CROSSLINKING REACTIONS OF CHITOSAN AND THEIR APPLICATIONS

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DECLARATION

*I hereby declare that the work presented in the thesis entitled "*Crosslinking reactions of chitosan and their applications*" submitted for Ph.D. degree to the University of Pune, has been carried out by me at the National Chemical Laboratory, Pune, under the supervision of Dr. A.J. Varma. The work is original and has not been submitted in part or full by me for any degree or diploma to this or any other University.*

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CERTIFICATE

Certified that the work incorporated in this thesis entitled "Crosslinking reactions of chitosan and their applications" submitted by Mrs. Kalpana Dhananjay Trimukhe, was carried out under my supervision. Such material as has been obtained from sources has been duly acknowledged in the thesis.

Dr. A.J. Varma

(Research Guide)

Dedicated to my mother

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Abstract of the thesis

Introduction:

Crosslinked polymers are a considered as a separate class of polymers on their own, due to very significant differences in properties from linear polymers. The effect of crosslinking a polymer is to render it insoluble in solvents, impart dimensional stability and after almost every property of the original linear polymers. Thus, the field of natural rubbers and synthetic elastomers, phenol-formaldehyde resins, epoxy resins etc. is some of the important classes of crosslinked polymers with specialized applications. Similarly, crosslinking of a linear polymer like polystyrene, a thermoplastic, by use of divinyl benzene, results in a resin with altogether different properties. In the field of biopolymers. For example starches natural polymers that are consumed as food by mankind. However, mild crosslinking of starch makes it stable to heat, pH change and thermal properties, and it is now suitable for canned food as well as for non-food uses such as papa coating/sizing, metal sequestering, etc.

In this dissertation we have reported new methods of crosslinking the biopolymers chitin and chitosan, so as to impart new structural and morphological features to the crosslinked properties of metal complexation, enzyme adsorption and thermal properties.

Chitin is a high molecular weight linear polymer of N-acetyl-D-glucosamine (N-acetyl-2-amino-2-deoxy-D-glucopyranose) units linked by β -D (1→4) bonds. It is the second largest biopolymer produced in nature; its deacetylation leads to the formation of chitosan. Chitin, chitosan and their derivatives have many current and potential uses because of the unusual combination of properties they passes which include toughness, biodegradability, and bioactivity, this combination makes these materials especially attractive. Chitosan is a useful material for applications in medicine, pharmacy, cosmetics, biotechnology, and agro-

chemicals. Chitosan and its derivatives have hemostatic, bacteriostatic, fungicidal, anti-cancer, and antichlolesteremic activities, as seen from published literature. However, these applications do not consume large amounts of chitosan, and there is severe need to develop the chemistry and applications for large scale use of this important biomaterial. One such important aspect of chitosan is its ability to bind a variety of cations. Therefore this research looks at developing crosslinked chitin and chitosan reactions to arrive at new polymers that are dimensionally stable and can complex cations. Research into new types of crosslinking reactions with chemical structure modification can lead to new and improved materials for specific cation complexation, material for protein immobilization, protein-purification, and elucidation of structure-property relationships. Therefore, research on this topic would be fruitful from a basic as well as applications point of view.

The work is presented in seven chapters. The outline of each chapter is as follows:

Chapter 1: Synthesis, applications and characterizations of crosslinked chitosan and their derivatives: A review

This chapter includes detailed literature search on crosslinked chitosan, different types of crosslinking agents, methods of crosslinking, metal complexation phenomena, varieties of applications and detailed characterization methods. The importance of research on crosslinked chitosan systems is brought out, due to the several potential applications.

Chapter 2: Complexation of heavy metals by crosslinked chitin and its deacetylated Derivatives.

This chapter describes the methods of crosslinking chitin/chitosan, and their detailed characterizations, followed by in depth study of metal complexations. Thus, Chitin was crosslinked using diisocyanatohexane (HDI), trimellitic

anhydride (TMA), and dibromodecane (DBD), then deacetylated in strong aqueous alkali. This led to a product with amine functional groups on the exposed surface of the crosslinked chitin, which could be utilized for complexation with heavy metals. Thus, a key feature of the crosslinked derivatives prepared was that only the hydroxy groups were utilized in the crosslinking reaction, and the acetylamino groups of chitin were hydrolyzed only after the crosslinking was accomplished. This ensured that all amino groups of the chitosans so produced would be available for metal complexation, and not partially used up in crosslinking. This proposed advantage was proved by the similar binding observed for heavy metals like Hg (348-372 mg/g), Cu (91-119 mg/g), Zn (71-92 mg/g), Mn (3-10 mg/g), Cd (121-160 mg/g), and Pb (32-86 mg/g). Where as the control polymer (uncrosslinked chitosan powder) had complexation values for Hg (348-361mg/g), Cu (100-106 mg/g), Zn (81-92 mg/g), Mn (4-7 mg/g), Cd (135 mg/g), and Pb (25-59 mg/g). Additionally, in a case where chitosan was crosslinked with HDI, the amino groups were consumed in the crosslinking reaction, and the metal complexation capacity has found to be decreased for Cu (91-109 mg/g), Cd 133 mg/g), and Zn (71-77 mg/g), while remaining nearly the same for Hg (362 mg/g). The literature value for Cu complexation is 59.67 mg/g for chitosan crosslinked with glutaraldehyde. The crosslinked derivatives have the added advantage of insolubility even in low pH aqueous media, making their repeated re-use possible. Further, these crosslinked derivatives could be used in powder form, and the additional step of preparing beads was found to be not necessary for ease of separation of the crosslinked powder by filtration. The binding capacity of various crosslinked chitin and deacetylated derivatives for Cu, Cd, Hg, Zn, Mn, and Pb was in the region of 100, 140, 360, 88, 5 and 60 mg/g (rounded off values) of polymer respectively, very close to the values obtained for uncrosslinked chitosan. The metal binding for crosslinked chitosan was slightly lower than that of crosslinked chitin and deacetylated derivatives, due to use of some amino groups in crosslinking. For Cu ions, the Langmuir equation was found to be the best fit for HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin. The morphological studies conducted using

WAXRD are in close agreement with the metal complexation data, showing complete loss of original chitosan peaks for the heavily complexed derivatives, and minor changes for the weakly complexed metals. The details are also discussed in a publication.

Chapter 3: A morphological study of heavy metal complexes of chitosan and crosslinked chitosans by SEM and WAXRD.

Metal complexes of salts of Hg, Cu, Cd, Pb, Zn and Mn with chitosan and crosslinked chitosans were prepared, and their morphologies were studied using scanning electron microscopy and wide angle X-ray diffraction. The metal ions, which were specifically and strongly complexed to the amino functions of chitosans, like Hg, showed smooth surface morphology inspite of large number of ions complexed (360 mg/g of chitosan). The presence of metal ions on the surface of the chitosans could be detected with decrease in metal ion binding, in the following sequence Hg > Cu > Cd > Zn > Pb > Mn. Particularly in the case of Pb ions, the presence of these ions is clearly seen on the surface of the polymer by SEM. The numbers of ions of Mn complexed on the polymers were too few (5 mg/g of chitosan) to be visible. These results are also in agreement with the morphologies studies by WAXRD. The metal complexation data for each of these metal ions was also in the same sequence. The details also discussed in a forthcoming publication

Chapter 4: Metal complexes of crosslinked chitosans: Part II. An investigation of their hydrolysis to chitooligosaccharides using chitosanase.

This chapter investigates the behavior of crosslinked chitosans and metal-complexed crosslinked chitosans under similar hydrolytic conditions. Crosslinked chitosans with trimellitic anhydride, diisocyanatohexane, and dibromodecane as crosslinking agents under heterogenous reaction conditions, were used as metal complexing agents by equilibrating them with metal salts such as $ZnCl_2$, $MnSO_4$,

$CuSO_4$, $CdSO_4$, $Pb(NO_3)_2$, and $HgCl_2$. Crosslinked chitosan without metal complexation had the same hydrolytic behavior as uncrosslinked chitosan. However, when the crosslinked chitosans were complexed with metals, their hydrolytic rates and extent of hydrolysis was significantly reduced. Thus while for chitosan about 840 µg/ml reducing sugar was produced in 4 hours time, and 780 µg/ml was produced for diisocyanatohexane crosslinked chitosan, only 400 µg/ml and 320 µg/ml was produced for cadmium sulfate with crosslinked chitosan and diisocyanatohexane crosslinked chitosan, respectively. Similar results are obtained for other crosslinking agents. Studies on preincubation of the metal with the enzyme show that of the metals studied, Mn has no effect on preincubatioin with the enzyme, Hg, Cd, Pb, and Cu completely deactivate the enzyme, while Zn reduces the enzyme activity by about 43.3%. Preincubation of the metal salts with the chitosan shows that Hg and Cu completely deactivate the molecule from enzyme hydrolysis, Cd and Zn inactivate it to the extent of 56.8% and 43.3% respectively, while Mn has no effect. Availability of the amino functions seems to be a key feature for the chitosanase to hydrolyze the chitosan polymer. This was also proved by the significant increase in the extent of hydrolysis for chitosan samples with 88% (final value 1120 µg/ml reducing sugar) and 85% deacetylation (final value 840 µg/ml reducing sugar). HPIC studies of the products show a variety of oligomers are produced in the chitosanase enzyme hydrolytic reaction. The details are also discussed in a publication

Chapter 5: Environment friendly crosslinked chitosan as a matrix for selective adsorption and purification of lipase of *Aspergillus niger.*

A series of ten crosslinked chitosans were synthesized with the specific objective of investigating the structure-property relationships for enzyme interactions, especially lipases, which are industrially most useful enzymes. Chitosan and its derivatives have been used as affinity matrices for purification of lipase from *Aspergillus niger* NCIM 1207. Ten derivatives of chitosan and crosslinked chitosan were evaluated for adsorption of lipase out of which trimellitic

anhydride-crosslinked deacetylated chitin adsorbed lipase selectively, which was eluted easily with 0.1M phosphate buffer, pH 7.0. Approximately 500 units of lipase were adsorbed per gram of trimellitic anhydride-crosslinked deacetylated chitin and 70% of the activity was eluted with increase in specific activity (63.17). These results suggested that trimellitic anhydride-crosslinked deacetylated chitin is an excellent substrate to get approximately 5.17 fold purification of the crude lipase with 70% yield. The details are also discussed in a publication

Chapter 6: Thermal properties of chitosans, crosslinked chitosans and metal complexed chitosans.

Thermal properties of polymer systems are important parameters for evaluating the structure-property relationships of chemically modified polymers. The thermal analysis of the crosslinked chitosan and its derivatives and metal-complexed chitosan was studied using thermogravimetry. Effect of the complexed metal on the thermal behavior throws useful light on catalysis of its degradation at high temperatures. These aspects are discussed in this work.

Chapter 7: Conclusions and suggestions for further work.

This thesis seeks to present a comprehensive documentation of the studies on crosslinked chitin/chitosan as it appears in published literature, as well as a detailed investigation of a series of newly synthesized and characterized crosslinked derivatives incorporating special structural features such as availability of all amino functional groups on the exposed surface of the crosslinked chitosan. These new polymers were then investigated for their heavy metal binding properties and specificities, the morphologies of the complexed polymers, applications in enzyme purification, and for enzyme mediated hydrolysis to produce chitooligosaccharides. All these studies showed that crosslinked chitosan is an important area for research and development, with several possibilities for developing uniquely tailored crosslinked polymer

structures for specific applications. Further work on purifications of a variety of different enzymes and specific uptake of specific metals from a mixture of metal ions would be a fruitful area of further research.

CHAPTER 1

"Synthesis, applications and characterizations of crosslinked chitosans and their derivatives : A review"

1.1. Introduction:

Chitosan, the *N*-deacetylated polysaccharide derived from chitin [poly (*N*-acetyl-d-glucosamine)], is attracting ever increasing attention from scientists due to its ever increasing potential applications, including in such areas as a constituent of biodegradable polymer system constituent, pharmaceutical and biomedical engineering, paper and textile finishes, heavy metal chelation, wastewater treatment, and fiber and film formation (Brine, Sandford & Zikakis 1992; Rathke & Hudson 1994Wan Ngah, Endud & Mayanar 2002).

In particular, the presence of amine groups makes chitosan unique among biopolymers, due to which it solubilises in acidic solutions, exhibits cationic behavior in acidic solutions, and possesses strong affinity for heavy metal ions (Crini, et al., 2005; Guibal, et al., 2005; Crini, et al., 2006). Being inexpensive, chitosan can thus also be used to make ion-exchange materials on an industrial scale.These chitosan based exchangers or adsorbents also have potential applications in enzyme immobilization (Krajewska, et al., 2004; Juang, Wu and Tseng 2002), base catalysis (Reddy, Rajgopal, Maheshwari & Kantam 2006), protein separation and purification (Zeng, & Ruckenstein 1998(a); Zeng & Ruckenstein 1998(b); Trimukhe & Varma 2007), sorption of precious metal ions for recovery or as catalysts after reduction (Ruiz, Sastre & Guibal 2000; Vincent & Guibal 2002; Chassary, Vincent, Marcano, Macaskie & Guibal 2005; Katarina, Takayanagi, Oshima. & Motomizu 2006), wastewater treatment to remove heavy metal ions (Crini, et al., 2005; Jin & Bai 2002; Dantas, Neto, Moura, Neto, & Telemaco 2001; Boddu, Abburi, Talbott & Smith 2003; Li & Bai 2005; Li, Bai, & Liu 2005) and acidic dyes (Crini, et al., 2006; Chiou & Chuang 2006; Chiou, Ho & Li 2004; Wong, Szeto, Cheung, & McKay 2004). Chitosan are generally used in “bead” form, which enables its easy application in sorption columns, since this beads also provides the potential for regeneration after adsorption and therefore reuse of the beads in several subsequent adsorption cycles.

Thus it is clear that a majority of applications of chitosans are based on crosslinking it in order to overcome its solubility at low pH and to improve its mechanical strength and dimensional stability. This review compiles the various crosslinking agents, crosslinking methods, and applications of chitosans and seeks to bring out the importance of these materials for a wide range of applications in fields as diverse as materials for human implant, medicine and industrial waste water treatment.

1.2 Different types of crosslinking agents for chitosan and their applications

Hydrogels of natural polymers have attracted much interest because they are biocompatible, and can be tried as candidates for wound healing (Bourke, Khalili, Briggs, Michniak, Kohn & Warren2003), carriers for the release of drugs (Peh & Wong 1999; Gariepy, Shive, Bichara, Berrada, Garrec, Chenite & Leroux 2004), agriculture and food processing industry. Chitosan is currently receiving a lot of interest in application because it is non-toxic, biocompatible and biodegradable (Borzacchiello, Ambrosio, Netti, Nicolais, Peniche, Gallardo & San 2001). A number of hydrogels containing chitosan have been reported and tested for various applications (Gibson, Walls, Kennedy, & Welsh 2003; Shin, Kim, Park, Lee & Kim 2002). Physically crosslinked chitosan hydrogels were synthesized by grafting d, l-lactic acid (LA) and/or glycolic acid (GA) onto chitosan. The physical crosslinking was possible due to the hydrophobic side chains aggregation and intermolecular interactions through hydrogen bonds between side and main chains. The crystallinity of the original chitosan decreased after grafting. Differential scanning calorimetry (DSC) used for probing the states of water in the chitosan hydrogels revealed, three types of water in the samples, i.e. freezing water (namely free water), non-freezing water (namely bound water), and freezing bound water (Qu, Wirsen & Albertsson 2001).

However, the hydrogel beads have the disadvantage of poor mechanical strength which reduces the recycle life.To improve these properties, crosslinking of

chitosan beads with glutaraldehyde, epichlorohydrin, ethylene glycol glycidyl ether has and several other crosslinking agents commonly been used. (Denkbas, & Odabas 2000; Ruiz, Sastre, & Guibal 2000; Wan Ngah, Endud & Mayanar 2002; Hsien, & Rorrer 1997). These crosslinked chitosan beads can swell in enormously, with over 90% water content. These crosslinking reactions generally utilize the amino functional groups of chitosan through a schiff base reaction. Consequently, most of the amino functional groups of chitosan are not available after crosslinking (Li, Cheng & Yan 2007).

In the above reaction methodology, the cross-linking reduces the adsorption capacity of metal ions, although it enhances the resistance of chitosan against acid, alkali and other chemicals (Inoue, Baba & Yoshiguza 1993). The mechanical strength also improves. These newly developed properties are very important for an adsorbent, so that it can be used in a wide pH range. Crosslinking also can alter the crystalline nature of chitosan and positively effect sorption abilities (Koyama & Taniguchi 1986).

Due to the inherent toxicity of glyoxal, glutaraldehyde and epichlorohydrin, these crosslinking agents, their use for improving the function of chitosan films is severly restricted. Dialdehyde starch (DAS), a polymeric aldehyde obtained by the reaction of native starch with periodic acid, which has much lower toxicity, has been shown to be useful in such cases. For improved mechanical and water-swelling properties of chitosan films, a series of transparent films were prepared with dialdehyde starch as a crosslinking agent. Fourier transforms infrared and X-ray studies clearly showed that the formation of Schiff's base decreased the crystallinity of chitosan, while the mechanical properties and water-swelling properties of the films were significantly improved. All the crosslinked films still retained obvious antimicrobial effects toward *S. aureus* and *E. coli*, and they have potential for biomedical applications (Tang, Du & Fan 2003). Considering the importance of the states and mobility of water molecules in crosslinked chitosan polyether semi-interpenetrating network (IPN) hydrogel for applications in the

biomedical field, the state and mobility of water was studied using differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR), respectively. The results show that with the increase of water content, the mobility of water molecules and the free volume of hydrogel network are enhanced (Yao, Liu & Liu 1999).

Due to the crystalline nature of chitosan, the highly crystalline portions in the chitosan membranes resist uptake of water and, in turn, hinder hydroxide ion transport in the membranes. To reduce the crystallinity of the membrane, chemical crosslinking of the chitosan membrane carried out (Wan, Creber, Peppley, & Bui 2003).

The adsorption of reactive dyes and divalent metals from aqueous solutions, onto raw chitosan flakes was studied (Juang, Tseng, Wu & Lee 1997; Juang, Wu, & Tseng 1999; Tseng, Wu & Juang 1999; Wu, Tseng & Juang 1999). The adsorption capacity was quit low, only comparable to those obtained while using commercially available activated carbons. However, the adsorption capacity was enhanced by crosslinking of chitosan chains (Guibal, Saucedo, Jansson-Charrier, Delanghe & Le Cloirec, 1994; Inoue, Yoshizuka & Ohto 1999; Kondo, Matsumoto & Okamoto 1999; Koyama, & Taniguchi, 1986; Rorrer, Hsien & Way 1993).

Recently many investigations (Uragami, Matsuda, Okuno & Miyata 1994; Uragami, Kato & Miyata 1997; Nawawi, Ghazali, & Huang 1997; Volkov, Skirda, Vasina, Korotchkova, Ohya & Soontarapa 1998; Ren & Jiang 1998; Qunhui, Ohya & Negishi 1995) have been directed to chitosan as a pervaporation membrane material due to its extremely high affinity to water, good film forming properties, functional groups which are easy to modify, and good mechanical strength and chemical stability. Since polysulfone is one of the most common commercial UF materials, several studies of chitosan or chitosan blend/polysulfone composite pervaporation membranes have been carried out to

dehydrate ethanol-water mixtures (Shieh, & Huang 1997) and isopropanol-water mixtures (Nawawi, Ghazali & Huang 1997) and to remove ethylene glycol from aqueous systems (Feng, & Huang 1996).

Cell adhesion and proliferation studies have been carried out using dicarboxylic acid crosslinked chitosans as a function of molecular weight, since this affects the hydrophobic/ hydrophilic balance of the material. Thus low molecular weight chitosans were crosslinked with sebacic acid, and the mechanical and cell-interaction properties of the resultant materials were studied. Specially, the adhesion and growth of lamb aortic smooth muscle cells (SMC) on these materials was examined (Piparia, & Mathew 2006).

Spreading and proliferation of vascular smooth muscle cells exhibited no significant changes as a function of molecular weight of chitosan, but both were inhibited at high crosslink densities. The results obtained are affected by the presence of hydrophobic domains contributed by the sebacic acid crosslinker. Results clearly showed that sebacic acid crosslinking of reduced molecular weight chitosans provides a mechanism for modulating both mechanical and cell adhesion/proliferation properties (Piparia & Mathew 2006).

The uptakes of Cu (II) ions on chitosan beads were 80.71 mg Cu (II) /g chitosan, on chitosan-glutaraldehyde beads it was 59.67 mg Cu (II) /g chitosan-glutaraldehyde, on chitosan-epichlorohydrin beads it was nearly the same, i.e., 62.47 mg Cu(II) /g chitosan- epichlorohydrin, and similarly on chitosan-ethylene glycol diglycidyl ether beads the value was 45.94 mg Cu(II) /g chitosan- ethylene glycol diglycidyl ether. Bound Cu (II) ions could be removed from the chitosan and cross-linked chitosan beads rapidly by treatment with an aqueous EDTA solution. These beads could be regenerated and re-used to adsorb heavy metal ions (Wan Ngah, Endud & Mayanar 2002).

The research field of metallo-organic chelating compounds is inspired by perspectives of biomedical, ecological and industrial applications. Transition metal-chitosan complexes are also metallo organic chelates (Ravi Kumar, et al., 2000). The complexes of chitosan with 3d metals, such as Cu, Ni and Fe, have been investigated both experimentally (Gamblin, Stevens, Wilson 1998; Bhatia & Ravi 2000 & 2003) and theoretically (Braier, & Jishi 2000). Recent investigations of the chitosan with iron show that both amino ($-NH_2$) and hydroxyl (-OH) groups chelate Fe(III) ions and more than one polymer chain is involved in the formation of these complexes. It has been indicated that Fe(III) ions are either penta or hexa coordinated, and exhibit magnetic ordering at low temperatures, leading to insights into the magnetic coupling and spin structure of the crosslinked Fe–chitosan.

Chitosan crosslinked by difunctional crown ethers have been of interest to expand the range of metal ions that can be complexed and to synergise then individual metal complexing abilities. Two novel chitosan derivatives-crosslinked chitosan dibenzo-16-*c*-5 acetate crown ether (CCTS-1) and crosslinked chitosan 3,5-di-tert-butyl dibenzo-14-*c*-4 diacetate crown ether (CCTS-2) were synthesized. Results showed that the two crosslinked chitosan crown ether derivatives had not only good adsorption capacities for Pb^{2+}, Cu^{2+}, but also high selectivity for Pb^{2+}, Cu^{2+} in the presence of Ni^{2+} (Tan, Wang, Peng, & Tang 1999).

A new type of crosslinked chitosan was prepared using Dihydroxy azacrown ether as the crosslinking agent. Its adsorption properties for Ag^{+}, Cd^{2+}, Hg^{2+}, and Co^{2+} showed that the compound had good adsorption capacities and high selectivity for adsorption of Ag^{+} in the presence of Hg^{2+} and Co^{2+} (Yang, Zhuang, & Tan 2002).

In another study N-benzylidene chitosan (CTB) was synthesized by the reaction of benzaldehyde with chitosan (CTS). Chitosan-dibenzo-18-crown-6 crown ether bearing Schiff-base group (CTBD) and chitosan-dibenzo-18-crown-6 crown ether

(CTSD) were prepared by the reaction of 4,4-dibromodibenzo-18-crown-6 crown ether with CTB and CTS, respectively. These novel CTS derivatives were used for separating and concentrating heavy or precious metal ions in aqueous environments. The experimental results showed that CTBD had better adsorption properties and higher selectivity for metal ions than CTSD. For aqueous systems containing Pb^{2+}–Ni^{2+} and Pb^{2+}–Cu^{2+}, the selectivity coefficients of CTSD and CTBD were 24.4 for Pb/Ni, 41.4 for Pb/Cu, and 35.5 for Pb/Ni, 55.3 for Pb/Cu respectively (Wan, Wang & Qian 2002).

Azacrown ethers are new functional compounds; they have specific selectivity and stability for heavy or precious metal ions, but their aqueous solubility is so high that it cannot be recovered after use. In one study crosslinked chitosan (CCTS) was first prepared then amino groups in CCTS were reacted with epoxy-activated azacrown ethers, to obtain crosslinked chitosan azacrown ethers (CCAE-I, CCAE-II). Their adsorption properties for Pb^{2+}, Cu^{2+}, Cr^{3+}, Cd^{2+}, and Hg^{2+} were also investigated (Yang, Wang & Tang 1999).

One investigation was aimed at designing the nickel (II)-containing, cross-linked chitosan prearranged for specific binding of proteins having terminal histidines ehich had affinity for Ni. It was found that these Ni containing crosslinked chitosans were indeed useful for such specific binding of biomacromolecules (Alexeev, et al., 1999).

2, 2'-Iminodibenzoic acid-crosslinked chitosan, prepared by reacting 2, 2'-iminodibenzoic acid salt with crosslinked chitosan-Cl which was obtained by chlorination of crosslinked chitosan. The adsorptivity of Pb(II), Cu(II), Cd(II) was studied on this adsorbent. Experimental results for the adsorption and the recovery characteristics showed that the more pH increases, the more the amount of adsorbed metal ion increases. An optimum adsorption time was 1 h, and adsorption capacity increased in order $Cu^{2+}<Cd^{2+}<Pd^{2+}$, and recovery capacity increased in order $Cd^{2+}<Cu^{2+}<Pb^{2+}$ (Shim & Ryu 1998).

Using Cu(II) as template and ethylene glycol diglycidyl ether as crosslinking agent, a novel adsorption resin of chitosan condensed with salicylaldehyde was synthesized. The adsorption capacity of the resin for Cu(II) was 177.78 mg/g and selectivity coefficient KCu(II)/Fe(III) was 4.6. The resin could be reused after simple regeneration (Zheng, Xingle & Xianming 1999).

Toluene diisocyanate(TDI)-crosslinked chitosan and its adsorption of metal ions including Hg^{2+}, Cd^{2+}, Cu^{2+}, Cr^{6+}, Pb^{2+}, Zn^{2+}, and Ni^{2+} were investigated, The results showed that the crosslinked chitosan could be easily regenerated and had greater adsorption selectivity for Hg^{2+}, Cd^{2+}, Cu^{2+} in a solution containing Hg^{2+}, Cd^{2+}, Cu^{2+}, Cr^{6+}, Pb^{2+}, Zn^{2+}, and Ni^{2+} ions (An, Zhang, Zheng & Xu 1999).
Acrylonitrile was grafted onto epichlorohydrin-crosslinked-chitosan using $FeSO_4$-H_2O_2 as the initiator, followed by saponification treatmentto produce a water soluble chitosan graft copolymer. The graft copolymer had good adsorption capacities for Pb^{2+}, the adsorption rate for Cd^{2+} was higher than that for Cu^{2+} (Peng, Wang Cheng & Tang 1998).

Two types of epichlorohydrin-crosslinked chitosan-crown ethers (CCTS-BX and CCTS-BY) were prepared by the reaction of 4'-formyl benzo-15-crown-5 or 4'-formyl benzo-18-crown-6 with crosslinked chitosan (CCTS), were investigated for adsorption and selectivity properties for metal ions [Pd(II), Ag(I), Pb(II), Cd(II), Cr(III)]. The results showed that the two adsorbents have high selectivity for Pd(II) and Ag(I) in the coexistence of Pb(II) and Cr(III). The coefficients of CCTS-BX and CCTS-BY were KPd2+/Pb^{2+} = 9.9, KAg+/Pb^{2+} = 9.8, KPd2+/Cr^{3+} = Y and KPd2+/Pb^{2+} = 11.5, KAg+/Pb^{2+} = 7.1, KPd^{2+}/Cr^{3+} = Y, respectively (Wang, Peng, Tang, Yang & Luan . 1998).

A novel chitosan-supported sulfonic acid resin modified by propane sulfone was prepared for studying the adsorption characteristics of metal ions. In the low acidity region, the metal selectivity was similar to that of a control sample of

crosslinked chitosan. From this one can inter that the selectivity of sulfonic acid resin was attributed not to the sulfonate group but to the chitosan matrix. The role of the sulfonate lay in increasing the metal concentration in the neighborhood of the chitosan matrix. The adsorption equilibrium constants of metal ion on the two resins were evaluated, and the maximum adsorption capacity for the sulfonic acid resin in the case of adsorption of Cu was1.6 times that of crosslinked chitosan (Kondo, Nakagawa, Matsumoto, Yamashita & Furukawa 1997).

A new type chitosan resin modified by o-aminophenol was prepared which did not dissolve in water, acid and alkali.The absorption amount of resin on Cu(II) which was 2.95 mmol/g was higher than that of Pb(II) (1.95 mmol/g) and Ca(II) (Liu, Xiao, Du, Xu & Qin 2002).

In another study of chitosan microparticles, two types of chitosan microparticles (CMs) and their silver-complexed CMs (SCMs) were investigated as novel types of adsorbents for pesticide removal. It is known that silver can function as a disinfectant, and it is widely applied in water treatment and some medical fields (Matsunaka, 1998; Emsley, et al., 1991). It is well known that chitosan adsorbs significant amounts of Ag(I) (Inoue, Baba, Yoshizuka, Noguchi & Yoshizaki 1988; Trimukhe & Varma 2008). Besides, Ag(I) can easily coordinate with ligands containing thiophosphate group (Yoshizuka, Koba, Yasukawa & Inoue 1993; Yoshizuka, Miyazaki, Wasai, Baba, Inoue 1989), because Ag(I) is a soft acid and thiophosphate type ligands are a soft base, respectively. From the combination of these coordination mechanisms, we expect that Ag(I) can link between chitosan and pestiside methyl parathion to enhance the adsorption capacity of the pesticide.

The epichlorohydrin-crosslinked CM apparently showed superior Ag(I) adsorption over glutaraldehyde-crosslinked CM, The MP release from the MP-loaded SCMs occurred without Ag(I) leakage from the particles. It was found from the iteration of adsorption and release experiments of MP, that

glutaraldehyde-crosslinked SCM provides good reusability for several cycles (Yoshizuka, Lou & Inoue 2000).

The high affinity of chitosan for metal ions has also resulted in increasing interest to using it as a support for heterogeneous catalysis (Vincent, Peirano & Guibal 2004). For example, in the case of precious metal catalysts such as gold, palladium, or platinum, sorption capacities as high as 1–2 mmol metal g^{-1} can be reached with crosslinked (Guibal, Vincent, Larkin & Tobin 1999; Ruiz, Sastre & Guibal 2000). In the case of other metals, the sorption capacity can reach levels as high as 7–8 mmol metal g^{-1} (example for molybdate or vanadate sorption) (Guibal, Milot & Roussy 2000; Guzman, Saucedo, Revilla, Navarro, & Guibal 2002). While some of these metals can easily be desorbed by changing the pH variation (alkaline desorption for metal anions, and acid desorption for metal cations), some others remain are strongly bound to the chitosan. Above a pH of 1, the amounts of palladium and platinum that can be desorbed do not exceed a few percent, thereby ensuring the stability of the catalyst. These catalysts are also considered environmentally friendly since chitosan is a natural polymer, and at the end of the life cycle, the polymer can be thermally degraded to recover precious metals with minimum contamination effect compared to some common resins and synthetic polymers which are petrochemical based polymers.

A chitosan-supported palladium catalyst, prepared by the immobilization of palladium on glutaraldehyde crosslinked chitosan followed by in situ chemical reduction has been reported. This catalyst was successfully used for the degradation of 4-nitroaniline (4-NA) in the presence of sodium formate, (used as the hydrogen donor), giving 1,4-phenylenediamine as the desired product (Vincent, Peirano, & Guibal 2004).

Crosslinked chitosan was synthesized with chitosan and ethylene glycol diglycidyl ether. The cross-linked chitosan adsorbed mercury and precious metals (Pd, Pt, and Au) at pH values from acidic to neutral. Especially, mercury in

concentrated hydrochloric acids could be adsorbed on cross-linked chitosan quantitatively more than 97% of mercury was removed from commercially available HCl and the residual mercury was found to be 0.15 ppb. Mercury adsorbed on the cross-linked chitosan could be easily desorbed with an eluent containing 1 M hydrochloric acid and 0.05 M thiourea. Thus the crosslinked chitosan could be repeatedly used for the removal of mercury in hydrochloric acid (Oshita, Oshima, Gao, Lee & Motomizu 2002).

Since the platinum is used in automobile exhaust catalytic converters and used as a catalyst in a wide variety of processes such as nitric acid production and petroleum reforming. And soluble platinum compounds are toxic and chronic, industrial exposure to them is responsible for the syndrome called platinosis. The small particles for the brasion of the catalytic surface can penetrate deeply in human lungs and are toxic (Kanitsar, Koellensperger & Hann 2003; Wei & Morrison 1994). The determination of platinum metals is thus of increasing interest in medical and environmental samples.

This interaction has led to the development of a novel method for the separation and preconcentration of platinum with crosslinked chitosan (CCTS) and determination by graphite furnace atomic spectrometry (GFAAS). CCTS was synthesized by reacting chitosan with epoxy chloropropane. The adsorption rate of CCTS for Pt (IV) was 100% at pH 3-4, and could be eluted off from CCTS with 5 mL mixture of 0.1 M HCl and 3% thiourea and determined by GFAAS. The detection limit for Pt (IV) was 0.43ng /mL with very low standard deviation. This method was applied to environmental water samples, resulting in recoveries of 90-94% (Guang, Shahua, Ganquan, Yan & Shaobo 2005).

Glutaraldehyde-crosslinked chitosan is effective at removing hexachloroplatinate ions in dilute effluents, with the maximum capacity exceeding 280 mg/g (or 1.4 mmol g-1). Sorption isotherms were not influenced by particle size nor crosslinking ratio. The sorption behavior for platinum is found to be significantly

different to that observed with other metals, and is speculated to be due to the speciation of metal ions: platinum forms only mononuclear species in contrast with molybdate or vanadate polynuclear hydroylsed anions (Guibal, Larkin, & Gillet 1998).

The adsorption of Sb (III)-ammonium pyrrolidine dithiocarbamate (APDC) and Sb (V)-APDC on cross-linked chitosan (CCTS) has been studied, and its mechanism is discussed. The adsorption rates of Sb (III) and Sb (V) are 96% and 98%, respectively. A new method was developed for the determination of aqueous Sb (III) and Sb (V) by hydride generation. The detection limit was 60 mg/L (Jiang, Huang, Qian, Wang & Wan 1998).

Separation of germanium (Ge) with a crosslinked derivative of 1,2-propanediol modified chitosan was studied successfully.The crosslinked derivative was prepared by the reaction of chitosan with 3-chloro-1,2-propanediol and then crosslinked through chloromethyloxirane or ethylene glycol diglycidyl ester (Yoshinari, Yasuhiko, Seji, 1998).

Chitosan resins, with crosslinking agents, methanal, glyoxal and glutaraldehyde, the glyoxal and glutaraldehyde crosslinked chitosan resins were reduced with $NaBH_4$ afterwards were used in the adsorption tests with some model uremic middle mol. toxins and BSA in vitro; all three adsorbents demonstrated significant adsorption capability to the model toxins but little adsorption to BSA. $NaBH_4$ reduction added chemical stability to the resins (Wang, Yuan, Liu, Teng & He 2001).

The degree of crosslinking of chitosan in the free base form was a crucial faction in the selective adsorption of Mo, W, and V oxoanions as binuclear complexes for obtaining metal concentrations <50 ppb in column operation (Matejka, Ruszova, Parschova, Jelinek & Kawamura 2002).

The crosslinking of chitosan by glutaraldehyde aliphatic chain and its secondary reduction by NaBH3CN increased the adsorption properties of this polymer for pentachlorophenol. And several organochlorinated xenobiotics in freshwater. The organochlorinated hydrocarbon adsorption rate is higher for the chitosan derivative than for the unmodified chitosan. A non-woven filter based on a cross-linked chitosan derivative was also tested for recovering organochlorinated hydrocarbons from freshwater (Thome & Weltrowski 1997).

In an important study, chitosan microspheres (CMSs) containing water-soluble gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) were prepared by an emulsion method using Span 80 as a surfactant and glutaraldehyde (GA) as a crosslinker and was used for the gadolinium neutron-capture therapy of cancer. The content of gadolinium (Gd) in CMS was saturated at about 13%. The electrostatic interaction between chitosan and Gd-DTPA and the preferential surface-hardening by GA contributed to the formation of fine, spherical, Gd-enriched and prolonged-releasing CMSs with a mass median diameter of 1.9 mm, a Gd content of 6.1% (Saha, Jono, Ichikawa & Fukumori 1998).

A method to immobilize anaerobic sludge using chitosan has been developed, based on the reaction of basic NH2 groups of chitosan with the acidic sulfonic groups of lignosulfonate. This simple method confirmed the effectiveness of the immobilization technique for maintenance of long-term stability of the polymer, by studying continuous operation in anaerobic upflow sludge bed and filteration reactor (Tartakovsky, Petti, Hawari & Guiot 1998).

Sulfonated hydroxyethylated crosslinked chitosan beads, can be prepared by the reaction of hydroxyethylated crosslinked chitosan with ClSO3H. These resins can selectively absorb triglycerides. Adsorption expteriments with resin showed that this type of adsorbent could cut down the concentration of triglyceride in plasma by 76.9%, while the concentration of total protein (TP) decreased only by 2.6%.

This novel adsorbent has potential application in curing hypertriglyceridemia (Yu, Sun, He & Gu 1997).

Microspheres of chitosan crosslinked with three different crosslinking agents viz, glutaraldehyde, sulphuric acid and heat treatment were prepared to encapsulate diclofenac sodium (DS). Chitosan microspheres are produced in a w/o emulsion followed by crosslinking in the water phase by one of the crosslinking methods. Encapsulation of DS has been carried out by soaking the already swollen crosslinked microspheres in a saturated solution of DS. Among all the systems studied, the 32% glutaraldehyde crosslinked microspheres have shown the slowest release and the fastest release of is shown by heat crosslinking. Drug release from the matrices deviated slightly from the Fickian process (Kumbar, Kulkarni & Aminabhavi 2002).

In order to avoid the use of toxic crosslinking agents such as glutaraldehyde, a new method has been developed to crosslink chitosan by heat treatment to produce microspheres for the controlled release (CR) of diclofenac sodium (DS). The advantages of such CR formulations containing non-steroidal anti-inflammatory drugs (NSAID) over the conventional dosage forms have been reported; such formulations minimize the serious gastric irritant side effects of the conventional NSAID preparations (Kumbar, Kulkarni & Aminabhavi 2002).

The glutaraldehyde-crosslinked counterparts were used as a control. Histological study of the genipin-crosslinked chitosan microspheres injected intramuscularly into the skeletal muscle of a rat model showed a less inflammatory reaction than its glutaraldehyde-crosslinked counterparts. However, the degradation of genipin-crosslinked chitosan microspheres was not significant even after 20 weeks of implantation. Thus the genipin-crosslinked chitosan microspheres have a superior biocompatibility and a slower degradation rate than the glutaraldehyde-crosslinked chitosan microspheres. Therefore it can be concluded that the genipin-crosslinked chitosan microspheres may be a suitable polymeric carrier for long-acting injectable drug delivery (Mi, Tan, Liang & Sung 2002).

Using sulfonated hydroxyethyl crosslinked chitosan beads as adsorbents, the concentration of low density lipoprotein (LDL) in plasma could be cut by 79.7% maximum, while the concentration of high density lipoprotein (HDL) and total protein (TP) decreased only by 7.3% and 2.6% at least resp., so this type of adsorbent can be used to cure hyperlipidemia in the future (Yu, Gu, He, Zhen, Bai & Wang 1997).

The effectiveness of chitosan, as a scaffold of hepatocyte attachment, was investigated. Since chitosan gel was found to be too fragile by itself to use for cell culture, it was crosslinked by glutaraldehyde to improve its strength. Rat hepatocytes was anchored onto glutaraldehyde-crosslinked chitosan (GA-chitosan) gel in bead form released a very small amount of lactate dehydrogenase during the 5 day culture period, Whereas hepatocytes on a collagen-coated surface spread flat, and much more lactate dehydrogenase was released. Hepatocytes on glutaraldehyde-crosslinked chitosan also retained higher urea synthesis activity, a liver-specific function, than those anchored on the collagen-coated surface. Thus, chitosan appears to be a promising biopolymer as a scaffold of hepatocyte attachment, for application as an effective bioartificial liver support system (Kawase, Michibayashi, Nakashima, Kurikawa, Yagi & Mizoguchi 1997).

Crosslinking of chitosan powder was carried out with glutaric dialdehyde followed by modifying with phenylalanine and tryptophan, made to be beads and used for studying adsorption capacities for cholesterol. The adsorption capacity of the crosslinked chitosan bead was decreased, and that of the modified chitosan bead was increased compared with that of simple chitosan. The removal rate of lipoprotein cholesterol in serum was 46.1% by using the adsorbers (Liu, Pei, Li & Yin 1999). There has been considerable interest in recent years in developing controlled drug delivery systems by using biopolymers (Desai & Park 2005).

Chitosan microspheres are most widely studied drug delivery systems for the controlled release of drugs viz. antibiotics, anti-hypertensive agents, anti-cancer agents, anti-inflammatory agents, proteins, peptide drugs and vaccines (Sinha, Singla, Wadhawan, Kaushik, Kumria, Bansal & Dhawan 2004). Chitosan microspheres can be synthesized by a number of different techniques such as solvent evaporation, spray drying, coacervation, emulsification/internal gelation and suspension cross-linking (Sinha, Singla, Wadhawan, Kaushik, Kumria, Bansal & Dhawan 2004).

In one study chitosan microspheres crosslinked with three different crosslinking agents viz, tripolyphosphate, formaldehyde and gluteraldehyde have been prepared by the spray drying technique and the influence of these cross-linking agents on the properties of spray dried chitosan microspheres were investigated. Those microspheres cross-linked with tripolyphosphate exhibited higher swelling capacity, % water uptake, % erosion and drug release rate when compared to those cross-linked with formaldehyde and gluteraldehyde. The sphericity and surface morphology of the spray dried chitosan microspheres was lost when the cross-linking extent was increased from 1 to 2%w/w as was the release rate of the drug. The physical state of the drug in chitosan-tripolyphosphate, chitosan-formaldehyde and chitosan- gluteraldehyde matrices was confirmed by the X-ray diffraction study, where it was confirmed that the drug remains in a crystalline state even after its encapsulation (Desai & Park 2005).

Similar to Chitosan microsystems chitosan macroparticles can also be employed in a wide range of biomedical application, such as drug (Hejazi, & Amiji 2003; Sinha, Singla, Wadhawan, Kaushik, Kumria, Bansal, Dhawan 2004; Mitra, Gaur, Ghosh, Maitra, 2001) or gene-delivery systems (Borchard et al., 2001; Janes, Calvo, Alonso, 2001). There is a report of a method for the preparation of nanoparticles based on chitosan by covalently crosslinking the chitosan chain with natural di- or tricarboxylic acids through the amino group of chitosan in aqueous media at room temperature by using water soluble carbodiimide.

This method permits the formation of polycations, polyanions, and polyampholyte nanoparticles in the size range 60-280nm which was stable in aqueous media at a wide range of pH conditions. The biodegradable crosslinked chitosan nanoparticles, as solutions or dispersions in aqueous media, are potentially useful for various biomedical applications (Magdolna Bodnar, Hartmann & Borbely 2005).

Crosslinked chitosans have also become a useful aid in brachytherapy. In one study crosslinked chitosan implants were investigated as potential biodegradable devices for brachytherapy. It is concluded that hydrogels made of crosslinked chitosan are potential novel, safe, degradable devices for brachytherapy (Azab, Orkin, Doviner, Nissan, Klein, Srebnik & Rubinstein 2006).

In an interesting study of the application of crosslinking of chitosan, the preparation of crosslinked chitosan resins with a high hydrophilicity backbone and better adsorption capacities for bilirubin was reported. The factors which affect the adsorption for bilirubin are also discussed.

As is well known, bilirubin (BR), a metabolite of heme in senescent red blood cells, is normally conjugated with albumin to form a water-soluble complex (J. Donald Ostrow (Ed), 1986; Broderson, & Brown 1982) and liver damage and related malfunctions can result in hyperbilirubinemia (Seligman et al., 1997). Excess free bilirubin (unconjugated bilirubin) tends to deposit in tissues, especially in the brain.

Various other materials, such as activated charcoal polar polymers and nonpolar polymers have been used for the adsorption of bilirubin. Activated charcoal is nonselective and can adsorb a variety of substances from the blood. It can also cause the loss of platelets.

Other adsorbents that have been tested for the adsorption of bilirubin include: (a) Amberlite XAD-2 and AR-l; (b) XAD-7; (c) agarose beads, or Sephadex (crosslinked dextran); (d) Ionex; (e) PAT (polyamine-triglycidyl isocyanurate).
By the reaction of glutaric dialdehyde with chitosan, a new type of resin was prepared which had high adsorption capacity for bilirubin and good blood compatibility (Yu, & He 1996).

Purification of proteins by removing endotoxins from albumin (Adachi, Ida & Hashimoto 1994) and chromatographic purification of lysozyme (Itagaki, Ishikawa & Ahiko, 1993) have been reported. Copolymers of chitosan with maleic anhydride and acrylamide or 1-vinyl-2-pyrrolidinone have been used for immobilizing enzymes (Berkovich, Tsyurupa, Davankov, Rogozhin, Gamzazade & Davidovich 1980).

Smooth, highly spherical, crosslinked chitosan microspheres loaded with progesterone, were prepared by glutaraldehyde crosslinking, of chitosan containing progesterone in a non-aqueous dispersion medium. The extent of drug release was highly dependent on the crosslinking density of the microspheres, the highly crosslinked spheres releasing only around 35% of the incorporated steroid in 40 days compared to 70% from spheres lightly crosslinked (Jameela, Kumary, Lal & Jayakrishnana 1998).

Some disadvantages owing to structural incompatibility of the two polymers, were noticed, when excessive swelling at the feed mixture of high water content when contacted with the composite membranes were found to be segregated in structure. Efforts to enhance the structural stability under various pervaporation operational conditions were made. A solution to the problem was found by crosslinking the chitosan layer with glutaraldehyde and H2SO4 in acetone solution to control the permselectivity (Huang, Pal & Moon 1999).

Tripolyphosphate (TPP) is a non-toxic polyanion which can bond with chitosan via electrostatic forces to form ionic crosslinks. Tripolyphosphate (TPP) can be used for the preparation of chitosan beads and microspheres because of its quickly gelling ability (Mi, Shyu, Lee & Wong 1999).

