รายงานวิจัยฉบับสมบูรณ์

โครงการ การปรับปรุงคุณสมบัติในการละลายน้ำและจับโลหะของไคโตซานให้ดีขึ้นโดยการต่อเดนดริเมอร์เข้ากับไคโตซาน

โดย ดร.รัตนา ฉันทเตยานนท์และคณะ

มิถุนายน 2552

สัญญาเลขที่ **MRG 4980123**

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

Project Code : **MRG4980123**

Project Title : Enhanced Solubility and Chelation Effects via Tethering of Dendrimers to Chitosan

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Abstract

In this work, an efficient and simple method to graft a vinylsulfonic acid sodium salt on a poorly water soluble chitosan is described. Commercially available low molecular weight chitosan is converted to water-soluble chitosan containing hyperbranched-vinylsulfonic acid sodium salt groups. The process comprises the following steps: Michael addition of methyl acrylate, amidation with ethylenediamine and Michael addition of vinylsulfonic acid sodium salt. A variety of chitosans containing vinylsulfonic acid sodium salt, with improved water solubility, is synthesized by repeating these three steps. The new chitosan derivatives show better antimicrobial activity against *Micrococcus luteus* ATCC 10240 and *Achromobacter xylosoxidans* ATCC 2706. In addition, they display better chelating behavior with heavy metals, like cadmium(II), copper(II), and nickel(II), than the starting chitosan.

Keywords: chitosan; solubility; antimicrobial activity; heavy metals

รหัสโครงการ : **MRG4980123**

ชื่อโครงการ : การปรับปรุงคุณสมบัติในการละลายน้ำและจับโลหะของไคโตซานให้ดีขึ้นโดยการต่อเดนดริเมอร์เข้ากับไคโตซาน

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บทคัดย่อ

ในงานวิจัยนี้ได้บรรยายถึงวิธีการต่อเกลือโซเดียมไวนิลซัลโฟนิกแอซิดเข้ากับไคโตซาน ซึ่งตามปกติจะละลายน้ำได้น้อยมาก ขั้นตอนการเตรียมนั้นมีด้วยกัน 3 ขั้นตอน เริ่มจากการนำไคโตซานที่มีน้ำหนักโมเลกุลต่ำและสามารถหาซื้อได้มาทำปฏิกิริยาไมเคิลแอดดิชันของเมทิลอะไครเลต ตามด้วยปฏิกิริยาอะมิเดชันกับเอทิลีนไดเอมีน จากนั้นจึงทำปฏิกิริยาไมเคิลแอดดิชันกับเกลือโซเดียมไวนิลซัลโฟนิกแอซิด อนุพันธ์ไคโตซานที่ประกอบด้วยเกลือโซเดียมไวนิลซัลโฟนิกแอซิดหลายชนิด สามารถเตรียมได้จากการทำปฏิกิริยาทั้งสามขั้นตอนนี้ซ้ำอีก และจากการทดสอบความสามารถในการละลายน้ำ ต้านเชื้อจุลินทรีย์ และจับโลหะของอนุพันธ์ไคโตซานเหล่านี้พบว่า อนุพันธ์ไคโตซานเหล่านี้สามารถละลายน้ำได้ดีขึ้นอย่างเห็นได้ชัด และมีความสามารถในการต้านเชื้อจุลินทรีย์ *Micrococcus luteus* ATCC 10240 และ *Achromobacter xylosoxidans* ATCC 2706. ได้ดีกว่าไคโตซานตั้งต้น นอกจากนี้ยังมีสมบัติในการจับโลหะหนัก เช่น แคดเมียม คอปเปอร์ และนิกเกิลได้ดีขึ้นเมื่อเปรียบเทียบกับไคโตซานตั้งต้น

Keywords: chitosan; solubility; antimicrobial activity; heavy metals

1. บทนำ

Chitosan is a polymer obtained by deacetylation of one of the most abundant biopolymers, chitin. Chitin is found widely in nature in the shells of shrimp and crab, and in the cuticles of insects. Chitosan is nontoxic and biodegradable so it is environmentally friendly.[1-5] Chitosan is composed of repeating units of anhydro-N-acetyl-D-glucosamine and anhydro-D-glucosamine (the latter has higher proportion as shown in Figure 1).

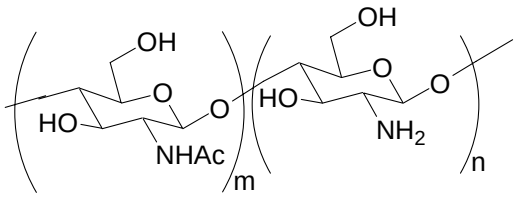


Figure 1 Chitosan (n > m)

Since Allan and Hadwiger found the antibacterial activity of chitosan in 1979, many continued studies have been made in this field.[1] Nevertheless, this application of chitosan as antibacterial agent is limited because of its water-insolubility. In addition, public concern over environmental problems has led to the minimization of waste in industry. Toxic metals, such as nickel and cadmium, which are often found in wastewater and appear to be an environmental health threat, have to be strictly controlled. Chitosan can bind to many metals owing to the presence of primary and secondary hydroxyl- and free amine- groups, which act as donor atoms. They have been thus attracted the attention of industry regarding the possible applications of chitosan in water and waste treatment. However, because the solubility and chelation behavior of chitosan are variable, this polymer is not seriously considered for use in industry.

Dendrimers are monodispersed polymers and have attracted great interest in the last 30 years. A significant advantage in the construction of dendrimers is the ease with which molecular size and shape can be controlled. The highly branched structure of dendrimers can be adjusted to modify their physical and electronic properties. There are therefore many potential applications of dendrimers, including the modification of chitosan. In recent work, dendrimers were connected to chitosan, yielding supported chitosan with improved water-solubility.[6-8] Although, hyperbranched chitosans, with

improved water-solubility, have been reported, there is a little study on the application of them, indicating a gap in the field, which underscores the potential significance of the present work.

2. **วัตถุประสงค์**

The objective of this work is to prepare dendrimers built on chitosan, which have improved water solubility. In addition, they are expected to exhibit a better antimicrobial activity and better chelating behavior with metals than the starting chitosan.

