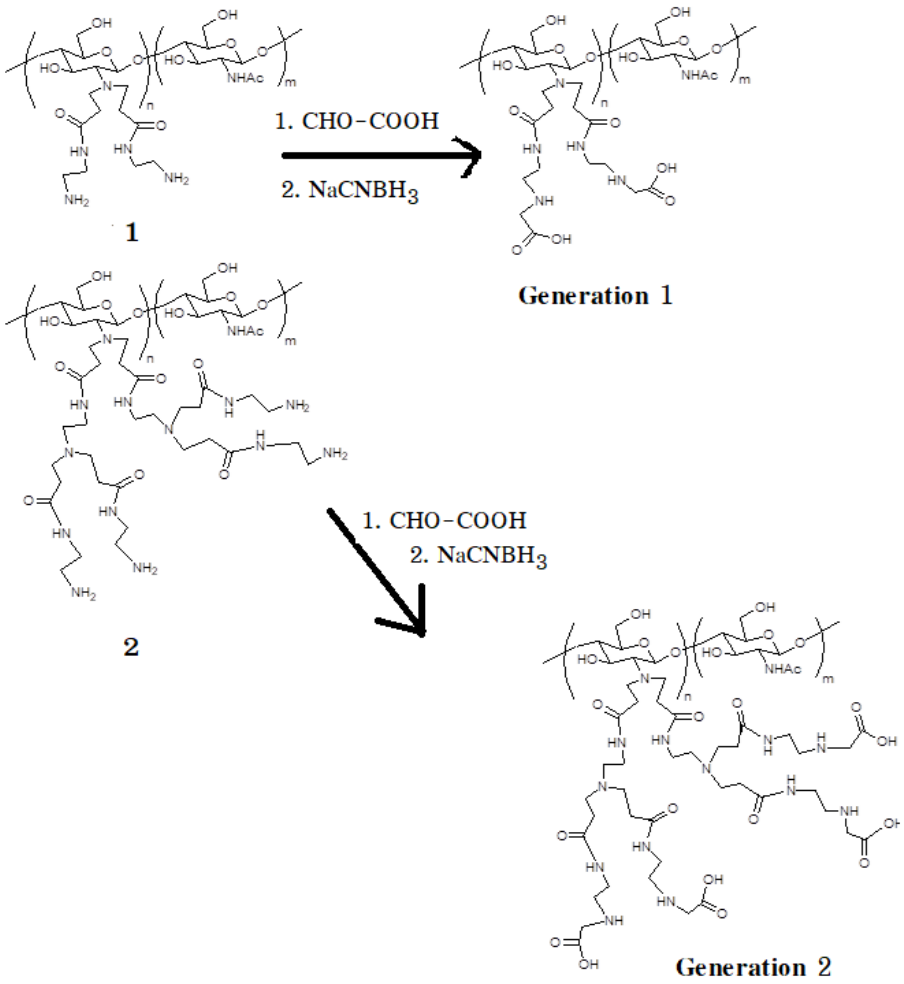
The procedure described in the literatures was used (Chanthateyanonth et al., 2010). Copper, cadmium, or nickel sulphate solutions (0.02 M, 10 mL) were passed slowly through the columns [glass tubing (ø = 0.6 cm), which were packed with chitosan or chitosan derivatives (100 mg)]. The adsorption of metals by chitosan and chitosan derivatives was determined by ICP analysis.

RESULTS AND DISCUSSION

Synthesis and characterization of generation one (G-1) (A, Mn = 150 kD), (B, Mn = 150 kD, further partial hydrolysis), (C, Mn = 50 kD), and (D, Mn = 50 kD, further partial hydrolysis) and generation two dendrimer (G-2) (E, Mn = 150 kD), (F, Mn = 150 kD, further partial hydrolysis), (G, Mn = 50 kD) and (H, Mn = 50 kD, further partial hydrolysis)

Compounds 1, 2, and partial hydrolysis of chitosan were synthesized according to the procedure described in the literatures (Baumann et al., 2001; Tsubokawa et al., 2000). Generation one dendrimer (G-1) (A), (B), (C) or (D) and generation two dendrimer (G-2) (E), (F), (G) or (H) were prepared by the analogous procedure reported in the publication (Colo et al., 2004), as illustrated in Scheme 1.