Tripolyphosphate (TPP) was employed as an ionic crosslinker while genipin, an ingradient of some herbal medicines, was used as a chemical crosslinker to produce a mixed ionic and covalently bonded crosslinked chitosan.

A coupled ionic and chemical co-crosslinking mechanism was used to prepare novel chitosan gel beads had been used in herbal medicine, were employed, respectively. The energy profiles of carbon. It was formed that chemical crosslinking dominates the co-crosslinking reaction at higher pH condition (pH 7.0 and 9.0) and ionic crosslinking dominates the co-crosslinking reaction at lower pH condition (pH 1.0, 3.0 and 5.0). The unique properties of pH-dependent ionic/chemical co-crosslinked chitosan can be exploided for novel applions, and may also be suitable for biomedical applications (Mi, Sung, Shyu, Su & Peng 2003).

Microspheres made of chitosan, were investigated as a potential carrier for therapeutic proteins, peptides and plasmid DNA for administration to the lung from a pressurized metered dose inhaler (pMDI). Different cross-linking agents and additives were used to improve the physicochemical properties of chitosan microspheres for compatibility in a pMDI delivery system. The surface hydrophobicity of the glutaraldehyde cross-linked chitosan microspheres was significantly greater than non cross-linked or tripolyphosphate (TPP) cross-linked chitosan microspheres.The non cross-linked and the glutaraldehyde cross-linked chitosan microspheres were found to be potential candidates for carrying biotherapeutic compounds to the lung via a pMDI system (Williams III, Barron, Alonso, & Lopez 1998).

Synthetic crosslinking reagents are all highly cytotoxic that may impair the biocompatibility of the crosslinked biomaterials (Speer, Chvapil, & Eskelson, 1980; Nishi, Nakajima & Ikada 1995). It is, therefore, desirable to provide a crosslinking reagent suitable for use in biomedical applications that is not cytotoxic and may form stable and biocompatible crosslinked products.

Genipin and its related iridoid glucosides extracted from the fruits of Gardenia jasminoides Ellis have been used as an antiphlogistic and cholagogue in herbal medicine (Akao, Kobashi & Aburada 1994). Further genipin can spontaneously react with amino acids or proteins to form dark blue pigments (Touyama, Takeda, Inoue, Kawamura, Yatsuzuka, Ikumoto, Shingu, Yokoi & Inouye 1994; Fujikawa, Fukui & Koga 1987). It was found that genipin is about 5000–10,000 times less cytotoxic than glutaraldehyde. In addition, it was reported that the genipin-fixed tissue had a comparable mechanical strength and resistance against in vitro enzymatic degradation as the glutaraldehyde fixed tissue (Sung, Huang, Huang, Tsai & Chiu 1998). Therefore the feasibility of using genipin to prepare biodegradable chitosan microspheres for long acting drug delivery application was investigated.

Chitosan microparticles and nanoparticles have been made by chemical cross-linking with glutaraldehyde, glyoxal, and ethylene glycol diglycidyl ether. Although these are efficient cross-linking agent, they are a cause of physiological toxicity, and there fore are not used in many applions. Chitosan is polycationic in acidic media (pKa 6.5) and can interact with negatively charged species such as TPP and sodium sulfate. This characteristic can be employed to prepare crosslinked chitosan nanoparticles. The interaction of chitosan with TPP leads to formation of biocompatible crosslinked chitosan nanoparticles, which can be used for protein and vaccine delivery (Bhumkar & Pokharkar 2006).

Chitosan / poly (vinyl pyrrolidone) (PVP) were used to prepare semi interpenetrating polymeric networks. The hydrogels were crosslinked using

genipin, a non-toxic herbal crosslinking agent extracted from the fruits of Gardenia jasminoides Ellis (Khurma, Rohindra & Nand 2005).

In recent years, considerable information on the subject of oppositely charged polyelectrolyte-surfactant interactions has accumulated. As a rule, polyelectrolyte-surfactant systems display a complicated phase behavior. Along with the expected cooperative binding behavior, crosslinked polyelectrolyte gels generally show a gel collapse behavior and a change in the absorptive properties, resulting from to the formation of micelle-like surfactant structures within the gel (Bae & Hudson 1997).

Biodegradation of chitosan and glutaraldehyde (GA)-crosslinked chitosan hydrogels was investigated, and it was found that these can be degraded by a fungus Penicillium caseicolum. It was found that when the chitosan gel had a high degree of crosslinking, both the biodegradability as well as the biodegrdation rate was slow. Thus, it is possible to control the biodegrdation of chitosan gel systems by crosslinking.The ultimate biodegrdation results using a soil microorganism point to the potential for use of chitosan and crosslinked chitosan hydrogels in biotechnology (Yamamoto & Amaike 1997).

Unsymmetrical dimethylhydrazine (UDMH) is an important liquid propellant used in space technology as a fuel for launching rockets and space crafts (Sridhar, Susheela, Jayasimha Reddy & Khan 2001; Schmidt et al., 1984). Chitosan was fully stable in anhydrous UDMH and hence was selected for dehydration of the propellant, keeping in minds its highly hydrophilic nature and good mechanical strength.

A chitosan support material made with simultaneous crosslinking and attaching a spacer arm between the chitosan matrix and an epoxy terminal group at the distal end of the spacer arm, endowed the crosslinked chitosan beads with enhanced binding specificity during chromatographic separations. A crosslinked support

material (I; R = CH2CHOHCH2OZOCH2CHOHCH2NR1R2; R1, R2 = C1-5 alkyl; Z = alkylene, poly(oxyalkylene); n > 3) and a method of separating solutes, e.g., proteins, enzymes, cells, antibodies, etc., from process liquor using crosslinked-I beads as chromatographic medium was achieved (Roy, Todd & Glasser 1998).

In another study, porous chitosan scaffolds were prepared by freeze dried method using $Na_5P_3O_{10}$ as a crosslinking agent. Mouse embryonic stem (E. S.) cells could grow on these crosslinked scaffolds. The growth rate of E. S. cells was improved by post surface treatment of the scaffolds with collagen. These porous chitosan scaffolds are a promising approach for tissue engineering applications (Lin, Chen, Chen, Lee, Chiou, Chang & Wu 2007).

1.2 Table 1: Literature review of various crosslinking agents of chitosan and their applications

Crosslinking agent and structure	Brief method of crosslinking	Application of crosslinked	Characterization	Remarks	Reference
Glutaraldehyde (GLA) See scheme 1	Chitosan-NH_2 + GLA-CHO → Chitosan-GLA beads (N=C)	Cross. chitosan can be used as a resin in ion exchange chromatogra-phy	Cross. Chitosan was insoluble in acidic & alkaline medium as well as distd.water. Adsorption isotherms & desorption studied.		Wan Ngah et al., 2002
Glutaraldehyde	Chitosan was first modified by reaction with ammonium thiocyanate and chloroacetic acid. (Ratio is 1:1.2:1.2) the temp. is at 50°, and the reaction time is 2 h. The modified chitosan contg. S=C and COOH groups was crosslinked with glutaraldehyde soln. at pH 8 to form 33% crosslinked chitosan	Crosslinked chitosan has high removal efficiency for Cu2+ (98.10%).	IR spectrometry studied. Cu^{++} binding capacity has 98.10%	It has excellent removal efficiency for Cu2+.	Liu et. al., 2004
Glutaraldehyde	Chitosan was dissolved in a $Fe(NO_3)_2$ 0.1 M aqueous solution for 4 h. An orange precipitate was obtained after addition of acetone. The solid was filtered and washed with acetone and dried in vacuum. Solid was placed in contact with glutaraldehyde 15% (in acetone) solution for 2 hr. chemical crosslinking of chitosan with glutaraldehyde occurs by Schiff's reaction	Chitosan complex compound is inspired by perpectives of biomedical, ecological and industrial applications.	Adsorption of Fe(III) was 118 mg/g		Nedelko et al., 2006

Glutaraldehyde	A mixture of 17.2 g of chitosan solution and 2.8 g of PVA soln. was heated to 70 °C for 12 h. A homogeneous polymer gel blend solution with 2/1 (w/w) ratio of chitosan to PVA was obtained. At pH 2 the resulting blended solution (~18.45 g) was suspended in 100 ml of toluene–chlorobenzene (1/3, v/v) containing 1.5 g of Span-80 with stirring speed of 230 rpm, temp. was raised to 90 °C and 13.1 ml of water as an azeotrope with toluene was distilled out slowly through a Barrett distilling receiver. A 0.33 g of 50% glutaraldehyde soln. was added to the flask and the stirring continued at room temperature for 8 h. The resulting beads washed with acetone, suspended in 4% NaOH solution for 8 h, and then rinsed with water until neutral pH.		Gelation time increases when pH values increase from 1 to 3. However, gelation time dramatically decreases when pH values increase to 4 and 5. Mechanical strength: Crosslinked chitosan beads possessed low water content and high mechanical strength	Crosslinking done to increase the mechanical strength. The crosslinking of PVA with glutaraldehyde was catalyzed by acid	Li et al., 2007
Glutaraldehyde (GA)	100mg chitosan dissolved in acetic acid solution and heated at 100^0C. Various amount of 25% aqueous solution of glutaraldehyde was added with stirring. A gel was formed immediately stirring was stopped. Excess amount of	Degradable crosslinked chitosan hydrogels are potential platforms for implantation in anatomical or post surgical cavities formed after resection of malignant tumors, to	Eosin adsorption: Crosslinked density was measured. Higher the ratio of glutaraldehyde: chitosan the lower the amount of eosin adsorbed. DSC: Crosslinking	Higher the crosslinking reaction at glutaraldehyde: chitosan ratio 10: 1	Azab et al., 2006

	glutaraldehyde was removed by dialysis	deliver adjuvant local radiotherapy. Therefore it is convinent tool for brachytherapy.	increase the thermal stability and exothermic heat change of the chitosan gel.		
Glutaraldehyde	Purified chitosan and glycine were dissolved in acetic acid under stirring for 3 hrs. at room temperature. The homogeneous mixture was extruded in the form of droplets using a syringe into NaOH-methanol solution (1: 20w/w). The beads were washed with hot and cold water, then placed in a water jacket containing various compositions of glutaraldehyde solution at 50^0C for about 10 min. Finally, beads were washed with hot and cold water and vacuum dried at 30^0C.	It is useful as a vehicle for controlled release of drugs.	Rate of swelling ratio decreases with increases the degree of crosslinking. IR: New peak at 1631 cm^{-1} in the crosslinked spectra is due to the formation of C=N because of imine reaction SEM of crosslinked chitosan observes the rough and folded surface of the beads; decreasing the concentrationof crosslinking agent contributed to an increase in swelling and a decrease in complexity of the surface folding.	Degradation of the polymer depends on the degree of crosslinking.	Gupta et al., 2000
Glutaraldehyde	A polysulfone substrate was immersed into hydrophilic binding polymer solutions such as polyvinyl alcohol, polyacrylic acid, and hydroxyethylcellulose before the casting of chitosan layer to increase the affinity between the thin chitosan layer and porous polysulfone layer. The chitosan layer was crosslinked	It is used for pervaporation dehydration			Huang et al., 1999

	with glutaraldehyde and sulfuric acid.				
Glutaraldehyde	Acetaminophen (0.5% w/v) was dissolved in acetic acid solution (300mL of 1% v/v). Then chitosan (1%w/v) was dissolved in above solution with 8hrs. To form a chitosan drug solution. 10mL of glutaraldehye GA) solution was added into the aqueous chitosan-drug solution with stirring at 8000rpm for 30 min to form a chitosan-GA drug solution, then spray dried to obtain the crosslinked chitosan microspheres loaded with the drug.	It is used in pharmaceutical.	To characterize their effects on the size, % encapsulation efficiency, % swelling, % erosion, surface morphology and release behaviour of the spray dried chitosan was studied.	Spray drying is a well known process to produce dry powders, granules or agglomerates from drug excipient solutions and suspensions.	Desai et al., 2005
Glutaraldehyde	A chitosan-supported Pd catalyst was prepd. by immobilization of Pd on glutaraldehyde-crosslinked chitosan, followed by in situ chem. redn.	As a catalyst: A chitosan-supported Pd catalyst was successfully used for hydrogenation of 4-nitroaniline in wastewater in the presence of Na formate to give p-phenylenediamine		A chitosan-supported Pd catalyst was prepd	Vincent et al., 2004
Glutaraldehyde	A different degree of crosslinking was performed in acetic acid soln. with glutaraldehyde, and the membranes were neutralized in NaOH soln.	Cytotoxicity screening, using cell culture tests, showed that eventually such materials could be adequate for use in biomedical	Mech. tensile tests: at higher crosslinking levels the membranes become stiffer but their strength decreases; these results are in agreement with swelling tests.		Silva et al., 2004

		applications.	All the membranes exhibited similar and significant damping properties in wet conditions, which were stable in a broad temp. range. Wt. loss measurements showed that the membranes degrade slowly over to 60 days.		
Glutaraldehyde			IR: The absorption of n(O-H) and v(N-H) at 3430-3440 cm^{-1}, n(C-O-C) at 1155 cm-1, n(C-OH) at 1030 cm^{-1} and n(O-O) of b-D-glucose at 899 cm^{-1} were not shifted obviously. The absorption of d(C-H) at 1400-1384 cm^{-1} and n(C-OH) at 1095 cm^{-1} varied markedly. The n(C-N) absorption of crosslinked chitosan was at 1640-1650 cm^{-1}. The skeleton vibration absorption of 1,3,5-triazine ring was at 803-812 cm^{-1} and 1584-1590 cm^{-1}. With different deacetylation or substitution, the amide I, II, III absorption at 1650, 600 and 1310 cm^{-1} varied obviously. When modified with 1-butylamine, dimethylamine, benzylamine, 4-		Feng et al., 2004

			methylaniline and 4-aminobenzenesulfonic acid, strong absorption of d(NH3+) appeared at 1517-1530 cm^{-1}, which was weakened or disappeared when modified with trimethylamine or triethylamine, but a series of new absorption of n(C-N) appeared at 1400-1500 cm^{-1}.		
Glutaraldehyde	Dimethylamine-modified glutaraldehyde-crosslinked chitosan resin was prepd	Resin can be used for the sepn. and purifn. of metal ions and proteins	IR: The adsorption capacities of the modified crosslinked chitosan resin to Cu(II) and bovine serum albumin (BSA) were 42 mg g-1 and 940 mg g-1, resp	Resin had good mech. strength	Feng et al., 2003
Glutaraldehyde	0.5-1.0w% chitosan membrane cast & dried, immersed in 2% aq NaOH soln & then neutralized with water washing Heterogeneous Water swollen chitosan membrane + 50to150 ppm glutaraldehyde sol. at ambient temp. for 24h →to form cross. Chitosan Homogeneous Chitosan (1gm) soln + glutaraldehyde (50 to 150ppm conc) for 30 min. stir filter, cast the		<u>IR:</u> Heterogeneous using GA-1665cm^{-1} (imine gr.). Homogeneous using GA-1725cm^{-1} (carbonyl gr) 1665cm^{-1} (amine gr.) XRD: The crystallinity of cross. Membrane was not significantly modified by heterogeneous crosslinked but the crystalline domains in the crosslinked membrane were partly destroyed by homogeneous crosslinking.		Ying Wan et al., 2003

	film. Crosslinked done at RT for 24h. membrane neutralized with ammonia/methanol soln.(1:8v/v) for 2h., washed with deionized water & dried it		Tensile test. Ionic conductivity: Swelling Index: studied		
1. Tripolyphosphate (TPP). and 2. Glutaraldehyde (GA)	Mixing the chitosan soln. with a tripolyphosphate (TPP) soln. to form ionic cross. chitosan beads, crosslinking the ionic cross-linked beads to form the cross-linked chitosan beads by adding NaOH and crosslinking agent and shaking for about a first period at temp. about 25-55°; and adding the cross-linked chitosan beads in dye soln. to adsorb the dye	The cross. chitosan beads is used in acid or neutral soln. to adsorb reactive type dye, acid type dye or direct type dye			Chiou et al., 2003
Glutaraldehyde	Chitosan was cross-linked with glutaraldehyde by using the Schiff reaction,	An adsorption capacity was determined.	Adsorption equil. fitted the Langmuir isotherm better than the Freundlich isotherm with a max. adsorption capacity of 458 mg Au/g chitosan and 5.5 h to reach equil		Peirano et al., 2003
Glutaraldehyde	Chitosan was cross-linked with different dosages of glutaraldehyde (1000-80,000mg/L).	The activity & lifetime of the immobilized enzyme were measured to evaluate the application potential.		The cross. equil. of chitosan with glutaraldehyde could be described by the Langmuir equation	Wu et al., 2002
Glutaraldehyde	Spray dried chitosan microsphere (0.15gm) dispersed in aqueous gluderaldehyde soln. (0.44M) to form or		Degradation studied	Spray dried chitosan microsphere have showd good sphericity	Mi et al., 2002

	glutaraldehyde cross. chitosan				
Glutaraldehyde	Glutaraldehyde crosslinked chitosan resins were reduced with NaBH4		FTIR and SEM it was found that the redn. treatment to the adsorbents efficiently improved the chem. stability of these chitosan resins, and the shifts in crosslinking agents exerted influences over the morphologies of the adsorbents obviously.studied		Wang et al., 2001
Glutaraldehyde	7% (w/w) chitosan solution was prepared by using 2% acetic acid solution.Then it was dispersed in light liquid paraffin in a 500mL beaker, stirred at 10000 rpm. The w/o emulsion formed was stabilized by adding 1% Tween 80 solution to form chitosan microsphere. To produce the Glutaraldehyde-crosslinked chitosan microspheres, three different amounts of GA added to the chitosan with stirring at 10000 rpm for 1 hr. Content transferred to beaker and stirr 4 hrs. then washed with hexane.	To encapsulate diclofenac sodium (DS)	FTIR, x-RD and SEM studied. XRD: Two new peaks appeared at 22^0C and 46^0C. SEM: Microspheres are almost spherical in nature and have smooth surfaces. It has very smaller particle size ranging from 83.7-73.5 µm.	Polymer crystallinity increases after crosslinking as determined by XRD. Particle size decreases with increasing extent of crosslinking. Drug loading, release of diclofenac sodium.system tested.	Kumbar et al., 2002
Glutaraldehyde See scheme 2	Chitosan flakes were dissolved in 2.5%w/v in 2% v/v aq. AcOH; filter it, 200ml of the clear light	Sorption study and pervaporation study	IR of chitosan: 1570-1655 cm^{-1} (amide I&II). 1383 cm^{-1} (-N-CH_3). 1160 cm^{-1}		Sridhar et al., 2001

	brownish soln. Obtained. 1ml of 25% aq. soln. Of glutaraldehyde was diluted with MeOH & added dropwise to the chitosan soln. with stirring, stir 30min. & then cast into glass plate, evaporate solvent (higher concn. Of glutaraldehyde = brittle membrane)		(saccharide structure). 2833 cm^{-1} (-CH_2 stret.). 3450cm^{-1} (-OH hydroxyl). Cross. Chitosan: 3430 cm^{-1} (v.sharp hydroxyl grp.). 1600 cm^{-1} (-C=N). 1545 cm^{-1} (weak -N-H deformation) WAXRD: $2\theta=10\text{-}11^0$ (responsible for separation as it comprises functional froup such as $-NH_2$ & -OH) Chitosan-$d_{eff.}$ $=8.67A^0$ (2θ $=10\text{-}11^0$) Crosslinked chitosan- $d_{eff.}$ $=7.8A^0$ (2θ $=11\text{-}12^0$). Swelling studied.		
Glutaraldehyde	Chitosan + glutaraldehyde (100-80,000 mg/l)	Activity and lifetime of the immobilized enzyme were measured for developing potential applications.		The crosslinking rate of chitosan with glutaraldehyde could be described by a pseudo-second-order equation and the crosslinking equil.by the Freundlich equation	Juang et al., 2001
Glutaraldehyde	A novel crosslinked chitosan resin was synthesized by suspension polymn. method.		SEM showed that surface structure of the resin was improved by increasing the concn. of the crosslinking reagent. Mechanical. strength and density of the resin increased		Yu et al., 2000

			with the chitosan concn. increased, and the porosity of the resin was only slightly affected.		
Glutaraldehyde	Glutaraldehyde-crosslinked CS	It was used in pervaporation studies.	Glutaraldehyde-crosslinked CS had higher sepn. factor and flux for propanol/water mixts. than for ethanol/water mixt.		Wang et al., 2001
Glutaraldehyde	Chitosan + glutaraldehyde (0 to 5.0%)		Crystallinity, swelling ratio and adsorption capacity reach the max. when glutaraldehyde content in the membrane is 0.25%.		Chen et al., 2000
Glutaraldehyde		Particle diam. and ion strength, and the adsorption capacities were studied			He et al., 2000
Glutaraldehyde	Chitosan membrane immersed in glutaraldehyde soln. for various periods of time & temp. (after cross. membrane becomes fragile & hydrophobic)	Ingredients in food & texture enhancers	SEM and at. force microscopy, potentiometric titrn., and FTIR-ATR spectroscopy.	The covalent bond between amino groups of chitosan and glutaraldehyde is irreversible and stable at high temp. and extreme pH.	Beppu et al., 1999
Glutaraldehyde (Glu)	Chitosan membrane + glutaraldehyde. Glu cross. Chitosan membrane is more stable toward heating than the uncross. Chitosan membrane. In addn., the crosslinked Chitosan membrane is also stable in acidic and basic media		Thermostability of the membrane were characterized by FTIR and TGA anal		Ning et al., 2000
Glutaraldehyde	Prepared 1.5 wt.%	Silver	Elemental		Yoshizuka

	of chitosan soln.30ml of the chitosan soln + 30 ml of hexane soln.containing 10 wt.% of Span 80. The mixture was stirred at 3000 rev./min for 5 min with homogenizer to form a water-in-oil (w/o) emulsion. Then the stirred at 500 rev./min & 5 ml of 50 vol.% aq. glutaraldehyde soln.was dropped into the w/o emulsion. After the crosslinking stirr at 500 rev./min for 20 min.	complexed chitosan (SCM) are used for pesticide removal. The glutaraldehy de-crosslinked SCM provides good reusability for MP removal	analysis shows that N% decreases in cross. chitosan		et al., 2000
Formaldehyde and glutaraldehyde	Optimum prepn. Condn. chitosan aq. soln. concn. 5%, liq. paraffin wax-aq. soln. vol. ratio 1:1.5, Span 80 concn. 6 drops, formaldehyde 10 mL, and glutaraldehyde 2 mL		The morphol., IR spectra were studied	Adsorption behavior of methylene blue were detd	Ding et al., 1998
Glutaraldehyde	A cross-linked chitosan resin was synthesized by chitosan and glutaraldehyde with Zn(II) as a template	It is used for adsorption capacity of Zn(II), Cd(II) and Hg(II)	IR studied	The resin was stable under acidic condition, and can be reused.	Huang et al., 2000
Glutaraldehyde	Chitosan and Cu^{2+} ion as a template, crosslinking with dialdehydes, and removal of Cu^{2+} ion.	Cu^{2+} adsorption studied		Crosslinked chitosan resin had very good reusability and stability under acidic condition.	Huang et al., 1998
Glutaraldehyde	3g chitosan dissolved in 96.0g. 0.25N AcOH & mixed with 2.8g polyether N330. Then 1.6g 0.5% glutaraldehyde soln. was added with agitation, mixture was poured into a	The state and mobility of water in cross. chitosan-polyether semiinter penetrating network was studied.	DSC & NMR studies		Yao et al., 1999

	frame mold & maintained at 45^0C for film formation.				
Glutaraldehyde		It is used for sustained release studies.			Huang et., al. 1999
Glutaraldehyde	Chitosan crosslinked with glutaraldehyde at amino:aldehyde reactant ratios of 1:0.05, 1:0.17, and 1:0.5 were studied			The nickel-contg. crosslinked chitosan was concluded to be a potentially good candidate for the specific binding of proteins having terminal histidines	Alexeev et al., 1999
Glutaraldehyde and glycine	1. Chitosan+ glycine + glutaraldehyde → Cross. chitosan-1 2. Chitosan + glutaraldehyde → Cross. chitosan-2		Structural changes during swelling were studied using IR and UV spectroscopy		Gupta et al., 1999
Glutaraldehyde	Chitosan + Glutaraldehyde → cross. chitosan, then modified with phenylalanine and tryptophan	Used for removal of lipoprotein cholesterol in serum	Adsorption capacity of the crosslinked chitosan bead was decreased.	The removal rate of lipoprotein cholesterol in serum was 46.1% by using the adsorbers.	Williams III et al., 1998
Glutaraldehyde		It is used in permeability study.	Characterized by DSC, IR, XRD & contact angle testing methods		Fang et al., 1998
Glutaraldehyde	Chitosan was crosslinked with glutaraldehyde in lactic acid, and the insol. polymer obtained	It is used in binding of C.I. Acid Orange 7 (I) and methyl orange (MO), in buffered solns. at pH 5 and 7.	The binding ability of crosslinked chitosan for methyl orange was also larger than those of bovine serum albumin and poly (N-vinylpyrrolidone).		Shimizu et al., 1998
Glutaraldehyde	Chitosan + glutaraldehyde using aq. acetic acid → cross. crosslinking	Used in biodegradable controlled delivery system		Smooth highly spherical, crosslinked chitosan	Jameela et al., 1998

	chitosan microsphere			microspheres in the size range of 45-300 µm loaded with progesterone were prepared	
Glutaraldehyde	Cross. chitosan microsphere prepared by emulsion method	It is useful formulations for mucosal administration of drugs	SEM and TEM analyses	It has Spherica shape and smooth surface	Genta et al., 1998
Glutaraldehyde	Beads were cross-linked with glutaraldehyde to avoid partial dissoln. in acidic or alk. media	It is used in removal of Cr VI from wastewater.			Bosinco et al., 1997
Glutaraldehyde	1gm chitosan dissolved in 60ml .25N AcOH + 0.8ml 0.5% glutaraldehyde soln. added with agitation → mixture poured into a frame mould & maintained at 45^0C for film formation. The film swollen in P^H 7.0using potassium phosphate buffer soln. at 37^0C for 4hrs. then dried it at temp. 70^0C →cross. chitosan	To study dynamic swelling behaviour of chitosan based hydrogels	^{1}HNMR: 4.8ppm-H_2O 2.5ppm-protons of the polymeric backbone. DSC analyzes		Yao et al., 1998
Glutaraldehyde (GA)	Chitosan in 10% acetic acid (20mg in 1ml) were added 2/5 (0.01ml, $5x10^{-5}$mole) & 4/5 (0.02ml, 10^{-4}mole) equivalent amount of 25% glutaraldehyde to chitosan residue with stirring for 5 min. at room temp.then without stirring kept for 24 hrs., gel formed , washed with P^H 6 sterlized water to remove acetic acid	Biodegradation study		Higher degree of cross. biodegradation rate is low & biodegradability is low.	Yamamoto et al., 1997
Glutaraldehyde	Chitosan soln.	To study	Cross.		Yu et al.,

(GA)	(using 5% aq. acetic acid) dispersed in 200ml chlorobenzene & toluene (3:1) containing 0.5gm span 80, stir at 140 rounds min^{-1} for 2min., then glutaraldehyde added stir at 40^0C for 1hrs. & 60-70^0C for 3hrs. (ratio of 2:1.221, 2:2.443, 2:3.664, 2:4.885 gelation time decreased with an increase in the amount of the aldehyde)	adsorption props. for bilirubin	chitosan→ SEM: Shows shape & porous structure of the beads. IR: 3400cm^{-1} (OH stret. Vib.) 2937.9 & 2869.5cm^{-1} (unreact.pendent aldehyde) 1652.1cm^{-1} (C=N bonds).		1996
Glutaraldehyde	Chitosan/poly(vinyl alc.) blends + glutaraldehyde→	Used in pervaporation study			Lee and Yoo et al., 1996
Glutaraldehyde	Chitosan + glutaraldehyde→cross. chitosan → grafted by acrylonitrileto form, cross. chitosan bead-g-AN →cyano group of chitosan bead-g-PAN copolymer with hydroxylamine → amidoximation of chitosan bead-g-PAN	Used in metal ion adsorption study. Zn2+, Cd2+ & Hg2+, Cu2+, Mn2+.			Kang et al., 1996
Glutaraldehyde	Glutaraldehyde with chitosan dispersed in acetic acid soln → cross. chitosan	Used in the removal of urea			Zhang et al., 1994.
Glutaraldehyde	Chitosan membrane + glutaraldehyde → cross. chitosan composite membrane	It is used for sepn. of water from aq. ethanol soln.	SEM. ATR-FTIR studied		Lee & Oh et al., 1996
Glutaraldehyde	Chitosan membrane, crosslinked with various ratio of glutaraldehyde/g polymer, has sepn. factor 510 and flux 140 g/m2h for 93 wt. % aq. pyridine soln. at 25°C.	Dehydration of aq. ethanol soln. through pervaporation performance & Swelling % studied.			Oh et al., 1993
Glutaraldehyde	Chitosan		Thermogravimet		Thacharodi

	membranes of 20 mm thickness + different concns. of glutaraldehyde→ membranes of various degrees of crosslinking		ric (TG) anal., DSC and tensile strength studies. decrease in the thermal stability of chitosan membranes due to crosslinking was obsd. The tensile strengths of the membranes was improved by crosslinking		et al., 1993
Glutaraldehyde and Butyraldehyde See scheme 3	Chitosan + glutaraldehyde and Bu aldehyde→ cross. chitosan by Schiff base bonds and had no pendant structure		Density and crystallinity of the crosslinked chitosan membranes decreased with increasing glutaraldehyde content in the membranes		Uragami et al., 1994
Glutaraldehyde	Dil. HCl soln. contg. chitosan was treated with a dil. NaOH to give smaller sizes of chitosan powder (av. diam. 5 mm). Then crosslinked with glutaraldehyde and bound to 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid at the wt. ratio of 95:5 to give a sunscreen	The deriv of cross. chitosan is a Sunscreen, which prevent skin disorders and coloration			Oka et al., 1987
Glutaraldehyde	Chitosan & glutaraldehyde (various amount) in a mixt. of 10% aq. AcOH and MeOH to give crosslinked chitosans.		Chitosan adsorbed only 74% of Cu^{2+}. aldehyde-amino group ratio of 0.7 (cross. chitosan) adsorbed 96% of Cu^{2+}, and then decreased as the ratio was increased. Crystallinity studied.		Koyama et al., 1986
Glutaraldehyde	Modified chitosan (using sulfites, sulfates, chlorides, borates, and hexafluorides) +	Useful in the removal of Cr or Mn from wastewater			Masri, et al., 1978

	glutaraldehyd				
Glutaraldehyde See scheme 4	Chtosan soln in AcOH + Polyether N330 + glutaraldehyde→ heat at 30^0C, 6hrs. → chitosan-Polyether-semi IPN				Yao et al., 1993
Glutaraldehyde	Chitosan gel beads are crosslinked by reaction with a 2.5 wt % glutaraldehyde solution within 24 h.	It is used for removal of cadmium ions from waste water Maximum adsorption capacities for the 1 and 3mm beads were 518 and 188 mg of mg of Cd/g of bead respectively.	To measure Particle size Porosity Pore size distribution SEM	Crosslinking made the gelled beads very elastic and resilient and Insoluble in low pH range	Rorrer et al., 1993
Glutaraldehyde	Ratio of glutaraldehyde to chitosan beads was approx. 15 mL g^{-1} of wet beads. Crosslinking occurred for 16 hrs. Three different particle size chitosan beads are used.	It is used for recovery of Molybdate and Vanadate. Uptake capacity of Molybdate and Vanadate reaches upto 7-8 mmol g^{-1} depending on the pH.		The optimum pH was 3 to 3.5	Guibal et al., 1998
Glycine	1. Chitosan+ glycine + glutaraldehyde → Cross. chitosan-1 3. Chitosan + glycine → cross. chitosan-3		Structural changes during swelling were studied using IR and UV spectroscopy		Gupta, et al., 1999
Terephthalaldehyde or Glyoxal	Chitosan membrane + terephthalaldehyde → cross. chitosan composite membrane	It is used for sepn. of water from aq. ethanol soln.	SEM. ATR-FTIR studied		Lee & Oh et al., 1996
Formaldehyde	Chitosan and Cu2+ ion as a template, crosslinking with formaldehydes, and removal of Cu2+ ion	Cu2+ adsorption studied		Crosslinked chitosan resin had very good reusability and stability under acidic condition.	Huang et al., 1998
Formaldehyde	Formaldehyde-	To study the	Adsorbing		Cao et al.,

	crosslinked chitosan was prepd. at pH 3-4 in 10 mL solvent by irradn. of microwave, and the irradn. time was 3 min.	adsorption capacity for Cu(II)	capacity for Cu(II) of crosslinked chitosan was two times as much as that of chitosan in acid soln.		1999
Formaldehyde	Chitosan + formaldehyde→ cross. chitosan.	To improve the water adsorption ability of the dried chitosan gel in salt solns			Jing et al., 1995
Dialdehyde starch (DAS)	2% chitosan soln. (2%AcOH) +DAS soln. (in H_2O) → mix well → cast onto glass plate → dried washed with 5w% aq. NaOH soln. For 30 mins. → film washed with H_2O & dried in air. (DAS concn. Used 1%, 3%, 5%, 7%, & 9%.)	Crosslinked film showed potential for biomedical application	FTIR: Chitosan film: 3400cm^{-1} –(NH stret. Vib. Bonded to –OH gr. Chitosan) 1599cm^{-1}-(Primary amine). 1637 , 1554 cm^{-1}-(amide I&II) DAS: 3400 cm^{-1}-(OH stret. Vib.) 1736 & 1645cm^{-1} – (carbonyl). Cross. –1599cm^{-1}-week. 1653cm^{-1} ($form^{n}$. of schiff base). 1093 & 665 cm^{-1}-disappearance of crystalline band. XRD: chitosan film:- $2\theta = 10.5^0$ 15.4^0 & 20.1^0 Cross. Chitosan film:-$2\theta = 10.5^0$ & 15.4^0 completely disappeared & $2\theta == 20.1^0$ rapidly weakened with increase in DAS content. Swelling prop. Improved. Mech. Prop. tensile strength improved.		Tang et al., 2003
Dialdehyde-starch	High strength antibacterial dialdehyde-starch-	It is used as biomedical materials for			Du et al., 2003

	crosslinked chitosan film is prepd. from 1-3% chitosan/1-4% acetic acid soln. with 2-7% dialdehyde starch soln. (at a ratio of 100:1-10),	decreasing the bacterial infection on trauma.			
Starch	chitosan-starch (4:1)	It is used in controlling membranes in isosorbide transdermal patche			Ritthidej et al., 1997
Epichlorohydrin (ECH) See scheme 1	Chitosan-CH_2OH + ECH→ Chitosan-ECH beads (CH_2O-CH_2)	Can be used as a resin in ion exchange chromatography	Insoluble in acidic & alkaline medium as well as distd.water. Adsorption isotherms & desorption studied.		Wan Ngah et al., 2002
Epichlorohydrin See scheme 5	1. The C2 amino group in chitosan was protected from the reactn. between benzaldehyde & chitosan to form N-benzylidene chitosan (CTB). 2. CTB. (5gm) powder swollen in 25ml dichloroethane at R.T. for 4hrs.+ epichlorohydrin (2gm) at 60^0C for 24hrs.→N-benzaldehyde chitosan (CCTB). 3. CCTB + dil. Ethanolic HCl soln. (HCl, 0.5M) at 65^0C for 2hrs. filter, washed with distd. water to make cross.chitosan (CCTS),	To study the adsorption props. For metal ion e.g. Pb^{2+}, Cu^{2+} Cd^{2+}, Cr^{2+}, Hg^{2+}, of cross. Chitosan (CCTS)	Elemental analysis shows that after cross. C & H % increases & N% decreases. XRD: CTS: $2\theta=10^0$ (presence of 001 & 100). $2\theta=20^0$ (101 & 002). In CCTS; peaks at $2\theta= 10^0$ & 20^0 decreased due to crystallinity decreases.		Yang et al., 1999
Epichlorohydrin and formaldehyde	The adsorption of Cu2+, Ni2+ and Co2+ onto chitosan crosslinked by formaldehyde and epichlorohydrin (AECTS)	It is used for adsorption study	The adsorption capacities of AECTS towards Cu2+, Ni2+ and Co2+ were 2.42, 1.37 and 0.39 mmol/g, resp		Shi et al., 2005

			FTIR: The FTIR anal. indicates that the adsorption was realized via the coordination of -NH2 and - OH groups in AECTS to the metal ions. WAXRD: WAXD results indicate metal ions adsorbed onto both the amorphous and crystal regions of AECTS, and the destructive degree of the crystal region was in direct proportion to the adsorption capacity. TG: TG anal. shows that the crosslinking and metal ions adsorption could change the three decompn. temps. of CTS to lower degree, depending on adsorption capacity change.		
Epichlorohydrin	Crosslinking chitosan with epichlorohydrin in alkaline condition.	It is applied to the determinatio n of Hg in H2O samples with recoveries of 92-108%.	Satn. adsorption capacity for Hg was 17.6 mg/g		Hou, et. al., 2004
Epichlorohydrin	0.5-1.0w% chitosan membrane cast & dried, immersed in 2% aq NaOH soln & then neutralized with water washing Heterogeneous Water swollen chitosan membrane + 0.001 to 0.05M		IR: Heterogeneous using ECH: $1650cm^{-1}$- (amide I carbonyl gr). $1600cm^{-1}$ (amide deformation) XRD: The crystallinity of cross. Membrane was not significantly		Ying Wan et al., 2003

	conc.epichlorohydrin by using 0.067M NaOH soln. (P^H10) for 40^0C for 2h → crosslinked chitosan.		modified by heterogeneous crosslinked		
Epichlorohydrin	Crosslinked chitosan (CCTS) was prepd. by protecting the amino group in chitosan with benzaldehyde and introducing epichlorohydrin		Characterized by FTIR and X-ray diffraction. & Adsorption properties studied.		Wang et al., 2003
Epichlorohydrin	A metal ion-absorbing resin was prepd. by crosslinking a chitosan resin Zn(II) complex with epichlorohydrin under microwave irradn, and then removing the Zn ions with dil. acid		The adsorption capacity of the resin for Zn2+, Cu2+, Ni2+ and Co2+ is reported.		Zhou, Yue et al., 2003
Epichlorohydrin	Chitosan soln. + silica gel(60-100 mesh), held for 12-24 h, vacuum-dried at 15°, mixed with DMSO for ultrasonic dispersion, mixed with epichlorohydrin at pH 6-8 for 12-30 h, filtered, dried.	Silica gel-cross. chitosan adsorbents for heavy metals.			Zeng et al., 2002
1.Tripolyphosphate (TPP). (Ionic.cross) 2.Epichlorohydrin (ECH)	Mixing the chitosan soln. with a tripolyphosphate (TPP) soln. to form ionic cross. chitosan beads, crosslinking the ionic cross-linked beads to form the cross-linked chitosan beads by adding NaOH and crosslinking agent and shaking for about a first period at temp. about 25-55°; and adding the cross-linked chitosan beads in dye soln.	The cross. chitosan beads is used in acid or neutral soln. to adsorb reactive type dye, acid type dye or direct type dye			Chiou et al., 2003

	to adsorb the dye.				
Epichlorohydrin.	A good-strength and large-porosity chitosan resin was obtained by crosslinking with epichlorohydrin			Cross. Chitosan resin possessed good capacity for adsorption of both copper ion and protein.	Wang & Sun et al., 2001
Epichlorohydrin	At low temp., the crosslinking reaction only occurred between -NH2 group of chitosan and epichlorohydrin. When the temp. was above 40°, -OH group of chitosan reacted with epichlorohydrin	It is used as controllable degrdn. biomaterial	FTIR, X-ray diffraction and SEM. SEM and measurement of mech. properties showed that the tensile strength of the crosslinked films was considerably improved, and the rate of degrdn. of crosslinked films by lysozyme was reduced		Zheng et al., 2000
Epichlorohydrin	Chitosan (6gm) was dissolved into 320ml of 1% (v/v) acetic acid. Then added 6ml epichlorohydrin (slowly, vigorous stirring), then added 50ml of 5% (w/v) NaOH added dropwise, stir 18hrs. at R.T.→ white solid ppt. forme, filter it, washed eith water, finally washed with propanol & dried to obtained cross. Chitosan.	Adsorption props. For Cr (VI) & Se (VI) Mechanism of adsorption crosslinked chitosan for Cr (VI) & Se (VI)	IR: Crosslinked chitosan after adsorption of Cr (VI): 3387 cm^{-1} (amino & hydroxyl gr.). 1724 cm^{-1} & 1662 cm^{-1} (carbonyl of amide). 1067 cm^{-1} (sec.alcohol) 1025 cm^{-1} (pri. Alcohol)		Qian et al., 2000
Epichlorohydrin	Epichlorohydrin cross. Chitosan: chitosan (5gm) &10 g of Cu(II) dichloride was dissolved in 400 ml of 0.3 mol/dm^3 aqueous acetic acid soln. This soln. was slowly dropped into 1500 ml of 0.2 mol/dm^3 aq. NaOH soln.for	Silver complexed chitosan (SCM) are used for pesticide removal Epichlorohydrin-crosslinked chitosan) has better adsorptivity	Elemental analysis shows that N% decreases in cross. chitosan		Yoshizuka et al., 2000

	1 hr. & stirred at 200 rev./min for 12 hr. to obtains Cu(II) complexed chitosan gel & this was cross. by 50 ml of epichlorohydrin in 300 ml of deionized water under reflux for 1 h. Then, 300 ml of 0.1 mol dm^3 aq.NaOH soln. was added to complete the crosslinking reaction.	of Ag(I) than glutaraldehy de-crosslinked chitosan.			
Epichlorohydrin	Chitin + epichlorohydrin→ cross. chitin→deacetylat ion to form cross. chitosan	It is used in to developed transdermal therapeutic systems			Nah et al., 1998
Epichlorohydrin	After cross. crown ether deriv. Studied.				Wang & Peng et al., 1998
Epichlorohydrin		To study adsorption properties.		Water sol. crosslinked chitosan polymers were prepd. by using epichlorohyd rin	Wang & Cheng et al., 1998
Epichlorohydrin	1gm chitosan film was placed in a flask containing 100ml aq. epichlorohydrin (three different concn. of ECH 0.05, 0.1, 0.2M resp.) were prepared three different level of cross. density. Catalyst: 0.067M NaOH (P^H10). Conditions: 40^0C, 2hrs., to obtained cross. chitosan.		FTIR: chitosan & cross. chitosan: 1650cm^{-1} (amide I carbonyl stret.). 1600cm^{-1} (amine deformation) Xray: chitosan films are amorphous. DSC: chitosan & cross. chitosan shows exo. peak at 270^0C due to degradation of the main chain. Glass transition temp. of chitosan has 80^0C.		Bae et al., 1997
Epichlorohydrin	Epichlorohydrin crosslinking agent	To study metal binding ability			Yoo et al., 1997.