3. วิธีการทดลอง แบ่งออกเป็น 5 ขั้นตอน

ขั้นตอนที่ 1. Compounds **1**, **2**, and further partial hydrolysis of chitosan were prepared according to the procedure described in the literature.[8]

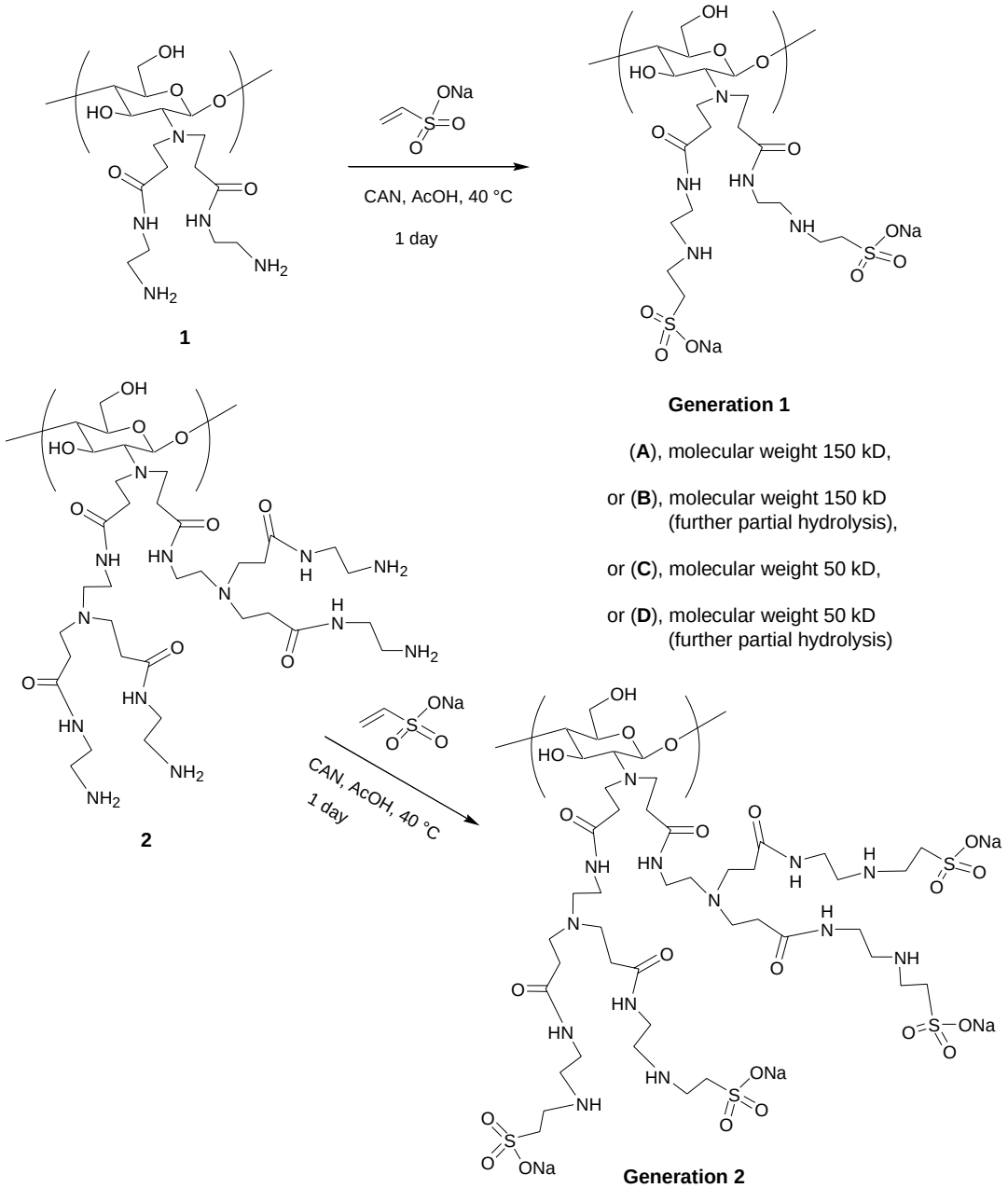
Synthesis of compound 1

A suspension of chitosan and methyl acrylate in methanol was stirred at 50 $^{\circ}$C under nitrogen for 3 days. The resulting mixture was cooled and the product was isolated by filtration and successive washing with methanol (at least five times) and dichloromethane (at least three times) until no impurities were left and dried *in vacuo* to afford compound **1**.

Synthesis of compound 2

Compound **1** and ethylenediamine in methanol were stirred at 50 $^{\circ}$C under nitrogen for 5 days. The suspension was filtered and the collected precipitate was washed with methanol (at least five times) and dichloromethane (at least three times) until no impurities were left and dried *in vacuo* to afford compound **2**.

ขั้นตอนที่ 2. Generation one dendrimer (**G-1**) (**A**), (**B**), (**C**) or (**D**) and generation two dendrimer (**G-2**) (**E**), (**F**), (**G**) or (**H**) were prepared by a similar strategy as described in the literature, illustrated in Scheme 1.



Scheme 1 Preparation of Chitosans Containing Hyperbranched-Vinylsulfonic Acid Sodium Salt (**A**), (**B**), (**C**), (**D**), (**E**), (**F**), (**G**), and (**H**)

Synthesis of a generation one dendrimer (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)

A modified literature procedure was used.[9] A solution of ceric ammonium nitrate (CAN) (5.9 mg) in 1 N HNO_3 (10 mL) was added dropwise to a solution of chitosan containing dendritic polyamidoamine (2 g) and 30 wt % vinylsulfonic acid sodium salt (4 mL) in 0.5 wt % acetic acid (100 mL) at 40 $^{\circ}$C under nitrogen. After the addition was complete, the solution was kept at that temperature for 1 day. The reaction mixture was cooled to room temperature and precipitated in acetone. The precipitated solid was isolated by filtration and successive washing with methanol at least five times and dichloromethane several times until no other polymers or impurities were left, and dried *in vacuo* to afford generation one dendrimer (**G-1**) as a pale brown powder.

Synthesis of a 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)

A modified literature procedure was used.[9] A solution of ceric ammonium nitrate (CAN) (5.9 mg) in 1 N HNO_3 (10 mL) was added dropwise to a solution of chitosan containing dendritic polyamidoamine (2 g) and 30 wt % vinylsulfonic acid sodium salt (8 mL) in 0.5 wt % acetic acid (100 mL) at 40 $^{\circ}$C under nitrogen. After the addition was complete, the solution was kept at that temperature for 1 day. The reaction mixture was cooled to room temperature and precipitated in acetone. The precipitated solid was filtered, washed first with methanol at least five times and dichloromethane several times until no other polymers or impurities were left, and dried *in vacuo* to afford generation two dendrimer (**G-2**) as a pale brown powder.

ขั้นตอนที่ 3. Investigation of the water solubility of chitosan and chitosan derivatives generation one dendrimer (**G-1**) (**A**), (**B**), (**C**), and (**D**) and generation two dendrimer (**G-2**) (**E**), (**F**), (**G**) and (**H**).

General procedure for estimation of water solubility

The chitosan derivatives (30 mg) were dispersed in water (10 mL) for 48 h and the pH of the suspensions was adjusted with 0.1 M HCl or 0.1 M NaOH. The solubility was determined at pH 5-9. The chitosan, which was not dissolved, was filtered, dried *in*

vacuo, and then the weight was determined. The aqueous solution was also evaporated, under reduced pressure, to afford the amount of dissolved chitosan. For each sample, at least two measurements were averaged in order to minimize the measurement error.