The identity of the modified chitosans is confirmed by FTIR-UATR. The characteristic absorption peaks of glyoxylic acid grafted dendritic hyperbranched chitosan presents two broad peaks at 3285 cm^{-1} (in the range 2400-3700 cm^{-1}) which attributed to O-H bond of carboxylic acid and at 1630 cm^{-1} (in the range 1600 and 1700 cm^{-1}) which attributed to C=O bond of carboxylic acid. Also, 13C CP/MAS NMR shows chemical shift at 164 ppm which represents the C=O moiety.



Scheme 1. Preparation of chitosan containing hyperbranched-N-carboxymethyl groups (G-1) and (G-2).

Water solubility

The water solubility of chitosan and new chitosan derivatives is shown in Table 1. It was found that chitosan derivatives containing hyperbranched-N-carboxymethyl groups had significantly higher water solubility compared to the starting material. This increase in water solubility was due to the hyper branched-N-carboxymethyl group that put on. It was also found that G-1 and G-2 had similar water-soluble properties to the chitosan derivatives. This might be due to the fact that the polarity of G-2 was decreased by extended chain length, although the numbers of carboxyl groups in G-2 is higher than G-1. Attempting to enhance water solubility by further partial hydrolysis of chitosan derivatives was carried out without success. There was no noticeable higher level of water solubility observed (A and B, C and D, E and F, and G and H). Quite possibly, the partial hydrolysis failed to produce any significant change in the degree of deacetylation. As expected, the solubility of lower molecular weight chitosan (50 kD) and its derivatives in water, at the same pH, is slightly higher than that of higher molecular weight chitosan (150 kD) and its derivatives.

Antimicrobial activity

Growth inhibition of bacteria in the presence of chitosan derivatives was observed compared with starting chitosan (Tables 2 and 3). The results indicated that both starting chitosans with concentration ranging from 625-2500 μg/ml have no antimicrobial activity against M. luteus, S. aureus and S. putrefaciens while the new chitosan derivatives containing hyperbranched-N-carboxymethyl groups show improved activity against these species with a minimum concentration 625 μg/ml. The chitosan derivatives have better antimicrobial activities than the starting chitosan. This is likely the contribution of their improved solubility in water.

Table 1. The water solubility of chitosan and new derivatives at various pHsa

Entry	Compound	Water solubility (%)				
		pH 5	pH 6	pH 7	pH 8	pH 9
1	Starting Chitosan (Mn = 150 kD)	19	19	11	12	12
2	Starting Chitosan (Mn = 50 kD)	21	21	21	24	22
3	A (G-1)	84	87	88	90	90
4	B (G-1)	86	83	89	87	89
5	C (G-1)	80	83	82	83	84
6	D (G-1)	84	90	87	84	75
7	E(G-2)	90	98	85	84	96
8	F (G-2)	74	76	84	90	90
9	G (G-2)	81	85	95	81	88
10	H (G-2)	70	75	78	75	79

[a] Samples (30 mg) were dispersed in water (10 mL) for 48 h and the pH of the suspensions were adjusted with 0.1 M HCl or 0.1 M NaOH.

Table 2. Antimicrobial activity of chitosan (Mn = 50 kD) and its derivatives

Entry	Compound	M. luteus	S. aureus	S. putrefaciens
1	Chitosan			
	625 µg/mL	4+	4+	4+
	1250 µg/mL	4+	4+	4+
	2500 µg/mL	4+	4+	4+
2	C (G-1)			
	625 µg/mL	1+	2+	2+
	1250 µg/mL	0	1+	1+
	2500 µg/mL	-[a]	0	0
3	G (G-2)			
	625 µg/mL	2+	1+	1+
	1250 µg/mL	1+	0	1+
	2500 µg/mL	0	-[a]	0

[a] Antimicrobial activity was not tested.