Epichlorohydrin	Chitosan first reacted with transition metallic ion (Cu2+ or Ni2+) and then epichlorohydrin to form chitosan-metal chelates→ then desorbing the metallic ion from chelate by dil. Acid, the chitosan resin were gained.		resins have good adsorption for Cu2+ and Ni2+		Qn et al., 1997
Epichlorohydrin	Metal-crosslinked chitosans (metal ion = Cu^{2+} Cd^{2+} Zb^{2+} Ni^{2+} and Fe^{3+}) + epichlorohydrin → cross. metal complexed chitosan	It is used for sepn. of Cu2+, Hg2+, and Cd2+ by using Cd-complexed resins			Ohga et al., 1987
Epichlorohydrinin	chitosan & epichlorohydrinin basic soln.heated at 40^0C to form Epichlorohydrin-crosslinked chitosan film			Water-insol. and biodegradable polymer formed	Mayer et al., 1991
Epichlorohydrin	Chitin + epichlorohydrin → Crosslinked chitin→deacetylation to form crosslinked chitosan and then synthesis of crosslinked chitosan phosphate.	To study adsorption characteristics of metallic ions			Choi et al., 1990
Epoxy chloropropane	6.0 g chitosan was dissolved in 320 mL of 1% (m/v) acetic acid + 6 mL of epoxy chloropropane was slowly added, vigorously stirring, followed by 50 mL of 5% (m/v) NaOH added in drops. Stir about 18 h at room temperature → White solid cross. Chitosan (CCTS) precipitated. The precipitate was filtered off, washed thoroughly with	It is used for sepn. and preconcn. of Pt The method was applied to environmental H2O samples with recoveries of 90-94%.	Adsorption rate of CCTS for Pt (IV) was 100% at pH 3-4 when the adsorption time was 20 min	The method was applied to environmental H2O samples with recoveries of 90-94%.	Wang et al., 2005

	distilled water and a little propanol and dried				
Epoxy chloropropane	Crosslinked chitosan (CCTS) was synthesized by the reaction of water-sol. chitosan with epoxy chloropropane	It is used for the determinatio n of mercury			Wang & Huang et al., 1997
Epoxypropane	Chitosan in AcOH, adding pore forming agent (PEG: polymn. degree of 400-6000) (the ratio of PEG:chitosan 5-50:100), reacting HCHO or MeCHO with chitosan amino groups to form Schiff bases for temporary protection of amino groups, adding epoxypropane and controlling crosslinking reaction only via OH groups at 40-80° and washing the product with acid to hydrolyze Schiff base and to obtain the product.				Xiao et al., 2001
Ethylene glycol diglycidyl ether (EGDE) See scheme 6	Chitosan reacted with benzaldehyde to form CTB. & NH_2 protected chitosan reacted with ethylene glycol diglycidyl ether to form crosslinked chitosan. Then this was deprotected using HCl	Removal of ultra-trace amounts ofmercury in conc. HCl			Oshita et al., 2002
1. Tripolyphosphate (TPP) 2. Ethylene glycol diglycidyl ether (EGDE)	Mixing the chitosan soln. with a tripolyphosphate (TPP) soln. to form ionic cross. chitosan beads, crosslinking the ionic cross-linked	The cross. chitosan beads is used in acid or neutral soln. to adsorb reactive type dye, acid type dye or			Chiou et al., 2003

	beads to form the cross-linked chitosan beads by adding NaOH and crosslinking agent and shaking for about a first period at temp. about 25-55°; and adding the cross-linked chitosan beads in dye soln. to adsorb the dye.	direct type dye			
Ethylene glycol diglycidyl ether (EGDE) See scheme 1	Chitosan-NH_2 + EGDH →Chitosan-EGDH beads (NH-CH_2)	Cross, chitosan can be used as a resin in ion exchange chromatography	Cross. Chitosan was insoluble in acidic & alkaline medium as well as distd.water. Adsorption isotherms & desorption studied.		Wan Ngah et al., 2002
Ethylene glycol diglycidyl ether	Ethylene glycol diglycidyl ether are excellent crosslinking agents	Particle diam. and ion strength, and the adsorption capacities were studied			He et al., 2000
Ethylene glycol diglycidyl ether	Reaction of chitosan gum with ethylene glycol diglycidyl ether in water,to form cross. chitosan				Kamya et al., 1997
Ethylene glycol diglycidyl ether	Chitosan crosslinked by ethylene glycol diglycidyl ether with chloroacetic acid to form Carboxymethyl crosslinked chitosan resin			The resin has good adsorption properties for metal ions like Cu2+, Ni2+, Co2+	Qu et al., 1997
Ethylene glycol diglycidyl ether	Chitosan acetylated using Ac_2O → chitin. Then chitin + ethylene glycol diglycidyl ether	Microparticles used for HPLC packing	Surface area 75.3 m2/g.		Itoyama et al., 1992
Ethylene glycol diglycidyl ether	Digesting caseins with protease → Phosphopeptides, then adsorbing the digestion products onto cross. chitosan at pH 1.5-5.0.	It is used in separation and concentration of phosphopeptides			Koide et al., 1991
Diethyleneglycol diglycidylether (DEDGE)	Chitosan membrane + DEDGE→	It is used for pervaporation study.			Uragami et al., 2001

	DEDGE cross. Chitosan	DEDGE-cross. Chtosan showed higher water permselectivity.			
Diethylene glycol bisglycidyl ether	Chitosan crosslinked by diethylene glycol bisglycidyl ether and Ni (II) as template		Template adsorbents showed better adsorption properties in Cu2+, Ni2+, Co2+		Qu & Xu et al., 1996.
Tridecaethylene glycol diglycidyl ether & hexamethylene - diamine	A soln. of chitosan in aq. AcOH was spun into aq. NaOH-EtOH mixt. → Chitosan fibers. 10gm of chitosan fibers +40 g tridecaethylene glycol diglycidyl ether in water at 60° for 2 h and further treated with 10 g hexamethylenediamine in water at 70° for 2 h to give an adsorbent	It is useful for treatment of wastewaters from dyeing processes		It was insol. in aq. AcOH, aq. HCl, and aq. NaOH	Togashi et al., 1991
Polyethylene glycol diglycidyl ether	Chitosan + PEG diglycidyl ether→cross chitosan gel	It is used in absorption study		Adsorbent has high selectivity for Cu2+ adsorption in the presence of Ni2+ and Co2+.	Qu and Liu et al., 1996
Genipin See scheme 7	Chitosan soln. (3g.chitosan) dispersed in to 500ml soybean oil, without adding surfactant at R.T., stir at 500 rpm for 30 mins. →to form water in dispersion, after that aq. genipin (1wt%) was slowly added with stirring & continued for several hrs.→ solid microspheres formed (blue colour), microspheres	It is used in biomedical application because low cytotoxicity & stable biocompatible	Chitosan: 905 cm^{-1} & 1153 cm^{-1} (saccharide structure). 1570 cm^{-1} (s. protonated amino peak). 1649 cm^{-1} (amide adsorption). 1270 cm^{-1} & 1110 cm^{-1} (hydroxyl group). Cross. Chitosan microsphere: 1643 cm^{-1} (increased the peak). 1570 cm^{-1}	crosslinked chitosan microsphere as a very promising polymeric carrier for drug release	Mi et al., 2001

	rinsed with ethyl acetate & dried in air for 24hrs.		(decreased the peak). ^{13}CNMR: 155-160ppm C-3 position og genipin is decreased after cross. (formation of nucleophilic reactn.). decrease C-6 at 62 ppm carbon atom of chitosan (heterocyclic amine). C-11 at 172 &-OCH_3 at 53 ppm (genipin decreased after cross.). 180 ppm formation of amide. C-4 at 81-ppm disappearence in cross. Chitosan.		
Genipin	The gelation kinetics of various concentrations (2.5 to 10%) of two water-soluble chitosan chlorides (low molecular weight potassium UP CL113 and high molecular weight Potassium UP CL213) and two chitosan glutamates (low molecular weight Potassium UP G113 and high molecular weight Potassium UPG213). Various concentrations (5 to 20%) of genipin, a naturally occurring crosslinking reagent used to prepare crosslinked chitosan hydrogels.	It might be a promising scaffold for disk tissue engineering.	It is biocompatible. 2.5% Potassium UP G213 crosslinked to 5% genipin was the best result.	The gel did not produce an inflammatory reaction when injected subcutaneously into C57BL/6 mice	Mwale et al., 2005
Genipin	Chitosan was dissolved in 1%		DSC: Separate peaks for	The swelling behavior of	Khurma et al., 2005

	aqueous acetic acid solution, a viscous solution was filter of mesh size 0.5mm. PVP was dissolved in deionized water at 85^0C with mechanical stirring for 1 hr to obtain 5% (w/v) solution. Varying amounts of 5% PVP solution was added to the chitosan solution to obtain mixtures having chitosan: PVP weight ratios of 1:3, 1:1 and 3:1. Genipin solution of concentration 0.5% (w/v), was slowly added to the mixtures under constant stirring. The pregel solutions were poured into polystyrene petri dishes and allowed to undergo gelation at room temp. for 12 hrs. then dried.		intermediate and free water were not observed. A broad melting endotherm around 0^0C is attributed to the free freezing and intermediate freezing water. Maximum swelling was observed at low pH and high temp.	the hydrogels was studied	
Genipin	Under basic conditions, genipin underwent a ring-opening polymn. prior to crosslinking with chitosan. At neutral and acidic conditions, genipin reacted with primary amino groups on chitosan to form heterocyclic amines. The heterocyclic amines were further assocd. to form crosslinked networks	biomedical applications	crosslinking value depends on pH values: 39.9 ± 3.8% at pH 5.0, 96.0 ± 1.9% at pH 7.4, 45.4 ± 1.8% at pH 9.0, and 1.4 ± 1.0% at pH 13.6 ($n = 5$, $p < 0.05$).	Basic condition: The crosslink bridges consisted of polymd. genipin macromers or oligomers (7 ~ 88 monomer units). This ring-opening polymn. of genipin was initiated by extg. proton from the hydroxyl groups at C-1 of deoxyloganin aglycon, followed by opening the	Mi et al., 2005

				dihydropyran ring to conduct an aldol condensation . At neutral and acidic conditions: crosslinked networks with short chains of dimmer, trimer, and tetramer bridges. An accompanied reaction of nucleophilic substitution of the ester group on genipin by the primary amine group on chitosan would occur in the presence of an acid catalysis.	
Genipin	Spray dried chitosan microsphere (0.15gm) dispersed in genipin soln (5ml) to form 30% genipin cross. chitosan		Degradation studied	Spray dried chitosan microsphere have showd good sphericity.	Mi et al., 2002
Genipin See scheme 8	Chitosan dissolved in 1.0 w% of aq. AcOH (the concn. & P^H of prepared chitosan is 1.5 w/v% & 5.0)+genipin, dissolved it at R.T., after certain time mixture turn blue & viscosity increases, to obtained cross. hydrogel, dialyzed against double distd. H_2O, to obtained blue color and elastic	It is applied to drug delivery & biomaterials preparation. The swelling ratio of the chitosan hydrogel increased at pH lower than 3 and higher than 11 due to the hydrolysis of amide linkage in the genipin crosslinked			Mi et al., 2000

		chitosan network by acid or alk. followed by the protonation of amine group or ionization of carboxyl acid group in the network.			
Sodium tripoly-phosphate (NaTPP) and genipin See scheme 9	0.1M NaTPP aq. Solution (P^H adjusted from 9.0 to 7, 5, 3, and 1) + genipin (added into diff. P^H values of TPP →TPP/genipin co crosslinker formed. The chitosan solution (3gm in 50ml water containing 0.5% acetic acid) added in TPP/genipin co-crosslinker and stirr for 24hrs. Solid beads formed washed with deionized water under stirring for two days. Then dried it.	It is useful for biomedical and drug delivery system	UV: Absorbance peak at 240nm decrease slowly at acidic P^H but quicker at neutral or basic P^H. IR: Saccharide peak at 905 &1153 cm^3. Resonated amino at 1570 cm^3. Amide peak at 1650cm^3. P=O group of TPP at 1150 cm^3 (intensity increases with the decrease in P^Hvalue of co-crosslink) EDAX: P%increases at lower pH	In basic or neutral condition chemical crosslinking dominated (genipin) beads are brittle colour blue to dark blue And in acidic condition ionic cross. dominated (TPP) white colour beads swelling behaviour and degradation studied.	Mi, et al., 2003
Sodium tri phosphate	Chitosan solution was firstly poured into the mold and rhen put it into a -20^0C refrigerator for 1 day and further placed it into a freeze dried for 2 days to dry the specimen completely. Then, the dried specimens were immersed into the 5% $Na_5P_3O_{10}$ solution for 4 h to process the crosslink reaction. Then, chitosan sponges were washed with double distilled	It is used in tissue engineering.	Degradation rate: 46% in weight of uncrosslinked scaffold after 30 days of exposure in PBS. However, this percentage could be increased upto 76% after crosslinked due to strong gel with three dimensional network was formed in the crosslinked scaffold which has strong ability to	The prepared porous scaffold with pore size between 40 and 100 µm has a three dimensional structure which makes cells grow easily. The weight of crosslinked chitosan scaffold can reach about 76% after being exposed to PBS. The	Lin et al., 2007

	water for 1 day. Then freeze dried for 1 day. To form porous chitosan scaffolds.		withstand the degradation environment. SEM: The interconnected pore structure was developed on both surface and cross section of the scaffold. IR: Absorption band at 1200 cm^{-1} which represents the presence of R-O-P-o group of $Na_5P_3O_{10}$	slow degradation rate is suitable for cell proliferation and growth. Cell numbers that grew on chitosan sponge were comparable to those of chitosan sponge treated with 10µl type 1 collagen and tissue culture plate. In animal test, chitosan wound dressing makes mice wound healed after 12 days and no inflammation was observed.	
Sodium tripoly-phosphate (NaTPP)	Chitosan (60mg) was dissolved in 20mL acetic acid (2% v/v) to obtain chitosan solution. TPP (0.1%) was added to chitosan solution with mild stirring until an opalescent suspension was obtained. The pH of TPP was adjusted from the original pH 9 to 3. The opalescent suspension of crosslinked chitosan particles was subjected to freeze dried.		The swelling behavior of crosslinked chitosan appeared to depend on the pH of TPP. Ionically crosslinked chitosan showed higher swelling ability (pH 3)	PH of TPP play a significant role	Bhumkar et al., 2006
Sodium tripolyphosphate (NaTPP)	Spherical chitosan-tripolyphosphate (TPP) chelating resin was prepd. by an in-liq. ionotropic crosslinking method. Chitosan	Cu2+ adsorption studied.			Lee et al., 1998

	gel beads cured at pH values <6 were highly ionic-crosslinked, and for those cured at pH values >7 were lower ionic-crosslinked.				
Sodium tripolyphosphate (NaTPP)	Synthesized by an in-liq. inotropic crosslinking method	Copper (II) ion adsorption studied		Chitosan gel beads curied in pH >6 was really ionic-crosslinking controlled, whereas, chitosan gel beads curied in pH 8.6 (pH < 7) was pH-dependent coacervation	Mi and Lee et al., 1999
pentasodium tripolyphosphate & glutaraldehyde	A chitosan in aq. AcOH soln. was crosslinked with Pentasodium tripolyphosphate, (ionic cross) then with Glutaraldehyde (covalent cross.) to give crosslinked product.		high surface area and low density		Smith et al., 1994
4,4'-dibromo dibenzo-18-crown-6 (Br-DBC) See scheme 10	Chitosan (CTS) (3gm.) dissolved in 160ml of 1.5% (v/v) AcOH). To this, 4,4'-dibromodibenzo-18-crown-6 (1.5gm) which was dissolved in chloroform (30ml) was slowly added with vigorous stirring.+15ml 15% (w/w) NaOH slowly added, stir at room temp. for 28hrs→white ppt. formed, filter, washed with water & little acetone,dried, to obtained dibenzo-18-crown-6-cross. Chitosan. (DCTS)	DCTS was applied to concentrate & analyze ultratrace elements inAntarctic water (such as lead, cadmium, cromium, copper & arsenic)			Zhang et al., 2003
4,4'-dibromodibenzo -	N-benzylidene chitosan (CTB)**:-**	It is used in environment	Crosslinked chitosans is		Wan et al., 2002

18-crown-6-crown ether See scheme 11	1gm chitosan (CTS) was dissolved in 60ml 0f 1wt% AcOH & diluted with MeOH + 1gm benzaldehyde diluted in 10ml MeOH → stirr well, 24hrs. → 1.3 gm CTB. Chitosan dibenzo-18-crown-6-crown ether (CTSD):- 1gm CTS + 50ml chloroform + 6 ml pyridine→stirr well 24hrs. at RT +4,4'-dibromodibenzo-18-crown-6 crown ether dissolved in 0.5gm in 30ml $CHCl_3$) →reflux 24hrs., filter, washed with water & extracted with $CHCl_3$ 4hrs., dried to form 1.1gm CTSD. Chitosan-dibenzo-18-crown-6 crown ether bearing Schiff base group (CTBD): -procedure is same instead of CTS here we used CTB	al analysis & hazardous waste remediation as toxic metal binding agents in aq. environments	insoluble in org. solvents and swollen in acetic acid soln. IR: CTB: -NH stret. Vib. (3150-3200 cm^{-1}) decreases & aromatic backbone vib. (1600 cm^{-1}) appeared. CTB & CTBD: C=N (1643 cm^{-1}). CTSD & CTBD:- Ar. Ether (1260 &1078 cm^{-1}) Ar. Backbone (1602 & 1444 cm^{-1}) CTB,CTBD, CTSD:- pyranoside vibration (900 cm^{-1}) XRD:- CTS:-2Θ = 10, 20 &28^0 CTB:- 2Θ = 10 & 28^0 disappear. &20^0 peak decreases. CTSD & CTBD:-2Θ= 28^0disappear & peaks at 10^0 & 20^0 decreases.		
4,4'-dibromodibenzo-18-crown-6	1.The C2 amino group in chitosan was protected from the reaction between benzaldehyde and chitosan to form N-benzylidene chitosan (CTB) 2. Chitosan-dibenzo-18-crown-6 bearing Schiff-base group(CTBD) was synthesized by the reaction of chitosan with 4,4'-		IR spectral. anal. and mass spectroscopic anal.		Wan et al., 2001

	dibromodibenzo-18-crown-6 3. Dibenzo-18-crown-6 crown ether crosslinked chitosan was synthesized by the removing of Schiff-base group of CTBD				
Epoxy azocrown ether (DEAC) See scheme 12	1. Protect the NH_2 group of chitosan by using benzaldehyde to form N-benzylidene chitosan (CTB) 2. Prepare Epoxy azocrown ether (DEAC) by reacting 3,7-dihydroxy-1,5-diazacyclic-octane dihybromic acid dissolved in trifluoroacetic acid +NaOH (10 mol/cm^{-3}) + epichlorohydrin in MeOH→stirr 48hrs.at 45^0C under N_2 atmp., cool,filter,washed with MeOH ether, dried to obtained light brown powder i.e. epoxy azocrown ether (DEAC). 3. <u>Prepn. of mesocyclic diamine cross. Chitosan (CCTS-AE):</u> CTB (3gm) swollen dichloroethane + DEAC (1.5gm) dissolved in EtOH → reflux under N_2 for 14hrs. cool, filter, washed with EtOH, ether, to obtain O-azacrown ether-N-benzylidene cross. Chitosan (CCTS-BA). Then	It is used in metal ion complex selectivity (Separation & concn. Of heavy metal ion)	Elemental analysis: IR: CTB & CCTS-BA →-C=N -(1635 cm^{-1}). Aromatic backbone (1560 cm^{-1}). CCTS-AE: disappear the peak at 1635 cm^{-1}. Two new peaks appeared i.e. -C-N-C- (1480 cm^{-1}). –C-O-C- (1080 cm^{-1}). XRD: <u>CTS:</u> $2\theta = 10^0, 20^0$ <u>CTB:-</u> $2\theta = 10^0$ - (decreased). $2\theta = 20^0$- disappeared. CCTS-BA: $2\theta = 20^0$-decreased. CCTS-AE: $2\theta = 20^0$- increased. ^{13}CNMR: CTB and CCTS-BA →128ppm (Ar.carbon). CCTS-BA & CCTS-AE → 42ppm (CH_2-N-gr.)		Yang et al., 2002

	this was deprotected by using dil. EtOH hydrochloride soln. To obtain CCTS-AE				
Dihydroxy mesocyclic diamine	A new type of azacrown ether crosslinked chitosan was synthesized by reaction of chitosan with dihydroxy mesocyclic diamine activated by epoxidn.		Characterization by FT-IR spectral anal. and X-ray diffraction anal.		Yang et al., 2000
3,5-di-tert-Bu dibenzo-14-c-4 dichloracetate crown ether See scheme 13	1. Chitosan + epichlorohydrin → cross. Chitosan (CCTS) 2. Cross. chitosan + 3,5-di-tert-Bu dibenzo-14-c-4 dichloracetate crown ether → cross. chitosan acetate crown ether (CCTS-2)	Presence of crown ether in cross. chitosan shows increase in adsorption props	Elemental analysis: it shows N% decreases in CCTS & CCTS-2 IR: CCTS & CCTS-2: 3150-3200 cm^{-1} (NH & OH stret. vib.decreases). 1595 cm^{-1} & 1500 cm^{-1} (Ar. Backbone appears). 1260 cm^{-1} (Ar ether) 900 cm^{-1} (pyanyl vib). XRD: CCTS: $2\theta = 10^0$, 20^0, 32^0, 46^0. CCTS-2: $2\theta = 10^0$, 32^0, 46^0, disappears & $2\theta = 20^0$ decreases therefore decreases the crystallinity.		Tan et al., 1999
Calixarene	Two calixarene-linked chitosans were synthesized by the reaction of chitosan with 1,3-diglycidyl calix[4] arene		The adsorption properties of these two cross-linked chitosan towards Co2+, Cu2-, Zn2+, Ni2+ and Na+, K+, Cs+ were studied. N-crosslinked chitosan (N-CC)		Gong et al., 2004

			exhibits a higher adsorption ability towards Ni2+, O-crosslinked chitosan (O-CC) has a higher adsorption ability towards Cu2+, and O-CC has a higher adsorption ability towards Co2+, Cu2+, Zn2+ than that of N-CC. The calixarene-linked chitosan exhibited much higher adsorption ability towards Na^+, K^+, and Cs^+ than its mother chitosan did. The adsorption properties of crosslinked chitosan towards Co^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} varied with the linked type.		
hexamethylene diisocyanate (HDI)	Chitosan fiber+HDI→ HDI-cross. Chitosan				Sakairi et al., 2002
Hexamethylene 1,6di(aminocarboxy-sulfonate) See scheme 14	HDI + $Na_2S_2O_5$→ Hexamethylene 1,6di(aminocarboxy-sulfonate) + Chitosan soln., mix.,degassed via centrifugation, cast film, air dried for 48h., then cured for 24h. at 60^0C under N_2 atmp. & then submerged in 0.1M $NaHCO_3$ & deionized water				Welsh et al., 2002
diisocyanates and/or dialdehydes	san in 1960 g H2O H 5.5 with HCl, lycerin, crosslinked e diisocyanate,	It is used in cosmetic prepns	The lyophilized block was elastic and water insol., and after rehydration resembled a sponge		Heilemann et al., 1998
Hexamethylene diisocyanate	Chitosan (I) soln. (70 g I dissolved		Granular porous chitosan was		Kawamura et al., 1986

(HDI)	in 930 g H2O contg. 35 g AcOH) infused into a soln. contg. NaOH 10, MeOH 50, and H2O 40% to give granular porous I granular porous I +100 mol. Me2CO contg. HDI → stirred for 1 h at 30°→ HDI-cross. I with		840-590 mm particle size and 89.4 m2/g sp. Surface HDI-cross. I with sp. surface <0.01 m2/g, and reestablishment of BED vol. 69.		
Toluene diisocyanate (TDI)	Chiotosan + TDI→ TDI-cross. chitosan	Adsorption of metal ions including Hg2+,Cd2+, Cu2+, Cr6+, Pb2+, Zn2+, and Ni2+ were studied	Modified chitosan is easy to be regenerated and less sol. in acid soln. and it has higher adsorption selectivity for Hg2+, Cd2+, Cu2+ in a soln. contg. Hg2+, Cd2+, Cu2+, Cr6+, Pb2+, Zn2+, and Ni2+ ions.		An et al., 1999
D, L-lactic acid See scheme 15	1.0gm chitosan powder dispersed in water and it was dissolved by adding D,L-lactic acid and at different feed ratios the solns. were poured in Teflon dish & dried it. And extracted with methanol	It is used in biomedical application such as artificial muscles & switches, biochemical separation systems, & controlled released system.	XRD shows crystallinity decreases after cross. FTIR shows two new peaks appeared i.e. 1735 cm^{-1} (ester or carbonyl group). 1655 cm^{-1} (amide I increases)		Qu et al., 2000
Glycolic acid See scheme 15	1.0gm chitosan powder dispersed in water and it was dissolved by adding glycolic acid at different feed ratios the solns. were poured in Teflon dish & dried it. And extracted with methanol.	It is used in biomedical application such as artificial muscles & switches, biochemical separation systems, & controlled released system	XRD shows crystallinity decreases after cross. FTIR shows two new peaks appeared i.e. 1735 cm^{-1} (ester or carbonyl group). 1655 cm^{-1} (amide I increases)		Qu et al., 2000
Acrylic acid (The addn. reaction of the amino group of chitosan to the double bond of acrylic acid and	Chitosan + acrylic acid membrane crosslinked by with and without inhibitor and Me acrylate in		Swelling-ratio test, tensile measurement, IR spectra		Zhong & Ge et al., 1996.

the neutral reaction between the amino group and the carboxyl group of acrylic acid)	homogeneous soln. → cross. membrane				
Acrylic acid	Acrylic acid-crosslinked chitosan membrane was prepd. through a homogeneous soln. reaction	It is used in pervaporation study			Zhong & Li et al., 1996.
H_2SO_4	7% (w/w) chitosan solution was prepared by using 2% acetic acid solution.Then it was dispersed in light liquid paraffin in a 500mL beaker, stirred at 10000 rpm. The w/o emulsion formed was stabilized by adding 1% Tween 80 solution to form chitosan microsphere. To produce the Sulphuric acid-crosslinked chitosan microspheres, three different amounts of SA added to the chitosan with stirring at 10000 rpm. Continued for 4 hrs at 50^0C. Content transferred to beaker and using magnetic stirrer stirr at 2 hrs. then washed with hexane	To encapsulate diclofenac sodium (DS)	FTIR, x-RD and SEM studied. XRD: Two new peaks appeared at 11^0C and 27^0C. SEM: Microspheres are almost spherical in nature and have smooth surfaces. The particle size ranging from 180-230 µm.	Polymer crystallinity increases after crosslinking as determined by XRD. Particle size decreases with increasing extent of crosslinking. Drug loading, release of diclofenac sodium.system tested.	Kumbar et al., 2002
H_2SO_4	Chitosan was crosslinked with H2SO4 and coated on the inner surface of the polyacrylonitrile ultrafiltration hollow-fiber membrane to form composite membrane	Membrane was used to sep. water-ethanol mixts. by pervaporation			Liu et al., 1994

Dicarboxylic acid (sebacic acid)	ssolved in 0.1M were reduced to nd 50 kDa using zation. Sebacic acid s solution with 1- opropyl) activated sebacic ith reduced an solutions for 30 ate-crosslinked olutions, air drying % ammonia S.	Sebacic acid crosslinking of reduced molecular weight chitosans provides a mechanism for modulating both mechanical and cell adhesion/proliferation properties.	FTIR spectra, aqueous swelling and contact angle studies done to characterize material properties as well as tensile strength, elastic modulus (at 20% strain) and breaking strain for the crosslinked and non crosslinked chitosan films determined.	Reduced the molecular weight of chitosan reduced the tensile strength at all crosslinking densities.	Piparia et al., 2006
Natural di- and tricarboxylic acids	The condensation reaction of carboxylic groups and pendant amino groups of chitosan was performed by using water-sol. carbodiimide	The biodegradable cross-linked chitosan nanoparticles, as solns. or dispersions in aq. media, might be useful for various biomedical applications.	NMR spectroscopy: Particle size: 1. Particle size measured by TEM varied in the range 60-280 nm. 2. The av. size of the particles measured by DLS was in the range 270-370 nm depending on the pH	It was found that particle size depends on the pH, but at a given pH, it was independent of the ratio of crosslinking and the crosslinking agent	Bodnar et al., 2005
Di- and tricarboxylic acids	The crosslinked chitosan nanoparticles were prepd. by condensation reaction with di- and tricarboxylic acids as crosslinking agents	Used in cosmetics, food technol., drug delivery systems			Bodnar et al., 2004
Dicarboxylic acids or anhydrides, e.g., itaconic anhydride	Chitosan support material is crosslinked using a heat-induced amidation reaction with dicarboxylic acids or anhydrides, e.g., itaconic anhydride, in nonaq. Solvent				Glasser et al., 1997
Anhydride borax	Homogenizing a mixt. of guar gum, water and propylene glycol and crosslinking with anhyd. borax	It is useful for wound dressings			Gilding et al., 1997

	gave a biocompatible hydrogel				
Lignosulfonate	NH2 groups of chitosan with the lignosulfonate to form sulfonylamide linkages. Reaction done under mild immobilization conditions				Tartakovsky et al., 1998
Chloromethyloxirane	reaction of chitosan with 3-chloro-1,2-propanediol in the presence of alkali to introduce 2,3-dihydroxypropyl groups, and then crosslinking through chloromethyloxirane or ethylene glycol diglycidyl ester	Cross. chitosan deriv. used in separation of germanium.			Inukai et al., 1998
PVA	chitosan-PVA (1:9)	It is used in controlling membranes in isosorbide transdermal patche			Ritthidej et al., 1997
Sodium sulfate	Chitosan-gelatin films dipped into sodium sulfate soln → Sulfate crosslinked chitosan-gelatin films (SCG)		Under acidic conditions pH less than 4, SCG swelled less than 120%, while under the conditions pH larger than 7.4, SCG swelled very significantly, the swelling ratio was over 350%	The lower concn. and the higher pH of sulfate soln. resulted in a larger swelling ratio	Xiao et al., 2004
Gamma irradiation	A solution containing 30:65:5 parts by weight of water, methanol and carbontetrachloride was prepared and to which chitosan was added till it is completely soaked by the solution. This mixture was kept overnight to allow the solvent to get	It is used for analysis of Cr(VI) in aqueous solution	To determine ionic capacity	The amino groups of chitosan are not appreciably affected by the time of irradiation crosslinking chitosan	Ramnani, et al. 2006

	diffuse into microstructure of chitosan. The mixture was then irradiated in Co^{60} gamma chamber for a required period of time. The irradiated mixture was washed first with methanol to remove any traces of carbon tetrachloride and other radiolytic products formed during the irradiation followed by washing with water. The mixture was repeatedly washed				
Heat treatment	7 %(w /w) chitosan solutions was prepared by using 2% acetic acid solution.Then it was dispersed in light liquid paraffin in a 500mL beaker, stirred at 10000 rpm. The w/o emulsion formed was stabilized by adding 1% Tween 80 solution to form chitosan microsphere. To produce the Sulphuric acid-crosslinked chitosan microspheres, the temperature of the emulsion was raised to 90^0C at 10000 rpm. The microspheres were separated at three different time intervals to obtained different extents of crosslinking. then washed with hexane	To encapsulate diclofenac sodium (DS)	FTIR, x-RD and SEM studied. XRD: Two new peaks appeared at 11^0C and 27^0C. SEM: Microspheres are almost spherical in nature and have smooth surfaces. The particle size ranging from 180-230 µm.	Polymer crystallinity increases after crosslinking as determined by XRD. Particle size decreases with increasing extent of crosslinking. Drug loading, release of diclofenac sodium.system tested.	Kumbar et al., 2002
Nitrilotriacetic acid	Chitosan (1g) , nitrilotriacetic	It may be useful as	Copper adsorption	1:1 type copper	Tikhonov et al., 1996

	acid (0.8g) and water (50mL)was stirred at 60^0C during 3h. to the resulting clear solution was added 1M NaOH (4mL). Stirring and heating were continued till full dissolution. Cool the solution to 40^0C, then at that temp. added 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide hydrochloride, with stirring until gelation was observed to start, stopped the stirring and continued the heating further 1 hr. The gel was formed. Cool to ambient temperature for 12hrs. Gel was filtered and washed with aq. AcOH and water	chromatography supports for lectin separations, enzyme immobilization and water treatment.	studied Degree of swelling studied	ligand complexes are formed during the copper ion uptake by the polymer network.	

1.3. Reaction Schemes:

Reaction Scheme 1:

Epichlorohydrin/ Glutaraldehyde / Ethylene glycol diglycidyl ether Crosslinked chitosan

(Ref. Reactive & Functional Polymers, 50(2), 181-190, 2002)

I) Chitosan + Epichlorohydrin (ECH) ⟶

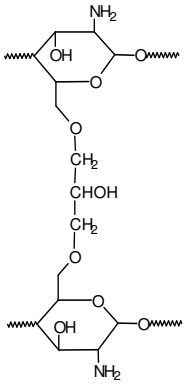
Epichlorohydrin Crosslinked chitosan

II) Chitosan + Glutaraldehyde (GLA) ⟶

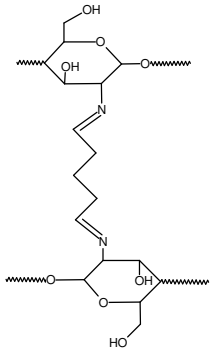
Glutaraldehyde Crosslinked chitosan

III) Chitosan + Ethylene glycol diglycidyl ether (EGDE) ⟶

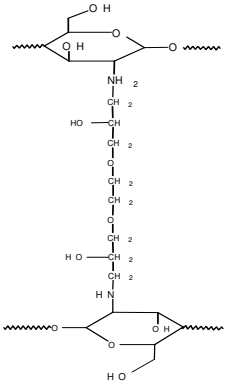
Ethylene glycol diglycidyl ether Crosslinked chitosan



ECH Cross. CTS



GLA Cross.CTS

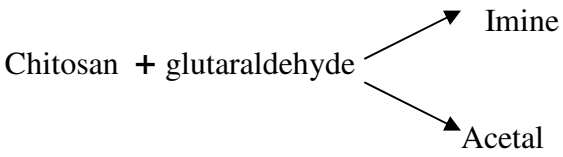


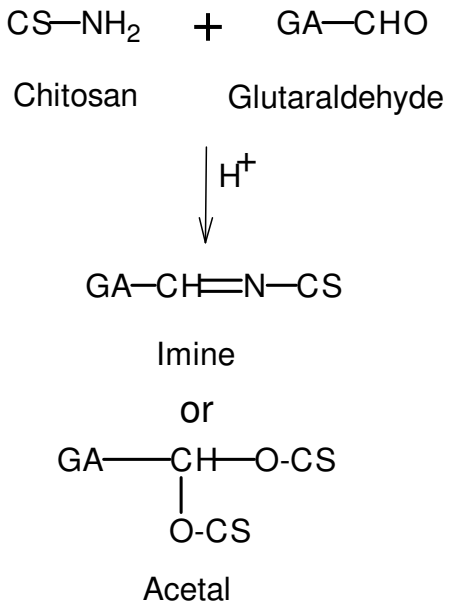
EGDE Cross. CTS

Reaction scheme 2:

Imine/acetal group crosslinked chitosan

(Ref. Polymer International, 50(10), 1156-1161, 2001)



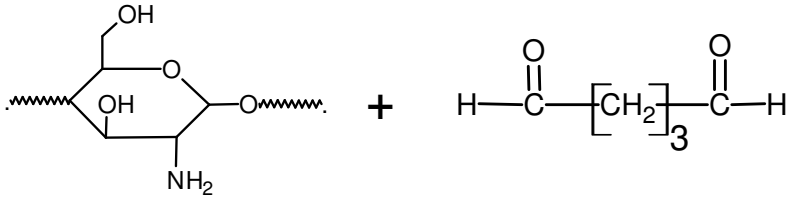


Reaction scheme 3:

Glutaraldehyde Crosslinked Chitosan 1

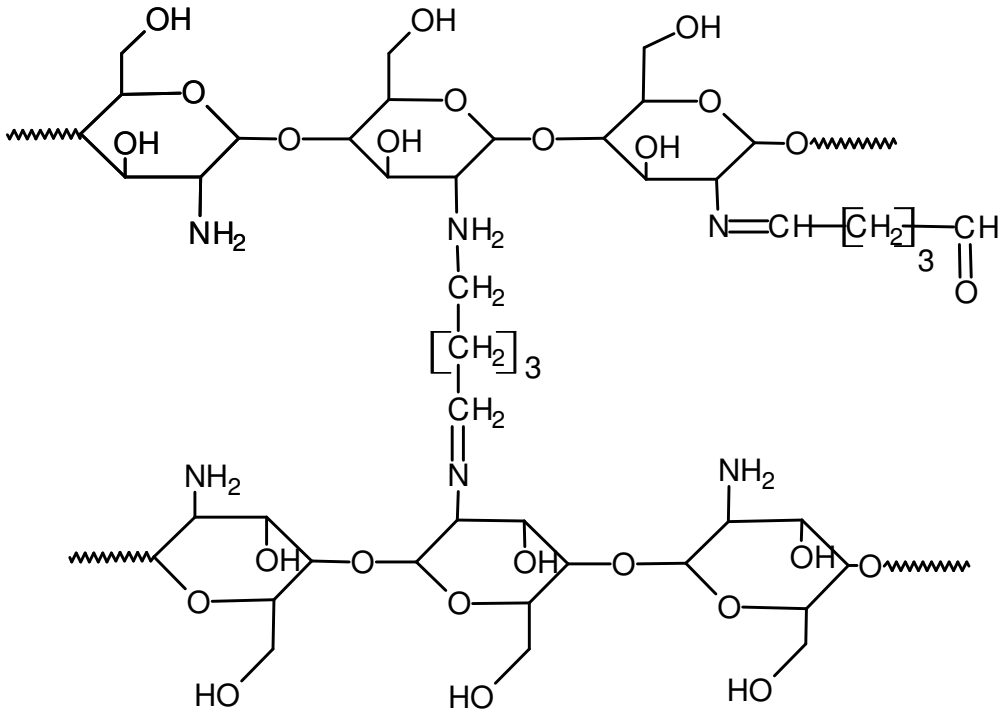
(Ref**.** J. Membr. Sci., 88(2-3), 243-51, 1994)

Chitosan + glutaraldehyde ⟶ Glutaraldehyde Crosslinked Chitosan



Chitosan Glutaraldehyde

Acid catalyst



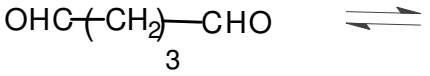
Glutaraldehyde Crosslinked Chitosan 1

Reaction scheme 4:

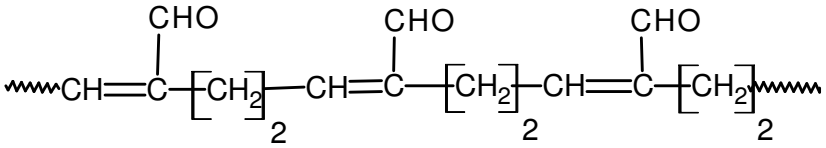
Glutaraldehyde crosslinked chitosan 2

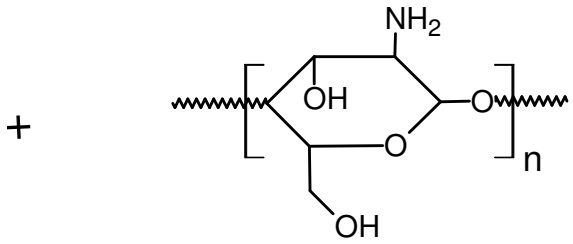
(Ref**.** J. Appl. Polymer Sci. 48, 343, 1997)

Chtosan + Glutaraldehyde ⟶ Glutaraldehyde crosslinked chitosan

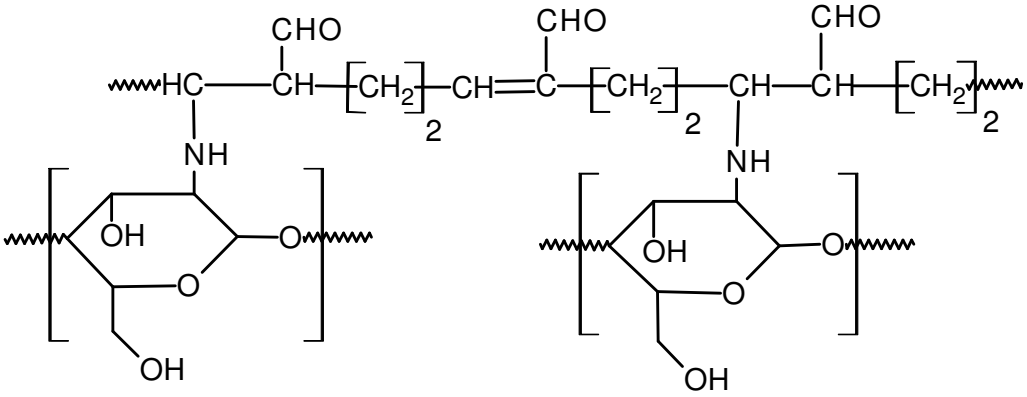


Glutaraldehyde





Chitosan



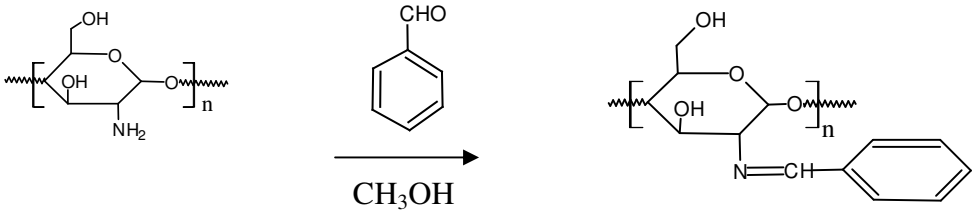
Glutaraldehyde crosslinked chitosan 2

Reaction scheme 5:

Crosslinked Chitosan (CCTS) by using N-benzylidene chitosan

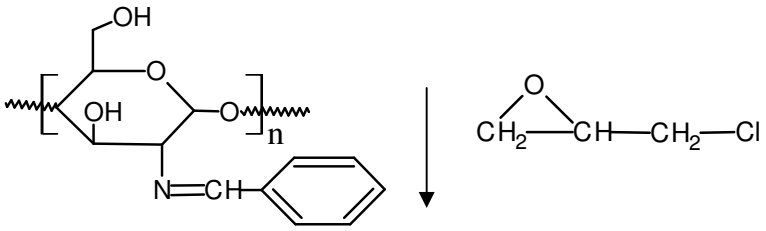
(Ref. J. Appl. Polym. Sci., 74(13), 3053-3058, 1999)

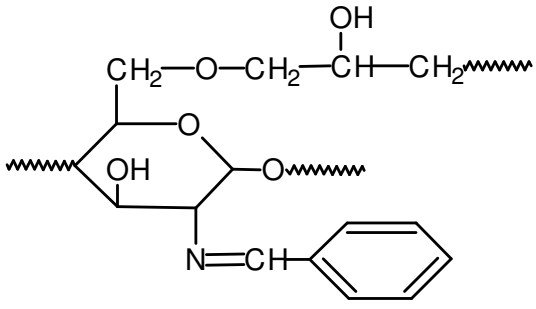
I) Chitosan **(CTS)** + benzaldehyde ⟶ N-benzylidene chitosan **(CTB)**



II) N-benzylidene chitosan (CTB) + Epichlorohydrin ⟶ N- benzaldehyde cross.