ขั้นตอนที่ 4. Investigation of the adsorption of metals by chitosan and chitosan derivatives generation one dendrimer (**G-1**) (**A**), (**B**), (**C**), and (**D**) and generation two dendrimer (**G-2**) (**E**), (**F**), (**G**) and (**H**).

General procedure for adsorption of metals at pH 7

Copper, cadmium, or nickel sulphate solutions (0.02 M, 10 mL) were passed slowly through the columns [glass tubings (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. The adsorption of metals by chitosan and chitosan derivatives was also determined by TGA**.** The values for the metal content obtained by TGA are slightly higher than those obtained by ICP (1-3%).

ขั้นตอนที่ 5. Investigation of the antimicrobial activity of chitosan and chitosan derivatives generation one dendrimer (**G-1**) (**A**), (**B**), (**C**), and (**D**) and generation two dendrimer (**G-2**) (**E**), (**F**), (**G**) and (**H**).

Antimicrobial activity

The antimicrobial activity of the chitosan derivatives was tested against Micrococcus luteus ATCC 10240, Candida albicans ATCC 90028, and Achromobacter xylosoxidans ATCC 2706 obtained from the Faculty of Medical Technology, Mahidol University, Thailand, using agar dilution method. 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 $^{\circ}$C for 24-48 h.

4. ผลการทดลอง และ บทวิจารณ์

Water solubility

The water solubility of starting chitosan (M_n = 150 kD and M_n = 50 kD) and chitosan containing vinylsulfonic acid sodium salt at various pH values was shown in Figure 2 and 3, respectively.

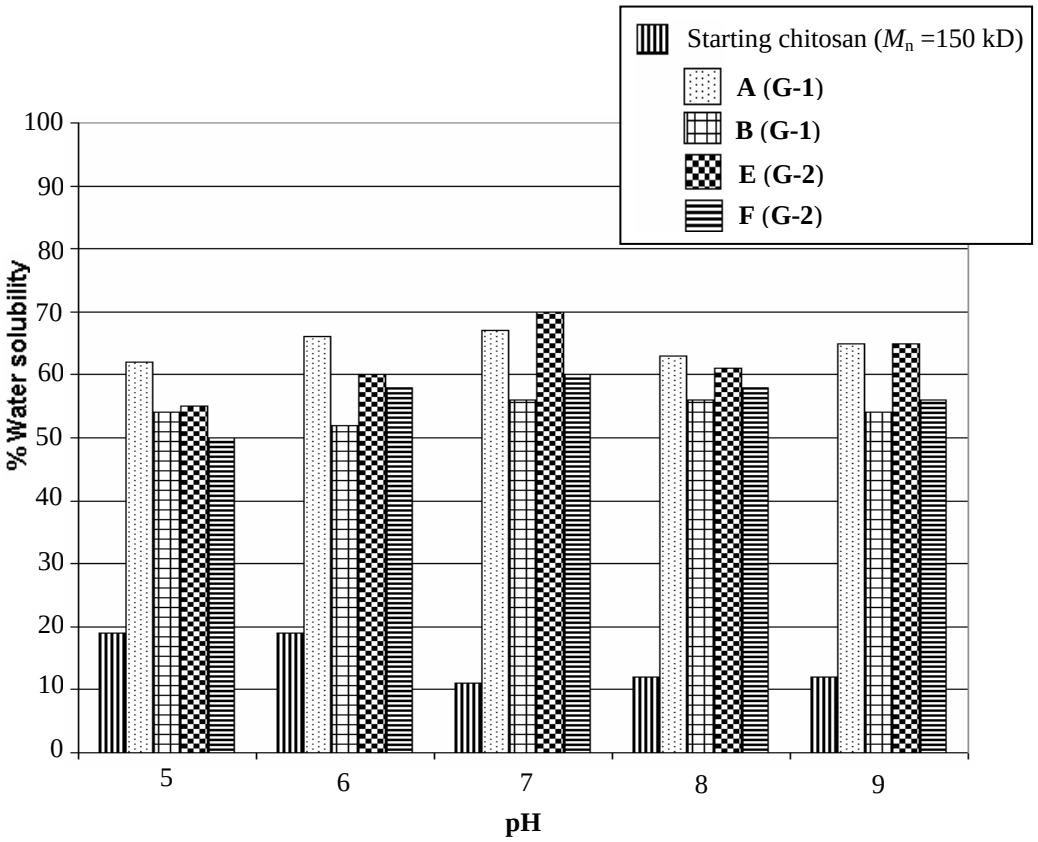


Figure 2 The water solubility of chitosan (M_n = 150 kD) and chitosan containing hyperbranched-vinylsulfonic acid sodium salt at various pH values.