Grading the growth of microorganisms, 4+ (100%), 3+ (75%), 2+ (50%), 1+ (25%), and no growth 0 (0%).

Table 3. Antimicrobial activity of chitosan (Mn = 150 kD) and its derivatives

Entry	Compound	M. luteus	S. aureus	S. putrefaciens
1	Chitosan			
	625 μg/mL	4+	4+	4+
	1250 μg/mL	4+	4+	4+
	2500 μg/mL	4+	4+	4+
2	A (G-1)			
	625 μg/mL	0	2+	0
	1250 μg/mL	-[a]	2+	-[a]
	2500 μg/mL	-[a]	1+	-[a]
3	E (G-2)			
	625 μg/mL	0	1+	1+
	1250 μg/mL	0	1+	0
	2500 μg/mL	-[a]	0	-[a]

[a] Antimicrobial activity was not tested.

Grading the growth of microorganisms, 4+ (100%), 3+ (75%), 2+ (50%), 1+ (25%), and no growth 0 (0%).

Table 4. Adsorption of metals by chitosan (Mn = 150 kD) and its derivatives at pH 7[a]

Entry	Compound	Cd (%)[b]	Cu (%)[b]	Ni (%)[b]
1	Chitosan	10.0	9.5	8.1
2	A (G-1)	15.1	11.3	10.3
3	B (G-1)	13.5	10.8	11.3
4	E (G-2)	16.0	11.6	9.7
5	F (G-2)	14.5	12.1	11.2

[a] Copper, cadmium, and nickel sulphate solutions were passed slowly through the columns [glass tubings (ø = 0.6 cm), which were packed with samples (100 mg)].

[b] Determined by inductively-coupled plasma (ICP) analysis.

Adsorption of metals

The adsorption/chelation of metals by chitosan and chitosan derivatives was investigated at pH 7 (Tables 4 and 5). Sulphate solution was used in this study since it has been reported that the metal uptake was higher from sulphate solution than solutions of chloride and nitrate, when nickel(II) and cadmium(II) are offered separately (Qin et al., 2004). In addition, sulphate anion differs from chloride and nitrate by its higher charge so it may be more effective in ionic binding. It was found that the new chitosan derivatives show good coordination ability to metals and have higher affinity to cadmium(II) than to nickel(II) and copper(II). The chelating behavior, with heavy metals, of lower molecular weight chitosan (50 kD) is slightly better than that of higher molecular weight chitosan (150 kD) and its derivatives.

Table 5. Adsorption of metals by chitosan (Mn = 50 kD) and its derivatives at pH 7[a]

Entry	Compound	Cd (%)[b]	Cu (%)[b]	Ni (%)[b]
1	Chitosan	11.2	10.8	9.4
2	C (G-1)	17.0	12.1	11.2
3	D (G-1)	19.4	14.0	11.7
4	G (G-2)	15.0	14.0	12.3
5	H (G-2)	15.6	14.5	11.1

[a] Copper, cadmium, and nickel sulphate solutions were passed slowly through the columns [glass tubings (ø = 0.6 cm), which were packed with samples (100 mg)].

[b] Determined by inductively-coupled plasma (ICP) analysis and thermo gravimetric analysis (TGA).

Conclusions

In this study, we present the successful preparation of the new chitosan derivatives containing hyperbranched-N-carboxymethyl groups by a robust method. Their higher water solubility and antimicrobial activities compared to the original chitosan at neutral pH range were demonstrated. These properties lead to varieties of potential usages for human safety against bacterial contamination. The new chitosan derivatives displayed improved chelation properties compared to the starting chitosan. They can be therefore applied for discarding heavy metal waste in the environment.

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