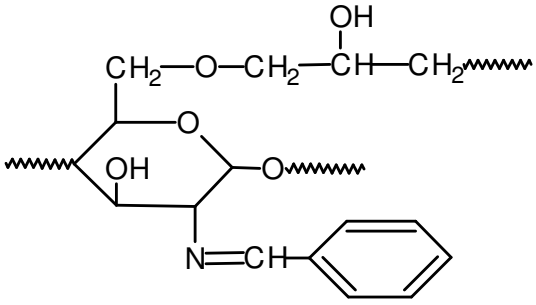
chitosan (CCTB)





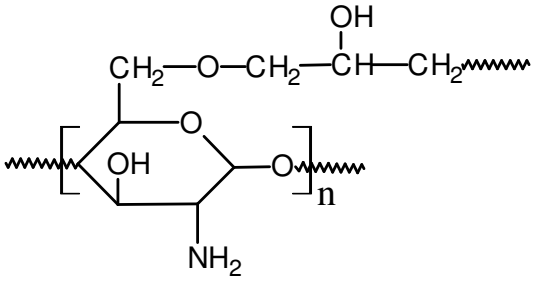
CCTB

III) N- benzaldehyde cross. chitosan **(CCTB)** -HCl → Crosslinked Chitosan **(CCTS)**



CCTB

-HCl



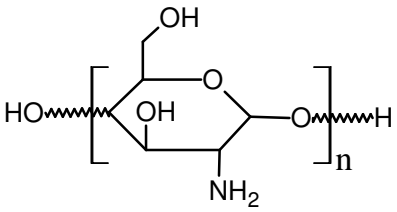
CCTS

Reaction scheme 6:

Ethylene glycol diglycidyl ether crosslinked chitosan

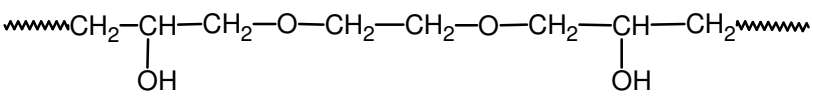
(Ref. Analytical Sciences, 18(10), 1121-1125, 2002)

Chitosan + Ethylene glycol diglycidyl ether ⟶

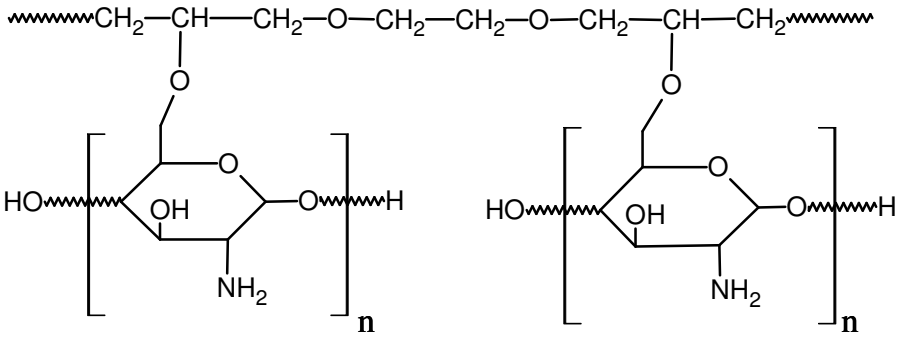


Chitosan

+



Ethylene glycol diglycidyl ether

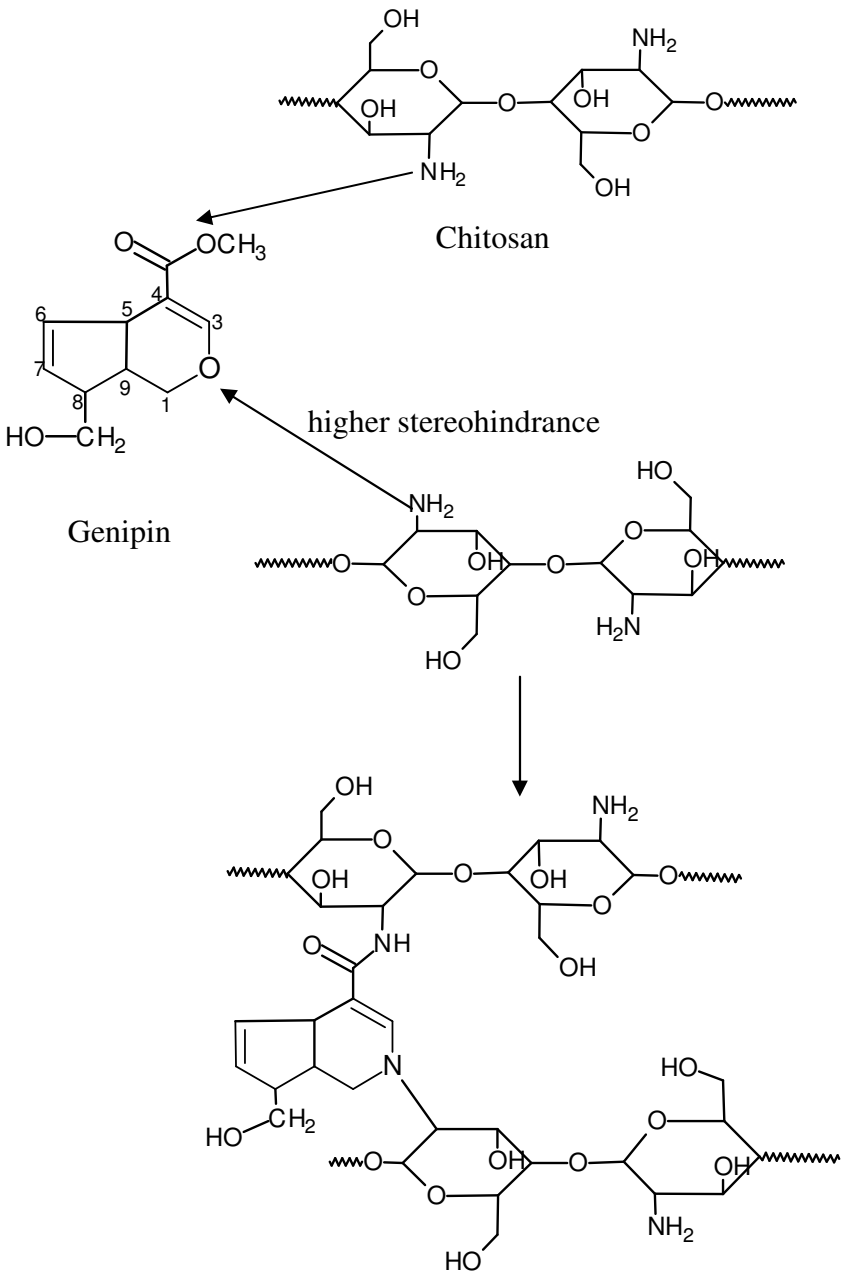


Reaction scheme 7:

Genipin crosslinked chitosan 1

(Ref**.** J. Appl. Polym. Sci., 81(7), 1700-1711, 2001)

Chitosan + Genipin

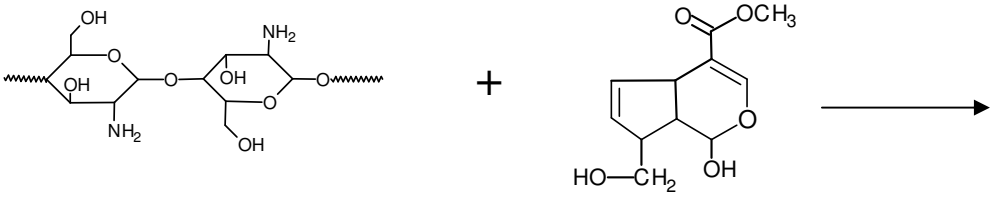


Genipin crosslinked chitosan 1

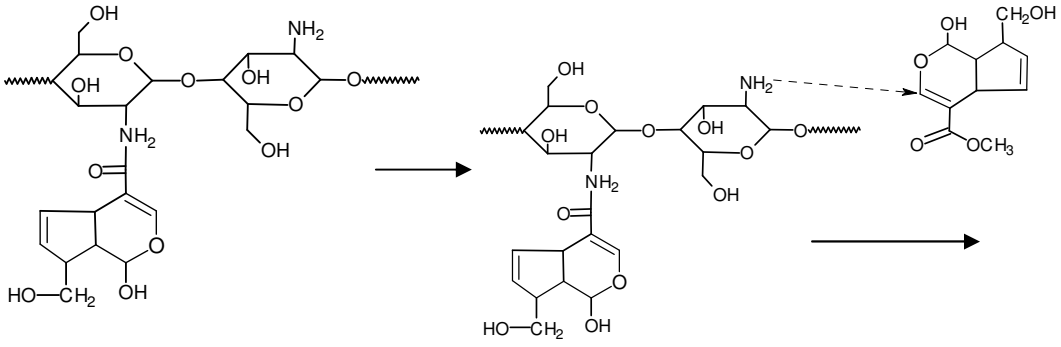
Reaction scheme 8:

Genipin crosslinked Chitosan-2

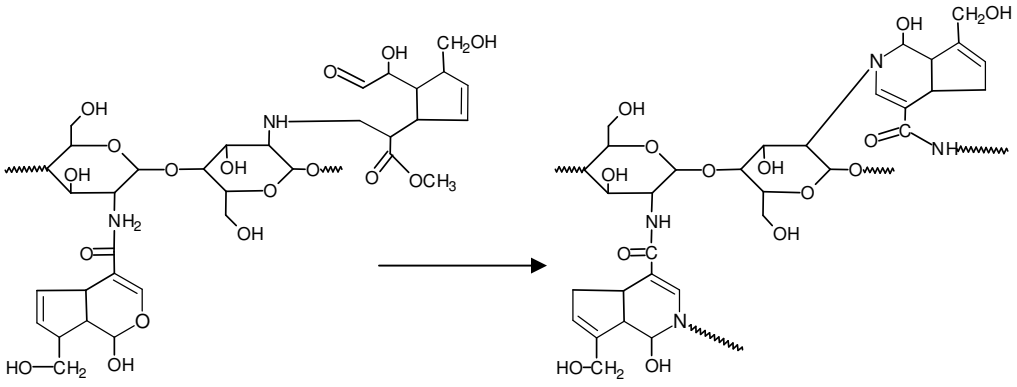
(Ref. J. Polym. Sci., Part A: Polym. Chem., 38(15), 2804-2814, 2000)



Chitosan Genipin



Seccondary amide (Nucleophilic attack)



(Heterocyclic amine) Genipin crosslinked Chitosan 2

Reaction scheme 9:

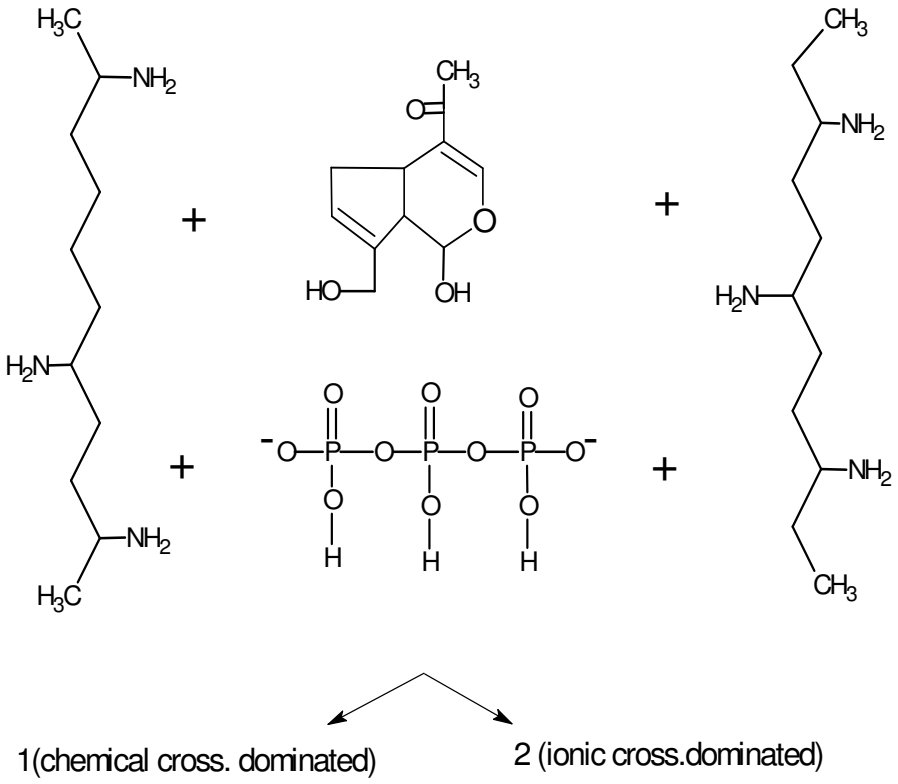
Chitosan and Genipin and sodium tripolyphosphate crosslinked chitosan

(Ref. Polymer, 44(21), 6521-6530 (English) 2003)

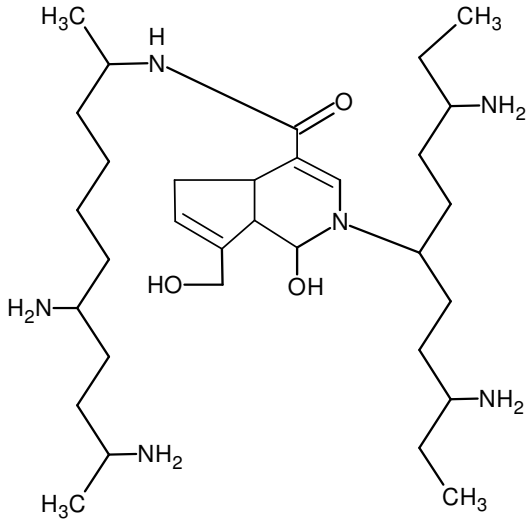
1 -Chemical crosslinking dominated
(In neutral or basic condition)

Chitosan + Genipin and sodium tripolyphosphate

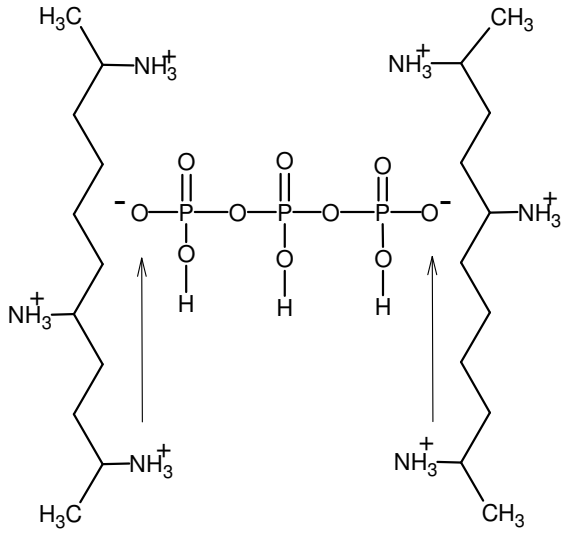
2-Ionic crosslinking dominated
(In acidic condition)



1-Chemical crosslinking dominated product: - (In neutral or basic conditions, beads are brittle blue to dark blue colour)



2-Ionic crosslinking dominated product: **-** (In acidic condition, white colour beads)



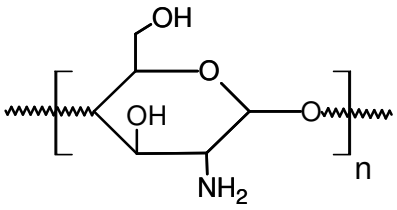
Reaction scheme 10:

Dibenzo-18-crown-6-crosslinked Chitosan

(Ref. Journal of Applied Polymer Science, 90(3), 806-809, 2003)

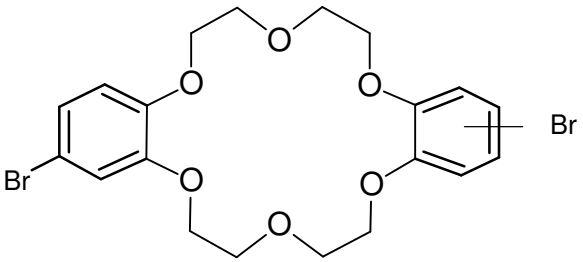
Chitosan + 4,4'-dibromo dibenzo-18-crown-6 (Br-DBC)

⟶ dibenzo-18-crown-6-crosslinked Chitosan (DCTS)



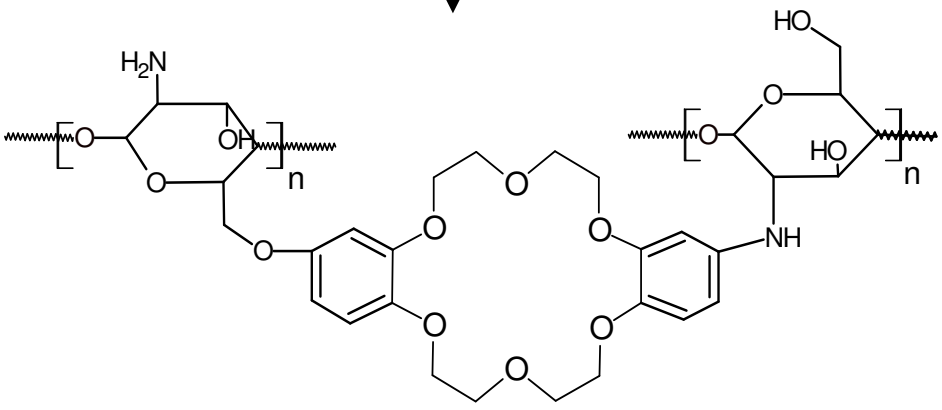
Chitosan

+



4,4'-dibromo dibenzo-18-crown-6 (Br-DBC)

NaOH, 28hrs, RT



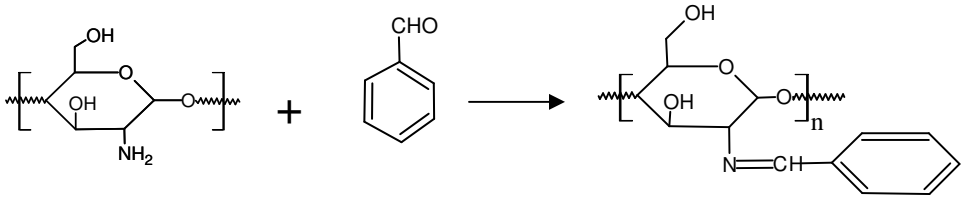
Dibenzo-18-crown-6-crosslinked Chitosan (DCTS)

Reaction Scheme 11:

Chitosan-dibenzo-18-crown 6-crown ether bearing Schiff base group (CTBD) and (CTSD)

(Ref. Journal of Applied Polymer Science, 84(1), 29-34 (English) 2002)

I) Chitosan (CTS) + benzaldehyde ⟶ N-benzylidene chitosan (CTB)

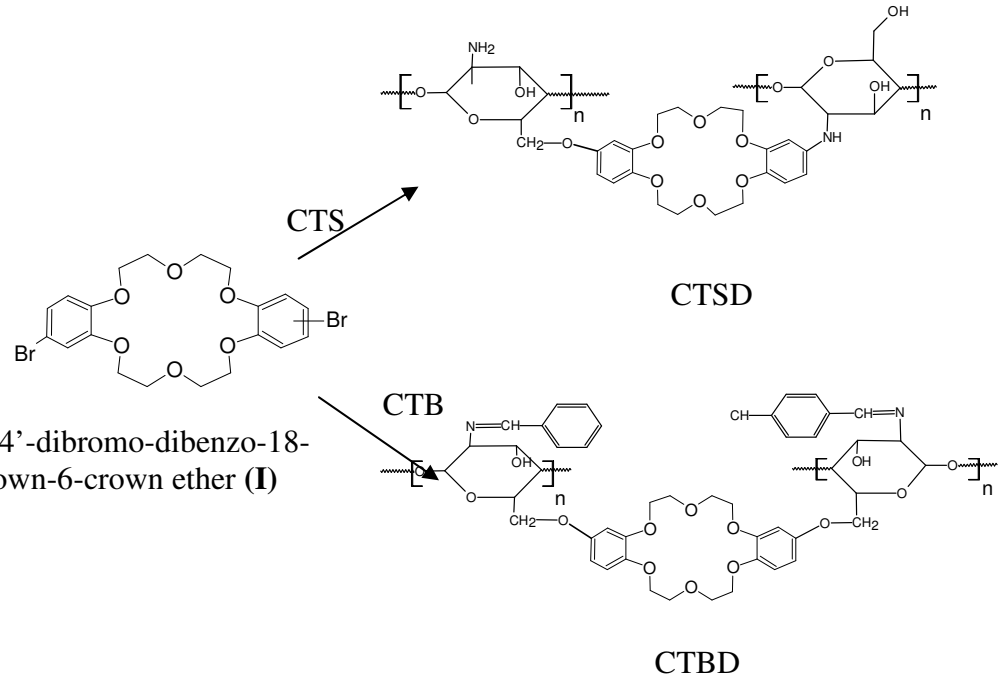


Chitosan (CTS) benzaldehyde N-benzylidene chitosan (CTB)

II) 4,4'-dibromo-dibenzo-18-crown-6-crown ether **(I)**

CTB → **1.** Chitosan-dibenzo-18-crown-6 crown ether bearing Schiff base group (CTBD)

CTS → **2.** Chitosan dibenzo-18-crown-6-crown ether (CTSD)



CTS → CTSD

CTB → CTBD

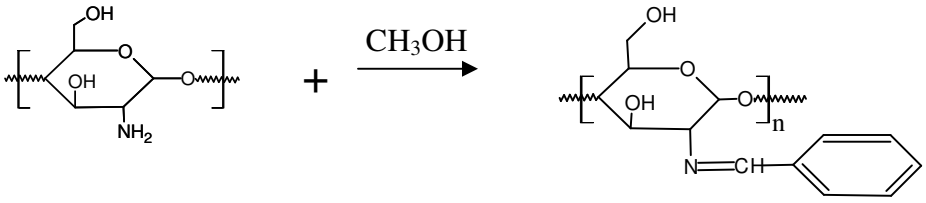
4,4'-dibromo-dibenzo-18-crown-6-crown ether **(I)**

Reaction scheme 12:

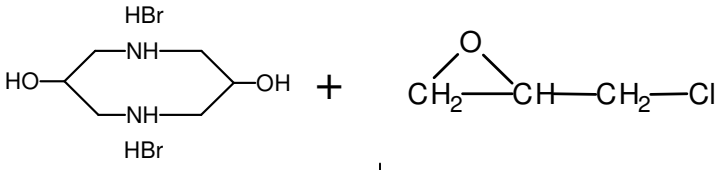
O-azacrown ether- crosslinked Chitosan (CCTS-AE)

(Ref: Journal of Applied Polymer Science, 85(3), 530-535, 2002)

I) Chitosan (CTS) + benzaldehyde ⟶ N-benzylidene chitosan (CTB)

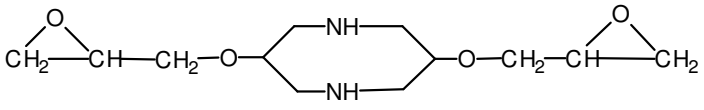


II) 3,7-dihydroxy-1,5-diazacyclic-octanedihybromic acid + epichlorohydrin ⟶ epoxy azocrown ether (DEAC).



3,7-dihydroxy-1,5-diazacyclic-octanedihybromic acid

Epichlorohydrin

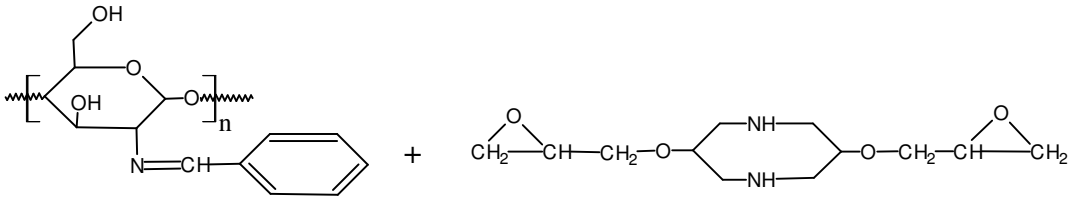


Epoxy azocrown ether (DEAC)

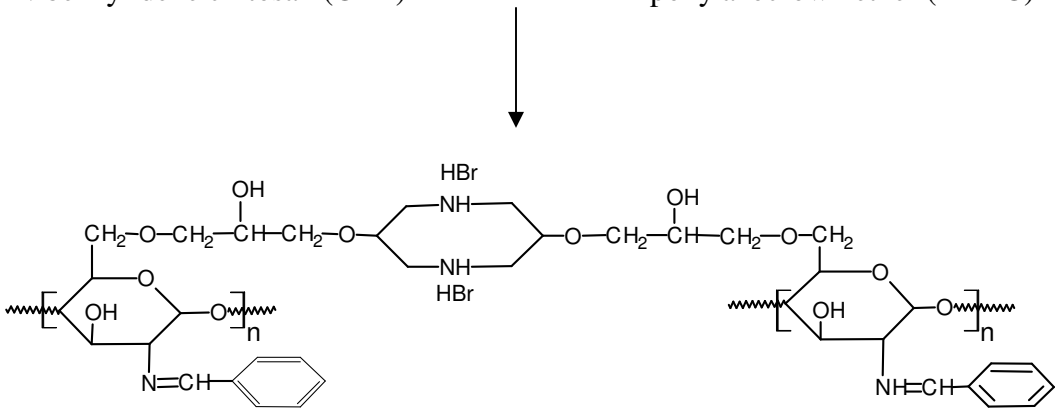
III) N-benzylidene chitosan (CTB) + Epoxy azocrown ether (DEAC)

⟶ O-azacrown ether-N-benzylidene crosslinked Chitosan (CCTS-BA)

HCl ⟶ O-azacrown ether- crosslinked Chitosan (CCTS-AE)

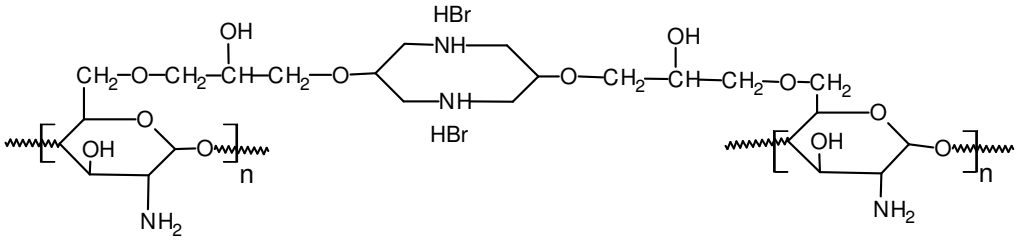


N-benzylidene chitosan (CTB) Epoxy azocrown ether (DEAC)



O-azacrown ether-N-benzylidene crosslinked Chitosan (CCTS-BA)

HCl



O-azacrown ether- crosslinked Chitosan (CCTS-AE)

Reaction scheme 13:

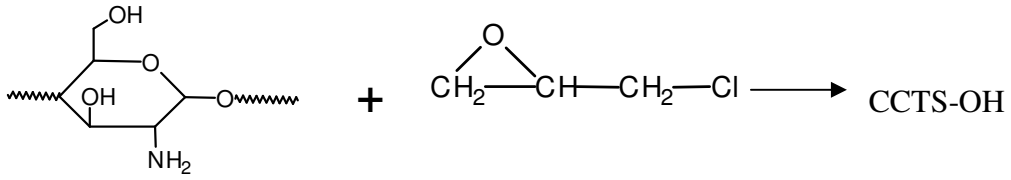
Crosslinked chitosan acetate crown ether (CCTS-2)

(Ref. Appl. Polym. Sci., 71(12), 2069-2074, 1999)

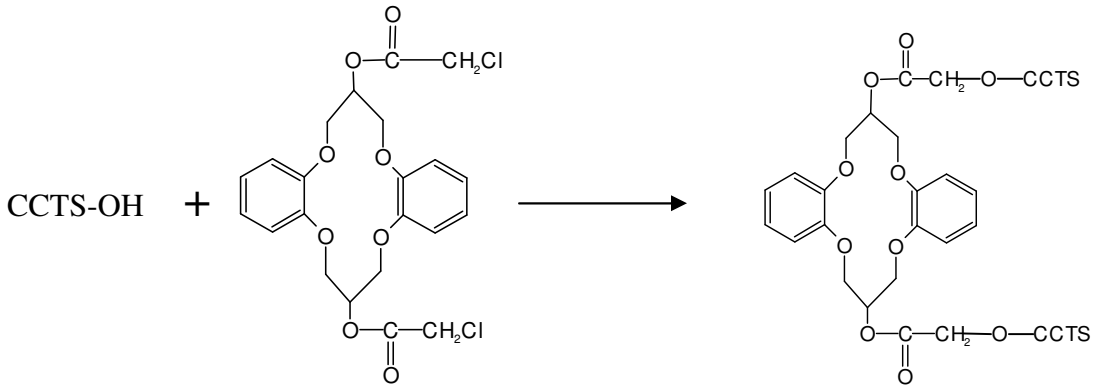
Chitosan + Epichlorohydrin ⟶ Crosslinked Chitosan (CCTS-OH)

↓ 3,5-di-tert-Bu dibenzo-14-c-4 dichloracetate crown ether

Crosslinked. chitosan acetate crown ether (CCTS-2)



Chitosan Epichlorohydrin Crosslinked chitosan



3,5-di-tert-Bu dibenzo-14-c-4 dichloracetate crown ether

Crosslinked chitosan acetate crown ether (CCTS-2)

Reaction Scheme 14:

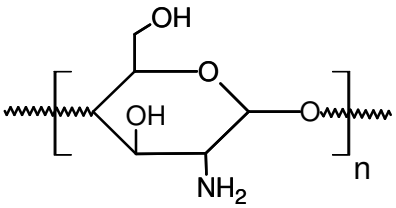
Hexamethylene 1,6di(aminocarboxysulfonate) crosslinked chitosan

(Ref. Biomacromolecules, 3(6), 1370-1374, 2002)

I) Diisocyanatohexane (HDI) + $Na_2S_2O_5$

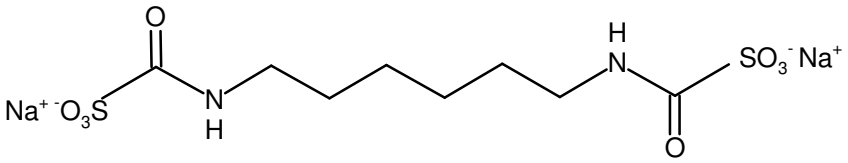
⟶ Hexamethylene 1,6di(aminocarboxysulfonate)

II) Chitosan + Hexamethylene 1,6di(aminocarboxysulfonate) ⟶



Chitosan

+



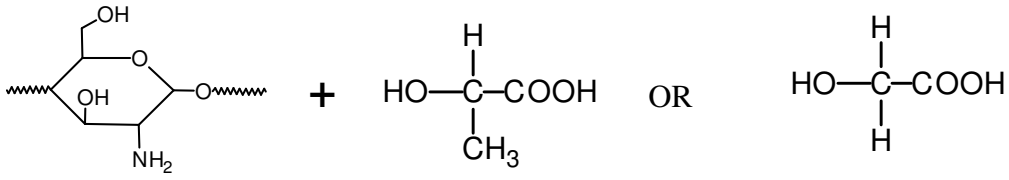
Hexamethylene 1,6di(aminocarboxysulfonate)

Reaction scheme 15:

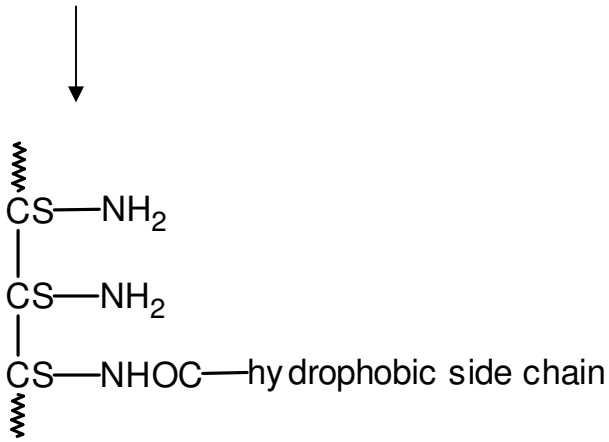
D, L-Lactic acid or Glycolic acid graft chitosan

(Ref. Polymer, 41(12), 4589-4598, 2000)

Chitosan + D, L-Lactic acid OR Glycolic acid ⟶ Graft chitosan



Chitosan D, L-Lactic acid Glycolic acid



Graft chitosan

1.4. References:

Adachi, T., Ida, J. & Hashimoto, M. (1994). Crosslinked chitosan for endotoxin removal. Jpn. Kokai Tokkyo Koho JP 06263799 A2 20 Sep 1994 Heisei, 6 pp.

Akao, T., Kobashi, K. & Aburada, M. (1994). Enzymatic studies on the animal and intestinal bacterial metabolism of geniposide. Biol. Pharm Bull., 17, 1573–1576.

Alexeev, V. L. (1999). Swelling Properties of a Nickel-containing Cross-linked Chitosan. Chem. Eng. Technol., 22(11), 963-965.

An, S., Zhang, L., Zheng, S. & Xu, J. (1999). Preparation of crosslinked chitosan modified by TDI and its adsorption properties. Changchun Keji Daxue Xuebao, 29(2), 197-199.

Azab, A. K., Orkin, B., Doviner, V., Nissan, A., Klein, M., Srebnik, M. & Rubinstein, A. (2006). Crosslinked chitosan implants as potential degradable devices for brachytherapy: In vitro and in vivo analysis. Journal of Controlled Release 111(3), 281–289**.**

An, S., Zhang, L., Zheng, S. & Xu, J. (1999). Preparation of crosslinked chitosan modified by TDI and its adsorption properties. Changchun Keji Daxue Xuebao, 29(2), 197-199.

Bae, H. S. & Hudson, S. M. (1997). The Cooperative Binding Behavior of Sodium Dodecyl Sulfate to Crosslinked Chitosan Films. Journal of Polymer Science: Part A: Polymer Chemistry, 35, 3755–3765.

Beppu, M. M., Arruda, E. J. & Santana, C. C. (1999). Synthesis and characterization of dense and porous structures of chitosan. Polim.: Cienc. Tecnol., 9(4), 163-169.

Berkovich, L. A., Tsyurupa, M. P., Davankov, V. A., Rogozhin, S. V., Gamzazade, A. I. & Davidovich, Yu. A. (1980). Crosslinked chitosan copolymers. Otkrytiya, Izobret., Prom. Obraztsy, Tovarnye Znaki, 15, 96.

Bhatia, S. C. & Ravi, N. (2000). A Magnetic Study of an Fe-Chitosan Complex and Its Relevance to Other Biomolecules. Biomacromolecules, 1, 413-417.

Bhatia, S. C. & Ravi, N. (2003). A Mossbauer Study of the Interaction of Chitosan and D-Glucosamine with Iron and Its Relevance to Other Metalloenzymes. Biomacromolecules, 4, 723-727.

Bhumkar, D. R. and Pokharkar, V. B. (2006). Studies on Effect of pH on Cross-linking of Chitosan with Sodium Tripolyphosphate: A Technical Note. AAPS PharmSciTech, 7(2) article 50.

Boddu, V. M., Abburi, K., Talbott, J. L. & Smith, E. D. (2003). Removal of Hexavalent Chromium from Wastewater Using a New Composite Chitosan Biosorbent. Environ. Sci. Technol., 37, 4449-4456.

Bodnar, M., Hartmann, J. F. & Borbely, J. (2004). Nanoparticles from chitosan. Polymer Preprints, 45(2), 307-308.

Bodnar, M., Hartmann, J. F. and Borbely, J. (2005). Preparation and Characterization of Chitosa-Based Nanoparticles. Biomacromolecules, 6(5), 2521-2527.

Borchard, G. (2001). Chitosans for gene delivery. Adv. Drug Delivery Rev., 52, 145-150.

Borzacchiello, A., Ambrosio, L., Netti, P. A., Nicolais, L., Peniche. C., Gallardo, A. & San, R. J. (2001). Chitosan based hydrogels: Dynthesis and Characterization. J. Mat. Sci. Materials in Medicine 12(10): 861-864.

Bosinco, S., Dambies, L., Guibal, E., Roussy, J., Le, C. & Pierre. (1997). Removal of Cr VI on chitosan gel beads. Kinetic modeling. Adv. Chitin Sci., 2, 453-461.

Bourke, S. L., Khalili, M. A., Briggs, T., Michniak, B. B., Kohn, J. & Warren, L. A. (2003). A Photo-Crosslinked Poly (vinyl Alcohol) Hydrogel Growth Factor Release Vehicle for Wound Healing Applications. AAPS Pharm. Sci. 5(4), article 33.

Braier, N. C. & Jishi, R. A. (2000). Density functional studies of Cu^{2+} and Ni^{2+} binding to chitosan. Journal of Molecular Structure, 499, 51-55.

Brine, C., Sandford, P., & Zikakis, J. (1992). Advances in Chitin and Chitosan. Eds.; Elsevier Applied Science: New York.

Broderson, R. Physical Chemistry of Bilirubin: Binding to macromolecules.
Brown, S. B. (1982). Bilirubin, 1, CRC Press, Florida, Chapter 3.

Cao, Z., Lai, S. & Ge, H. (1999). Preparation of formaldehyde crosslinked chitosan by microwave irradiation and Cu(II) adsorption properties of crosslinked chitosan. Xiandai Huagong, 19(6), 24-26.

Chassary, P., Vincent, T., Marcano, J. S., Macaskie, L. E. & Guibal, E. (2005). Palladium and platinum recovery from bicomponent mixtures using chitosan derivatives. Hydrometallurgy, 76, 131-147.

Chen, X., Shao, Z. Z., Huang, Y. G. Huang, Y., Zhou, P. & Yu, T. Y. (2000). Influence of crosslinking agent content on structure and properties of glutaraldehyde crosslinked chitosan membranes. Huaxue Xuebao, 58(12), 1654-1659.

Choi, K. S. & Ahn, H. S. (1990). A study of synthesis of crosslinked chitosan phosphate and adsorption characteristics of metallic ions. Pollimo, 14(5), 516-526.

Chiou, M. S. & Chuang, G. S. (2006). Competitive adsorption of dye metanil yellow and RB15 in acid solutions on chemically cross-linked chitosan beads. Chemosphere 62, 731–740.

Chiou, M. S., Ho, P. Y. & Li, H. Y. (2004). Adsorption of anionic dyes in acid solutions using chemically cross-linked chitosan beads. Dyes and Pigments, 60, 69–84.

Chiou, M. S. & Li, H. Y. (2003). Method of adsorbing dye in aqueous solution by chemical cross-linked chitosan beads. U.S. Pat. Appl. Publ. US 2003101521.

Crini, G. (2005). Recent developments in polysaccharide-based materials used as adsorbents in wastewater treatment. Progress in Polymer Science, 30, 38-70.

Crini, G. (2006). Non-conventional low-cost adsorbents for dye removal: A review. Bioresource Technology, 97, 1061-1085.

Dantas, T. N. C., Neto, A. A. D., Moura, M. C. P. A., Neto, E. L. B. & Telemaco, E. P. (2001). Chromium Adsorption by Chitosan Impregnated with Microemulsion. Langmuir**,** 17, 4256-4260.

Denkbas, E. B. & Odabas, M. (2000). Chitosan Microspheres and Sponges: Preparation and Characterization. Journal of Applied Polymer Science, 76, 1637–1643.

Desai, K. G. H. & Park, H. J. (2005). Preparation of cross-linked chitosan microspheres by spray drying: Effect of cross-linking agent on the properties of spray dried microspheres. Journal of Microencapsulation, 22(4), 377-395.

Ding, M., Shi, J., Huangpu, L. & Gao, J. (1998). Study on preparation of chitosan microspheres. Huaxue Shijie, 39(12), 636-640.

Du, Y., Tang, R. & Fan, L. (2003). High-strength antibacterial dialdehyde starch crosslinked chitosan film, its preparation and application. Faming Zhuanli Shenqing Gongkai Shuomingshu CN 1434072.

Fang, Y. E., Lu, X. B., Wang, H. T., Cheng, Q., Chen, B. J., Chen, G. J., Wang, Y. J. & Wang, S. G. (1998). Studies of permeabilities of metallic ions through crosslinked chitosan membranes. Chem. Res. Chin. Univ., 14(4), 408-413. CA 130:239086.

Feng, C.G., Bai, L.S., & Ren, Q.S. (2004). Preparation and FTIR Properties of Amino Compounds Modified Glutaraldehyde Crosslinked Chitosan Resin Activated with Cyanuric Chloride. Guangpuxue Yu Guangpu Fenxi, 24(11), 1315-1318.

Feng, C. G., Bai, L. S., & Ren, Q. S. (2003). Preparation and properties of dimethylamine modified glutaraldehyde-crosslinked chitosan resin activated with cyanuric chloride. Lizi Jiaohuan Yu Xifu, 19(3), 262-268.

Feng, X. & Huang, R.Y.M. (1996) Pervaporation with chitosan membranes. I. Separation of water from ethylene glycol by a chitosan/polysulfone composite membrane, J. Membr. Sci., 116, 67-76.

Fujikawa, S., Fukui, Y. & Koga, K. (1987). Structure of genipocyanin G1, a spontaneous reaction product between genipin and glycine. Tetrahedron Lett., 28, 4699–4700.

Gamblin, B. E., Stevens, J. G. Wilson, K. L. (1998). Structural investigations of chitin and chitosan complexed with iron or tin. Hyperfine Interact. 112, 117-122.

Gariepy, E. R., Shive, M., Bichara, A., Berrada, M., Garrec, D. L., Chenite, A. & Leroux, J. C. (2004). A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel. European Journal of Pharmaceutics and Biopharmaceutics, 57, 53-63.

Genta, I., Costantini, M., Asti, A., Conti, B. & Montanari, L. (1998). Influence of glutaraldehyde on drug release and mucoadhesive properties of chitosan microspheres. Carbohydrate Polymers, 36, 81-88.

Gibson, S. L., Walls, H.J., Kennedy, S. B. & Welsh, E. R. (2003). Polymeric Materials Science and Engineering, 88, 199.

Gilding, D. K. & Al-Lamee, K. G. (1997). Biocompatible hydrogels made from crosslinked chitosan and galactomannan-type or/and pectic substances and their manufacture and uses. PCT Int. Appl. WO 9730092.

Glasser, W. G. & Jain, R. K. (1997). Manufacture of ester-crosslinked chitosan hydrogels as support material and quaternized, cyclodextrin-modified chitosan. PCT Int. Appl., WO 974545.

Gong, S., Hu, C., Wang, W. & Liu, Q. (2004). Preparation and adsorption properties of calixarene cross-linked chitosan. Wuhan Daxue Xuebao, Lixueban, 50(4), 458-462.

Guang, W., Shahua, Q., Ganquan, H., Yan, L. & Shaobo, M. (2005). Preconcentration of trace Pt on crosslinked chitosan and its determination by graphite furnace atomic absorption spectrometry, Chemical J. on internet, 7(1), 3

Guibal, E. (2005). Heterogeneous catalysis on chitosan-based materials: a review. Progress in Polymer Science, 30, 71-109.

Guibal, E., Larkin, A., Contandriopoulos, Y. & Gillet, D. (1998). Platinum removal from dilute effluents using chitosan-derived sorbents. Chitin Sci., 3, 456-462.

Guibal, E., Milot, C. & Roussy, C. (2000). J. Sep. sci. Technol., 35, 1021.

Guibal, E., Milot, C. & Tobin, J.M. (1998). Metal-anion sorption by Chitosan Beads: equilibrium and kinetic Studies. Ind. Eng. Chem. Res., 37, 1454-1463.

Guibal, E., Saucedo, I., Jansson-Charrier, M., Delanghe B. & Le Cloirec, P. (1994). Uranium and vanadium sorption by chitosan and derivatives. Water Science and Technology, 30, 183–190.

Guibal, E., Vincent, T., Larkin, A. & Tobin, J. M. (1999). Chitosan Sorbents for Platinum Sorption from Dilute Solutions. Ind. Eng. Chem. Res., 38, 4011-3638.

Gupta, K.C. & Ravi Kumar, M. N. V. (2000). Semi-interpenetrating polymer network beads of crosslinked chitosan-glycine for controlled release of chlorphenramine maleate. Journal of Applied Polymer Science, 76, 672-683.

Gupta, K. C. & Ravi Kumar, M.N.V. (1999). Structural changes and release characteristics of crosslinked chitosan beads in response to solution pH. J. Macromol. Sci., Pure Appl. Chem., A36(5 & 6), 827-841.

Guzman, J., Saucedo, I., Revilla, J., Navarro, R. & Guibal, E. (2002). Vanadium Interactions with Chitosan: Influence of Polymer Protonation and Metal Speciation. Langmuir**,** 18, 1567-1573.

Itoyama, M. & Fujii, T. (1992). Crosslinked, porous chitin microparticles useful for molecular fractionation. Jpn. Kokai Tokkyo Koho JP 04132701.

He, X., Tan, T., Qi, Y., Li, W. & Liu, Q. (2000). Study on synthesis of chitosan chelated resin and its adsorption properties to metal. Lizi Jiaohuan Yu Xifu, 16(1), 47-53.

Heilemann, A., Holzer, J., Horlacher, P., Sander, A. and Wachter, R. (1998). Collagen-free cosmetic preparations obtained from crosslinked chitosan hydrogels. PCT Int. Appl. WO 9817245.

Hejazi, R. & Amiji, M. (2003). Chitosan-based gastrointestinal delivery systems. J. Controlled Release, 89, 151-165.

Hou, M., Hu, C. & Qian, J. (2004). Adsorption properties of the crosslinked chitosan for mercury. Guilin Gongxueyuan Xuebao, 24(2), 223-226.

Hsien, T. Y. and Rorrer, G. L. (1997). Heterogeneous Cross-Linking of Chitosan Gel Beads: Kinetics, Modeling, and Influence on Cadmium Ion Adsorption Capacity. Ind. Eng. Chem. Res., 36, 3631-3638.

Huang, R. Y. M., Pal. R. & Moon, G. Y. (1999). Crosslinked chitosan composite membrane for the pervaporation dehydration of alcohol mixtures and enhancement of structural stability of chitosan/polysulfone composite membranes. Journal of Membrane Science 160(1), 17-30.

Huang, R. G., Schwartz, J. B. and Ofner, C. M. 3rd. (1999). Microencapsulation of chlorpheniramine maleate-resin particles with crosslinked chitosan for sustained release. Pharm. Dev. Technol., 4(1), 107-115.

Huang, X., Yuan, G. & Wang, A. (2000). Adsorption of transition metal ions on cross-linked chitosan resin prepared in presence of Zn(II) template. Lizi Jiaohuan Yu Xifu, 16(3), 262-266.

Huang, W., Han, L., Li, C., Yin, S. & He, B. (1998). Crosslinked chitosan tempolated by Cu^{2+} ion for adsorption of Cu^{2+}, Ni^{2+} and Co^{2+}. Lizi Jiaohuan Yu Xifu, 14(4), 329-336.

Inoue, K., Baba, Y. & Yoshiguza, K. (1993).Adsorption of Metal Ions on Chitosan and Crosslinked Copper (II) –Complexed Chitosan. Bull. Chem. Soc. Jpn., 66, 2915-2921.

Inoue, K., Baba, Y., Yoshizuka, K., Noguchi, H. & Yoshizaki, M. (1988). Selectivity Series in the Adsorption of Metal Ions on a Resin Prepared by Crosslinking Copper (II)-Complexed Chitosan. Chemistry Letters, 1281-1284.

Inoue, K., Yoshizuka, K. & Ohto, K. (1999). Adsorptive separation of some metal ions by complexing agent types of chemically modified chitosan. Analytica Chimica Acta, 388, 209-218.

Inukai, Y., Kaita, Y. & Yasuda, S. (1998). Method for separation of germanium. Jpn. Kokai Tokkyo Koho JP 10195555.

Itagaki, K., Ishikawa, H. & Ahiko, K. (1993). Chromatographic purification of lysozyme. Jpn. Kokai Tokkyo Koho JP 05260966 A2 12 Oct 1993 Heisei, 6 pp.

J. Donald Ostrow (Ed), Bile Pigments and Jaundice. Marcel Dekker, New York, NY, 1986.

Jameela, S. R., Kumary, T. V., Lal, A.V. & Jayakrishnana, A. (1998). Progesterone-loaded chitosan microspheres: a long acting biodegradable controlled delivery system. Journal of Controlled Release, 52, 17–24.

Janes, K. A., Calvo, P., Alonso, M. J. (2001). Polysaccharide colloidal particles as delivery systems for macromolecules. Adv. Drug Delivery Rev., 47, 83-97.

Jiang, J., Huang, G., Qian, S., Wang, M. & Wan, Y. (1998). Application of crosslinked chitosan in speciation analysis of antimony. Fenxi Ceshi Xuebao, 17(4), 1-4.

Jin, L. and Bai, R. (2002). Mechanisms of Lead Adsorption on Chitosan/PVA Hydrogel Beads. Langmuir**,** 18, 9765-9770.

Jing, S. B., Ida, S. & Yamaguchi, T. (1995). Water absorption of chitosan crosslinked by formaldehyde. Kichin, Kitosan Kenkyu, 1(2), 106-107.

Juang, R. S., Wu, F. C. and Tseng, R. L. (2002). Adv. Environ. Res., 171-177.

Juang, R. S., Tseng, R. L., Wu, F. C. & Lee, S. H. (1997). Adsorption behavior of reactive dyes from aqueous solutions on chitosan. J. Chem. Technol. Biotechnol., 70, 391-399.

Juang, R. S., Wu, F. C. & Tseng, R. L. (1999). Adsorption removal of Cu(II) using chitosan from a simulated rinse solution containing chelating agents. Water Res., 33, 2403-2409.

Juang, R. S., Wu, F. C. & Tseng, R. L. (2001). Solute adsorption and enzyme immobilization on chitosan beads prepared from shrimp shell wastes. Bioresource Technology, 80, 187-193.

Kamya, H. & Kita, K. (1997). Novel chitosan derivatives for aqueous solution with good transparency and viscosity stability. Jpn. Kokai Tokkyo Koho JP 09003101.

Kang, D. W., Choi, H. R. & Kweon, D. K. (1996). Selective adsorption capacity for metal ions of amidoximated chitosan bead-g-PAN copolymer. Pollimo, 20(6), 989-995.

Kanitsar, K., Koellensperger, G. & Hann, S. (2003). Determination of Pt, Pd and Rh by inductively coupled plasma sector field mass spectrometry (ICP-SFMS) in size-classified urban aerosol samples. J. Anal. At. Spectrom, 18, 239-246.

Katarina, R. K., Takayanagi, T., Oshima, M. & Motomizu, S. (2006). Synthesis of a chitosan-based chelating resin and its application to the selective concentration and ultratrace determination of silver in environmental water samples. Analytica Chimica Acta, 558, 246-253.

Kawamura, Y., Tanibe, H., Kurahashi, I., Seo, H. and Nakajima, S. (1986). Granular porous chitosan. Ger. Offen. DE 3527482.

Kawase, M., Michibayashi, N., Nakashima, Y., Kurikawa, N., Yagi, K. & Mizoguchi, T. (1997). Application of glutaraldehyde-crosslinked chitosan as a scaffold for hepatocyte attachment. Biol. Pharm. Bull., 20(6), 708-710.

Khurma, J. R., Rohindra, D. R. & Nand, A. V. (2005). Swelling and Thermal Characteristics of Genipin Crosslinked Chitosan and Poly(vinyl pyrrolidone) Hydrogels. Polymer Bulletin, 54, 195–204.

Koide, K., Itoyama, K., Fukushima, T., Miyazawa, F. & Kuwata, T. (1991). Method for separation and concentration of phosphopeptides. Eur. Pat. Appl. EP 443718.

Kondo, K., Matsumoto, M. & Okamoto, K. (1999). Enhanced adsorption of copper(II) ion on novel amidoxime chitosan resin. J. Chem. Eng. Jpn., 32, 217-222.

Kondo, K., Nakagawa, S. I., Matsumoto, M., Yamashita, T. & Furukawa, I. (1997). Selective adsorption of metal ions on novel chitosan-supported sulfonic acid resin. J. Chem. Eng. Jpn., 30(5), 846-851.

Koyama, Y. & Taniguchi, A. (1986). Studies on Chitin X. Homogeneous Cross-linking of Chitosan for Enhanced Cupric Ion Adsorption. Journal of Applied Polymer Science, 31, 1951-1954.

Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. Enzyme and Microbial Technology, 35, 126-139.

Kumbar, S. G., Kulkarni, A. R. and Aminabhavi, T. M. (2002). Crosslinked chitosan microspheres for encapsulation of diclofenac sodium: effect of crosslinking agent. Journal of Microencapsulation, 19(2), 173-180.

Lee, S. T., Mi, F. L., Shen, Y. J., Shyu, S. S. & Tang, I. H. (1998). The copper (II) ion uptake by chitosan-tripolyphosphate chelating resin and related kinetic studies. Adv. Chitin Sci., 3, 475-480.

Lee, Y. M., Nam, S.Y., Oh, B.K., Lee, B.R., Woo, D.J., Lee, K.H., Won, J.M. & Ha, B.H. (1996). Dehygration of alcohol solutions through crosslinked chitosan composite membranes. I. Preparation of chemically crosslinked chitosan composite membranes and ethanol dehydration. Memburein, 6(1), 37-43.

Lee, Y. M., Nam, S. Y., Yoo, J. K. & Yoo, K. O. (1996). Dehydration of alcohol solutions through crosslinked chitosan composite membranes. II. Dehydration of ethanol solutions through modified chitosan composite membranes. Memburein, 6(4), 242-249.

Li, B. & Bai, R. (2005). A Novel Amine-Shielded Surface Cross-Linking of Chitosan Hydrogel Beads for Enhanced Metal Adsorption Performance. Ind. Eng. Chem. Res., 44, 6692-6700.

Li, M., Cheng, S., & Yan, H. (2007). Preparation of crosslinked chitosan/poly(vinyl alcohol) blend beads with high mechanical strength. Green Chemistry, 9, 894-898.

Li, N., Bai, R. & Liu, C. (2005). Enhanced and Selective Adsorption of Mercury Ions on Chitosan Beads Grafted with Polyacrylamide via Surface-Initiated Atom Transfer Radical Polymerization. Langmuir**,** 21, 11780-11787.

Lin, H. R., Chen, K. S., Chen, S. C., Lee, C. H., Chiou, S. H., Chang, T. L. & Wu, T. H. (2007). Attachment of stem cells on porous chitosan scaffold crosslinked by Na5P3O10. Materials Science and Engineering C 27, 280–284.

Liu, Hui (2004). Synthesis of crosslinked chitosan and its adsorption power for Cu2+. Shanxi Daxue Xuebao, Ziran Kexueban, 27(3), 268-272.

Liu, M., Pei, Z., Li, J. & Yin, C. (1999). Adsorption of cholesterol on chitosan beads modified with phenylalanine and tryptophan. Lizi Jiaohuan Yu Xifu, 15(1), 90-94.

Liu, X., Xiao, L., Du, Y., Xu, H. & Qin, C. (2002). Study on adsorption characteristics of o- aminophenol modified chitosan resin. Lizi Jiaohuan Yu Xifu, 18(5), 399-405.

Liu, Y., Lin, Y. & Jiang, C. (1994). Study of chitosan crosslinked with H_2SO_4 hollow fiber composite membrane for pervaporation. Mo Kexue Yu Jishu, 14(1), 21-7.

Magdolna Bodnar, M., Hartmann, J. F. and Borbely, J. (2005). Preparation and Characterization of Chitosan-Based Nanoparticles. Biomacromolecules, 6, 2521-2527.

Masri, M. S. & Randall, V. G. (1978). Chitosan modified with anionic agent and glutaraldehyde. U.S. US 4125708.

Matejka, Z., Ruszova, P., Parschova, H., Jelinek, L. & Kawamura, Y. (2002). Selective uptake of (Mo, V, W, As)- oxoanions by crosslinked chitosan; beads vs. fibers. Advances in Chitin Science, 6, 213-216.

Matsunaka, S., Shin-noyakugaku, Soft Science, Tokyo, 1998. & Emsley, J. in: 2nd ed, The Elements, Clarendon Press, Oxford, 1991

Mayer, J. M. & Kaplan, D. L. (1991). Epichlorohydrin-crosslinked chitosan polymers. U.S. US 5015293.

Mwale, F., Iordanova, M., Demers, C. N., Steffen, T., Roughley, P. & Antoniou, J. (2005). Biological Evaluation of Chitosan Salts Cross-Linked to Genipin as a Cell Scaffold for Disk Tissue Engineering. Tissue Engineering, 11(1-2), 130-140.

Mi, F. L., Lee, S. T. & Shen, Y. J. (1999). Adsorption of copper (II) ion on chitosan-tripolyphosphate chelating resin. Huaxue, 57(1), 11-24.

Mi, F. L., Shyu, S. S., Lee, S. T. & Wong, T. B. (1999). Kinetic Study of Chitosan-Tripolyphosphate Complex Reaction and Acid-Resistive Properties of the Chitosan-Tripolyphosphate Gel Beads Prepared by in-Liquid Curing Method. J. Polym. Sci. Part B: Polym. Phys., 37, 1551-1552.

Mi, F. L., Shyu, S. S. & Peng, C. K. (2005). Characterization of ring-opening polymerization of genipin and pH-dependent cross-linking reactions between chitosan and genipin.Journal of Polymer Science, Part A: Polymer Chemistry, 43(10), 1985-2000.

Mi, F. L., Sung, H. W., Shyu, S. S., Su, C. C. & Peng, C. K. (2003). Synthesis and characterization of biodegradable TPP/Genipin co-crosslinked chitosan gel beads. Polymer, 44(21), 6521-6530.

Mi, F. L., Sung, H. W. & Shyu, S. S. (2000). Synthesis and Characterization of a Novel Chitosan-Based Network Prepared Using Naturally Occurring Crosslinker. Journal of Polymer Science: Part A: Polymer Chemistry, 38, 2804-2814.

Mi, F. L., Sung, H. W. & Shyu, S. S. (2001). Release of indomethacin from a Novel Chitosan Microsphere Prepared by a naturally Occuring Crosslinker: Examination of Crosslinking and Polycation-Anionic Drug Interaction. Journal of Applied Polymer Science, 81, 1700-1711.

Mi, F. L., Tan, Y. C., Liang, H. F. & Sung, H. W. (2002). In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant. Biomaterials, 23, 181–191.

Mitra, S., Gaur, U., Ghosh, P. C., Maitra, A. N. J. (2001). Tumour targeted delivery of encapsulated dextran–doxorubicin conjugate using chitosan nanoparticles as carrier. Controlled Release, 74, 317-323.

Nah, J. W. (1998). Development of transdermal therapeutic systems using crosslinked chitosan and chitosan. Hwahak Konghak, 36(3), 484-490.

Nawawi, M., Ghazali, M. & Huang, R.Y.M. (1997). Pervaporation dehydration of isopropanol with chitosan membranes, J. Membr. Sci., 124, 53-62**.**

Nedelko, N., Lawska-Waniewska, A., Kamierczak, J., Rodrigues, C.A. & Bordini, C. (2006). Magnetic studies of Fe(III)-crosslinked chitosan. Physica Status Solidi (C), Applied Research, 3(1), 126-129.

Ning, G. H., Lu, J. R., Fang, Y. & Hu, D. D. (2000). Preparation and properties of medium polarity sensitive membranes. Gaodeng Xuexiao Huaxue Xuebao, 21(8), 1196-1199.

Nishi, C., Nakajima, N. & Ikada, Y. (1995). In vitro evaluation of cytotoxxicity of diepoxy compounds used for biomaterial modification. J Biomed. Mater. Res., 29, 829–834.

Oh, B. K., Park, C. K. & Lee, Y. M. (1993). Dehydration of aqueous pyridine solution through crosslinked chitosan membrane. Polym. Bull. (Berlin), 31(6), 723-728.

Ohga, K., Kurauchi, Y. & Yanase, H. (1987). Adsorption of copper(II) or mercury(II) ion on resins prepared by crosslinking metal-complexed chitosans. Bull. Chem. Soc. Jpn., 60(1), 444-446.

Oka, K. (1987). Sunscreen and cosmetic powders containing polymers modified with anionic compounds. Jpn. Kokai Tokkyo Koho JP 62198609.

Oshita, K., Oshima, M., Gao, Y. H., Lee, K. H. & Motomizu, S. (2002). Adsorption Behavior of Mercury and Precious Metals on Cross-linked Chitosan and the Removal of Ultrace Amounts of Mercury in Concentrated Hydrochloric Acid by a Column Treatment with Cross-linked Chitosan. Analytical Science, 18, 1121-1125**.**

Peh, K. K., Wong, C. F. (1999). J. Pharm. Pharmaceuicalt Science 2(2) 53-61.

Peirano, F., Flores, J. A., Rodriguez, A., Borja, N. A. Ly, M. & Maidonado, H. (2003). Adsorption of gold(III) ions by chitosan biopolymer. Revista de la Sociedad Quimica del Peru, 69(4), 211-221.

Peng, C., Wang, Y., Cheng, G. & Tang, Y. (1998). Synthesis of carboxyl-containing grafted chitosan polymer and its adsorption properties for heavy metal ions. Huanjing Kexue, 19(5), 29-33.

Piparia, R. & Mathew, H. W. (2006). Optimizing mechanical and cell adhesion properties of chitosan through simultaneous manipulation of molecular weight

and crosslinking. Transaction of 31st Annual Meeting for society for Biomaterials, 29, 278.

Qian, S., Huang, G., Jiang, J., He, F. & Wang, Y. (2000). Studies of Adsorption Behavior of Crosslinked Chitosan for Cr(VI), Se(VI). Journal of Applied Polymer Science, 77, 3216-3219.

Qn, R. & Wang, C. (1997). Studies on natural macromolecular adsorbents. (X). Synthesis and adsorption property of crosslinking chitosan resin. Shuichuli Jishu, 23(4), 230-235.

Qu, R. & Liu, Q. (1996). Preparation and adsorption properties for metal ions of chitosan crosslinked by PEG diglycidyl ether. Huanjing Huaxue, 15(1), 41-46.

Qu, R., Liu, Z., Wang, C., Zhang, P. & Liu, Q. (1997). Synthesis and adsorption characteristics of carboxymethyl crosslinked chitosan resin. Huanjing Kexue Xuebao, 17(1), 121-125.

Qu, X., Wirsen, A. & Albertsson, A. C. (2001). Novel pH-sensitive chitosan hydrogels: swelling behavior and states of water. Polymer, 41, 4589–4598.

Qunhui, G., Ohya, H. & Negishi, Y. (1995). Investigation of the permselectivity of chitosan membrane used in pervaporation separation II. Influences of temperature and membrane thickness. J. Membr. Sci., 98, 223-232.

Ramnani, S. P. & Sabharwal, S. (2006). Adsorption behavior of Cr(VI) onto radiation crosslinked chitosan and its possible application for the treatment of wastewater containing Cr(VI). Reactive and Functional Polymers, 66, 902-909.

Rathke, T., & Hudson, S. (1994). J. Macromol. Sci. Rev. Macromol. Chem. Phys., 34, 375.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. React. Funct. Polym. 46, 1-27.

Reddy, K. R., Rajgopal, K., Maheshwari, C. U. and Kantam, M. L. (2006). Chitosan hydrogel: A green and recyclable biopolymer catalyst for aldol and Knoevenagel reactions. New J. Chem., 30, 1549-1552.

Ren, J. & Jiang, C. (1998). Transport phenomena of chitosan membrane in pervaporation of water-ethanol mixture. Sep. Sci. Tech., 33, 517-535.

Ritthidej, G. C. & Viyoch, J. (1997). Isosorbide dinitrate transdermal patches using crosslinked chitosan-PVA and chitosan-starch membranes. Proc. Int. Symp. Controlled Release Bioact. Mater., 24th, 705-706.

Rorrer, G. L., Hsien, T. Y. & Way, J. D. (1993). Synthesis of porous magnetic chitosan beads for removal of cadmium ions from waste water. Ind. Eng. Chem. Res., 32, 2170-2178.

Roy, S. K., Todd, J. G. & Glasser, W. G. (1998). Manufacture of crosslinked hydrogel beads from chitosan. U.S. US 5770712 A 23 Jun 1998, 14 pp.

Ruiz, M., Sastre, A. M., & Guibal, E. (2000). Palladium sorption on glutaraldehyde-crosslinked chitosan. Reactive and Functional Polymers, 45, 155-173.

Saha, T. K., Jono, K., Ichikawa, H. & Fukumori, Y. (1998). Preparation and evaluation of glutaraldehyde crosslinked chitosan microspheres as a gadolinium reservoir for neutron-capture therapy. Chem. Pharm. Bull., 46(3), 537-539.

Sakairi, N. (2002). Polymeric inclusion compound-based fibers for toxic substance removal, preparation method thereof, and uses therewith. Jpn. Kokai Tokkyo Koho JP 2002327338.

Schmidt, E. W., (1984). Hydrazine and its derivatives, John Wiley and sons Inc, New York, 73.

Shimizu, Y., Takeda, H., Higashimura, T. & Takagishi, T. (1998). Binding of acid dyes by chitosan crosslinked with glutaraldehyde. Nippon Kagaku Kaishi, (9), 637-641.

Seligman, J. W. (1997). Pediatr. Clin. North Am., 24, 509.

Shi, G., Yuan, Y. C., Chen, B. B. & Wang, R. X. (2005). Structure and adsorption property of crosslinked chitosan towards metal ions. Yingyong Huaxue, 22(2), 195-199.

Shieh, J. J. & Huang, R.Y.M. (1997). Pervaporation with chitosan membranes. II. Blend membranes of chitosan and polyacrylic acid and comparison of homogeneous and composite membrane based on polyelectrolyte complexes of chitosan and polyacrylic acid for the separation of ethanol-water mixtures. J. Membr. Sci., 127, 185-202.

Shim, S. K. & Ryu, J. J. (1998). Synthesis of chelating adsorbent (2, 2'-iminodibenzoic acid-crosslinked chitosan) and adsorptivity of Pb(II), Cu(II), Cd(II). Anal. Sci. Technol., 11(6), 452-459.

Shin, M. S., Kim, S. J., Park, S. J., Lee, Y. H. & Kim, S. I. (2002). Synthesis and Characteristics of the Interpenetrating Polymer Network Hydrogel Composed of Chitosan and Polyallylamine. J. Appl. Polym. Sci. 86, 498-503.

Silva, R. M., Silva, G. A., Coutinho, O. P., Mano, J. F. & Reis, R. L. (2004). Preparation and characterization in simulated body conditions of glutaraldehyde crosslinked chitosan membranes. Journal of Materials Science: Materials in Medicine, 15(10), 1105-1112.

Sinha, V. R., Singla, A.K., Wadhawan, S., Kaushik, R., Kumria, R., Bansal, K. & Dhawan, S. (2004). Review Chitosan microspheres as a potential carrier for drugs. International Journal of Pharmaceutics 274, 1–33.

Smith, T. (1994). Rigid materials having high surface area and low density from crosslinked chitosan-like polymers. U.S. US 5322935.

Speer, D. P., Chvapil, M. & Eskelson, C. D. (1980). Ulreich J. Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. J. Biomed. Mater. Res., 14, 753–764.

Sridhar, S., Susheela, G., Jayasimha Reddy, G. & Khan, A. A. (2001). Crosslinked chitosan membranes: characterization and study of dimethylhydrazine dehydration by pervaporation, Polymer International, 50, 1156-1161.

Sung, H. W., Huang, R. N., Huang, L. L. H., Tsai, C. C. & Chiu, C. T. (1998). Feasibility study of a natural crosslinking reagent for biological tissue fixation. J. Biomed. Mater. Res., 42, 560–567.

Tan, S., Wang, Y., Peng, C. & Tang, Y. (1999). Synthesis and Adsorption Properties for Metal Ions of Crosslinked Chitosan Acetate Crown Ethers. Journal of Applied Polymer Science, Vol. 71, 2069–2074.

Tang, R., Du, Y. & Fan, L. (2003). Dialdehyde Starch-Crosslinked Chitosan Films and Their Antimicrobial Effects. Journal of Polymer Science: Part B: Polymer Physics, 41, 993–997.

Tartakovsky, B., Petti, L., Hawari, J. & Guiot, S. R. (1998). Immobilization of anaerobic sludge using chitosan crosslinked with lignosulfonate. J. Ind. Microbiol. Biotechnol., 20(1), 45-47.

Thacharodi, D. & Rao, K. P. (1993). Propranolol hydrochloride release behavior of crosslinked chitosan membranes. J. Chem. Technol. Biotechnol., 58(2), 177-81.

Thome, J. P. & Weltrowski, M. (1997). Affinity of a cross-linked chitosan derivative for organochlorinated xenobiotics in freshwater. Adv. Chitin Sci., 2, 830-836.

Tikhonov, V. E., Radigina, L. A. & Yamskov, Y. A. (1996). Metal chelating chitin derivatives via reaction of chitosan with nitrilotriacetic acid. Carbohydrate Research, 290, 33-41.

Togashi, M. & Hayashi, S. (1991). Dye adsorbents for wastewater treatment. Jpn. Kokai Tokkyo Koho JP 03068446.

Touyama, R., Takeda, Y., Inoue, K., Kawamura, I., Yatsuzuka, M., Ikumoto, T,, Shingu, T., Yokoi, T. & Inouye, H. (1994). Studies on the blue pigments produced from genipin and methylamine. I. Structures of the brownish-red pigments, intermediates leading to the blue pigments. Chem. Pharm. Bull., 42, 668–673.

Trimukhe, K. D., Bachate, S., Gokhale, D. V. & Varma, A. J. (2007). Metal complexes of crosslinked chitosans Part II. An investigation of their hydrolysis to

chitooligosaccharides using chitosanase. International Journal of Biological Macromolecules, 4, 491-496.

Trimukhe, K. D. & Varma, A. J. (2008). Complexation of heavy metals by crosslinked chitin and its deacetylated derivatives. Carbohydrate Polymers, 71, 66-73.

Tseng, R. L., Wu, F. C. & Juang, R. S. (1999). Effect of complexing agents on liquid-phase adsorption and desorption of copper(II) using chitosan. J. Chem. Technol. Biotechnol. 74, 533-538.

Uragami, T., Shirakawa, T. & Miyata, T. (2001). Relationship between structures of cross-linked chitosan membranes and their permeation and separation characteristics for an aqueous solution containing urine components. Nettowaku Porima, 22(4), 212-217**.**

Uragami, T., Kato, S. & Miyata, T. (1997). Structure of N-alkyl chitosan membranes on water-permselectivity for aqueous ethanol solutions, J. Membr. Sci., 124, 203-211.

Uragami, T., Matsuda, T., Okuno, H. & Miyata, T. (1994). Structure of chemically modified chitosan membranes and their characteristics of permeation and separation of aqueous ethanol solutions, J. Membr. Sci., 88, 243-251.

Vincent, T. & Guibal, E. (2002). Chitosan-Supported Palladium Catalyst. 1. Synthesis Procedure. Ind. Eng. Chem. Res., 41, 5158-5164.

Vincent, T., Peirano, F. & Guibal, E. (2004). Chitosan Supported Palladium Catalyst. VI. Nitroaniline Degradation. Journal of Applied Polymer Science, 94(4), 1634–1642**.**

Volkov, V., Skirda, V.D., Vasina, E.N., Korotchkova, S.A., Ohya, H. & Soontarapa, K. (1998). Self-diffusion of water±ethanol mixture in chitosan membranes obtained by pulsed-field gradient nuclear magnetic resonance technique. J. Membr. Sci., 138, 221-225.

Wan Ngah, W. S., Endud, C. S., & Mayanar, R. (2002). Removal of copper (II) ions from aqueous solution onto chitosan and crosslinked chitosan beads. Reactive & Functional Polymers, 50(2), 181-190.

Wan, L. & Wang, Y. (2001). Synthesis of new crown ether crosslinked chitosan. Huaxue Shiji, 23(1), 6-7.

Wan, L., Wang, Y. & Qian, S. (2002). Study on the Adsorption Properties of Novel Crown Ether Crosslinked Chitosan for Metal Ions. Journal of Applied Polymer Science, 84, 29–34.

Wan, Y., Creber, K. A. M., Peppley, B. & Bui, V.T. (2003). Ionic Conductivity and Related Properties of Crosslinked Chitosan Membranes. Journal of Applied Polymer Science, 89(2), 306-317**.**

Wang, G., Qian, S., Huang, G., Yan, L. and Mo, S. (2005). Preconcentration of trace Pt on crosslinked chitosan and its determination by graphite furnace atomic absorption spectrometry. Chemical Journal on Internet, 7(1).

Wang, H., Yuan, Z., Liu, X., Teng, X. & He, B. (2001). Investigations on the adsorbents for uremic middle molecular toxins (II) -Influences of crosslinking agent chain length on the adsorption capacities of crosslinked chitosan adsorbents. Science in China, Series B: Chemistry, 44(5), 457-464.

Wang, M., Huang, G., Qian, S., Jiang, J., wan, Y. & Chau, Y.K. (1997). Fresenius Journal Anal. Chem., 358, 856-858.

Wang, W. & Wang, X. (2001). Studies on separation performance of crosslinked chitosan membrane for various alcohol/water mixtures. Shuichuli Jishu, 27(4), 192-195.

Wang, Y., Peng, C., Tang, Y., Yang, Q. & Luan, Z. (1998). Synthesis of crosslinked chitosan-crown ethers and its adsorption properties for metal ions. Huanjing Huaxue, 17(4), 349-354.

Wang, Y., Cheng, G., Zhu, H., Tang, Y., Feng, X. & Zhao, Y. (1998). Study on properties of crosslinked chitosan polymers for adsorbing metal ions. Huanjing Wuran Yu Fangzhi, 20(1), 1-3.

Wang, Y., Peng, C., Tang, Y., Yang, Q. & Luan, Z. (1998). Synthesis of crosslinked chitosan-crown ethers and its adsorption properties for metal ions Huanjing Huaxue, 17(4), 349-354.

Wang, Y. & Sun, Y. (2001). Preparation of crosslinked chitosan resins suitable for protein adsorption. Tianjin Daxue Xuebao, Ziran Kexue Yu Gongcheng Jishuban, 34(6), 819-822.

Wang, Z., Li, Q. & Wang, S. (2003). Adsorption properties of a new polyaminated and crosslinked chitosan for Cd(II). Beijing Huagong Daxue Xuebao, Ziran Kexueban, 30(2), 93-96.

Wei, C. & Morrison, G. M. (1994). Platinum analysis and speciation in urban gullypots. Anal. Chim. Acta, 284, 587-592.

Welsh, E. R., Schauer, C. L., Qadri, S.B. & Price, R. R. (2002). Chitosan Crosslinking with a Water-Soluble, Blocked Diisocyanate. 1. Solid State. Biomacromolecules, 3, 1370-1374.

Williams III, R. O., Barron, M. K., Alonso, M. J. & Lopez, C. R. (1998). Investigation of a pMDI system containing chitosan microspheres and P134a. International Journal of Pharmaceutics, 174, 209–222.

Wong, Y. C., Szeto, Y.S., Cheung, W.H. & McKay, G. (2004). Adsorption of acid dyes on chitosan equilibrium isotherm analyses. Process Biochemistry, 39, 693–702.

Wu, F. C. & Tseng, R. L. (2002). Adsorption abilities and b-glucosidase immobilization efficiency of glutaraldehyde crosslinked chitosan bead. Taiwan Nongye Huaxue Yu Shipin Kexue, 40(6), 420-426.

Wu, F. C., Tseng, R. L. & Juang, R. S. (1999). Role of pH in metal adsorption from aqueous solutions containing chelating agents on chitosan. Ind. Eng. Chem. Res. 38, 270-275.

Xiao, L., Yu, Z., Yang, C., Zhu, H. & Du, Y. (2004). Swelling studies of chitosan-gelatin films cross-linked by sulfate. Wuhan University Journal of Natural Sciences, 9(2), 247-251.

Xiao, L., Du, Y., Shi, X. & Xue, B. (2001). Manufacture of porous microspheres from crosslinked chitosan with high amino group content. Faming Zhuanli Shenqing Gongkai Shuomingshu CN 1328083.

Yamamoto, H. & Amaike, M. (1997). Biodegradation of Cross-linked Chitosan Gels by a Microorganism. Macromolecules, 30(13), 3936-3937.

Yang, Z., Yuan, Y., Wang, Y. & Tan, G. (2000). Study on azacrown ether grafted chitosan (III) synthesis of dihydroxy mesocyclic diamine crosslinked chitosan. Huaxue Shiji, 22(2), 68-69.

Yang, Z., Wang, Y. & Tang, Y. (1999). Preparation and Adsorption Properties of Metal Ions of Crosslinked Chitosan Azacrown Ethers. Journal of Applied Polymer Science, 74, 3053–3058.

Yang, Z., Zhuang, L. & Tan, G. (2002). Preparation and Adsorption Behavior for Metal of Chitosan Crosslinked by Dihydroxy Azacrown Ether. Journal of Applied Polymer Science, 85, 530–535**.**

Yao, K. D., Liu, J., Cheng, G. X., Zhao, R. Z., wang, W. H. & Wei, L. (1998). The Dynamic Swelling Behavior of Chitosan-based Hydrogels. Polymer International, 45, 191-194.

Yao, K. D., Liu, W. G. & Liu, J. (1999). The Unique Characteristics of Water in Chitosan–Polyether Semi-IPN Hydrogel. Journal of Applied Polymer Science, 71, 449–453.

Yao, K. D., Peng, T., Goosen, M. F. A., Min, J. M. & He, Y. Y. (1993). pH-sensitivity of Hydrogels Based on Complex Forming Chitosan: Polyether Interpenetrating Polymer Network. Journal of Applied Polymer Science, 48, 343-354.

Yang, Z., Wang, Y. & Tang, Y. (1999). Preparation and adsorption properties of Metal Iona of Crosslinked Chitosan azacrown Ethers. Journal of Applied Polymer Science, 74, 3053-3058.

Yang, Z., Zhuang, L. & Tan, G. (2002). Preparation and adsorption behavior for Metal of Chitosan Crosslinked by Dihydroxy Azacrown Ether. Journal of Applied Polymer Science, 85, 530-535.

Ying Wan, Creber, K. A. M., Prppley, B. & Tam Bui, V. (2003). Ionic Conductivity and Related Properties of Crosslinked Chitosan Membranes. Journal of Applied Polymer Science, 89, 306-317.

Yoo, H. J. & Lee, H. J. (1997). Preparation of crosslinked and phosphorylated chitosans and their metal binding properties. Han'guk Somyu Konghakhoechi, 34(7), 451-458.

Yoshinari, I., Yasuhiko, K., Seji, Y. (1998). Method for separation of germanium. Jpn. Kokai Tokkyo Koho JP 10195555 A2 28 Jul 1998.

Yoshizuka, K., Koba, M., Yasukawa, R. & Inoue, K. (1993). In: Solvent Extraction in the Process Industries, 651–658.

Yoshizuka, K., Lou, Z. & Inoue, K. (2000). Silver-complexed chitosan microparticles for pesticide removal. Reactive & Functional Polymers, 44, 47–54.

Yoshizuka, K., Miyazaki, H., Wasai, K., Baba, Y., Inoue, K. (1989). Adsorption equilibrium of silver(I) from aqueous ammoniacal solutions on chelating resins. J. Chem. Eng. Japan, 22, 424–426.

Yu, Y., Gu, H., He, B., Zhen, Z., Bai, T. & Wang, Z. (1997). Adsorption properties of sulfonated hydroxyethyl crosslinked chitosan beads for low-density lipoprotein in plasma. Gaofenzi Xuebao, 5, 606-610.

Yu, Y. H. & He, B. L. (1996). A new type of ALSS - the preparation of crosslinked chitosan resins and its adsorption properties for bilirubin. Reactive & Functional Polymers, 31, 195-200.

Yu, Y. H., Sun, J. T., He, B. L. & Gu, H. Q. (1997). Preparation of a new adsorbent for triglyceride and its adsorption properties for triglyceride in serum. Gaodeng Xuexiao Huaxue Xuebao, 18(10), 1729-1733.

Yu, Y., Sun, Y. & He, B. (2000). Preparation and characterization of crosslinked chitosan resin. Tianjin Daxue Xuebao, Ziran Kexue Yu Gongcheng Jishuban, 33(1), 113-117.

Zhang, S. Q., Wang, Y. T. and Tang, Y. R. (2003). Studies of Some Ultrasonic Elements in Antarctic Water via Crown Ether Crosslinked Chitosan. Journal of Applied Polymer Science, 90, 806-809.

Zhang, Y., Zhang, Y. and He, B. (1994). Study on synthesis of crosslinked chitosan polymer and its use in the removal of urea. Chin. J. React. Polym., 3(1-2), 47-53.

Zeng, W., Wan, Y. & Li, J. (2002). Synthesis of silica gel-crosslinked chitosan adsorbents for heavy metals. Faming Zhuanli Shenqing Gongkai Shuomingshu CN 1359750.

Zeng, X. F. and Ruckenstein, E. (1998a). Cross-linked macroporous chitosan anion-exchange membranes for protein separations. J. Membr. Sci., 148, 195-206.

Zeng, X. F. and Ruckenstein, E. (1998b). Trypsin Purification by *p*-Aminobenzamidine Immobilized on Macroporous Chitosan Membranes. Ind. Eng. Chem. Res., 37, 159-165.

Zheng, H., Du, Y. M., Yu, J. H.& Xiao, L. (2000). Properties and preparation of crosslinked chitosan films. Gaodeng Xuexiao Huaxue Xuebao, 21(5), 809-812.

Zheng, L., Xingle, T. & Xianming, J. (1999). Synthesis of chelating resin of crosslinked chitosan condensed with salicylaldehyde by Cu(II) as template. Yingyong Huaxue, 16(3), 79-80.

Zhong, W., Li, W. J., Ge, C. J. & Chen, X. (1996). Studies on new acrylic acid crosslinked chitosan pervaporation membrane. (II). Prevaporation separation of ethanol-water mixtures. Gaodeng Xuexiao Huaxue Xuebao, 17(8), 1327-1329.

Zhong, W., Ge, C. J., Chen, X. & Li, W. J. (1996). Studies on new acrylic acid crosslinked chitosan pervaporation membrane. (I). Membrane structure and crosslinking mechanism. Gaodeng Xuexiao Huaxue Xuebao, 17(3), 470-473.

Zhou, Yue. (2003). Preparation of resins under microwave irradiation and their properties for adsorbing metal ions. Hunan Ligong Xueyuan Xuebao, Ziran Kexueban, 16(3), 61-64.

CHAPTER 2

"Complexation of heavy metals by crosslinked chitin and its deacetylated Derivatives"

Abstract:

Chitin was crosslinked using diisocyanatohexane (HDI), trimellitic anhydride (TMA), and dibromodecane (DBD), then deacetylated in strong aqueous alkali. This led to a product with amine functional groups on the exposed surface of the crosslinked chitin, which could be utilized for complexation with heavy metals. Thus, a key feature of the crosslinked derivatives prepared was that only the hydroxy groups were utilized in the crosslinking reaction, and the acetylamino groups of chitin were hydrolyzed only after the crosslinking was accomplished. This ensured that all amino groups of the chitosans so produced would be available for metal complexation, and not partially used up in crosslinking. This proposed advantage was proved by the similar binding observed for heavy metals like Hg (348-372 mg/g), Cu (91-119 mg/g), Zn (71-92 mg/g), Mn (3-10 mg/g), Cd (121-160 mg/g), and Pb (32-86 mg/g) using these crosslinked polymers (max. and min. values taken from Table 1), whereas the control polymer (uncrosslinked chitosan powder) had complexation values for Hg (348-361mg/g), Cu (100-106 mg/g), Zn (81-92 mg/g), Mn (4-7 mg/g), Cd (135 mg/g), and Pb (25-59 mg/g). Additionally, in a case where chitosan was crosslinked with HDI, the amino groups were consumed in the crosslinking reaction, and the metal complexation capacity has found to be decreased for Cu (91-109 mg/g), Cd 133 mg/g), and Zn (71-77 mg/g), while remaining nearly the same for Hg (362 mg/g). The literature value for Cu complexation is 59.67 mg/g for chitosan crosslinked with glutaraldehyde. The crosslinked derivatives have the added advantage of insolubility even in low pH aqueous media, making their repeated re-use possible. Further, these crosslinked derivatives could be used in powder form, and the additional step of preparing beads was found to be not necessary for ease of separation of the crosslinked powder by filtration. The binding capacity of various crosslinked chitin and deacetylated derivatives for Cu, Cd, Hg, Zn, Mn, and Pb was in the region of 100, 140, 360, 88 , 5 and 60 mg/g (rounded off values) of polymer respectively, very close to the values obtained for uncrosslinked chitosan. The metal binding for crosslinked chitosan was slightly lower than that of crosslinked chitin and deacetylated derivatives, due to use of

some amino groups in crosslinking. For Cu ions, the Langmuir equation was found to be the best fit for HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin. The morphological studies conducted using WAXRD are in close agreement with the metal complexation data, showing complete loss of original chitosan peaks for the heavily complexed derivatives, and minor changes for the weakly complexed metals.

2.1. Introduction

A key property of soluble polymers endowed with functional groups is their ability to complex with a variety of metal ions in solution. For water soluble polysaccharides, the optimum binding of a particular metal to a particular polymer having suitable ligands (such as carboxyl or amino groups) occurs under specific conditions of pH, polymer concentration, metal concentration, temperature, and so on. However, crosslinked polymers offer flexibility in metal binding conditions, as polymer solubility, conformation, molecular weight and concentration is not an issue. Here, the surface area, concentration of metal complexing ligands on the surface of the crosslinked polymer and porosity of the crosslinked polymer will affect the extent of binding. A very large number of publications have dwelt on the complexation ability of chitosan and its crosslinked derivatives with complex transition metals, organic species like dyes, and enzymes (Varma, Deshpande, & Kennedy, 2004; Juang, Wu, & Tseng, 2002; Merrifield, Davids, MacRae, & Amirbahman, 2004; Tan, Wang, Peng, & Tang, 1999; Li, Chen, & Liu, 2003; Taboada, Cabrera, & Cardenas, 2003; Rhazi, Desbrieres, Tolaimate, Rinaudo, Vottero, Alagui, El Meray, 2002; Domard & Piron, 2000; Dobetti & Delben, 1992; Schmuhl, Krieg, & Keizer, 2001; Bassi, Prasher, & Simpson, 2000). However, we believe this to be the first study where chitin was first crosslinked and then deacetylated to give crosslinked chitosans which have been used here to study heavy metal complexation without bead formation. Results of crosslinked chitosans with these new structures and morphologies will help in gaining new insights into factors affecting metal

binding to chitosans. A recent study on glutaraldehyde crosslinked chitosan (Webster, Halling & Grant, 2007) infers that though the surface area of the crosslinked polymer increases, the effect of crosslinking is to increase the competion of metal-binding N sites, which in turn decreases the metal uptake. Using our methodology, the crosslinked polymer does not exhibit decrease in metal uptake. Another recent paper (Kopecky, Kopecka, & Misikova, 2005) showed the importance of the counterion in metal complexation, where the changes for CU can range from 140 mg/g for $Cu(NO_3)_2$ to 190 mg/g for $CuSO_4$. However, this aspect was not investigated in the present research.

Investigations on many other materials like fly ash, silica gel, zeolites, lignin, seaweed, wool wastes, agricultural wastes, clay materials, sugarcane bagasse, etc. have also been reported for applications like the removal of pollutants from aqueous streams, especially for heavy metals like Cd^{2+}, Cr^{3+}, Cr^{6+}, $Hg^{2+}$and Pb^{2+} (Varma, Deshpande, & Kennedy, 2004). A search of the published literature shows that maximum adsorption capacities for complexing Cd^{2+}, Cr^{3+} and Hg^{2+} are 558, 92, 1123 mg/g of chitosan respectively, which are higher than that of other polysaccharide materials studied (Bailey, Olin, Bricka, & Adrian, 1999). For example, sugarcane bagasse and its oxidized product (Filho, Winkler-Hechenleitner, & Gomez-Pineda, 1996) can bind Cu^{2+}; the adsorption capacity being 0.1292 mmol/g (8.21 mg/g), while the value for chitosan beads is 80.71 mg/g and 59.67 mg/g for chitosan crosslinked with glutaraldehyde (Ngah Wan, Endud, & Mayanar, 2002). Similarly, lignin from bagasse can bind Cd^{2+} and Pb^{2+} (Peternele, Winkler-Hechenleitner, & Gomez-Pineda, 1999). Analysis of these studies make it clear that chitosan and its crosslinked derivatives have greater capacity for complexing heavy metals, and are also amenable to easy functionalization by specific ligands for building specificity into the molecule.

In this present study, the complexation of a series of heavy metal salts of Cu^{++}, Zn^{++}, Mn^{++}, Pb^{++}, Hg^{++}, Cd^{++} to a series of crosslinked chitosans were investigated by a variety of techniques like UV spectroscopy, wet chemical

methods (appropriate titration methods for each case), atomic absorption spectroscopy, Langmuir adsorption studies, and wide angle X-ray diffractometry. Evidence for specific as well as non-specific interaction between the metal ion and the polymer could be detected. The ligands on the crosslinked polymers were predominantly amino groups, along with hydroxyl groups (and, in the case of trimellitic anhydride crosslinking, some carboxyl groups were also present). All the chosen heavy metals have implications in toxic interactions in living systems, in addition to industrial applications like ion-exchange media and pollution control. This comprehensive investigation seeks to simplify the metal complexation methodology and obtain an understanding of the morphology of the metal complexes.

2.2. Experimental Section:

2.2.1. Materials

The chitin and chitosan used in this study are commercial products of Meron Biopolymers, Cochin, Kerala, India. (^{13}C-CPMAS NMR are shown in Fig. 1(1) and 1(2). D-glucosamine was obtained from Sigma Chemical Co. (St. Louis, MO). Diisocyanatohexane (HDI) was obtained from Aldrich Chemical Co., trimellitic anhydride (TMA) and dibromodecane (DBD) was obtained from Merck. Dimethylformamide, toluene and sodium hydroxide pellets were AR grade chemicals, obtained from SD fine chemicals, Mumbai. Sodium hydride was obtained from Merck. 4-Dimethylaminopyridine was purchased from Lancaster Company. All metal salts were AR grade materials and used without further purification. The salts $ZnCl_2$, $MnSO_4$, $CdSO_4$, $Pb(NO_3)_2$ were obtained from Loba Chemie, Mumbai, $CuSO_4$ was from SD Fine Chemicals, Mumbai, and $HgCl_2$ was from Merck.

2.2.2. Synthetic procedures

2.2.2.1. General procedure for deacetylation of chitin and crosslinked chitin:

10g chitosan was dispersed in 200 ml of 50% NaOH solution (100 g NaOH dissolved in 153 ml distilled water). The reaction was carried out in a high pressure Parr reactor by first bubbling in nitrogen gas through the reaction mixture for 5 minutes, followed by maintaining nitrogen gas pressure of 60 psi in the reactor. The temperature was set at 135^0C and maintained at 135^0C for 3.5 hours, with stirring at 200 rpm. Under these conditions, the gage pressure reading was 100 psi. The whole reaction mixture was transferred into 4 l of distilled water and left as such for 2 hours. Then the water layer was decanted and the solid separated was washed with distilled water and methanol till the pH of the wash water was neutral. Finally solid was washed with methanol and dried under vacuum. Fig. 1(3) shows the ^{13}C-CPMAS NMR of deacetylated chitin.

Calculations for the degree of deacetylation:

Degree of acetylation (DA) and degree of deacetylation (DD) calculated from ^{13}C CP/MAS NMR spectroscopy (J. of Applied Polymer Science, Vol. 93, 1876-1885 (2004)

$$DA\% = \frac{I_{CH3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6} \times 100$$

$$DD\% = 100\text{-}DA$$

By this method, the degree of deacetylation of chitin to produce chitosan using the above deacetylation procedure was 88%. By this same procedure we calculated the degree of deacetylation of commercial chitosan (obtained from Meron, India) as 85%.

2.2.2.2. Diisocyanatohexane-crosslinked deacetylated chitin:

40gm (0.1970 M) dry chitin was stirred in dry and distilled toluene (300mL) and shaken well. It was left for 2 hours at room temperature. It was filtered to obtain moisture free chitin. This moisture free chitin was dispersed in 500 ml dry distilled toluene in a round bottom flask equipped with reflux condenser and

calcium guard tube. It was stirred vigorously at 50^0C using a mechanical stirrer. 35mL (0.2167 M) diisocyanatohexane (HDI) diluted in 100ml dry distilled toluene was added dropwise over a period of one hour at 50^0C. After completion of addition, the reaction was continued at 50^0C for 5 h.

The reaction mixture was then filtered. The solid part was washed with toluene two to three times and finally dried. Fig. 1(4) shows the ^{13}C-CPMAS NMR of HDI crosslinked chitin

Deacetylation of HDI–crosslinked chitin:

10gm HDI–crosslinked chitin was deacetylated by the above mentioned deacetylation procedure. The weight of the deacetylated product was 8.6 g. The CPMAS ^{13}C-NMR is shown in Fig. 1(5).

2.2.2.3. Trimellitic anhydride-crosslinked deacetylated chitin:

20gm (0.0985 M) dry chitin was dispersed in dry and distilled DMF (150ml) and shaken well. It was left for 2 hours at room temperature. It was filtered to obtain moisture free chitin. This moisture free chitin was dispersed in 300ml dry distilled DMF taken in round bottom flask equipped with a reflux condenser and guard tube. It was stirred vigorously at 110^0C using a mechanical stirrer. 200mg DMAP was added in the reaction flask and 4gm (0.0202 M) trimellitic anhydride (TMA) diluted in 20ml dry distilled DMF was added dropwise over a period of 40 min. at temp.110^0C. Reaction was continued at 110^0C.for 6 hours. The reaction mixture was filtered and the solid was separated. and washed with methanol several times and finally dried. The CPMAS ^{13}C-NMR is shown in Fig. 1(6).

Deacetylation of trimellitic anhydride–crosslinked chitin:

10g Trimellitic anhydride–crosslinked chitin was deacetylated by the general procedure outlined earlier. Weight of the TMA-crosslinked deacetylated chitin obtained was 6.85gm. The CPMAS ^{13}C-NMR is shown in Fig.1(7).

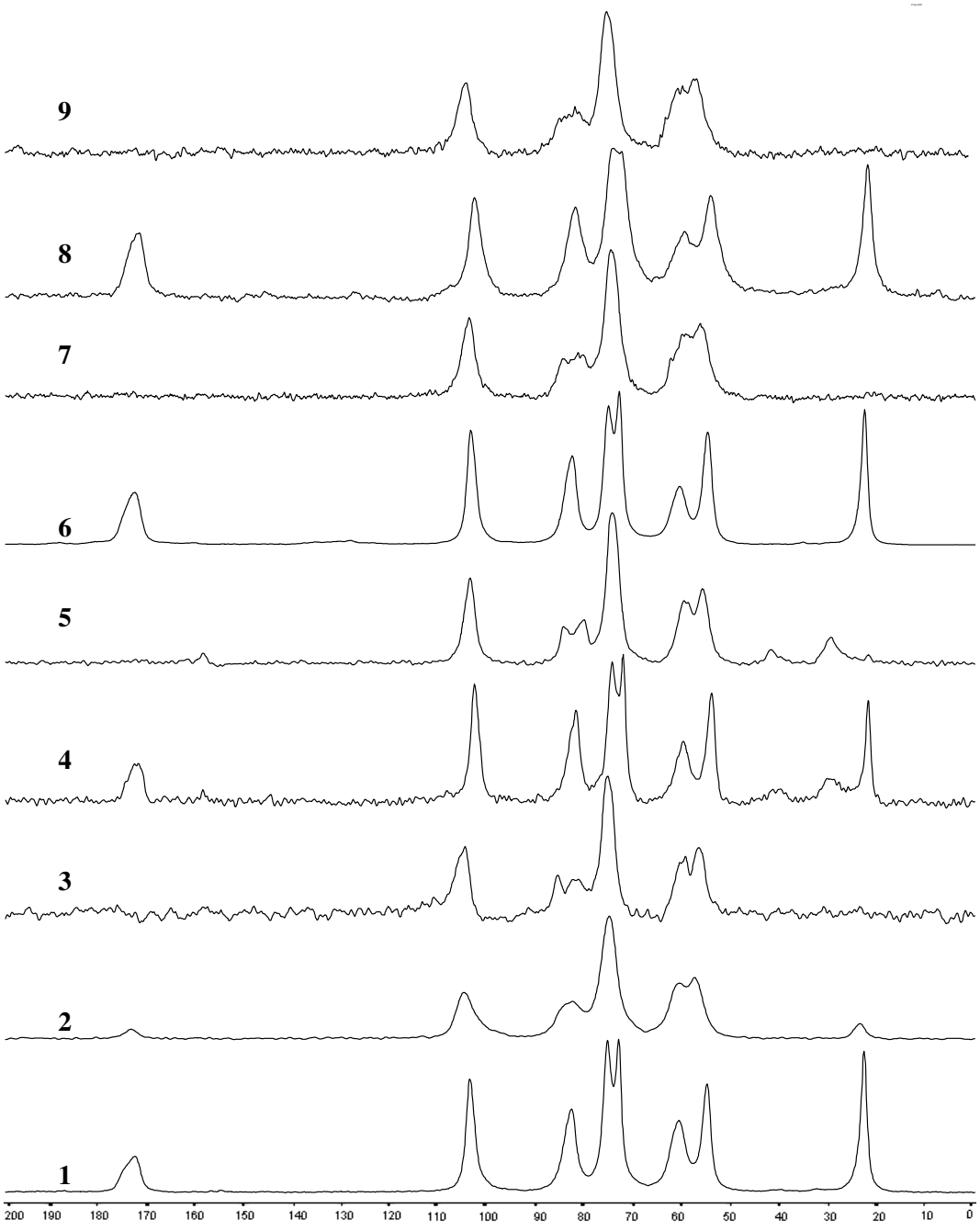


Figure 1: CPMAS ^{13}C-NMR of (1) chitin (Meron), (2) commercial chitosan (Meron), (3) deacetylated chitin (chitosan), (4) HDI crosslinked chitin, (5) HDI crosslinked deacetylated chitin (chitosan), (6) TMA crosslinked chitin, (7) TMA crosslinked deacetylated chitin (chitosan), (8) DBD crosslinked chitin, (9) DBD crosslinked deacetylated chitin (chitosan).