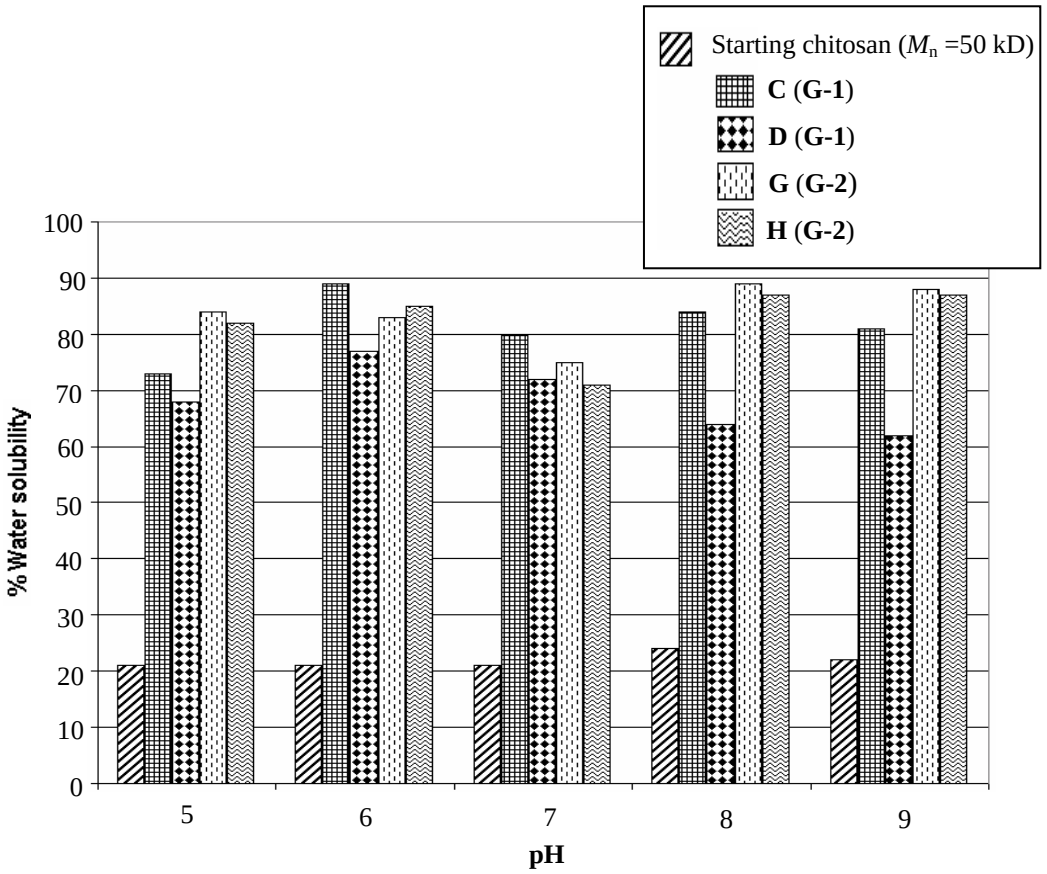


Figure 3 The water solubility of chitosan (M_n = 50 kD) and chitosan containing hyperbranched-vinylsulfonic acid sodium salt at various pH values.

It was found that new chitosan derivatives containing vinylsulfonic acid sodium salt have significantly enhanced water solubility. These results clearly confirm that the vinylsulfonic groups increase the solubility of the chitosan. **G-1** and **G-2** have similar solubility properties, which might be due to the fact that the polarity is decreased by increasing the chain length, although the amount of the vinylsulfonic acid sodium salt groups in **G-2** is higher than in **G-1**. Attempting to enhance water solubility, further partial hydrolysis chitosan derivatives have been prepared but no significant change in the solubility was noticed (**A** and **B**, **C** and **D**, **E** and **F**, and **G** and **H**). Quite possibly, the further 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.

Adsorption of metals

The adsorption of metals by chitosan and chitosan derivatives was investigated at pH 7 (Tables I and II). The chelating behavior, with heavy metals, of lower molecular weight chitosan (50 kD) is better than that of higher molecular weight chitosan (150 kD) and its derivatives. The metal adsorption capacities of **G-2** are similar to those observed for **G-1**, which might due to the increasing steric hindrance when higher generation was prepared.

TABLE I

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)	10.1	10.8	8.2
3	B (G-1)	14.6	11.0	10.1
4	E (G-2)	15.7	11.0	11.2
5	F (G-2)	15.0	11.0	11.9

[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.*

TABLE II

Adsorption of Metals by Chitosan (*M*n = 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)	15.7	11.4	12.3
3	D (G-1)	16.0	17.4	11.7
4	G (G-2)	13.5	13.3	11.7
5	H (G-2)	15.8	16.4	12.0

[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.*

Antimicrobial activity

Antimicrobial activity was evaluated (Agar dilution) by observing the growth of microorganisms on Muller Hinton Agar (MHA at pH 5.75) and grading 4+ (100%), 3+ (75%), 2+ (50%), 1+ (25%), and no growth (0) (0%). Growth inhibition of chitosan derivatives was observed compared with starting chitosan (Tables III and IV). The results show that the new chitosan derivatives containing vinylsulfonic acid sodium salt perfectly inhibit the growth of *M. luteus* ATCC 10240 with minimum inhibitory concentration 625 µg/mL while both starting chitosans show no antimicrobial activity against *M. luteus* ATCC 10240. The antibacterial activities of new derivatives against *A. xylosoxidans* ATCC 2706 were improved with minimum concentration 1250 or 2500 µg/mL. This might be due to the fact that *M. luteus* ATCC 10240 is a Gram-positive bacteria which has a cell wall mainly composed of peptidoglycan layer, which has a lot of pores while *A. xylosoxidans* ATCC 2706 is a Gram-negative bacteria which has a cell wall that consists of thin peptidoglycan and an outer layer of lipoproteins, lipopolysaccharides and phospholipids

TABLE III

Antimicrobial Activity of Chitosan (*Mn* = 150 kD) and Its Derivatives

Entry	Compound	M. luteus	A. xylosoxidans
1	Chitosan		
	625 μg/mL	4+	4+
	1250 μg/mL	4+	4+
	2500 μg/mL	4+	4+
2	**A** (**G-1**)		
	625 μg/mL	0	4+
	1250 μg/mL	0	4+
	2500 μg/mL	-[a]	1+
3	**B** (**G-1**)		
	625 μg/mL	0	4+
	1250 μg/mL	0	1+
	2500 μg/mL	-[a]	1+
4	**E** (**G-2**)		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	4+
	2500 μg/mL	-[a]	0
5	**F** (**G-2**)		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	4+
	2500 μg/mL	-[a]	0

[a]Antimicrobial activity was not tested

TABLE IV

Antimicrobial Activity of Chitosan (*Mn* = 50 kD) and Its Derivatives

Entry	Compound	M. luteus	A. xylosoxidans
1	Chitosan		
	625 μg/mL	4+	4+
	1250 μg/mL	4+	4+
	2500 μg/mL	4+	4+
2	**C** (**G-1**)		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	1+
	2500 μg/mL	-[a]	1+
3	**D** (**G-1**)		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	1+
	2500 μg/mL	-[a]	1+
4	**G** (**G-2**)		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	4+
	2500 μg/mL	-[a]	1+
5	**H** (**G-2**)		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	4+
	2500 μg/mL	-[a]	1+

[a]Antimicrobial activity was not tested

5. สรุป

We have shown that the new chitosan derivatives containing vinylsulfonic acid sodium salt show markedly improved water solubility compared to chitosan at neutral pH range. The new chitosan derivatives also display improved antimicrobial activity and chelation behavior compared with the starting chitosan.

6. หนังสืออ้างอิง

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Preparation of New Water-Soluble Chitosan Containing Hyperbranched-Vinylsulfonic Acid Sodium Salt and Their Antimicrobial Activities and Chelation with Metals คาดจะตีพิมพ์ได้ใน Journal of Applied Polymer Science

ภาคผนวก

Preparation of New Water-Soluble Chitosan Containing Hyperbranched-Vinylsulfonic Acid Sodium Salt and Their Antimicrobial Activities and Chelation with Metals

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Preparation of New Water-Soluble Chitosan Containing Hyperbranched-Vinylsulfonic Acid Sodium Salt and Their Antimicrobial Activities and Chelation with Metals

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Abstract: In this work, an efficient and simple method to graft a vinylsulfonic acid sodium salt on a poorly water-soluble chitosan is described**.** Commercially available low molecular weight chitosan is converted to water-soluble chitosan containing hyperbranched-vinylsulfonic acid sodium salt groups. The process comprises the following steps: Michael addition of methyl acrylate, amidation with ethylenediamine and Michael addition of vinylsulfonic acid sodium salt. A variety of chitosans containing vinylsulfonic acid sodium salt, with improved water solubility, is synthesized by repeating these three steps. The new chitosan derivatives show better

Dendrimers and hyperbranched polymers can be synthesized by repetition of similar steps. The possibility of modifying their physical and electronic properties has led to many potential applications. Dendrimer-functionalized chitosan showed improved water-solubility,[10-16] compare with chitosan. The grafting of dendritic hyperbranched polyamidoamine onto the surface of chitosan powder has been reported (Fig. 2).[10]

Insert Figure 2 here

Chitosan can also bind to many metals owing to the presence of primary and secondary hydroxyl and free amine groups, which act as donor atoms.[17-23] These properties attracted the attention of industry regarding the possible applications of chitosan in water and waste treatment.[19-23]

We report the successful immobilization of vinylsulfonic acid sodium salt onto dendritic hyperbranched chitosan by a readily accessible method (Fig. 3). The new chitosan derivatives show improved water solubility as compared to the starting material. The antimicrobial activity and chelating behavior with cadmium(II), copper(II), and nickel(II) of these new derivatives are found to be better than starting chitosan.

Insert Figure 3 here

EXPERIMENTAL

Materials

All chemicals were obtained from Sigma-Aldrich and Fluka Chemical companies and were used without further purification. Low molecular weight chitosan was purchased from Sigma-Aldrich (degree of deacetylation = > 85%, *M*n = 50 kD) and Fluka Chemical (degree of deacetylation = 72%, *M*n = 150 kD) companies. Compounds **1** and **2** were prepared according to literature method.[10]

All reactions were carried out under an atmosphere of nitrogen. Solvents were purified and dried following standard procedures.[24]

ICP

Inductively-coupled plasma mass spectrometer (ICP-MS) is a well established analytical technique for characterization of trace metals. ICP sources are used to excite atom for atomic-emission spectroscopy and to ionize atoms for mass spectrometry. The ions are separated on the basis of their mass-to-charge ratio and a detector receives an ion signal proportional to the concentration in the sample. It is highly sensitive and capable of the determination of a range of metals and several non-metals at concentration below one part in 10^{12}.

ICP-MS analyses were carried out by Mahidol University. The amount of chemisorbed metals was determined by quantitative extraction at the end of the experiment. Modified chitosan (10-20 mg) was treated with 2 ml aqua regia (3/1 mixture of HCl/HNO_3) and heated for 15 minutes at 90°C. The solution was diluted to 5 ml with distilled water and analyzed by ICP-MS.

TGA

Thermogravimetric analysis (TGA) is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the

weight change that occurs as a specimen is heated. The measurement is normally carried out in air or in an inert atmosphere. It is usually used to determine characteristics of materials such as polymers, to determine the degradation temperatures, absorbed moisture content of materials, the level of inorganic and organic components in materials.

TGA was carried out on Metter Toledo TGA/SDTA 851^e. The thermograms were obtained at uniform heating rate of 20 °C/min in the temperature range 40-600 °C under nitrogen and in the temperature range 600-850 °C under oxygen.

IR

Fourier transform infrared (FT-IR) spectra were recorded on a Perkin Elmer System 2000FT-IR spectrometer by Chulabhorn Research Institute. Samples for IR were examined using a Universal Attenuted Total Reflectance, solid (UATR-solid).

^{13}C CP/MAS

^{13}C Cross polarization/magic angle spinning (^{13}C CP/MAS) NMR spectra were recorded on a Bruker DPX-300 spectrometer by National Metal and Materials Technology Center, Thailand.

Synthesis of a generation one dendrimer (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)

A modified literature procedure was used.[17] A solution of ceric ammonium nitrate (CAN) (5.9 mg) in 1 N HNO_3 (10 mL) was added dropwise to a solution of chitosan containing dendritic polyamidoamine (2 g) and 30 wt % vinylsulfonic acid sodium salt (4 mL) in 0.5 wt % acetic acid (100 mL) at 40 °C under nitrogen. After the

addition was complete, the solution was kept at that temperature for 1 day. The reaction mixture was cooled to room temperature and precipitated in acetone. The precipitated solid was isolated by filtration and successive washing with methanol at least five times and dichloromethane several times until no other polymers or impurities were left, and dried *in vacuo* to afford generation one dendrimer (**G-1**) as a pale brown powder.

Generation one dendrimer (**G-1**) (**A**) (2.8 g) IR (cm^{-1}) 3297, 2877, 1642, 1551, 1370, 1155, 1036, 898, 749; ^{13}C CP/MAS NMR δ (ppm) 174.7, 105.5, 84.2, 75.1, 61.2, 45.2, 38.4, 23.6.

Generation one dendrimer (**G-1**) (**B**) (2.9 g) IR (cm^{-1}) 3363, 2871, 1639, 1557, 1377, 1133, 1037; 895, 752; ^{13}C CP/MAS NMR δ ((ppm) 175.2, 105.4, 83.5, 75.6, 61.3, 58.2, 47.0, 39.7.

Generation one dendrimer (**G-1**) (**C**) (2.6 g) IR (cm^{-1}) 3287, 2885, 1643, 1552, 1377, 1156, 1035; 894, 751; ^{13}C CP/MAS NMR δ (ppm) 174.6, 98.4, 75.1, 61.8, 55.9, 38.5, 24.5.

Generation one dendrimer (**G-1**) (**D**) (2.6 g) IR (cm^{-1}) 3298, 2876, 1639, 1556, 1373, 1150, 1032; 897, 740; ^{13}C CP/MAS NMR δ (ppm) 175.6, 105.5, 84.2, 75.3, 61.3, 58.1, 38.7.

Synthesis of a 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)

A modified literature procedure was used.[17] A solution of ceric ammonium nitrate (CAN) (5.9 mg) in 1 N HNO_3 (10 mL) was added dropwise to a solution of chitosan containing dendritic polyamidoamine (2 g) and 30 wt % vinylsulfonic acid sodium salt

(8 mL) in 0.5 wt % acetic acid (100 mL) at 40 °C under nitrogen.. After the addition was complete, the solution was kept at that temperature for 1 day. The reaction mixture was cooled to room temperature and precipitated in acetone. The precipitated solid was filtered, washed first with methanol at least five times and dichloromethane several times until no other polymers or impurities were left, and dried *in vacuo* to afford generation two dendrimer (**G-2**) as a pale brown powder.

Generation two dendrimer (**G-2**) (**E**) (2.6 g) IR (cm^{-1}) 3282, 2873, 1635, 1543, 1370, 1153, 1031; 893, 744; ^{13}C CP/MAS NMR δ (ppm) 174.5, 105.2, 84.1, 74.9, 61.6, 46.8, 38.5, 23.8.