2.2.2.4. Dibromodecane–crosslinked deacetylated chitin:

40gm (0.1970 M) dry chitin was dispersed in 300 mL dry distilled DMF taken in a round bottom flask and shaken well. It was left over night at room temperature. It was filtered to obtain moisture free chitin. This moisture free chitin was dispersed in 500 ml dry distilled DMF taken in a round bottom flask equipped with a reflux condenser and calcium guard tube. It was stirred vigorously at room temperature using a mechanical stirrer.10ml (13.35 g) (0.0445 M) 1,10 dibromodecane added dropwise at room temperature. Then slowly increase the temperature to110^0 C. Then added portion wise 2.2g (0.092) of NaH (3.7 g of 60% dispersion of NaH in mineral oil). At that time maintain the temperature to 110^0C. Complete addition of NaH took 6h. After completion of addition the reaction was continued at 110^0C for one hour.

The reaction mixture was filtered. The solid was washed with distilled water to remove NaBr. The solid was washed with distilled water till the filtrate shows neutral and finally solid washed with acetone and dried. The CPMAS ^{13}C-NMR is shown in Fig.1(8).

Deacetylation of dibromodecane – crosslinked chitin:

10g Dibromodecane–crosslinked chitin was deacetylated by the general deacetylated procedure outlined earlier. Weight of the Dibromodecane-crosslinked deacetylated chitin obtained was 7.25 g. The CPMAS ^{13}C-NMR is shown in Fig.1 (9).

2.2.2.5. Diisocyanatohexane-crosslinked chitosan:

40g (0.1970M) dry chitosan was dispersed in dry and distilled toluene (300mL) taken in round bottom flask and shaken well. It was left for 2 hours at room temperature. It was filtered to obtain moisture free chitosan. This moisture free chitosan was dispersed in dry distilled toluene (500mL) taken in a round bottom flask equipped with a reflux condenser and calcium guard tube. It was stirred vigorously at 50^0C using a mechanical stirrer. 35mL (0.2167M) diisocyanatohexane (HDI) diluted in 100ml dry distilled toluene was added

dropwise over a period of one hour at 50^0C. Reaction was continued at 50^0C for 5 hours. The reaction mixture was filtered and the solid separated. The solid was washed with toluene two to three times and finally dried.

2.2.3. Methods for evaluation of metal complexation:

2.2.3.1. Determination of the metal complexation capacity by titration method:

Chitosan and crosslinked derivatives prepared as above (approx.100mg) were dispersed in 20ml of 0.01M metal solutions (eg.$CuSO_4.5H_20$, $MnSO_4.H_2O$, $ZnSO_4.H_2O$, $CdSO_4.8/3H_2O$, $Pb(NO_3)_2$, $HgCl_2$) and were shaken at 90 rpm in a lab shaker bath overnight at room temperature. After that it was filtered, and the filtrate was used for titration as well as UV study and AAS study.

Determination of copper complexation

10 ml of filtrate was taken in a conical flask and diluted with 40 ml deionized water. Adjust the pH of the solution to 10-11 by the addition of 1 ml of 25 % aqueous ammonia solution. Add few drops of fast sulphon black F as an indicator and titrate against 0.01M EDTA solutions until the color changes from purple to green. For blank titration use 0.01M $CuSO_4.5H_2O$ solutions instead of filtrate.

Determination of zinc complexation

10 ml of filtrate was taken in a conical flask and diluted with 40 ml deionized water. Adjust the pH of the solution to 10 by the addition of 4 ml of ammonia and ammonium chloride buffer solution (pH is approx 10). Add few drops of solochrome black T as an indicator and titrate against 0.01M EDTA solutions until the color changes from purple to light green color. For blank titration use 0.01M ZnSO4 solutions instead of filtrate.

Determination of manganese complexation

10 ml of filtrate was taken in a conical flask and diluted with 40 ml deionized water, then add 0.1 gm ammonium hydroxide hydrochloride and 1.5 ml triethanol

amine. Adjust the pH of the solution to 10 by the addition of 4 ml of ammonia and ammonium chloride buffer solution (pH is approx.10). Add few drops of solochrome black T as an indicator. Titrated it against 0.01M EDTA solutions until the color changes from purple to bluish green color. For blank titration use 0.01M ZnSO4 solutions instead of filtrate.

Determination of lead complexation

10 ml of filtrate was taken in a conical flask and diluted with 40 ml deionized water. Adjust the pH of the solution to 6 by the addition of 4 ml of 30% aqueous hexamine buffer solution (pH is 6-7). Add few drops of 0.2% aqueous xylenol orange as an indicator. Titrated it against 0.01M EDTA solutions until the color changes from purple to light yellow color. For blank titration use 0.01M $Pb(NO_3)_2$ solution instead of filtrate.

Determination of mercury complexation

10 ml of filtrate was taken in a conical flask and diluted with 40 ml deionized water. Adjust the pH of the solution to 6 by the addition of 4 ml of 30% hexamine buffer solution (pH is 7 to 8). Add few drops of methyl thymol blue as an indicator. Titrate it against 0.01M EDTA solutions until the color changes from light purple to t yellow color by literature it was blue to yellow color. For blank titration use 0.01M $HgCl_2$ solutions instead of filtrate.

Determination of cadmium complexation

10 ml of filtrate was taken in a conical flask and diluted with 40 ml deionized water. Adjust the pH of the solution to 5 by the addition of 4 ml of 30% hexamine buffer solution (pH is 6 to 7). Add few drops of 0.2% xylenol orange as an indicator and titrate against 0.01M EDTA solutions until the color changes from light purple to yellow color by literature it was blue to yellow color. For blank titration use 0.01M $CdSO_4 8/3H_2O$ solutions instead of filtrate.

Table 1:

Removal of heavy metal by chitosan and crosslinked chitosan for metal ion solutions
mg/g.

(Titr^{n}. - by titration method.

UV - by Ultraviolet spectrophotometer method.

AAS - by Atomic Absorption spectrophotometer method

a - λ_{max} shows below 190 nm.

b - due to unavailability of particular metal lamp)

Sample Name.	Cu^{++} mg/g			Cd^{++} mg/g			Hg^{++} mg/g			Zn^{++} mg/g			Mn^{++} mg/g			Pb^{++} mg/g		
	Titr^{n}	UV	AAS	Titr^{n}	UV	AAS	Titr^{n}.	UV	AAS	Titr^{n}	UV	AAS	Titr^{n}	UV	AAS	Titr^{n}	UV	AAS
Chitosan (Meron)	100	104	106	135	a	b	348	361	b	92	a	81	07	a	04	59	25	46
Deacetylated Chitin	107	111	119	121	a	b	356	372	b	88	a	73	05	a	04	53	65	33
HDI-crosslinked deacetylated Chitin	103	107	115	145	a	b	348	370	b	85	a	77	05	a	08	55	49	33
Dibromo-decane-crosslinked deacetylated Chitin	104	104	115	160	a	b	361	362	b	87	a	79	06	a	04	51	32	33
Trimellitic anhydride-crosslinked deacetylated Chitin	100	111	116	134	a	b	360	361	b	90	a	84	03	a	10	69	82	33
HDI-crosslinked Chitosan	091	099	109	133	a	b	362	362	b	77	a	71	05	a	06	67	86	33

2.2.3.2. Metal adsorption capacity by UV spectrophotometry:

The filtrates from above section (c) were used for calculating the value of absorbance from the standard calibration curves generated earlier. Results are reported in Table 1.

2.2.3.3. By Atomic Absorption spectrophotometer:

The filtrates from above section (c) were used for calculating the concentration of the metal ion in mg/l in the solution, after preparing standard calibration curves. Results are reported in Table 1.

2.2.4. Adsorption experiments

Langmuir and Freundlich adsorption equilibrium studies were conducted for the specific case of copper ion complexation using diisocyanatohexane-crosslinked deacetylated chitin and trimellitic anhydride-crosslinked deacetylated chitin powder. The isotherm studies were conducted with a constant weight of crosslinked deacetylated chitin powder weight (100 mg) and adding varying initial concentration of Cu (II) ions in the range 125-2500 ppm in aqueous media, with contact time of 24 h and pH 7.0.

Langmuir adsorption: The extent of adsorption was calculated based on the difference of Cu (II) concentration in the aqueous solution before and after adsorption, as follows (Atia, A.A., Ahmed, M.D., & Elwakeel, K.Z., 2005).

$$\text{Adsorption Capacity (X)} = \frac{(Co - Ce)}{W} V$$

Where, Co is the initial Cu (II) concentration (ppm), Ce is the final or equilibrium concentration (ppm), V is the volume of Cu (II) solution (in liters), and W is the weight of the crosslinked deacetylated chitin powder (g).

The basic assumption in Langmuir adsorption isotherm is that the adsorbed layer is one molecule thick and that all sites are equal, thereby resulting in equal energies and enthalpies of adsorption (Ngah, W.S.Wan, & Musa, A, 1998). The strength of the intermolecular attractive forces is believed to fall of rapidly with distance. The sorption data were analyzed according to the linear form of the Langmuir isotherm

$Ce/X = Ce/X_{max} + 1/X_{max}\, b$

Where, Ce is the equilibrium or final concentration of M (II) (ppm), X is the amount of M (II) adsorbed per unit weight of chitosan at equilibrium concentration (mg/g), X_{max} is the maximum adsorption at monolayer coverage (mg/g) and b is the Langmuir adsorption equilibrium constant (ml/mg) and is a measure of the energy of adsorption. Figs. 2 and 3 show the experimental equilibrium isotherms for adsorption of M on HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin

Uptake measurements:

Uptake experiments of the Cu (II) ions were done by placing 100 mg of dry HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin in a series of flasks containing 20ml Cu (II) solution in the concentration range 125-2500 ppm. The flasks were kept at room temperature with shaking over a period of 24 hrs. After the completion of equilibration time solution was filtered and 10 ml of filtrate was titrated against 0.01M EDTA using fast sulphon black F as indicator.

From figure 2 it is seen that the adsorption capacity of HDI-crosslinked deacetylated chitin and TMA-crosslinked deacetylated chitin increases as the concentration of Cu (II) ion increases until an equilibrium concentration of about 490 ppm.

From table 2 it is seen that the maximum uptake capacity of HDI-crosslinked deacetylated chitin-Cu (II) was 176.1mg/g and TMA-crosslinked deacetylated chitin-Cu (II) was 159.12 mg/g. These are much higher than the values reported by other workers, such as 59.67 mg/g for chitosan crosslinked with glutaraldehyde (Ngah Wan, Endud, & Mayanar, 2002).

Figure 2: Adsorption isotherm of Cu (II) ion on HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin

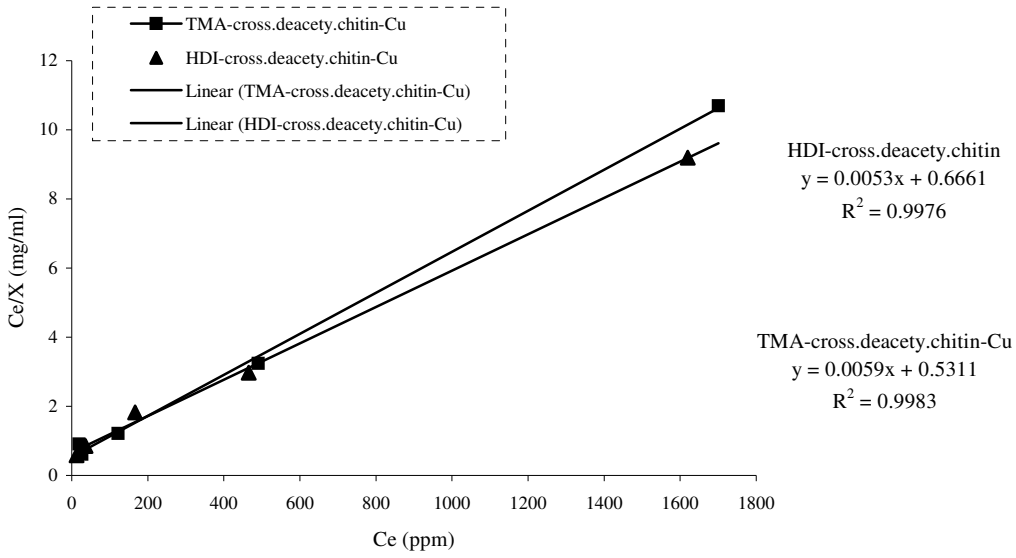


Figure 3: Adsorption isotherms of Cu (II) by the HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin, linearized according to the Langmuir isotherm.

Table 2:

Uptake capacity or adsorption capacity (X) (mg/g) of HDI-crosslinked deacetylated chitin and TMA-crosslinked deacetylated chitin using Cu (II) towards different initial concentrations.

Initial Concentration (Ce) ppm	Uptake capacity or adsorption capacity (X) mg/g	
	HDI-crosslinked deacetylated chitin	TMA-crosslinked deacetylated chitin
2500	176.1	159.12
1250	156.38	151.42
625	91.37	100.24
250	42.37	44.39
125	22.32	21.09

Uptake capacity increases with the increase of equilibrium concentration until reaching a saturation value 156.38 mg/g for HDI-crosslinked deacetylated chitin-Cu (II) and 151.42 mg/g for TMA-crosslinked deacetylated chitin-Cu (II) after that concentration has no longer effect on uptake capacity.

Plotting the graph of Ce versus Ce/X gives X_{max} and b (Figure 3) shows that the experimental adsorption isotherm values are fitted into the linearized forms of the Langmuir equation (correlation coefficient was found to be $R^2 > 0.99$). From the slope and the intercept of the straight lines the numerical values of Langmuir constants were obtained are shown in table 3.

Freundlich Isotherm

The Freundlich isotherm is another form of the Langmuir approach used for studying adsorption on amorphous surfaces (Ng, J.C.Y., 2002). The Freundlich equation shows that the metal concentrations on the adsorbent will increase as

long as there is an increase in the metal ions concentrations in the solution. The equation can be represented by

$X = K_F Ce^{bF}$

Where $b_F = 1/n$

Where, K_F is the Freundlich constant and represents the adsorption capacity (mg/g), b_F is the Freundlich exponent and "n" is a constant, which represents adsorption intensity.

If it is in the linear form of the Freundlich equation, it will yield the constants K_F and b_F.

In this case, $\text{Log } X = 1/n \log Ce + \log K_F$

Table 3: Experimental Langmuir isotherm constants and correlation coefficients

Sample used	Metal ion	Adsorption constant		R^2
		X_{max}	b (L/mmol)	
HDI-crosslinked deacetylated chitin	Cu (II)	188.67	0.008	0.9976
TMA-crosslinked deacetylated chitin	Cu (II)	169.49	0.011	0.9983

Table 4: Freundlich isotherm constant for Cu (II) ion sorption onto HDI-crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin

Sample-Cu (II)	$1/n = b_F$ (mg/g)	K_F (dm^3/g)	R^2
HDI-crosslinked deacetylated chitin - Cu (II)	0.4457	8.2281	0.9566
TMA-crosslinked deacetylated chitin - Cu (II)	0.422	9.2512	0.8495

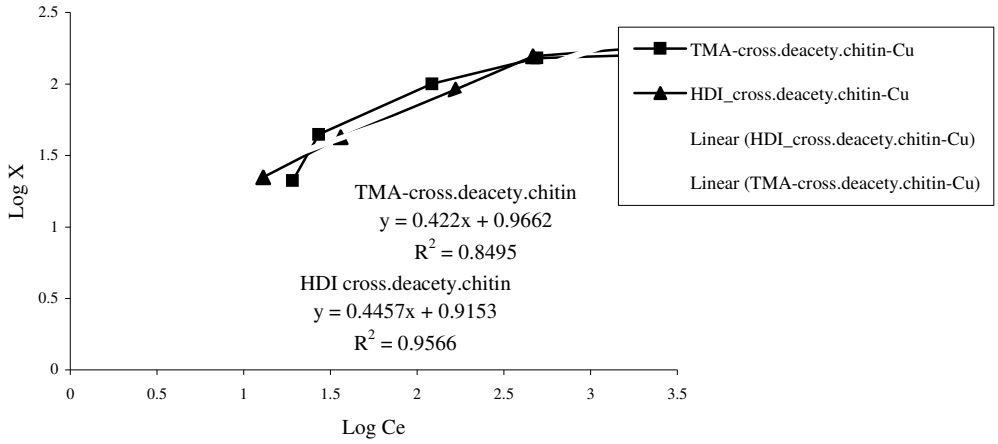


Figure 4: Adsorption isotherm of Cu (II) ion with HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin on the linearized form of Freudlich equation.

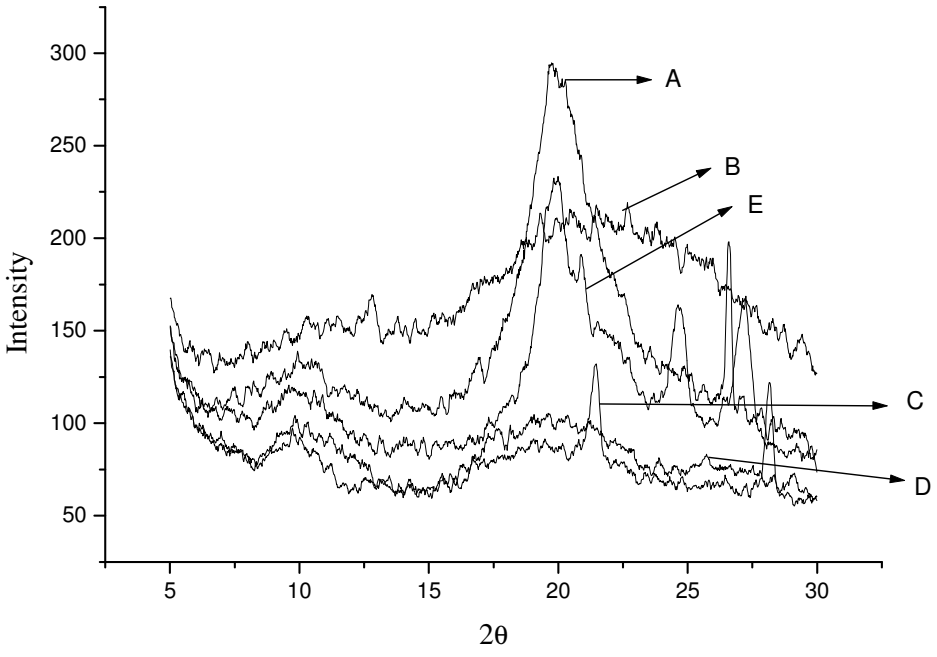


Figure 5: WAXRD of (A) Chitosan, (B) chitosan-Cu complex, (C) chitosan-Hg complex, (D) chitosan-Cd complex, (E) chitosan-Pb complex.

Figure 4 shows the adsorption isotherm of Cu (II) ion with HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin on the linearized form of Freudlich equation. The graph is linear for the HDI crosslinked material, but not so with the TMA crosslinked material.

Wide-angle X-ray diffraction:

From fig. 5(A) it is seen that chitosan shows three characteristic peaks of crystalline nature at 9.85, 19.70 and 26.57^0 but the chitosan-Cu complex (Fig. 5(B)) is characterized by a broad amorphous peak at 22.16. A similar spectrum has been reported earlier (Yin, X, et al., 2004). Thus, complexation of Cu (II) leads to very significant changes in the morphology of the chitosan, indicating complete disruption of the interpolymer bonds.

Similarly, the chitosan-Hg complex (Fig. 5(C)) shows three peaks of crystalline region at 9.76, 21.50 and 28.17^0. From literature $HgCl_2$ shows three characteristic peaks of crystalline region at 20.40, 29.46 and 21.66^0. The main chitosan peak characteristic of chitosan disappears in chitosan-Hg complex due to disruption of the interpolymer bonds**.**

In the case of chitosan-Cd complex (Fig. 5(D)), one characteristic peak of crystalline region at 9.83^0 of chitosan is observed, while the major characteristic peaks of chitosan as well as CdSO4.8/3H2O disappear. This is reflected in the lower extent of Cd complexation to chitosan as compared to Cu and Hg.

In Fig.5 (E), the very small extent of complexation of Pb with chitosan is further proved by the negligible change in the WAXRD spectrum.

Exactly the same WAXRD results were obtained with TMA-crosslinked deacetylated chitin, and are therefore not reproduced here.

2.3. Conclusions:

The studies carried out indicate that simple crosslinking of chitin with crosslinking agents like HDI, TMA, and DBD followed by deacetylation affords a simple method of obtaining a resin with amino groups at the surface which give high degrees of complexation with heavy metals like Cu and Hg, comparable to the highest values reported in literature for chitosans, crosslinked chitosans, and chitosan beads. For binding of copper ions, we found the Langmuir equation to be the best fit for HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin. The morphology of the metal complexes was studied in a facile manner by WAXRD, which afforded an easy tool to evaluate the extent of binding. Thus, high values of metal complexation lead to WAXRD spectra where the original chitosan peaks substantially disappear. Thus a study of WAXRD is in itself a powerful tool to study the extent of metal binding by polymer ligands where the key functional groups are extensively utilized in binding, thereby disrupting the original polymer structure and the consequent changes in the WAXRD spectra. Weak complexes like that of Pb do not lead to any changes in the WAXRD spectra.

2.4. References:

Atia, A. A., Ahmed, M. D., & Elwakeel, K. Z. (2005). Selective separation of mercury (II) using a synthetic resin containing amine mercaptan as chelating groups. Reactive & Functional Polymers, 65, 267-275.

Bailey, S. E., Olin, T. J., Bricka, R. M., & Adrian, D. D. (1999). A review of potentially low-cost sorbents for heavy metals. Water Research, 38(11), 2469-2479.

Bassi, R., Prasher, S. O., & Simpson, B. K. (2000). Removal of selected metal ions from aqueous solutions using chitosan flakes. Sep. Sci. Technol., 35(4), 547-560.

Dobetti, L., & Delben, F. (1992). Binding of metal cations by N-carboxymethyl chitosans in water. Carbohydrate Polymers, 18(4), 273-82.

Domard, A., & Piron, E. (2000). Recent approach of metal binding by chitosan and derivatives. Adc. Chitin science, 4(EUCHIS '99), 295-301.

Filho, N. C., Winkler, Hechenleitner, A. A., & Gomez-Pineda, E. A. (1996). Copper (II) Adsorption onto Sugar Cane Bagasse. International Journal of Polymer Materials, 34(3-4), 211-218.

Juang, R. S., Wu, F. C., Tseng, R. L. (2002). Use of chemically modified chitosan beads for sorption and enzyme immobilization. Advances in Environmental Research, 6, 171-177.

Kopecky, F., Kopecka, B., & Misikova, E. (2005). Sorption of Copper (II) on chitosan from solutions of copper sulfate, copper perchlorate, and copper nitrate. Acta Facultatis Pharmaceuticae Universitatis Comenianae, Tomus LII, 125-135.

Li, H. B., Chen, Y. Y., & Liu, S. L. (2003). Synthesis, characterization, and metal ions adsorption properties of chitosan-calixarenes (I). Journal of Applied Polymer Science, 89, 1139-1144.

Merrifield, J. D., Davids, W. M., MacRae, J. D., & Amirbahman, A. (2004). Uptake of mercury by thiol-grafted chitosan gel beads. Water Research, 38, 3132-3138.

Ngah Wan, W. S., & Musa, A. (1998). Adsorption of Humic Acid onto chitin and chitosan. Journal of Applied Polymer Science, 69, 2305-2310.

Ngah Wan, W.S., Endud, C.S., & Mayanar, R. (2002). Removal of copper (II) ions from aqueous solution onto chitosan and crosslinked chitosan beads. Reactive & Functional Polymers, 50, 181-190.

Ng, J. C. Y., Cheung, W. H., & Mckay, G. (2002). Equilibrium studies of the sorption of Cu (II) Ions onto chitosan. Journal of Colloid and Interface, 255, 64-74.

Peternele, W. S., Winkler-Hechenleitner, A. A., & Gomez-Pineda, E. A. (1999). Adsorption of Cd(II) and Pb(II) onto functionalized formic lignin from sugar cane bagasse. Bioresource Technology, 68(1), 95-100.

Rhazi, M., Desbrieres, J., Tolaimate, A., Rinaudo, M., Vottero, P., Alagui, A., & EI Meray, M. (2002). Influence of the nature of the metal ions on the complexation with chitosan. Application to the treatment of liquid waste. European Polymer Journal, 38(8), (English). 1523-1530.

Schmuhl, R., Krieg, H. M., & Keizer, K. (2001). Adsorption of Cu(II) and Cr(VI) ions by chitosan: Kinetics and equilibrium studies. Water SA, 27(1), 1-7.

Taboada, E., Cabrera, G., & Cardenas, G. (2003). Retention capacity of chitosan for copper and mercury ions. Journal of the Chilean Chemical Society, 48(1), 7-12.

Tan, S., Wang, Y., Peng, C., & Tang, Y. (1999). Synthesis and adsorption properties for metal ions of crosslinked chitosan acetate crown ether. Journal of Applied Polymer Science, 71, 2069-2074.

Webster, A., Halling, M.D., & Grant, D.M. (2007). Metal complexation of chitosan and its glutaraldehyde cross-linked derivative. Carbohydr. Res., 342, 1189-1201.

Varma, A. J., Deshpande, S. V., & Kennedy, J. F. (2004). Metal complexation by chitosan and its derivatives: a review. Carbohydrate Polymers, 55, 77-93.

Yin, X., Zhang, X., Lin, Q., Feng, Y., Yu, W., & Zhang, Q. (2004). Metal-coordinating controlled oxidative degradation of chitosan and antioxident activity of chitosan-metal complex. ARKIVOC, ix, 66-78.

2.6. Supplementary data:

2.6.1. FTIR spectral data:

FTIR spectroscopy of the chitin, deacetylated chitin, chitosan, crosslinked chitin, crosslinked deacetylated chitin and their metal ions

The FTIR spectra of all the chitin, deacetylated chitin, chitosan and various types of crosslinked chitin and crosslinked deacetylated chitin were recorded in the diffused reflectance mode on Perkin Elmer FTIR Spectrophotometer. 3mg of each vacuum oven dried polymer was mulled with 100mg of anhydrous KBr and the spectra recorded at a resolution of 2 cm^{-1} and the numbers of scans were 60.

FTIR spectrum of chitin and deacetylated chitin was recorded, and peak assignments are based on reported spectral assignments of chitin and chitosan.

Table 5: Infrared Spectrum of chitin (Ref. Pearson, F. G., Marchessault, R. H. & Liang, C. Y. (1960). Journal of Polymer Science Vol.XLIII, 101-116)

Peak at (cm^{-1})	**Assignments**
685	OH out of plane bending
730	NH out of plane bending
890	Ring stretching
915, 952 & 975	CH_3 wagging along chain
1013, 1020, 1025, 1065 & 1070	C-O stretching
1110	Asymmetric in phase ring stretching mode
1155	Asymmetric bridge oxygen stretching
1203, 1230 and 1257	Same bands observed in cellulose
1310	Amide III band and CH_2 wagging
1378	CH bending and symmetric CH_3 deformation
1420 & 1430	CH_2 bending & CH_3 deformation
1555	Amide II band
1619	C=N
1652	Amide I band
2840	CH_2 symmetric stretching
2878 & 2890	CH stretching
2929	Symmetric CH_3 stretching and symmetric CH_2 stretching
2962	CH_3 stretching
3106 & 3264	N-H stretching
3447 & 3480	O-H stretching

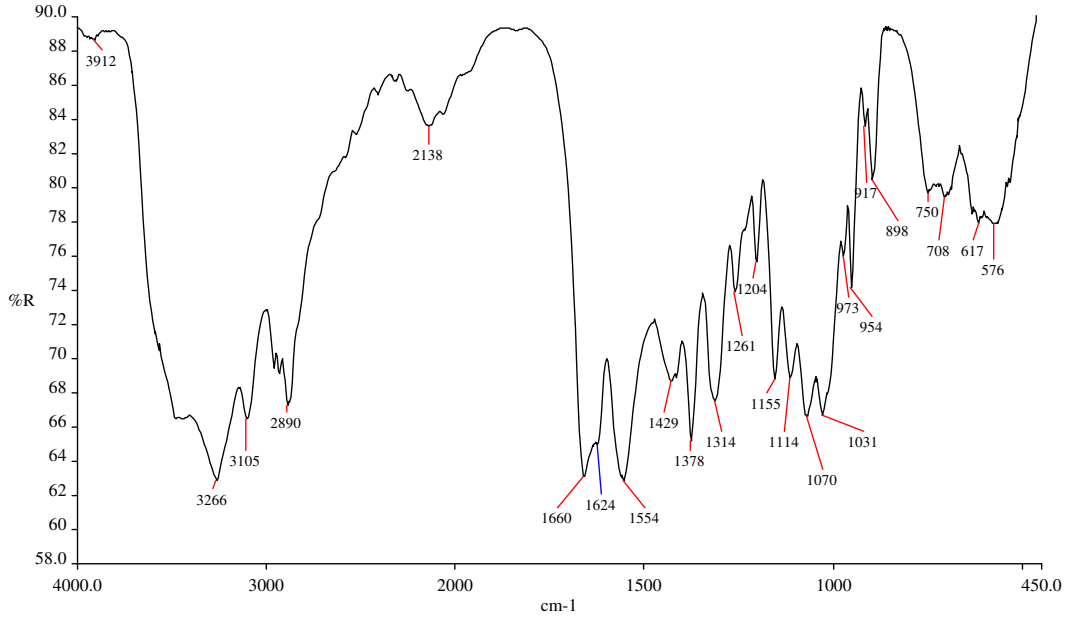


Figure 6: FTIR spectrum of chitin (Meron)

The FTIR spectrum of chitin was recorded, and peak assignments are based on reported spectral assignments.

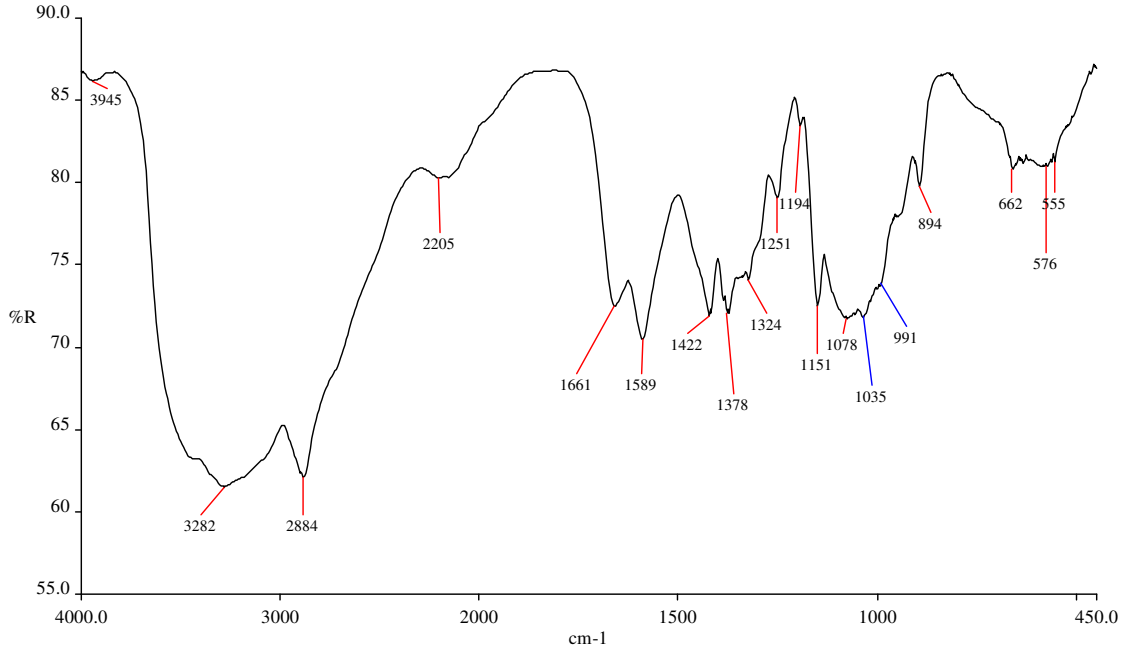


Figure 7: FTIR spectrum of deacetylated chitin (chitosan)

Figure 7 shows the FTIR spectrum of deacetylated chitin. Conversion into deacetylated chitin from chitin, peaks that are related to secondary amides and thus to chitin are reduced or disappear like 3480, 3266, 3105, 1554, 1314, and 1261 cm^{-1}. Primary amide such as in chitosan appears at 1661 and 1589 cm^{-1}. Some peaks are related to C-H bonds and which are more present in chitin are reduced or disappear like 2962, 2934, 1378 and 1204 cm^{-1}. (Ref. Van de Velde K. and Paul K. (2004). Structure analysis and degree of substitution of chitin, chitosan and dibutyryl chitin by FTIR spectroscopy and solid state ^{13}C-NMR. Carbohydrate Polymers, 58, 409-416.)

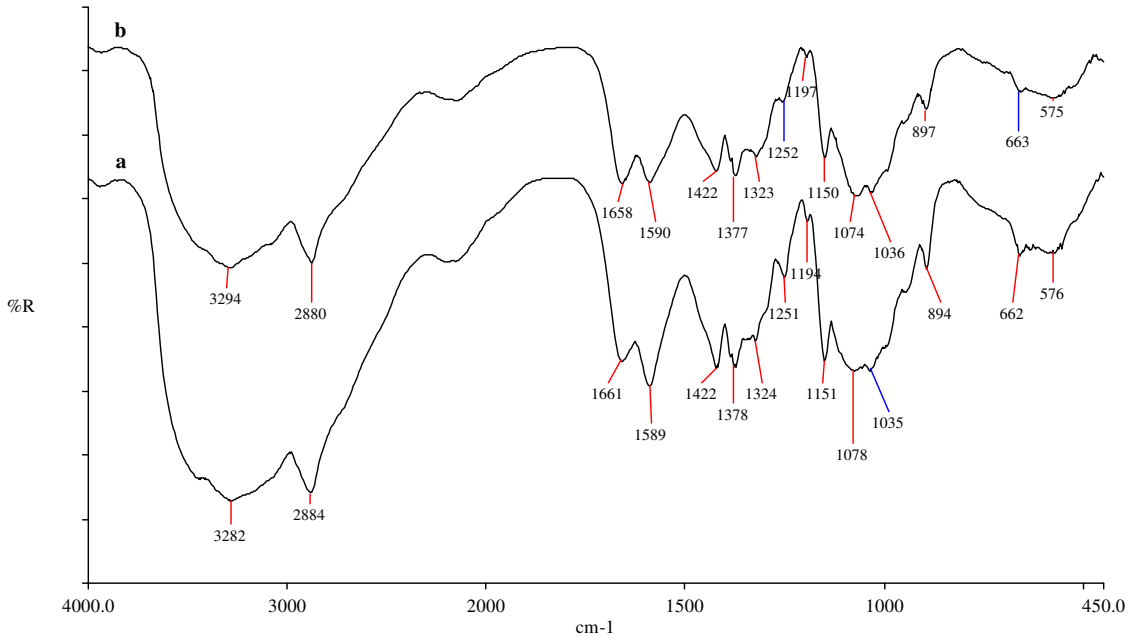


Figure 8: Overlapping FTIR spectra of (a) chitosan (Meron) and (b) deacetylated chitin (chitosan)

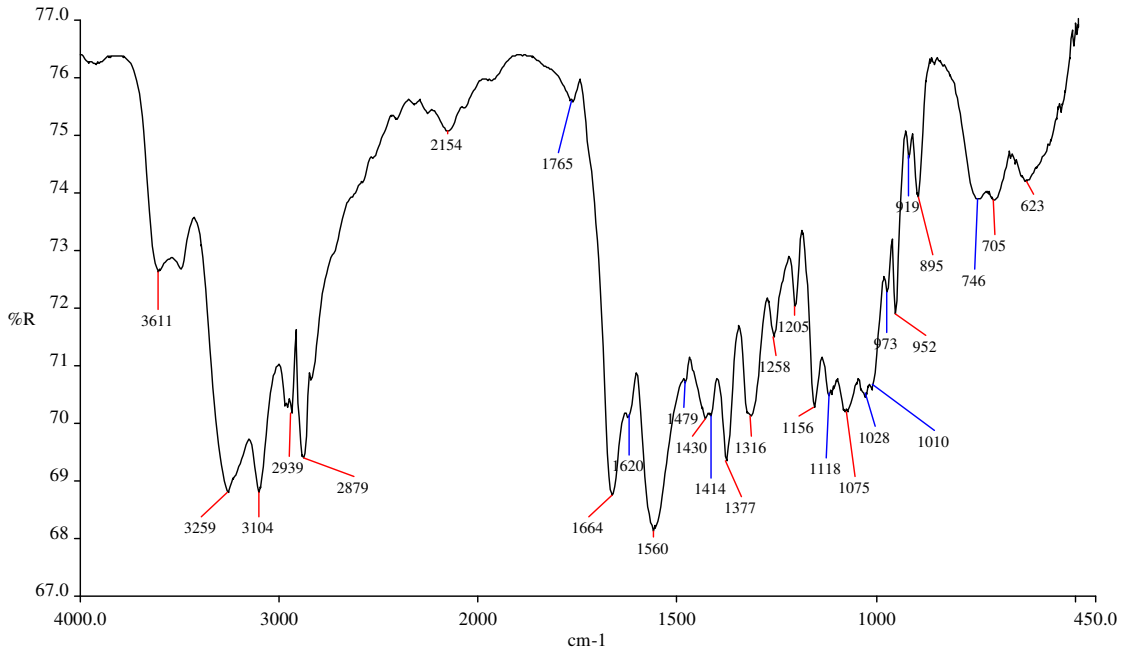


Figure 9: FTIR spectra of HDI-crosslinked chitin

Figure 9 shows the FTIR spectrum of the HDI-crosslinked chitin. The spectrum shows the increase in the intensity of the N-H peak at 3105 cm^{-1} and appearance of a new peak at 1765 cm^{-1}due to carbonyl ester peak. It also shows the slightly increase in the intensity of carbonyl amide peak

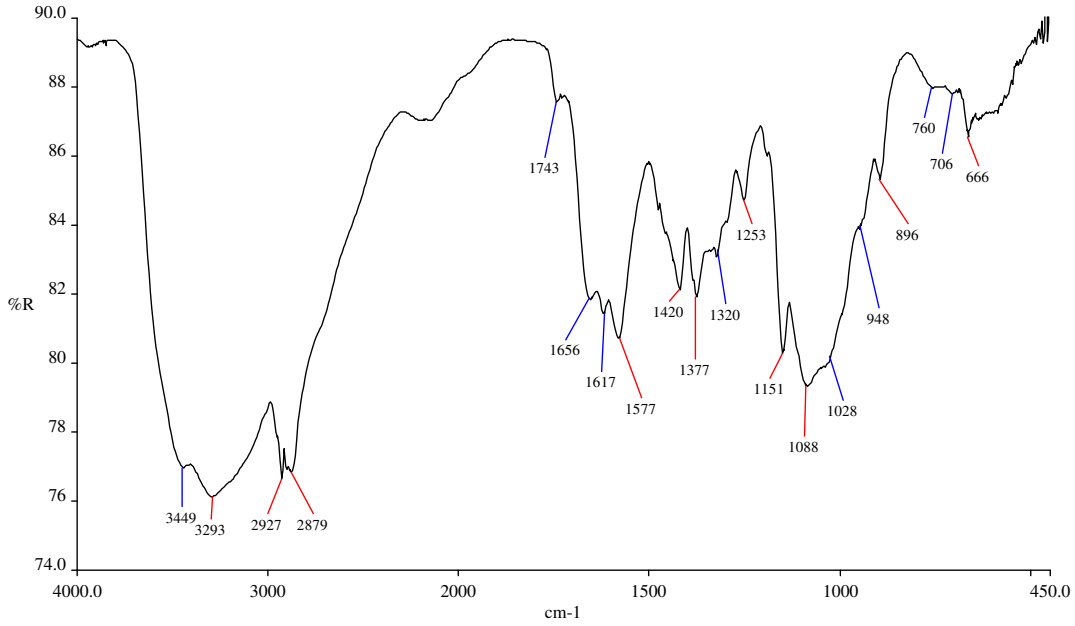


Figure 10: FTIR spectra of HDI-crosslinked deacetylated chitin

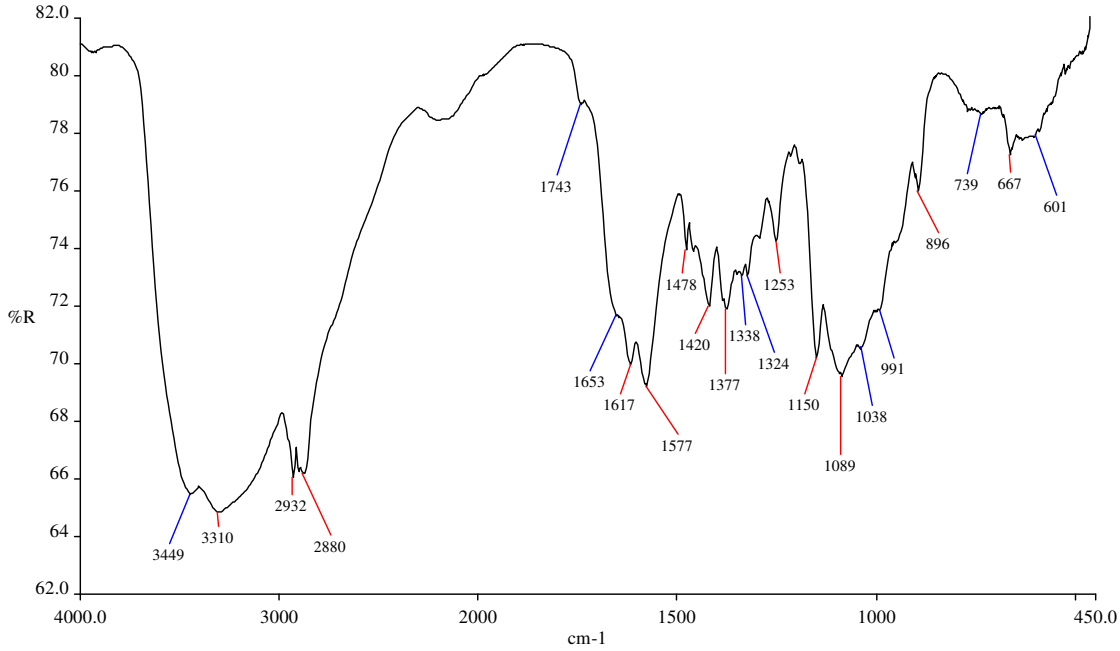


Figure 11: FTIR spectra of DBD-crosslinked chitin

Figure shows the FTIR spectrum of the DBD-crosslinked chitin. The spectrum shows broad peak at 3310 cm^{-1} due to overlapping of N-H and hydroxyl peak (in chitin sharp peak at 3266 cm^{-1}) and appearance of two new peaks at 1743 and 1478 cm^{-1} due to ether peak and CH_2 bending of alkane peak.

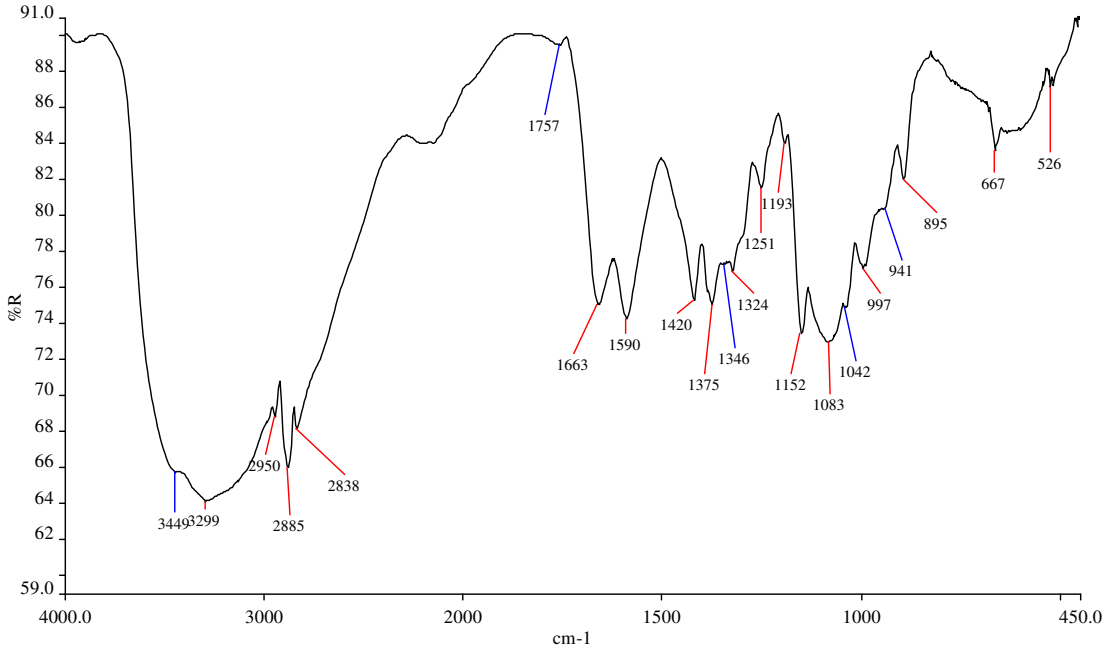


Figure 12: FTIR spectra of DBD-crosslinked deacetylated chitin

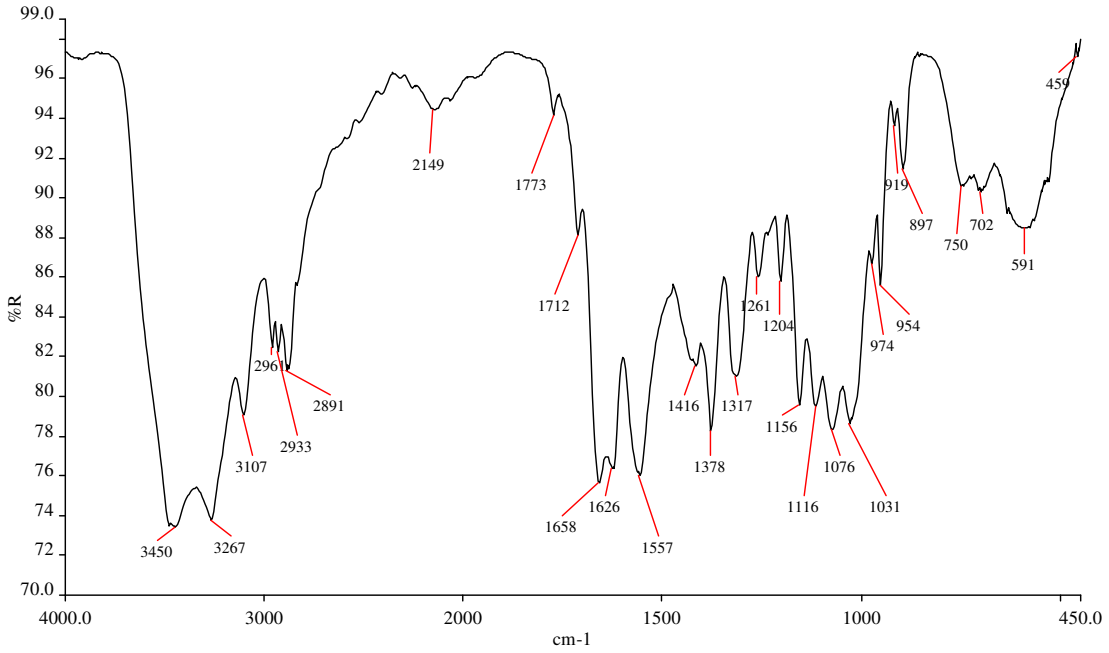


Figure 13: FTIR spectra of TMA-crosslinked chitin

Figure shows the FTIR spectrum of the TMA-crosslinked chitin. The spectrum shows the increase in the intensity of the hydroxyl peak at 3450 cm^{-1} and appearance of two new peaks at 1773 and 1712 cm^{-1} due to ester and carboxylic acid carbonyls. It also shows overlapping N-H and aromatic peak at 1626 cm^{-1}.