Generation two dendrimer (**G-2**) (**F**) (2.9 g) IR (cm^{-1}) 3273, 2887, 1647, 1543, 1373, 1154; 1030, 894, 750; ^{13}C CP/MAS NMR δ (ppm) 174.6, 104.8, 85.0, 75.3, 62.2, 58.1, 39.6.

Generation two dendrimer (**G-2**) (**G**) (2.7 g) IR (cm^{-1}) 3283, 3083, 2928, 1637, 1549, 1370, 1164; 1036, 894, 749; ^{13}C CP/MAS NMR δ (ppm). 174.7, 105.7, 84.8, 75.9, 55.2, 47.0, 36.6, 23.7

Generation two dendrimer (**G-2**) (**H**) (2.6 g) IR (cm^{-1}) 3284, 2867, 1636, 1549, 1367, 1136, 1110; 1042, 893, 760; ^{13}C CP/MAS NMR δ (ppm) 174.2, 105.3, 83.5, 74.9, 62.1, 47.2, 38.4.

General procedure for estimation of water solubility

The chitosan derivatives (30 mg) were dispersed in water (10 mL) for 48 h and the pH of the suspensions was adjusted with 0.1 M HCl or 0.1 M NaOH. The solubility was determined at pH 5-9. The chitosan, which was not dissolved, was filtered, dried *in vacuo,* and then the weight was determined. The aqueous solution was also evaporated, under reduced pressure, to afford the amount of dissolved chitosan. For

each sample, at least two measurements were averaged in order to minimize the measurement error.

Antimicrobial activity

The antimicrobial activity of chitosan and its derivatives was tested against *Micrococcus luteus* ATCC 10240 and *Achromobacter xylosoxidans* ATCC 2706 obtained from the Faculty of Medical Technology, Mahidol University, Thailand, using agar dilution method. *Micrococcus luteus* ATCC 10240 is a Gram-positive bacteria, which is an opportunistic pathogen that can compromise immune systems such as HIV patients.[25] *Achromobacter xylosoxidans* ATCC 2706 is a Gram-negative bacteria, which can cause some severe diseases in humans, especially in immunocompromised hosts.[26]

The tested compounds were individually mixed with Muller Hinton Broth (MHB), a medium containing beef infusion, peptone or casamino acids, and starch, to obtain a final volume of 2 ml. The microorganisms cultured in MHB at 37 °C for 24 h, were 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 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

Copper, cadmium, or nickel sulphate solutions (0.02 M, 10 mL) were passed slowly through the columns [glass tubings (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. The adsorption of metals by chitosan and chitosan derivatives was also determined by TGA**.** The values for the metal content obtained by TGA are slightly higher than those obtained by ICP (1-3%). The results shown in Table III, IV, and V are based only on 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 further partial hydrolysis of chitosan were prepared according to the procedure described in the literature.[10,27] Generation one dendrimer (**G-1**) (**A**), (**B**), (**C**) or (**D**) and generation two dendrimer (**G-2**) (**E**), (**F**), (**G**) or (**H**) were prepared by a similar strategy as described in the literature,[17] illustrated in Scheme 1.

Insert Scheme 1 here

FTIR spectroscopy was used as a tool for the determination of the successful binding to the chitosan. The characteristic absorption peaks are at 1723 cm^{-1} (ester group), 1635 cm^{-1} (amide group), and 750, 1030, and 1154 cm^{-1} (symmetric and asymmetric stretching S=O in sulfonate anion). Figure 4 shows IR spectra of the starting chitosan (Mn = 150 kD), methyl propylaminopropionate grafted chitosan, amidoamine grafted chitosan, and vinylsulfonic acid sodium salt grafted chitosan.

Insert Figure 4 here

The percentage of vinylsulfonic acid sodium salt grafted onto dendritic hyperbranched chitosans shown in Tables I and II was calculated using eq.2.

Insert Table I here

Insert Table II here

Insert Equation 2 here

All these reactions are chemioselective due to their characteristic difference in reactivity of primary amine and hydroxyl groups.[27] It was also found that compounds **1** and **2** could be prepared in higher yields of amidoamine grafting by using strategies which involved time-sequenced propagation techniques (increasing the reaction time for the Michael addition of methyl acrylate from 24 h to 3 days and that of the amidation with ethylenediamine step from 24 h to 5 days).[28]

Another preparation of **G-1** and **G-2** was also attempted, under heterogeneous conditions with longer reaction time (Scheme 2). The product could be easily

separated but the percentage of vinylsulfonic acid sodium salt grafting was much lower than that obtained under homogeneous conditions (10-15%). No reaction occurred in the absence of CAN under either condition.

Insert Scheme 2 here

Water solubility

The water solubility of chitosan and chitosan derivatives is shown in Figures 5 and 6. New chitosan derivatives containing vinylsulfonic acid sodium salt have significantly enhanced water solubility. These results clearly confirm that the vinylsulfonic groups increase the solubility of the chitosan. **G-1** and **G-2** have similar solubility properties, which might be due to the fact that the polarity is decreased by increasing the chain length, although the amount of the vinylsulfonic acid sodium salt groups in **G-2** is higher than in **G-1**. Attempting to enhance water solubility, further partial hydrolysis chitosan derivatives have been prepared but no significant change in the solubility was noticed (**A** and **B**, **C** and **D**, **E** and **F**, and **G** and **H**). Quite possibly, the further 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.

Insert Figure 5 here

Insert Figure 6 here

Antimicrobial activity

Growth inhibition of chitosan derivatives was observed compared with starting chitosan (Tables III and IV). The results show that the new chitosan derivatives containing vinylsulfonic acid sodium salt perfectly inhibit the growth of *M. luteus* ATCC 10240 with minimum inhibitory concentration 625 µg/mL while both starting chitosans show no antimicrobial activity against *M. luteus* ATCC 10240. The antibacterial activities of new derivatives against *A. xylosoxidans* ATCC 2706 were improved with minimum concentration 1250 or 2500 µg/mL. This might be due to the fact that *M. luteus* ATCC 10240 is a Gram-positive bacteria which has a cell wall mainly composed of peptidoglycan layer, which has a lot of pores while *A. xylosoxidans* ATCC 2706 is a Gram-negative bacteria which has a cell wall that consists of thin peptidoglycan and an outer layer of lipoproteins, lipopolysaccharides and phospholipids.[29-31]

Insert Table III here

Insert Table IV here

Adsorption of metals

The adsorption of metals by chitosan and chitosan derivatives was investigated at pH 7 (Tables V and VI). Sulphate solution was used in this study since it has been reported that the metal uptake is higher from sulphate solution than from solutions of chloride and nitrate, when nickel(II) and cadmium(II) are offered separately.[19] 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. The metal adsorption capacities of **G-2** are similar to those observed for **G-1**, which might due to the increasing steric hindrance when higher generation was prepared.

Insert Table V here

Insert Table VI here

The possibility of metal leaching, from the chitosan derivatives after the absorption with the metal, was examined. ICP analysis showed that the content of metal in the derivatives was not markedly decreased after stirring the derivatives in water at pH 7 for 3 days. It is possible that stable six-membered ring metallacycles could be formed as shown in figure 7.

Insert Figure 7 here

Waste water usually contains various metals, therefore, a study of the selective removal of cadmium(II), copper(II), or nickel(II) using chitosan derivatives was also undertaken. A control experiment was carried out by stirring chitosan derivatives (50 mg) in copper, cadmium, and nickel sulphate solutions (0.02 M, 10

mL) at pH 7. It was found that these chitosan derivatives have good selectivity for Cu^{2+}over Cd^{2+} and Ni^{2+} (e.g. 10.8 % for Cu^{2+}, 0.3 % for Cd^{2+} and 0.2 % for Ni^{2+}).

Since wastewater, which contains metal ions, is sometimes acidified; another control experiment for metal adsorption at pH 2 with further partially hydrolysed derivatives of chitosan was also carried out. It was found that chitosan derivatives have a higher efficiency than the starting chitosan, which the amino groups might be protonated in acidic aqueous solution (Table VII). Nonetheless, the metal uptake at pH 7 of the chitosan derivatives is higher than that of at pH 2, which suggests that some amino groups in the new derivatives are protonated in acidic aqueous solution as well.

Insert Table VII here

It is known that there is a shift in the absorption peak in the FTIR spectrum of a compound chelated with a metal. The characteristic peaks of amide and sulfonate groups of the chitosan derivatives were shifted. For example, for generation two dendrimer (**G-2**) (**H**), the amide peak at 1635 cm^{-1} and sulfonate peak at 750 cm^{-1} were shifted to 1628 and 736 cm^{-1}, respectively, after chelating with nickel(II). This result supports the fact that amide and sulfonate anionic groups are involved in chelate formation.

CONCLUSIONS

We have shown that the new chitosan derivatives containing vinylsulfonic acid sodium salt show markedly improved water solubility compared to chitosan at neutral

pH range. The new chitosan derivatives also display improved antimicrobial activity and chelation behavior compared with the starting chitosan.

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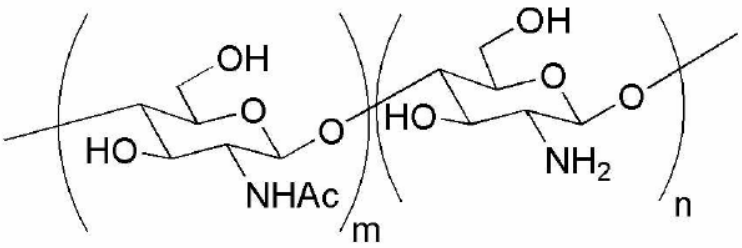

Figure 1 Chitosan (n > m)

71x31mm (300 x 300 DPI)

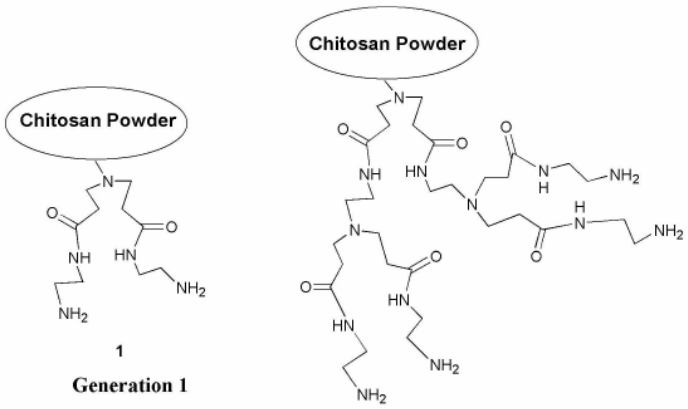

Figure 2 Theoretical illustration of polyamidoamine dendrimer grafted chitosan powder

150x104mm (300 x 300 DPI)

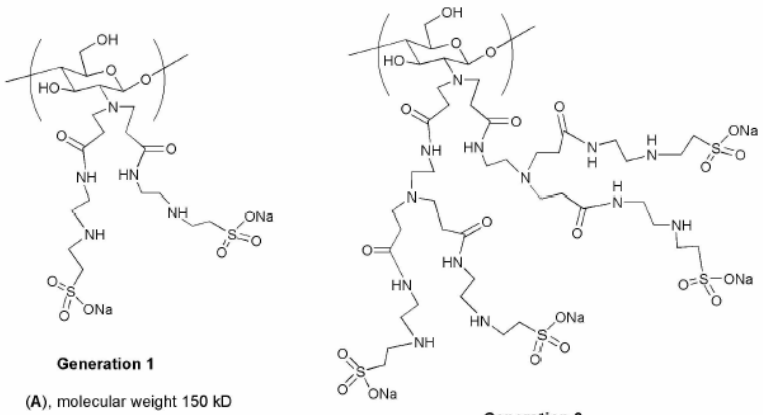

Figure 3 Vinylsulfonic acid sodium salt was grafted onto dendritic hyperbranched chitosan

165x147mm (300 x 300 DPI)

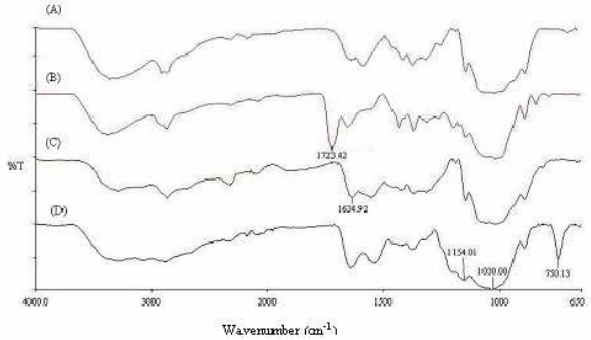

Figure 4 IR spectra of (A) starting chitosan (Mn = 150 kD), (B) methylpropylaminopropionate grafted chitosan, (C) amidoamine grafted chitosan, and (D) vinylsulfonic acid sodium salt grafted chitosan

150x103mm (96 x 96 DPI)

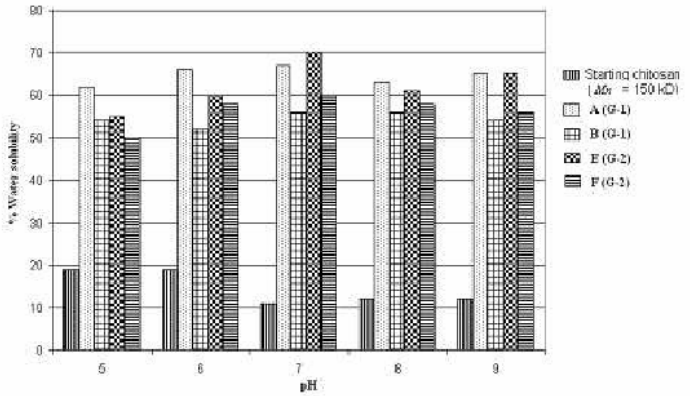

Figure 5 The water solubility of chitosan ($M\bar{n}$ = 150 kD) and chitosan containing vinylsulfonic acid sodium salt at various pH values