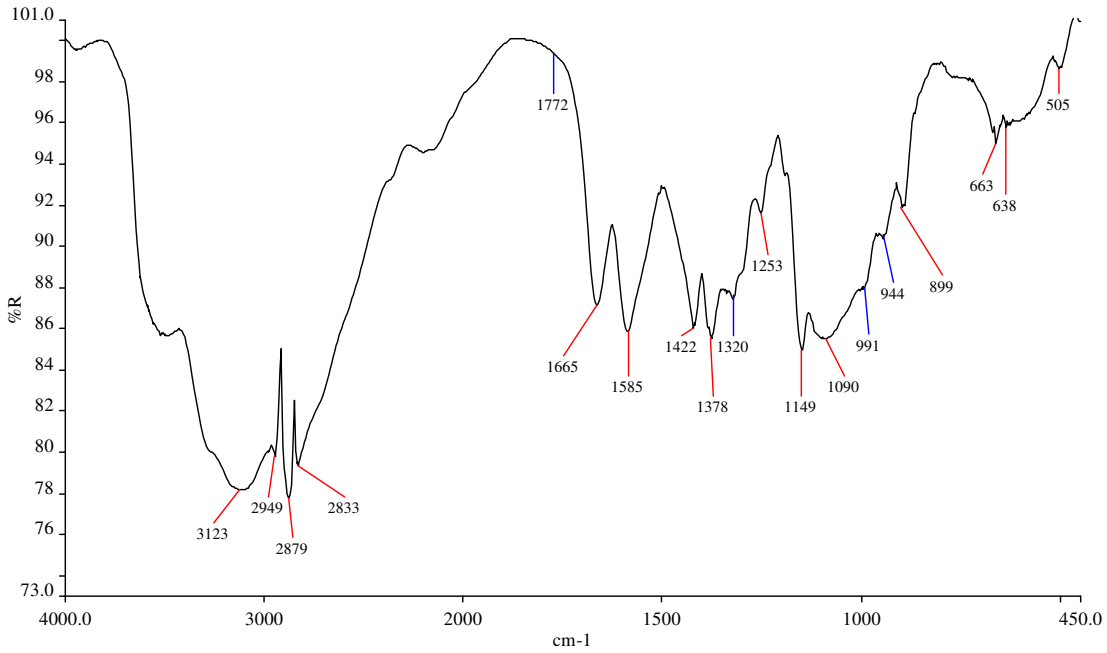


Figure 14: FTIR spectra of TMA-crosslinked deacetylated chitin

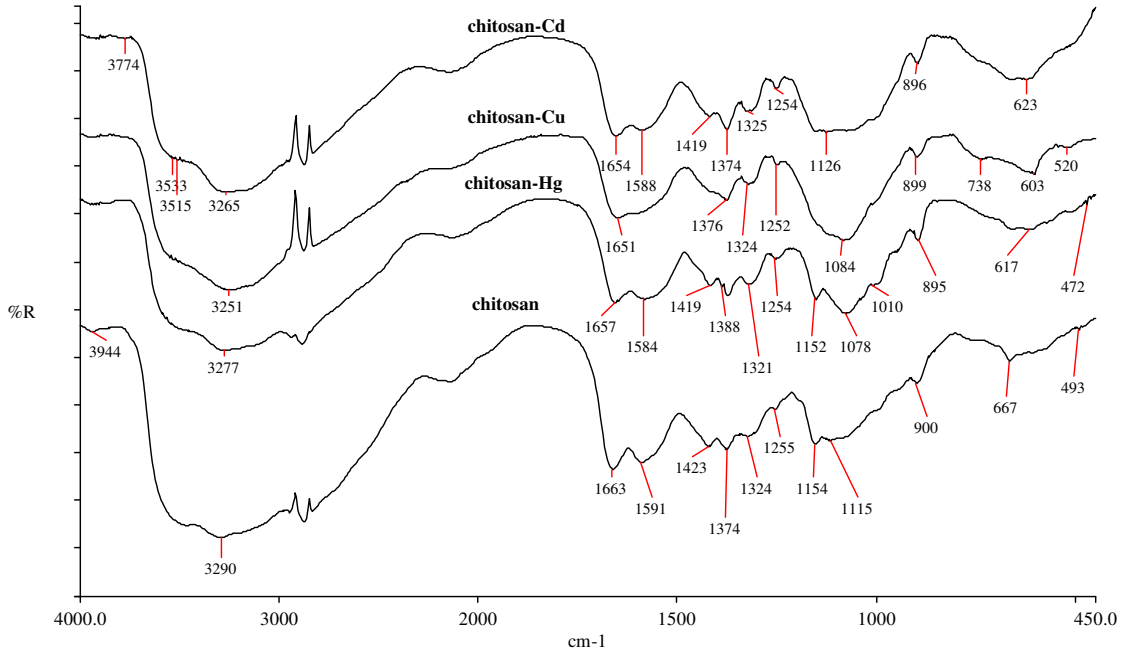


Figure 15: FTIR spectrum of (1) commercial chitosan (Meron), (2) chitosan-Hg complex, (3) chitosan-Cu complex, (4) chitosan-Cd complex

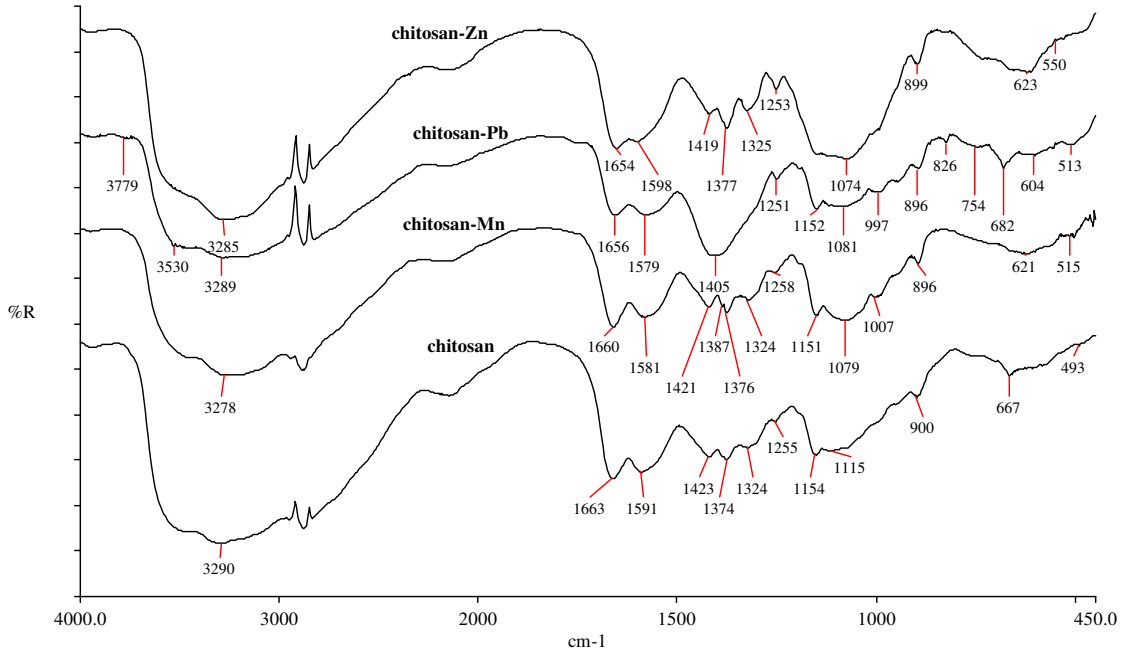


Figure 16: FTIR spectrum of (1) commercial chitosan (Meron), (2) chitosan-Mn complex, (3) chitosan-Pb complex, (4) chitosan-Zn complex

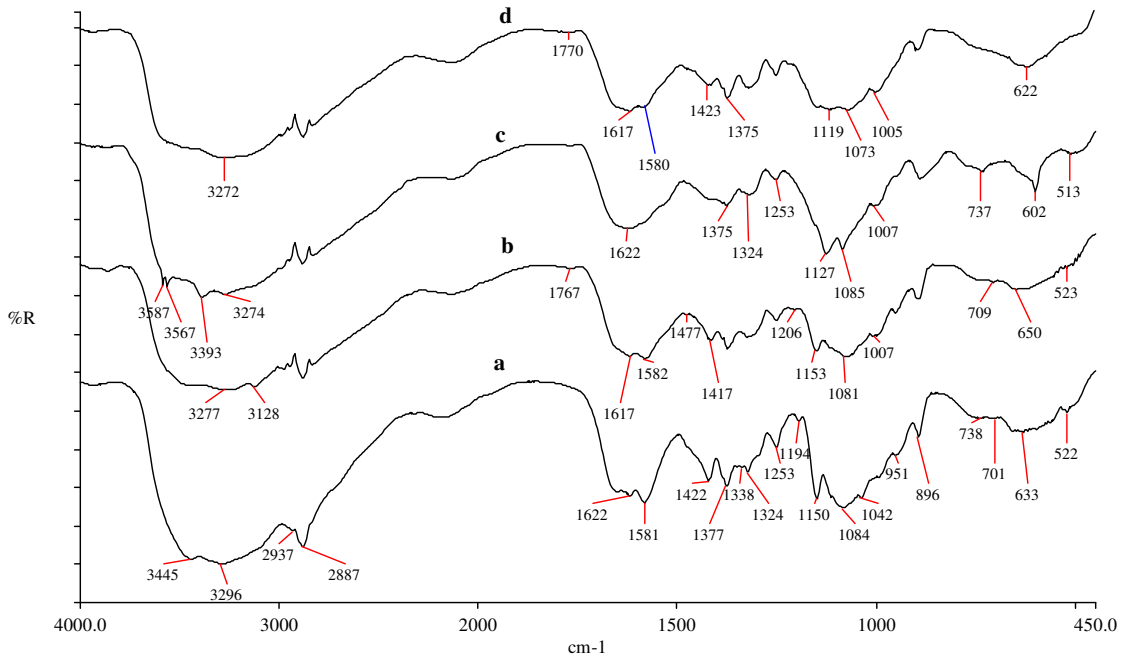


Figure 17: FTIR spectrum of (a) HDI-crosslinked deacetylated chitin, (b) HDI-crosslinked deacetylated chitin-Hg complex, (c) HDI-crosslinked deacetylated chitin-Cu complex, (d) HDI-crosslinked deacetylated chitin-Cd complex.

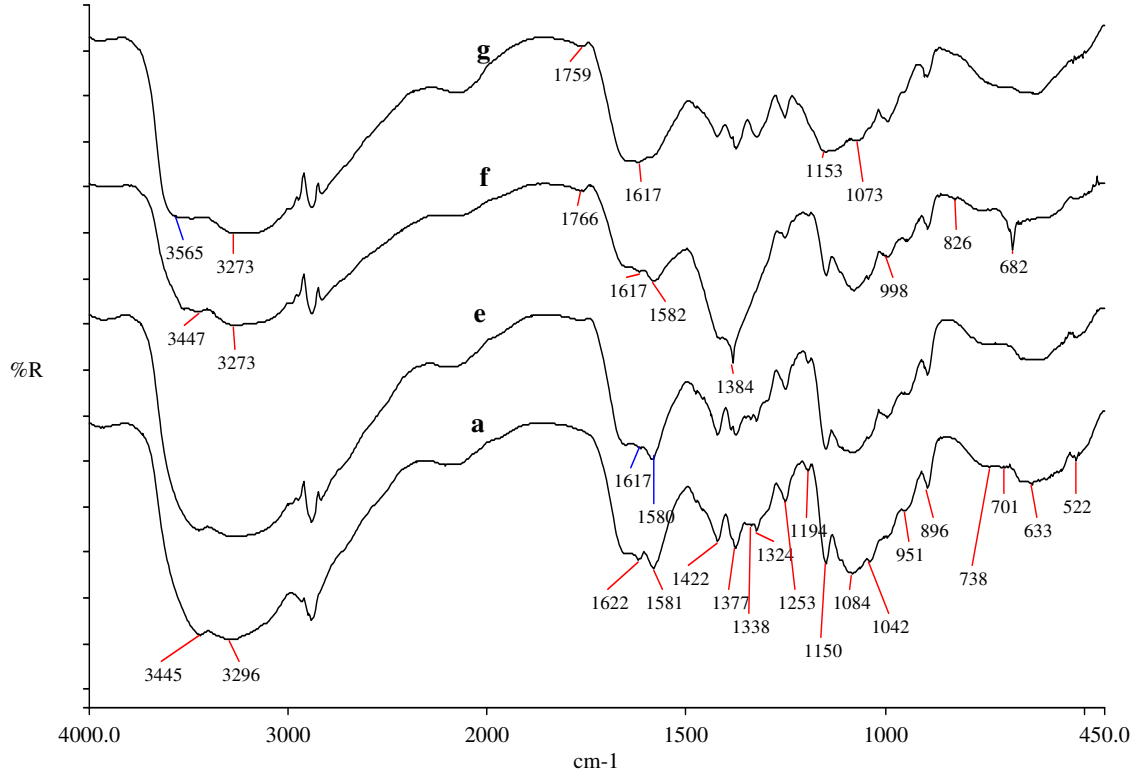


Figure 18: FTIR spectrum of (a) HDI-crosslinked deacetylated chitin, (e) HDI-crosslinked deacetylated chitin-Mn complex, (f) HDI-crosslinked deacetylated chitin-Pb complex, (g) HDI-crosslinked deacetylated chitin-Zn complex.

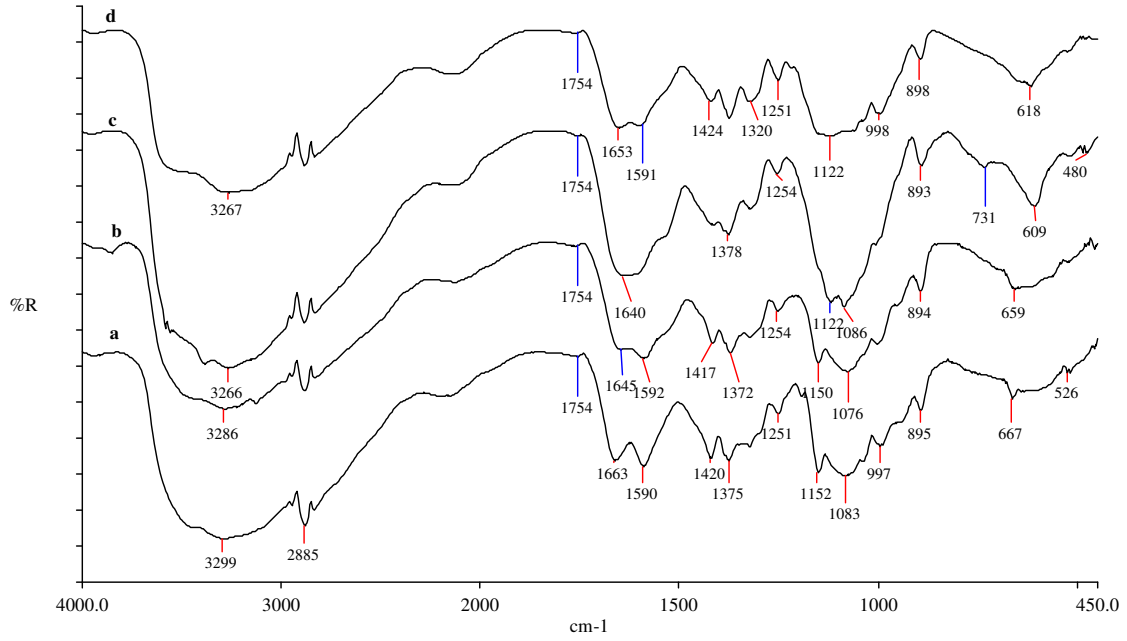


Figure 19: FTIR spectrum of (a) DBD-crosslinked deacetylated chitin, (b) DBD-crosslinked deacetylated chitin-Hg complex, (c) DBD-crosslinked deacetylated chitin-Cu complex, (d) DBD-crosslinked deacetylated chitin-Cd complex.

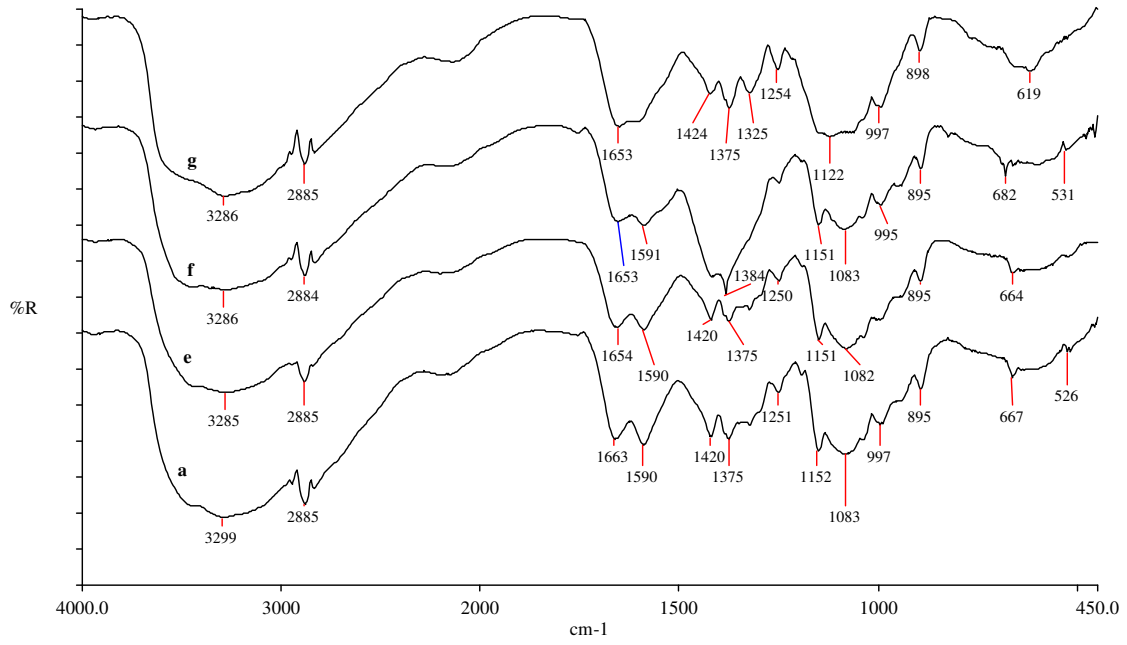


Figure 20: FTIR spectrum of (a) DBD-crosslinked deacetylated chitin, (e) DBD-crosslinked deacetylated chitin-Mn complex, (f) DBD-crosslinked deacetylated chitin-Pb complex, (g) DBD-crosslinked deacetylated chitin-Zn complex.

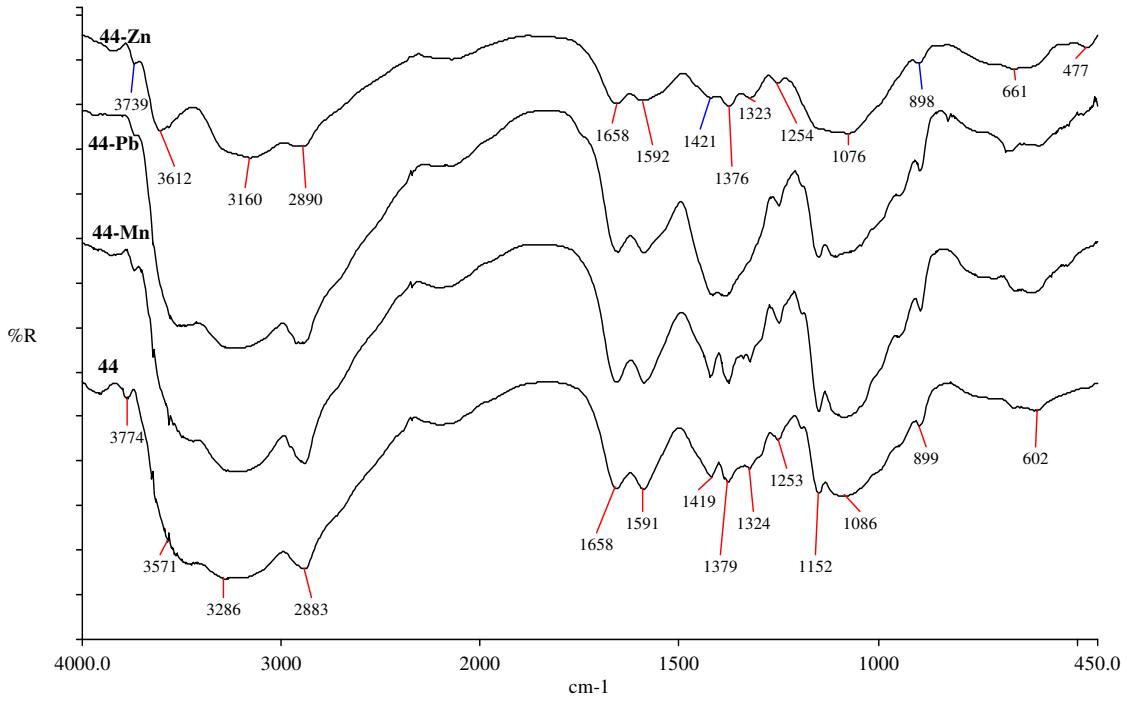


Figure 21: FTIR spectrum of (1) TMA-crosslinked deacetylated chitin, (2) TMA-crosslinked deacetylated chitin-Mn complex, (3) TMA-crosslinked deacetylated chitin-Pb complex, (4) TMA-crosslinked deacetylated chitin-Zn complex

2.7. Appendix 1: Schematic structure of crosslinked deacetylated chitins

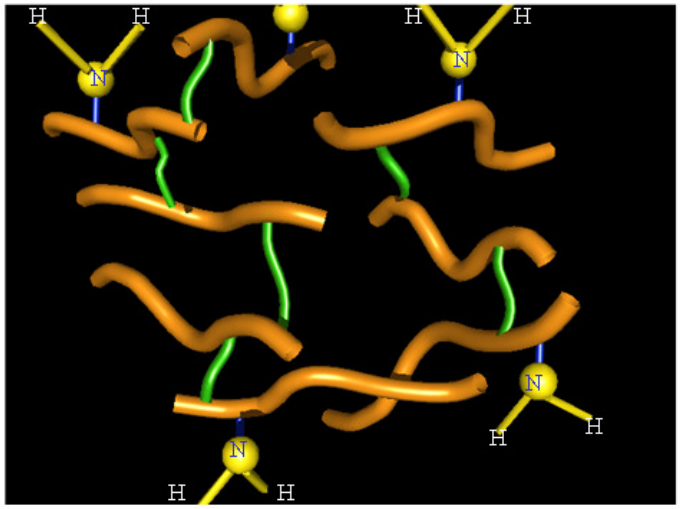


Figure 22: Schematic structure of Diisocyanatohexane-crosslinked deacetylated chitin

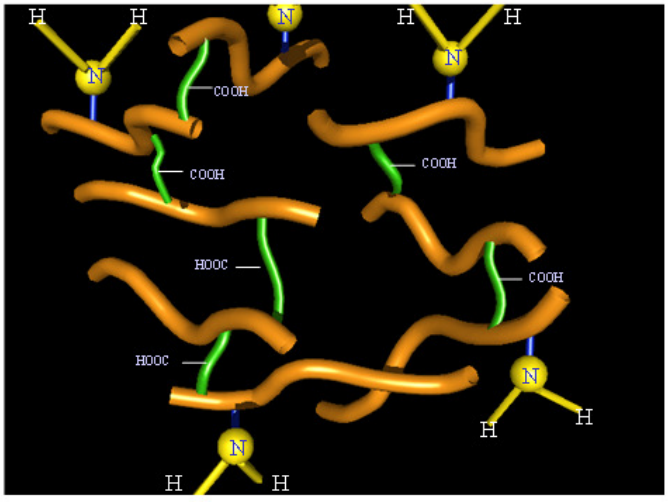
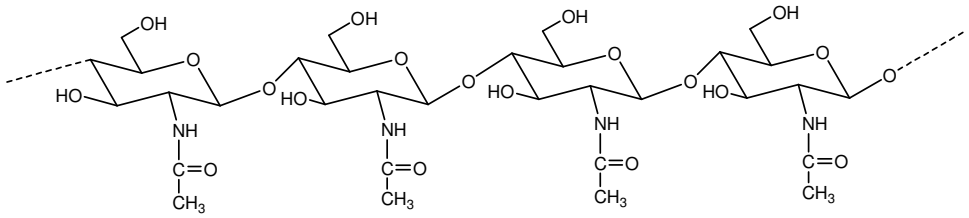
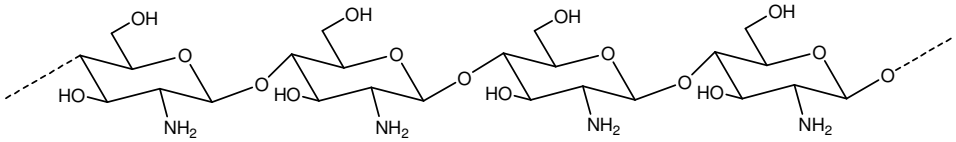


Figure 23: Schematic structure of Trimellitic anhydride-crosslinked deacetylated chitin

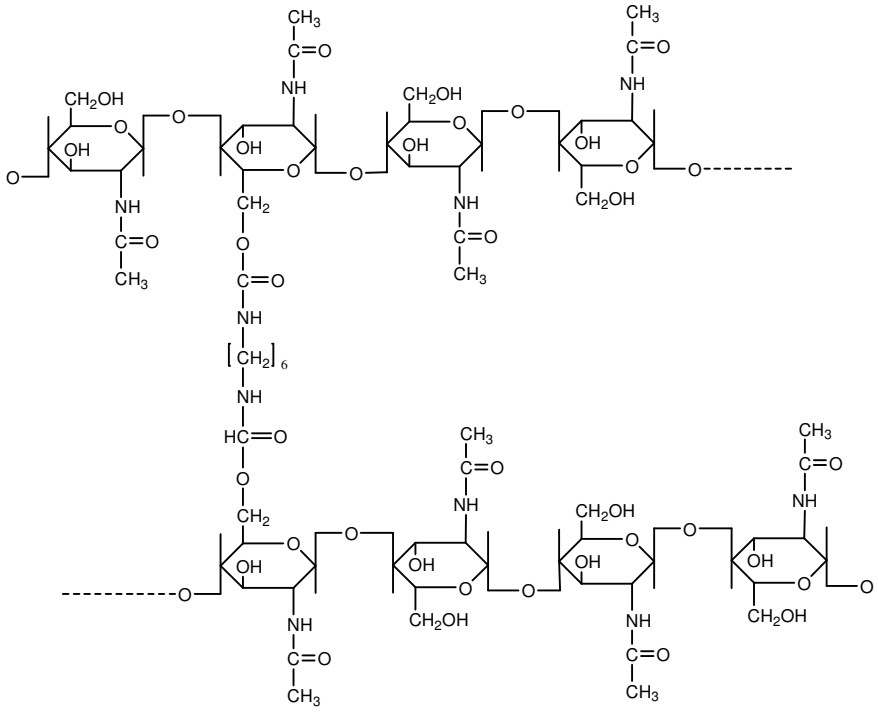
Figure 24: Schematic structure of chitin, chitosan, crosslinked chitosan with metal ions



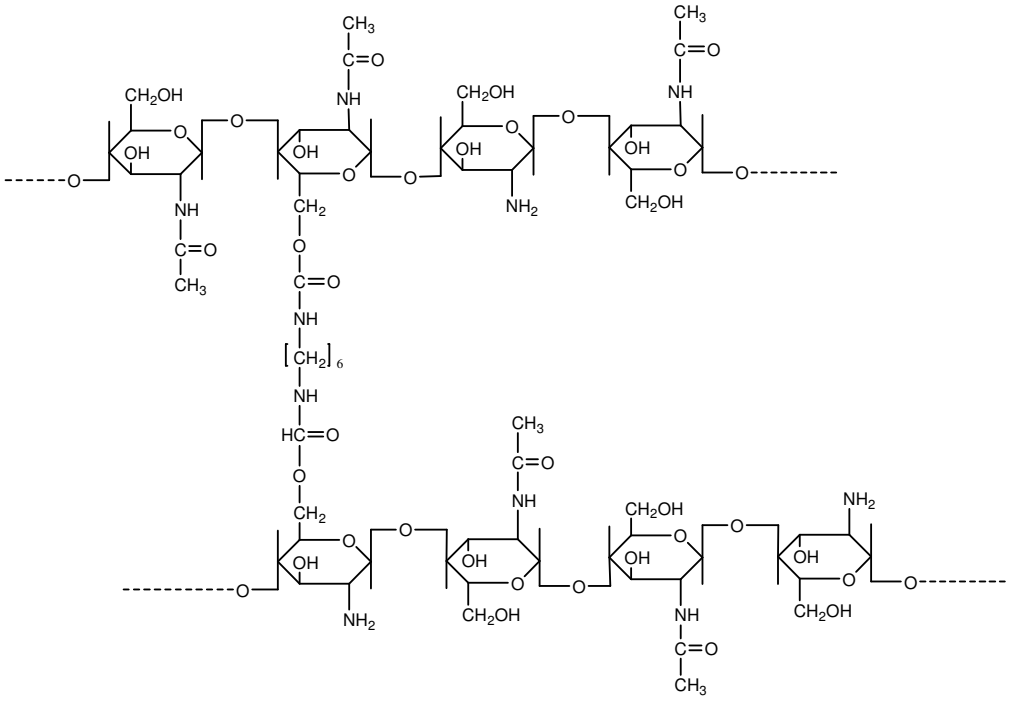
Chitin



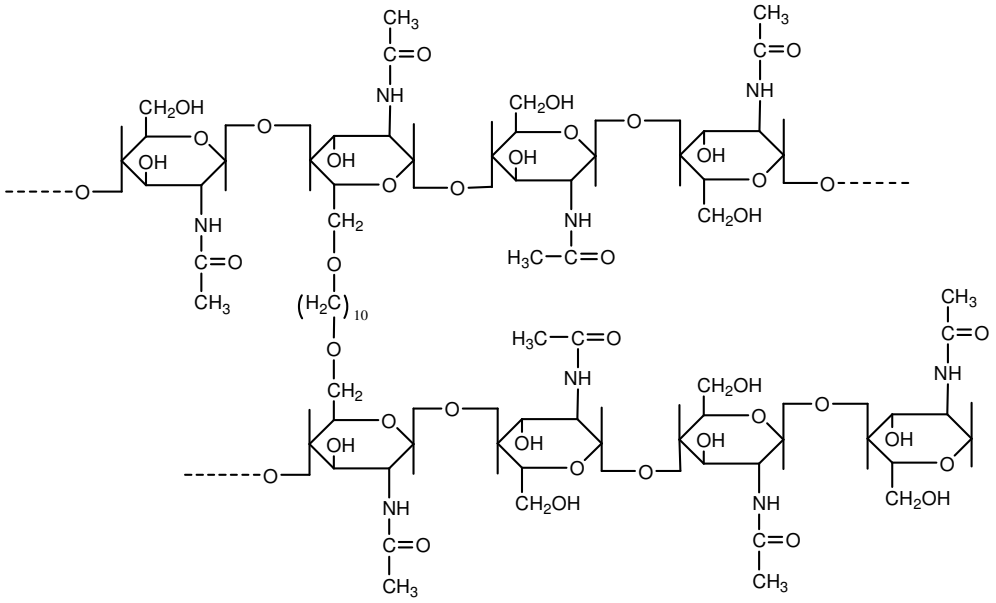
Chitosan



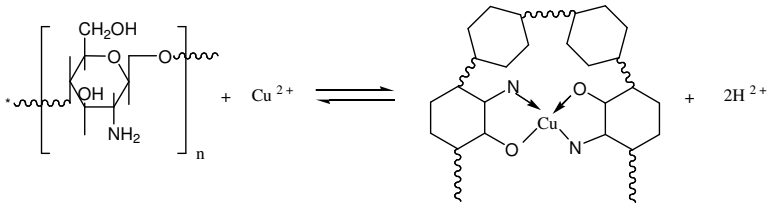
HDI-crosslinked chitin



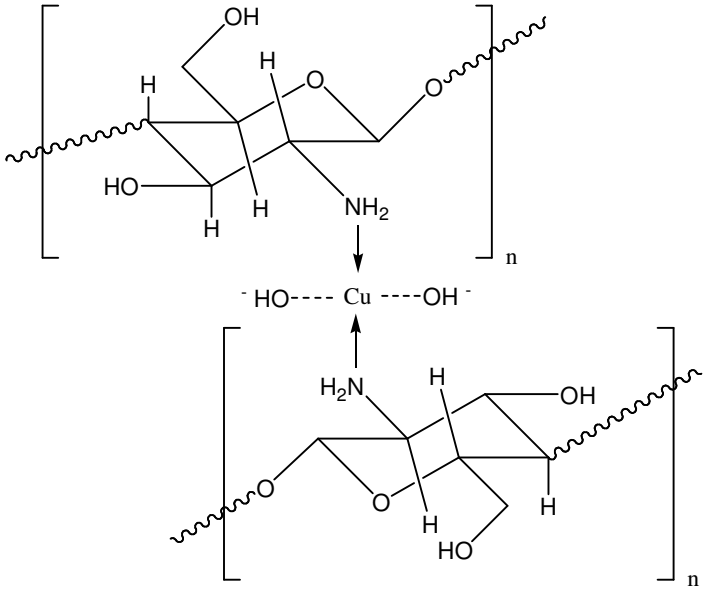
HDI-crosslinked deacetylated chitin



DBD-crosslinked chitin

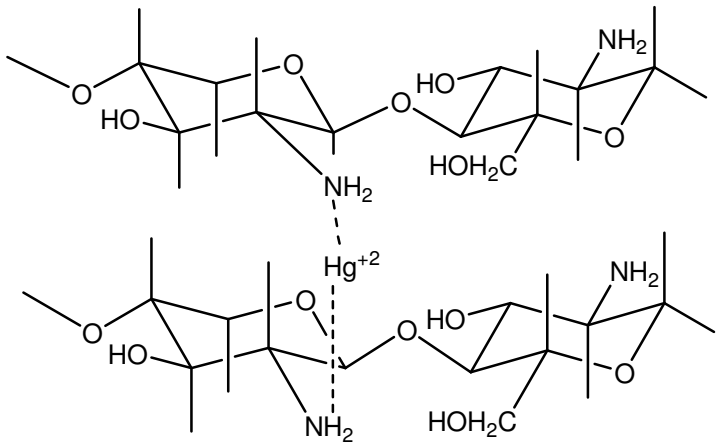


Cu complexed chitosan (Ref. Separation science & technology, 40, 2005, 1483-1495.

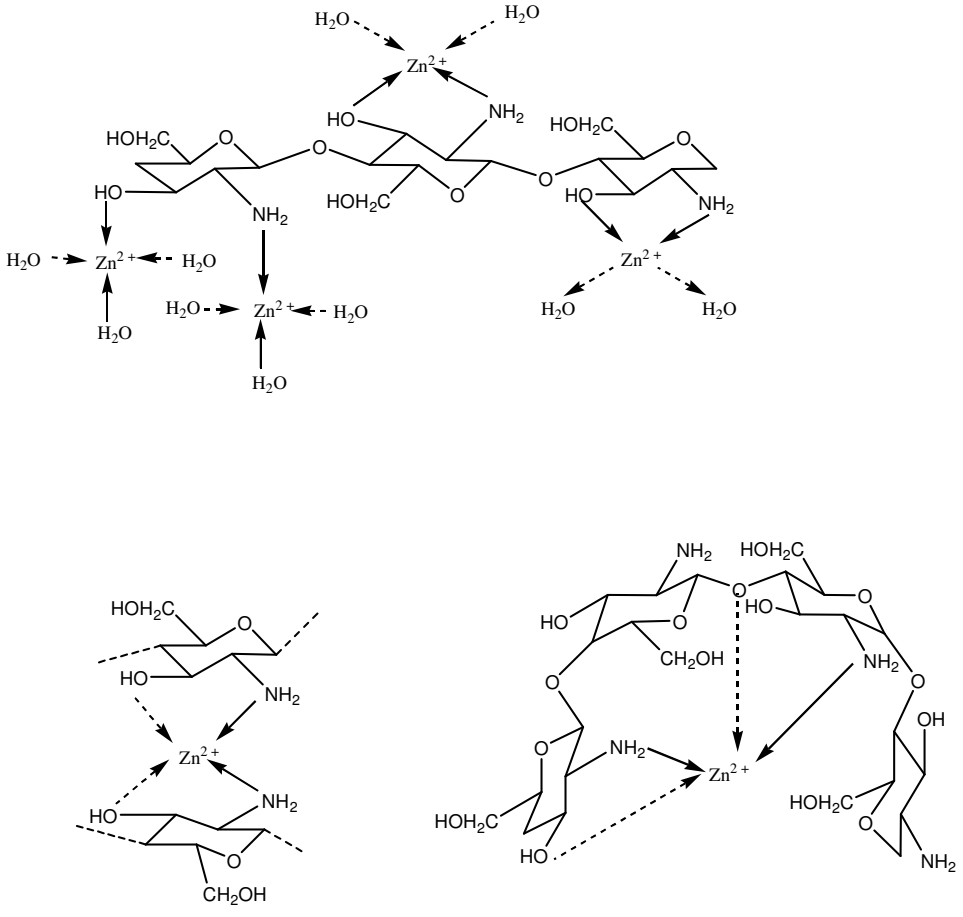


B

Cu complexed chitosan (Ref. Surface & coating technology 201(5-6),2007, 5973-5978).



Hg complexed chitosan (Ref. Macromolecular Bioscience, 1, 2001, 233-248).



Zn complexed chitosan (Ref. Carbohydrate Polymers, 56(1), 2004, 21-26)

2.8. Appendix 2:

CPMAS ^{13}CNMR Spectroscopy of chitin, chitosans and crosslinked chitosans:

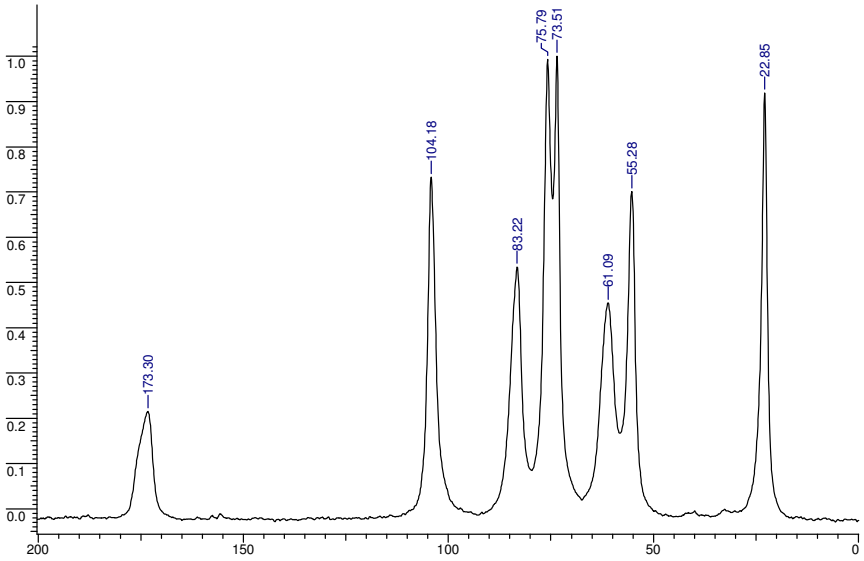


Figure 25: CPMAS ^{13}C-NMR of chitin (Meron)

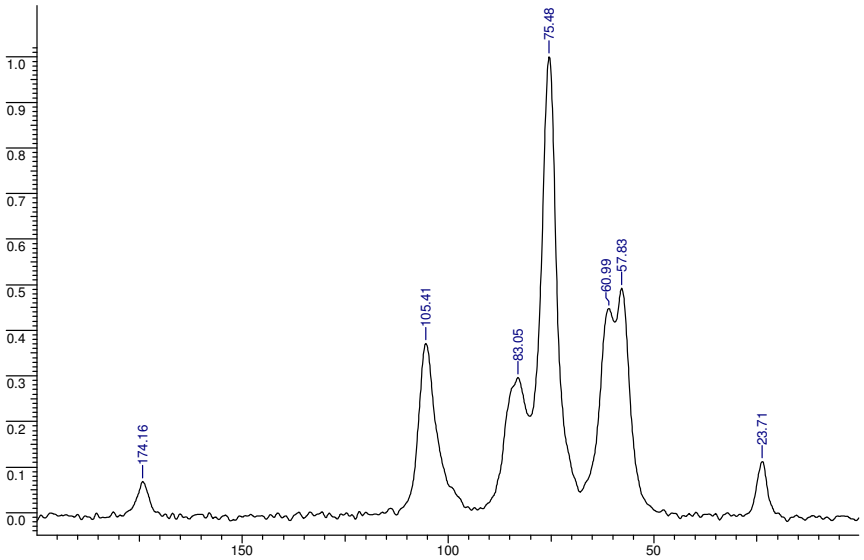


Figure 26: CPMAS ^{13}C-NMR of chitosan (Meron)