135x101mm (96 x 96 DPI)

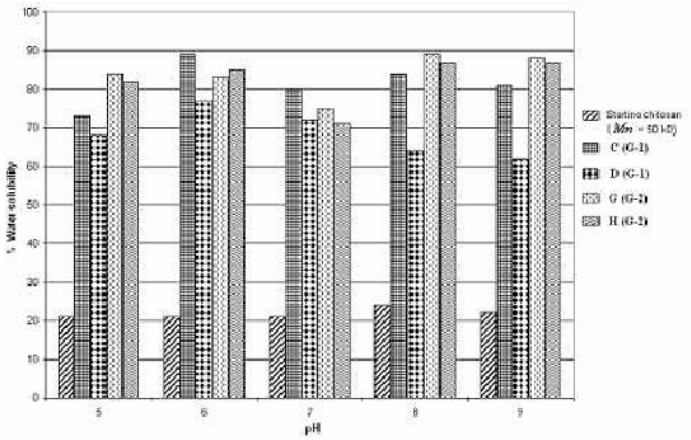

Figure 6 The water solubility of chitosan ($\bar{M}n$ = 50 kD) and chitosan containing vinylsulfonic acid sodium salt at various pH values

135x101mm (96 x 96 DPI)

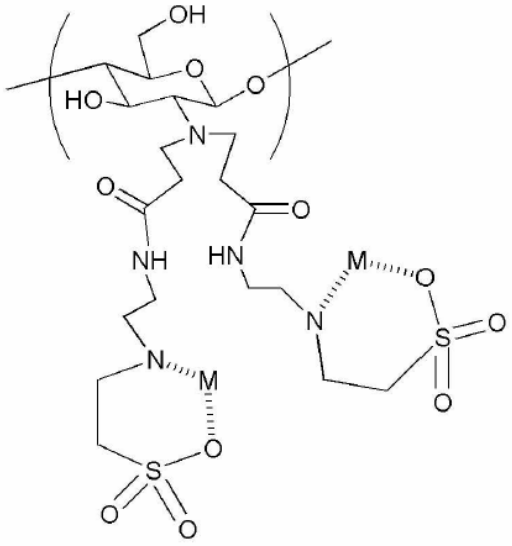

Figure 7 Postulated six-membered ring metallacycle

90x79mm (300 x 300 DPI)

TABLE I
Percentage of vinylsulfonic acid sodium salt grafting onto dendritic hyperbranched chitosan (M_n = 150 kD)

Entry	Compound	Vinylsulfonic Acid Sodium Salt Grafting (%)		
		Experimental Amount	Theoretical Amount	Yield
1	**A (G-1)**	40	56	71
2	**B (G-1)**	45	67	67
3	**E (G-2)**	30	56	54
4	**F (G-2)**	45	61	74

TABLE II
Percentage of vinylsulfonic acid sodium salt grafting onto dendritic hyperbranched chitosan (M_n = 50 kD)

Entry	Compound	Vinylsulfonic Acid Sodium Salt Grafting (%)		
		Experimental Amount	Theoretical Amount	Yield
1	**C (G-1)**	30	61	49
2	**D (G-1)**	30	67	45
3	**G (G-2)**	35	60	58
4	**H (G-2)**	30	61	49

TABLE III
Antimicrobial Activity of Chitosan (*Mn* = 150 kD) and Its Derivatives

Entry	Compound	M. luteus	A. xylosoxidans
1	Chitosan		
	625 μg/mL	4+	4+
	1250 μg/mL	4+	4+
	2500 μg/mL	4+	4+
2	**A (G-1)**		
	625 μg/mL	0	4+
	1250 μg/mL	0	4+
	2500 μg/mL	-[a]	1+
3	**B (G-1)**		
	625 μg/mL	0	4+
	1250 μg/mL	0	1+
	2500 μg/mL	-[a]	1+
4	**E (G-2)**		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	4+
	2500 μg/mL	-[a]	0
5	**F (G-2)**		
	625 μg/mL	0	4+
	1250 μg/mL	-[a]	4+
	2500 μg/mL	-[a]	0

[a]Antimicrobial activity was not tested

TABLE IV
Antimicrobial Activity of Chitosan (*Mn* = 50 kD) and Its Derivatives

Entry	Compound	M. luteus	A. xylosoxidans
1	Chitosan		
	625 µg/mL	4+	4+
	1250 µg/mL	4+	4+
	2500 µg/mL	4+	4+
2	**C (G-1)**		
	625 µg/mL	0	4+
	1250 µg/mL	-[a]	1+
	2500 µg/mL	-[a]	1+
3	**D (G-1)**		
	625 µg/mL	0	4+
	1250 µg/mL	-[a]	1+
	2500 µg/mL	-[a]	1+
4	**G (G-2)**		
	625 µg/mL	0	4+
	1250 µg/mL	-[a]	4+
	2500 µg/mL	-[a]	1+
5	**H (G-2)**		
	625 µg/mL	0	4+
	1250 µg/mL	-[a]	4+
	2500 µg/mL	-[a]	1+

[a]Antimicrobial activity was not tested

TABLE V
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)	10.1	10.8	8.2
3	B (G-1)	14.6	11.0	10.1
4	E (G-2)	15.7	11.0	11.2
5	F (G-2)	15.0	11.0	11.9

[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.*

TABLE VI

Adsorption of Metals by Chitosan (*M*n = 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)	15.7	11.4	12.3
3	D (G-1)	16.0	17.4	11.7
4	G (G-2)	13.5	13.3	11.7
5	H (G-2)	15.8	16.4	12.0

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

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

TABLE VII

Adsorption of Metals by Chitosan and Chitosan Derivatives at pH 2[a]

Entry	Compound	Cd (%)[b]	Cu (%)[b]	Ni (%)[b]
1	Chitosan (*M*n = 150 kD)	5.6	5.7	5.3
2	Chitosan (*M*n = 50 kD)	6.7	6.4	5.9
3	**B (G-1)**	12.4	8.9	7.0
4	**D (G-1)**	10.1	8.3	6.5
5	**F (G-2)**	9.0	8.9	7.0
6	**H (G-2)**	14.6	8.9	7.6

[a]*Copper, cadmium, and nickel sulphate solutions which were adjusted to the pH 2 with 0.1 M HCl were passed slowly through the columns [glass tubings (ϕ = 0.6 cm), which were packed with samples (100 mg)].*

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