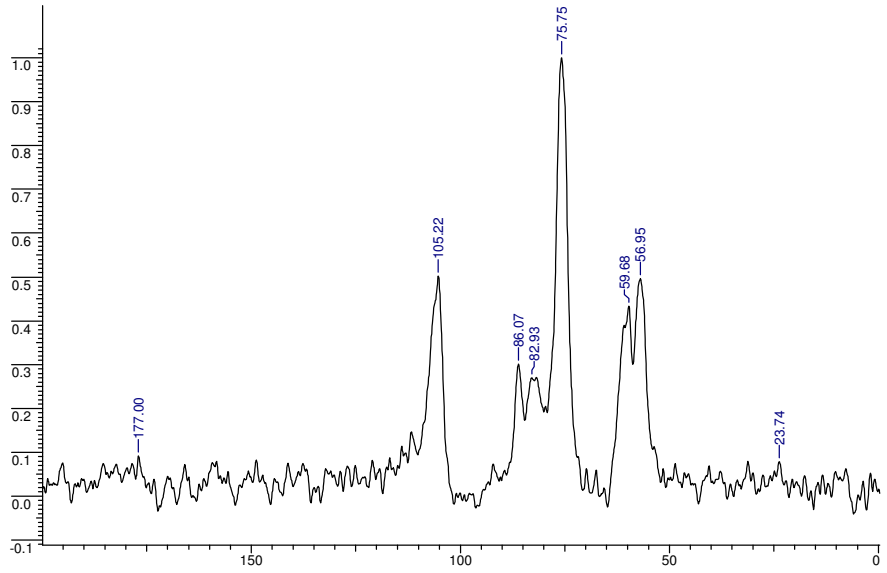


Figure 27: CPMAS ^{13}C-NMR of deacetylated chitin

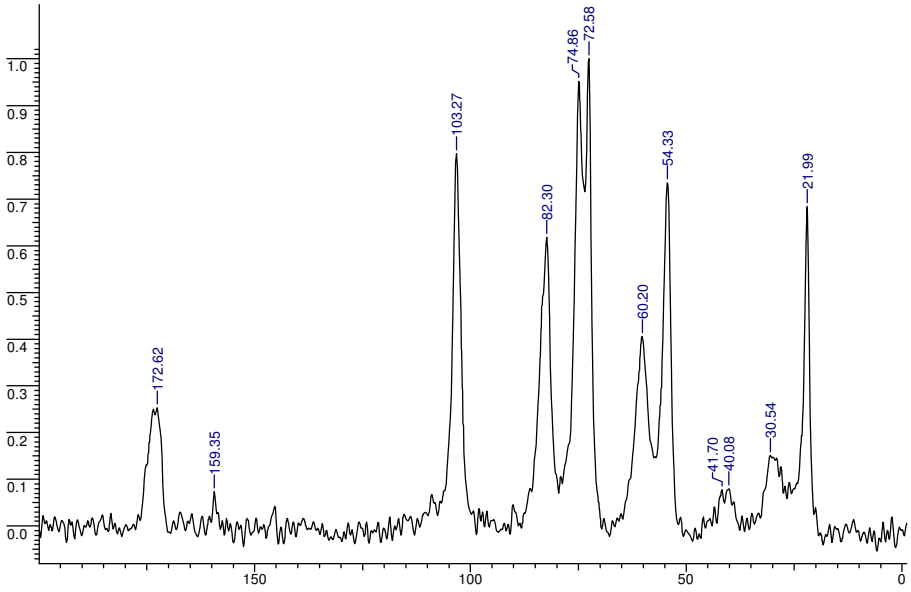


Figure 28: CPMAS ^{13}C-NMR of HDI-crosslinked chitin

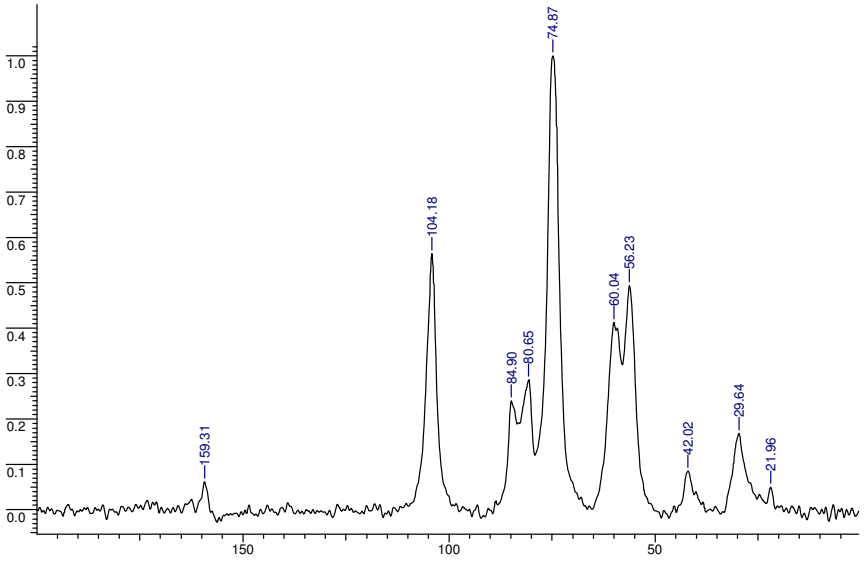


Figure 29: CPMAS ^{13}C-NMR of HDI-crosslinked deacetylated chitin

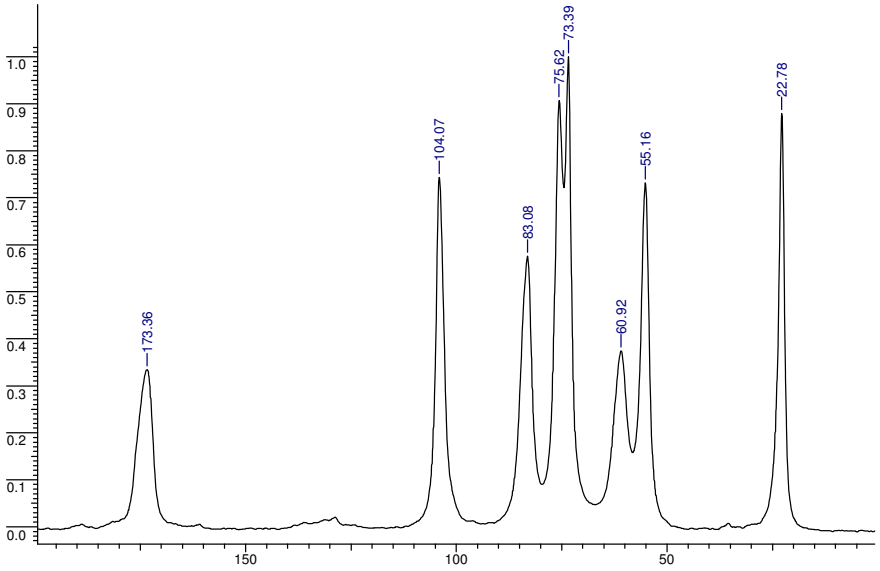


Figure 30: CPMAS ^{13}C-NMR of TMA-crosslinked chitin

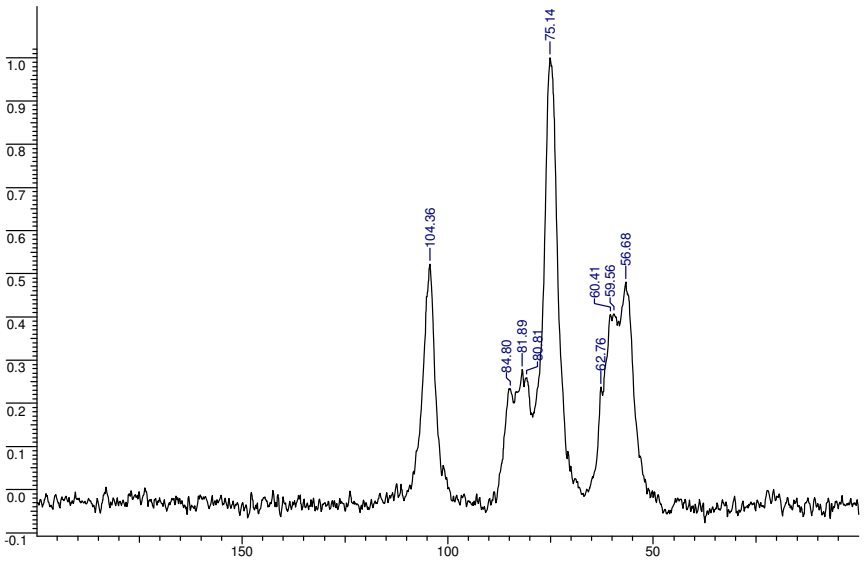


Figure 31: CPMAS ^{13}C-NMR of TMA-crosslinked deacetylated chitin

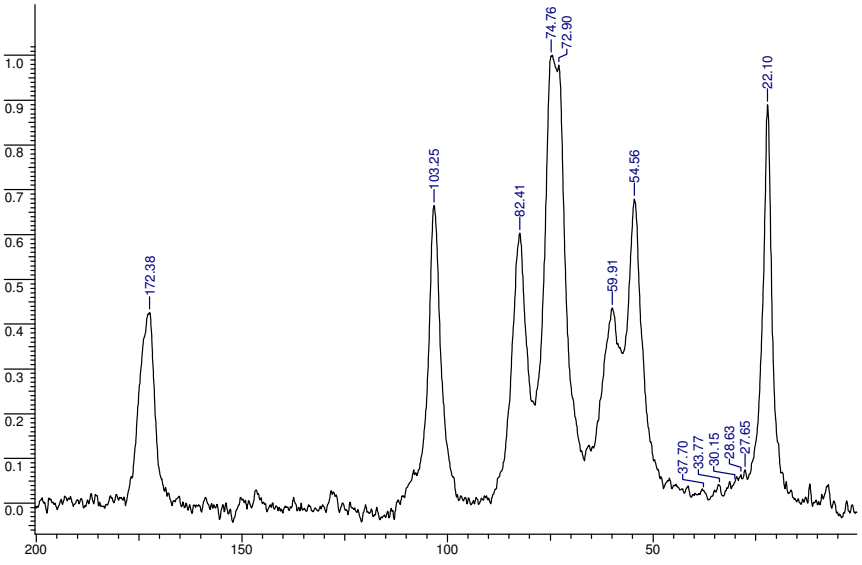


Figure 32: CPMAS ^{13}C-NMR of DBD-crosslinked chitin

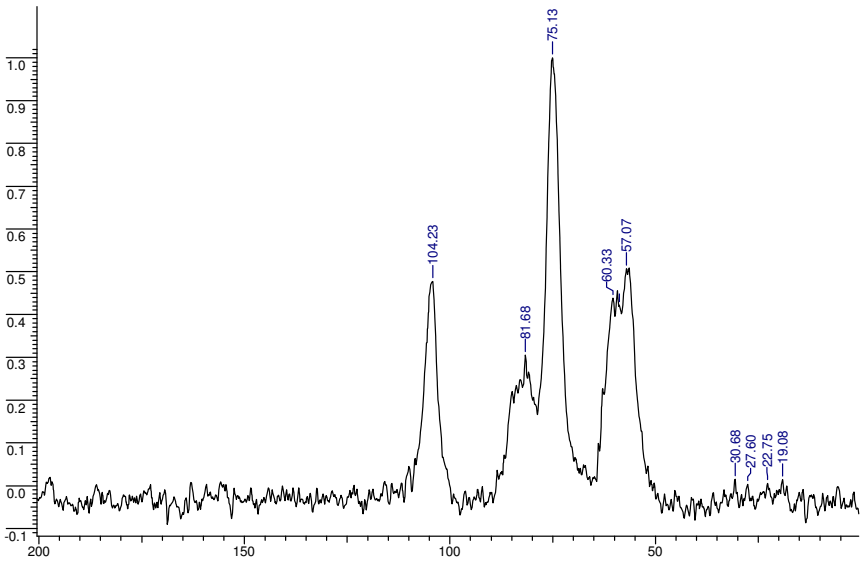


Figure 33: CPMAS ^{13}C-NMR of DBD-crosslinked deacetylated chitin

2.9. Appendix 3: UV spectroscopy of chitosan deacetylated chitin and crosslinked deacetylated chitins and their metal ions:

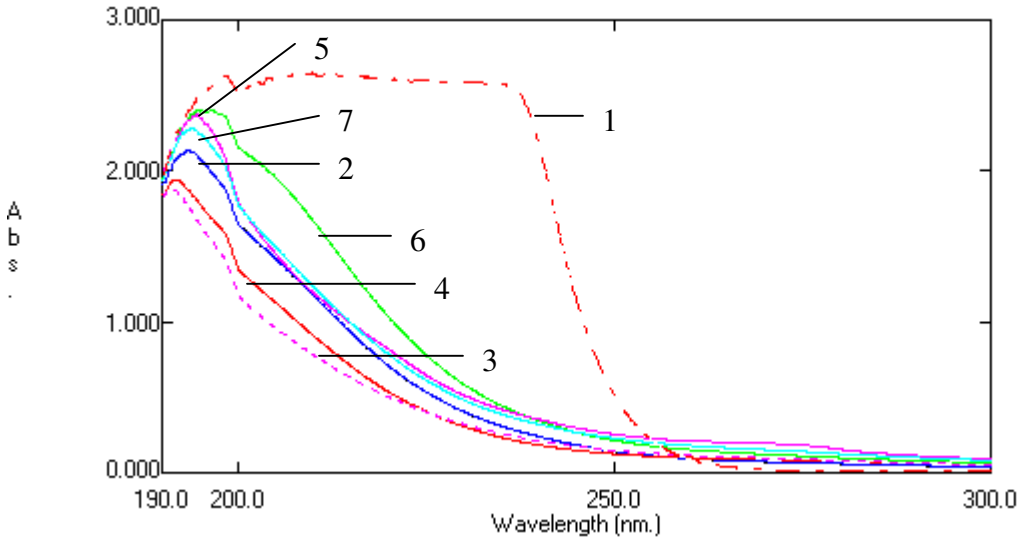


Figure 34: UV of $HgCl_2$ (0.01M) complexation study, (1) $HgCl_2$ (0.01M),
(2) Chitosan-Hg complex, (3) deacetylated chitin-Hg complex,
(4) diisocyanatohexane-crosslinked deacetylated chitin-Hg complex,
(5) dibromodecane-crosslinked deacetylated chitin-Hg complex,
(6) trimellitic anhydride-crosslinked deacetylated chitin-Hg complex and
(7) diisocyanatohexane-crosslinked chitosan-Hg complex.

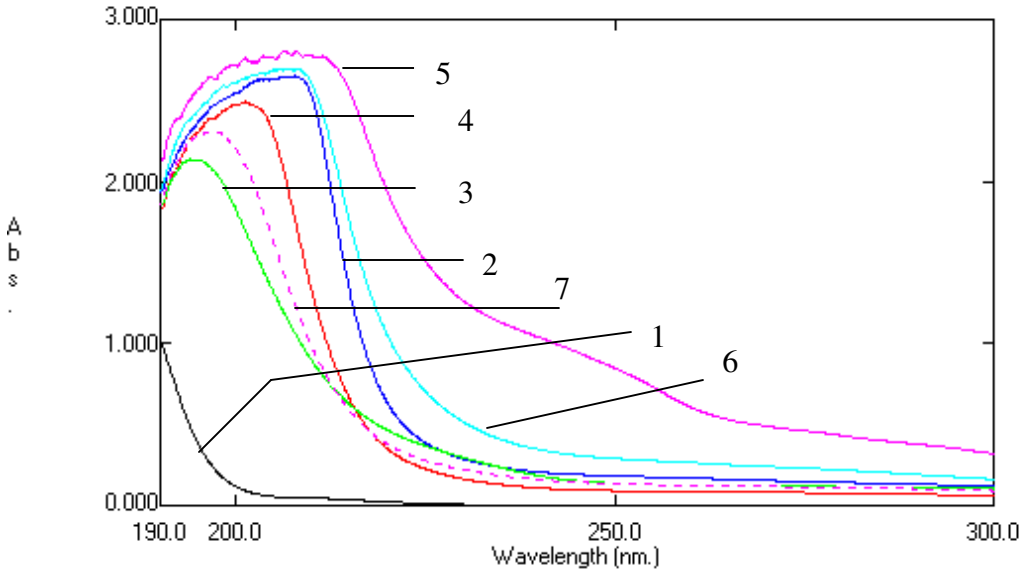


Figure 35: UV of $CdSO_4$ (0.01M) complexation study, (1) $CdSO_4$ (0.01M),
(2) Chitosan-Cd complex, (3) deacetylated chitin-Cd complex,
(4) diisocyanatohexane-crosslinked deacetylated chitin-Cd complex,
(5) dibromodecane-crosslinked deacetylated chitin-Cd complex,
(6) trimellitic anhydride-crosslinked deacetylated chitin-Cd complex and
(7) diisocyanatohexane-crosslinked chitosan-Cd complex.

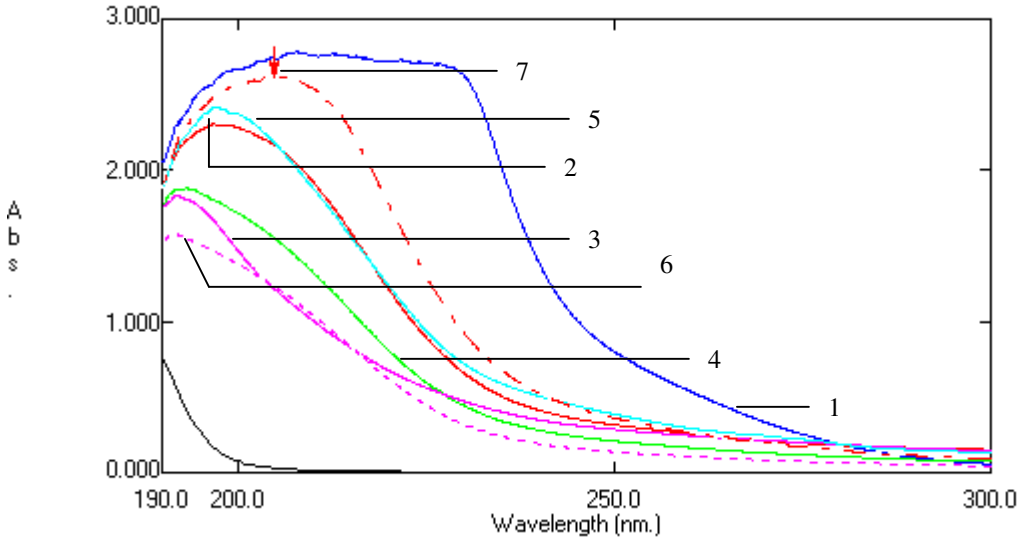
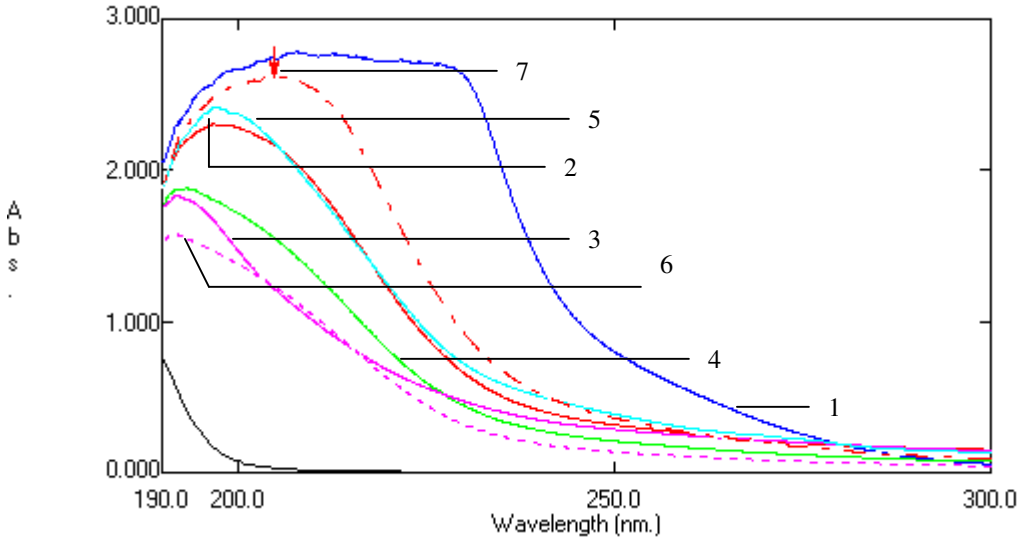


Figure 36: UV of $CuSO_4$ (0.01M) complexation study, (1) $CuSO_4$ (0.01M),
(2) Chitosan-Cu complex, (3) deacetylated chitin-Cu complex,
(4) diisocyanatohexane-crosslinked deacetylated chitin-Cu complex,
(5) dibromodecane-crosslinked deacetylated chitin-Cu complex,
(6) trimellitic anhydride-crosslinked deacetylated chitin-Cu complex and
(7) diisocyanatohexane-crosslinked chitosan-Cu complex.



Figuer 37: UV of $ZnSO_4$ (0.01M) complexation study, (1) $ZnSO_4$ (0.01M), (2) Chitosan-Zn complex, (3) deacetylated chitin-Zn complex, (4) diisocyanatohexane-crosslinked deacetylated chitin-Zn complex, (5) dibromodecane-crosslinked deacetylated chitin-Zn complex, (6) trimellitic anhydride-crosslinked deacetylated chitin-Zn complex and (7) diisocyanatohexane-crosslinked chitosan-Zn complex.

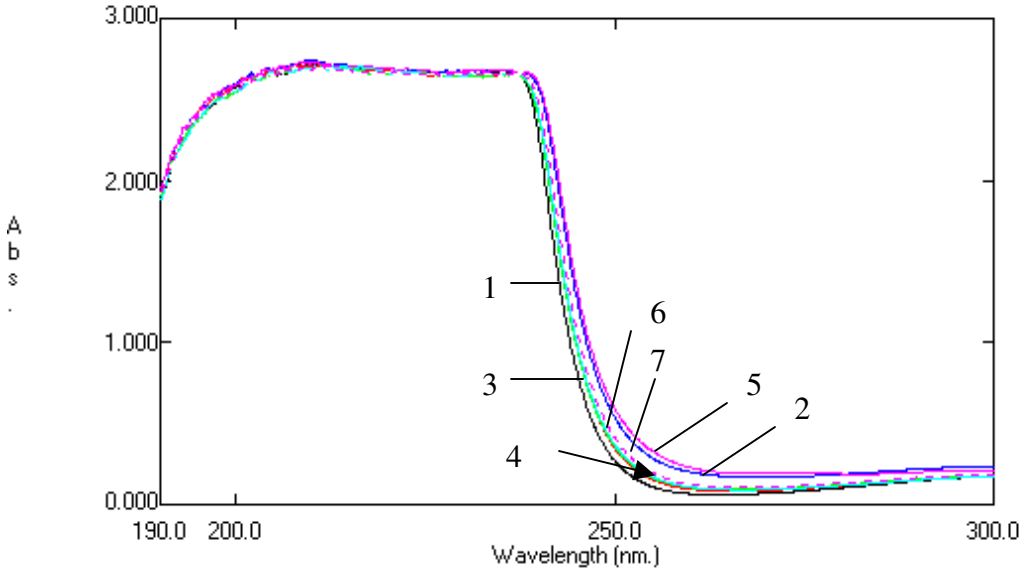


Figure 38: UV of $Pb(NO_3)_2$ (0.01M) complexation study, (1) $Pb(NO_3)_2$ (0.01M),
(2) Chitosan-Pb complex, (3) deacetylated chitin-Pb complex,
(4) diisocyanatohexane-crosslinked deacetylated chitin-Pb complex,
(5) dibromodecane-crosslinked deacetylated chitin-Pb complex,
(6) trimellitic anhydride-crosslinked deacetylated chitin-Pb complex and
(7) diisocyanatohexane-crosslinked chitosan-Pb complex.

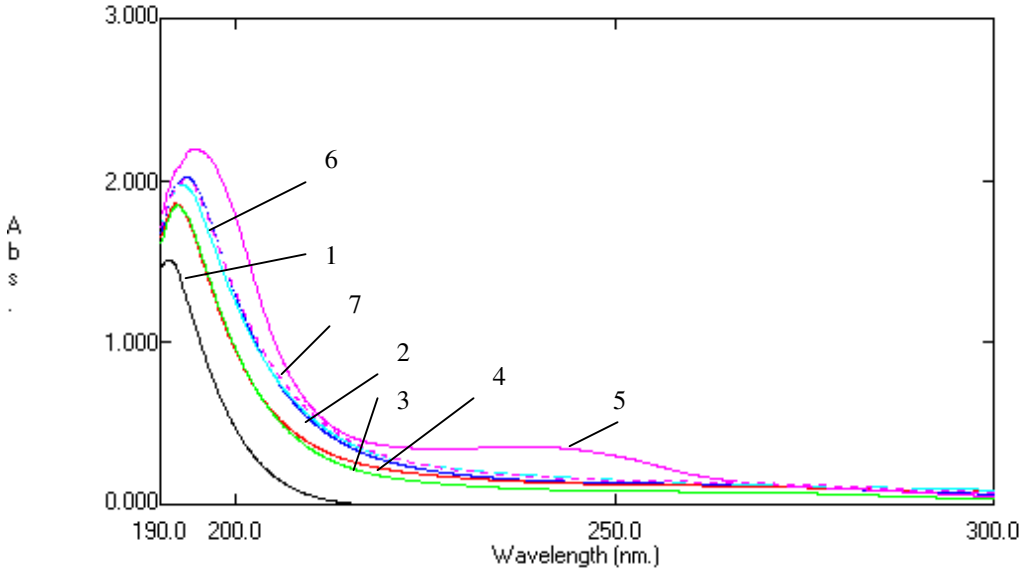


Figure 39: UV of $MnSO_4$ (0.01M) complexation study, (1) $MnSO_4$ (0.01M), (2) Chitosan-Mn complex, (3) deacetylated chitin-Mn complex, (4) diisocyanatohexane-crosslinked deacetylated chitin-Mn complex, (5) dibromodecane-crosslinked deacetylated chitin-Mn complex, (6) trimellitic anhydride-crosslinked deacetylated chitin-Mn complex and (7) diisocyanatohexane-crosslinked chitosan-Mn complex.

CHAPTER 3

"A morphological study of heavy metal complexes of chitosan and crosslinked chitosans by SEM and WAXRD"

Abstract:

Metal complexes of salts of Hg, Cu, Cd, Pb, Zn and Mn with chitosan and crosslinked chitosans were prepared, and their morphologies were studied using scanning electron microscopy and wide angle X-ray diffraction. The metal ions which were specifically and strongly complexed to the amino functions of chitosans, like Hg, showed smooth surface morphology inspite of large number of ions complexed (360 mg/g of chitosan). The presence of metal ions on the surface of the chitosans could be detected with decrease in metal ion binding, in the following sequence Hg > Cu > Cd > Zn > Pb > Mn. Particularly in the case of Pb ions, the presence of these ions is clearly seen on the surface of the polymer by SEM. The number of ions of Mn complexed on the polymers were too few (5 mg/g of chitosan) to be visible. These results are also in agreement with the morphologies studies by WAXRD. The metal complexation data for each of these metal ions was also in the same sequence.

3.1. Introduction:

One of the key properties of chitosan is its abilty to complex strongly with heavy metal ions, especially with Hg, Cu, Pb, Zn, Ni, Cr, and so on, and several applications proposed are in water purification for removing toxic metals by complexation (Varma, Deshpande, & Kennedy, 2004; Bailey et al., 1999; Muzzarelli and Rocchetti, 1974; Elson et al., 1980) Further, crosslinked polymers offer flexibility in metal binding conditions, as polymer solubility, conformation, molecular weight and concentration is not an issue. Here, the surface area and surface morphology, concentration of metal complexing ligands on the surface of the crosslinked polymer and porosity of the crosslinked polymer will affect the extent of binding. Recently we reported (Trimukhe & Varma, 2007) our detailed investigation into the heavy metal ion binding (Hg, Cu, Cd, Pb, Zn, Mn) to a series of crosslinked chitosans, using trimelitic anhydride, diisocyanatohexane, and dibromodecane as crosslinking agents. This is the first study wherein chitin was first crosslinked and then deacetylated to give crosslinked chitosans retaining

all the amino groups, which are crucial functional groups for specific heavy metal ion complexation. The present study reports the morphologies of the metal complexes obtained. Results of crosslinked chitosans with these new structures and morphologies will help in gaining new insights into factors affecting metal binding to chitosans.

3.2. Experimental Section:

3.2.1. Materials

The chitin and chitosan used in this study are commercial products of Meron Biopolymers, Cochin, Kerala, India. Diisocyanatohexane (HDI) was obtained from Aldrich Chemical Co., trimellitic anhydride (TMA) and dibromodecane (DBD) was obtained from Merck. Dimethylformamide, toluene and sodium hydroxide pellets were AR grade chemicals, obtained from SD fine chemicals, Mumbai. Sodium hydride was obtained from Merck. 4-Dimethylaminopyridine was purchased from Lancaster Company. All metal salts were AR grade materials and used without further purification. The salts $ZnCl_2$, $MnSO_4$, $CdSO_4$, $Pb(NO_3)_2$ were obtained from Loba Chemie, Mumbai, $CuSO_4$ was from SD Fine Chemicals, Mumbai, and $HgCl_2$ was from Merck.

3.2.2. Synthetic procedures

These are reported in detail in a previous publication (Trimukhe & Varma, 2007).

3.2.3. Scanning Electron microscopy studies :

The surface morphology of chitosan and various types of crosslinked deacetylated chitin was carried out before and after various types of metal binding by using Leica stereoscan 440 SEM.

3.2.4. X-Ray Diffraction :

XRD of TMA-crosslinked deacetylated chitin with Cu^{++}, Hg^{++}, Cd^{++}, Pb^{++} metals and without metals were carried out using Philips 1830 XRD

3.3. Results and Discussion

Figure 1 shows the surface structure of commercial chitosan (85% deactylated), deacylated chitin (chitosan) with 88% deacetylation prepared in our laboratory, as well as HDI and TMA crosslinled chitosans. The chitosan surfaces are seen to be smooth, but the crosslinked chitosans have large pore structures which are clearly seen. Figure 2 shows Hg complexed chitosan and crosslinked chitosan. The very strong and specific binding of Hg (360 mg/g chitosan) (Trimukhe & Varma, 2007 and refs. therein) involving most of the amino groups again leads to a smooth surface structure. Cd , Cu, and Zn which are also strongly bound (~145, 100, and 90 mg/g chitosan, respectively) with the amino groups, but not as strongly as Hg, shows the presence of some Cd and Cu salts on the surface (Figs. 3, 4, 5). Pb, on the other hand is more weakly bound, and shows more Pb salt on the surface (fig. 6). One would expect Mn, which is very weakly bound to the chitosan (5 mg/g) to also show up on the surface, but the very small amount of Mn present precludes this observation.

These morphological studies are well supplemented by our previous morphological studies using WAXRD of chitosan complexed with these same metal ions (Trimukhe & Varma, 2007). Here we clearly showed that the chitosan-Hg complex shows three peaks of crystalline region at 9.76, 21.50 and 28.17^0. The main chitosan peak characteristic of chitosan disappears in chitosan-Hg complex due to disruption of the interpolymer bonds**.** Similarly, the chitosan-Cu complex was characterized by a single broad amorphous peak at 22.16. Thus, complexation of Cu (II) leads to very significant changes in the morphology of the chitosan, indicating complete disruption of the interpolymer bonds. In the case of chitosan-Cd complex we showed one characteristic peak of crystalline region at 9.83^0 of chitosan is observed, while the major characteristic peaks of chitosan as well as $CdSO_4.H_2O$ disappear. This is reflected in the lower extent of Cd complexation to chitosan as compared to Cu and Hg.

The very small extent of complexation of Pb with chitosan is further proved by the negligible change in the WAXRD spectrum (Trimukhe & Varma, 2007). Exactly the same WAXRD results were obtained with TMA-crosslinked deacetylated chitin, and are shown in Figure 7 (A-E). The chitosan peaks are seen to disappear with Cu (curve B), Hg (curve C), and Cd (curve D), but not with Pb (curve E) complex.

3.4. Conclusions:

Morphological information gleaned from SEM studies indicate that metal ions like Hg which are very strongly bound to the chitosan and crosslinked chitosans, even to the extent of 360mg/g, are not seen as distinct moieties on the surface of the polymer, whereas with decreasing extent of binding, in the order Hg > Cu > Cd > Zn > Pb > Mn, we begin to observe the increasing presence of metal on the surface. These results agreed with the morphological information obtained with WAXRD studies, as well as with the metal complexation data of these metals with these same chitosan polymer / crosslinked polymer systems.

3.5. References:

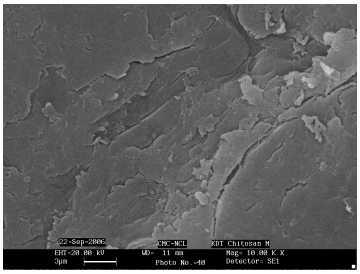
Bailey, S. E., Olin, T. J., Bricka, R. M., Adrian, D. D. (1999). A review of potentially low-cost sorbents for heavy metals. *Water Res,* 33(11), 2469-2479.

Muzzarelli, R. A. A., Rocchetti, R. (1974). The use of chitosan columns for removal of mercury from waters. *J. Chromatogr.,* 96(1), 115-121.

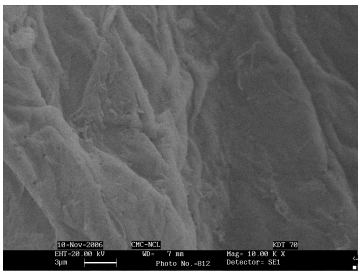
Elson C. M., Davies D. M., Hayes E. R. (1980). Removal of arsenic from contaminated drinking water by a chitosan/chitin mixture. *Water Res,* 14(9), 1307 - 1311, CA 93:245181.

Varma, A.J., Deshpande, S.V., Kennedy, J.F. (2004). Metal complexation by chitosan and its derivatives: a review. Carbohydrate Polymers, 55, 77–93.

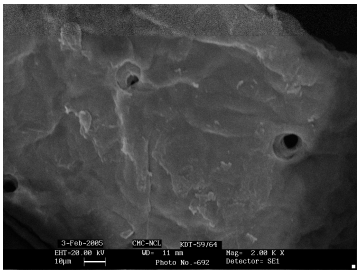
Trimukhe, K.D. and Varma, A.J. (2007). Complexation of Heavy Metals by Crosslinked Chitin and its Deacetylated Derivatives. Carbohydrate Polym. (in press, 2007)



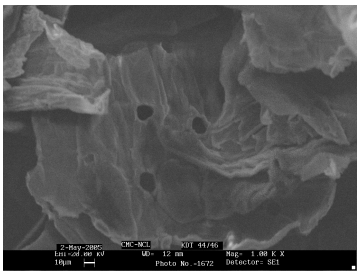
Chitosan



Deacetylated chitin

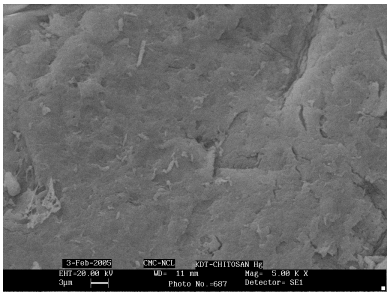


HDI crosslinked deacetylated chitin

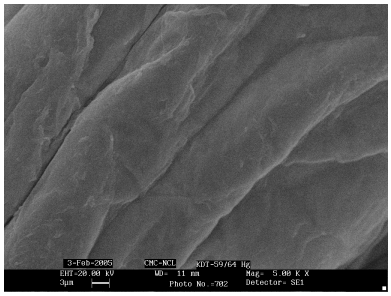


TMA crosslinked deacetylated chitin

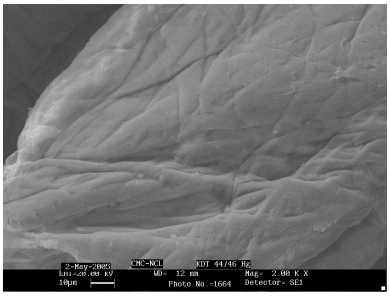
Fig. 1 : SEM of chitosan, deacetylated chitin and various crosslinked deacetylated chitin



Chitosan Hg^{++}

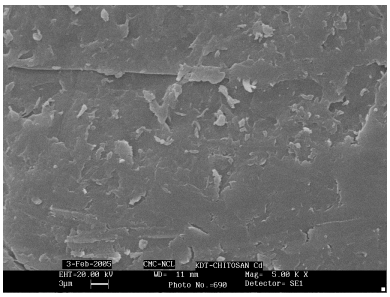


HDI-crosslinked deacetylated chitin Hg^{++}

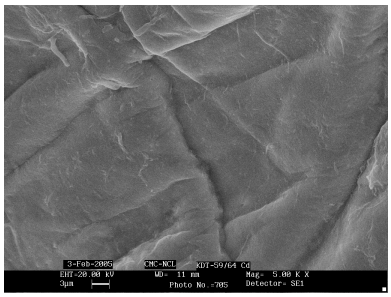


TMA-crosslinked deacetylated chitin Hg^{++}

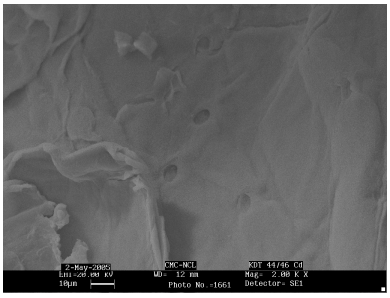
Fig. 2 : SEM of Hg^{++} binding on chitosan, and various crosslinked deacetylated chitin



Chitosan Cd^{++}

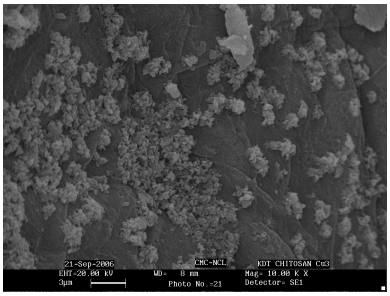


HDI-crosslinked deacetylated chitin Cd^{++}

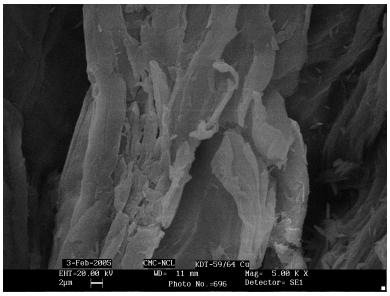


TMA-crosslinked deacetylated chitin Cd^{++}

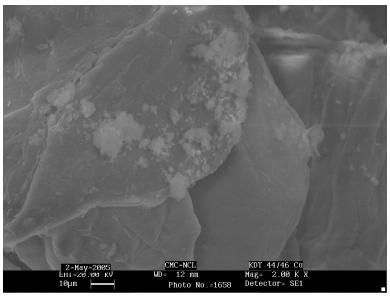
Fig. 3 : SEM of Cd^{++} binding on chitosan, and various crosslinked deacetylated chitin



Chitosan- Cu^{++}

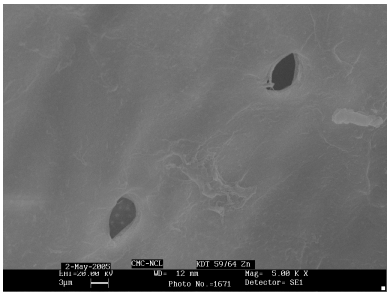


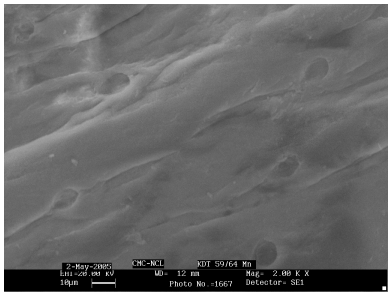
HDI-crosslinked deacetylated chitin Cu^{++}



TMA-crosslinked deacetylated chitin Cu^{++}

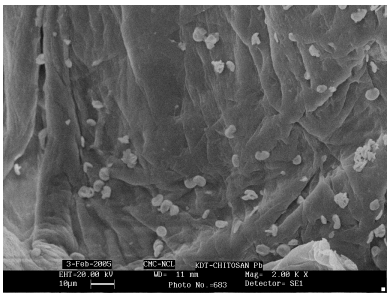
Fig. 4 : SEM of Cu^{++} binding on chitosan, and various crosslinked deacetylated chitin



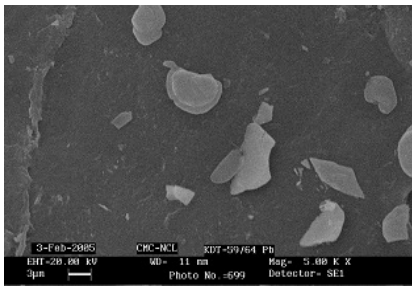


HDI-crosslinked deacetylated Zn^{++} HDI-crosslinked deacetylated Mn^{++}

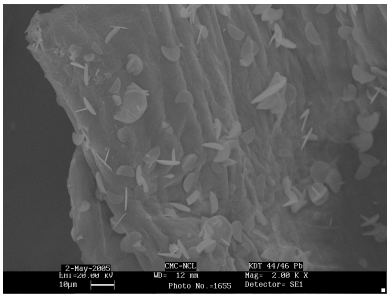
Fig. 5 :SEM of Zn^{++} and Mn^{++} binding on HDI crosslinked deacetylated chitin



Chitosan Pb^{++}



HDI-crosslinked deacetylated chitin Pb^{++}



TMA-crosslinked deacetylated chitin Pb^{++}

Fig. 6 : SEM of Pb^{++} binding on chitosan, and various crosslinked deacetylated chitin

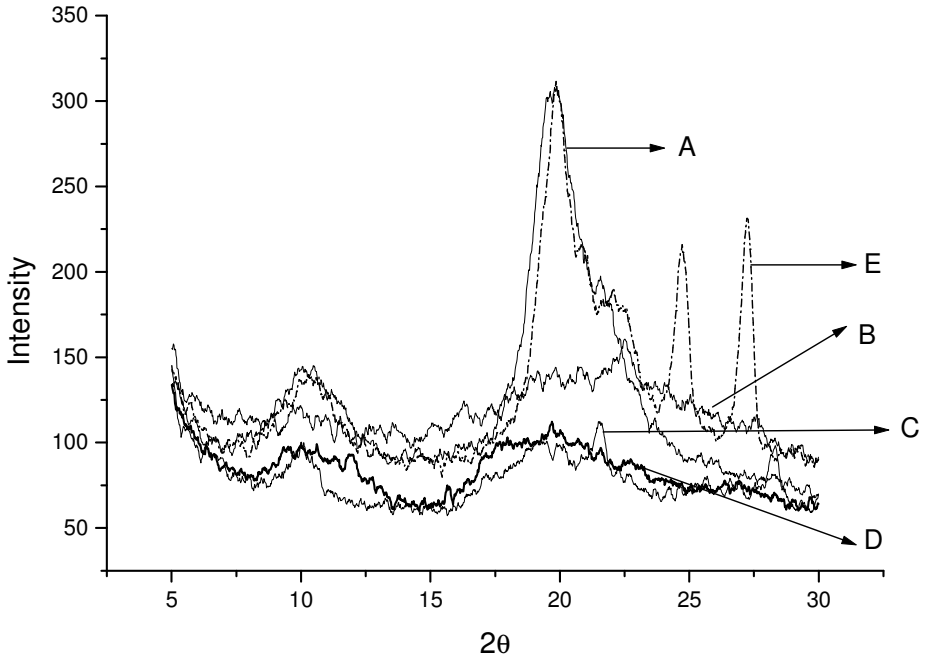


Figure 7: XRD of (A) TMA-crosslinked deacetylated chitin (B) TMA-crosslinked deacetylated chitin -Cu complex (C) TMA-crosslinked deacetylated chitin -Hg complex (D) TMA-crosslinked deacetylated chitin -Cd complex (E) TMA-crosslinked deacetylated chitin -Pb complex.

3.6. Appendix 4: XRD of TMA-crosslinked deacetylated chitin with metal ions

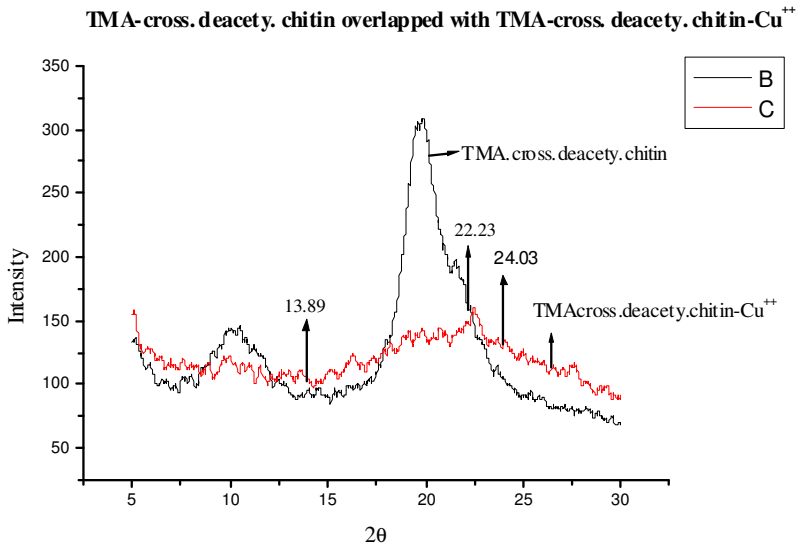


Figure 8: TMA-crosslinked deacetylated chitin overlapped with TMA-crosslinked deacetylated chitin-Cu^{++}

Trimellitic anhydride-crosslinked deacetylated chitin shows three characteristic peaks of crystalline region at 9.95, 19.78 and very small peak at 21.58^0. But Trimellitic anhydride-crosslinked deacetylated chitin-Cu complex gives a without characteristic peak of crystalline region. Therefore chitosan-Cu complex have more amorphous, and it may be due to higher percentage of metal complexation. (From literature $CuSO_4.5H_2O$ shows three characteristic peaks of crystalline region at 13.86, 22.20 and 24.02^0).

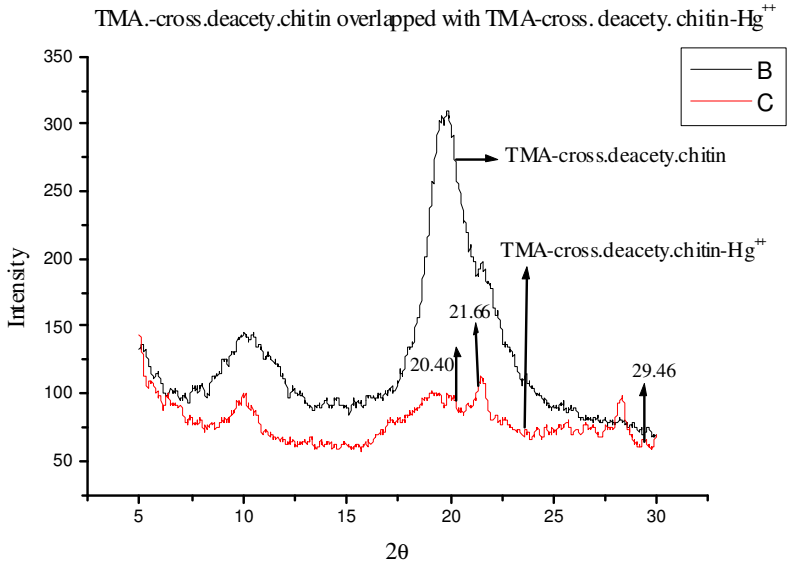


Figure 9: TMA-crosslinked deacetylated chitin overlapped with TMA-crosslinked deacetylated chitin-Hg^{++}

TMA-crosslinked deacetylated chitin gives three characteristic peaks of crystalline region at 10.03, 19.78 and very small peak at 21.57^0. But in TMA-crosslinked deacetylated chitin-Hg^{++} complex shows five peaks of crystalline region at 10.03, 19.16, 19.97, 21.57, 28.33^0. From literature $HgCl_2$ shows three characteristic peaks of crystalline region at 20.40, 29.46 and 21.66^0. $HgCl_2$ characteristic peaks slightly shifted and main TMA-crosslinked deacetylated chitin characteristic peak intensity was very less in TMA-crosslinked deacetylated chitin-Hg complex due to complex formation.

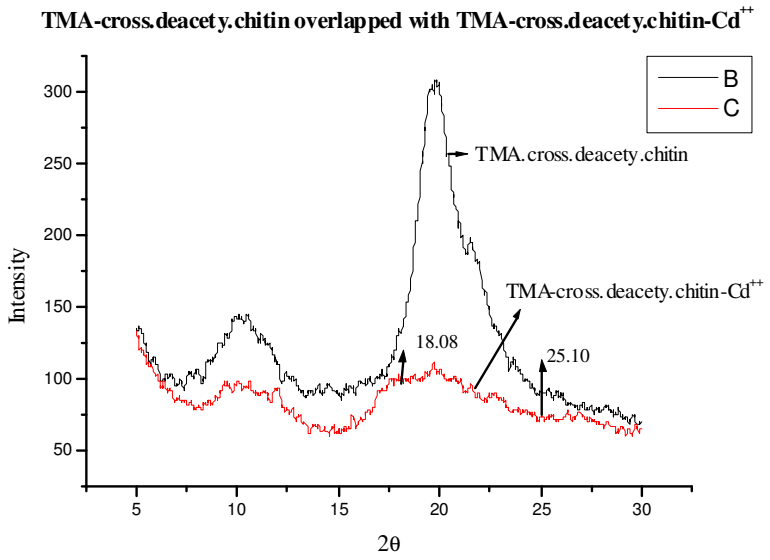


Figure 10: TMA-crosslinked deacetylated chitin overlapped with TMA-crosslinked deacetylated chitin-Cd^{++}

Trimellitic anhydride-crosslinked deacetylated chitin shows three characteristic peaks of crystalline region at 9.95, 19.78 and very small peak at 21.58^0.But Trimellitic anhydride-crosslinked deacetylated chitin-Cd complex has no peak. Therefore chitosan-Cd complex has more amorphous. From literature $CdSO_4.H_2O$ shows three characteristic peaks of crystalline region at 25.06, 18.08 and 35.74^0.

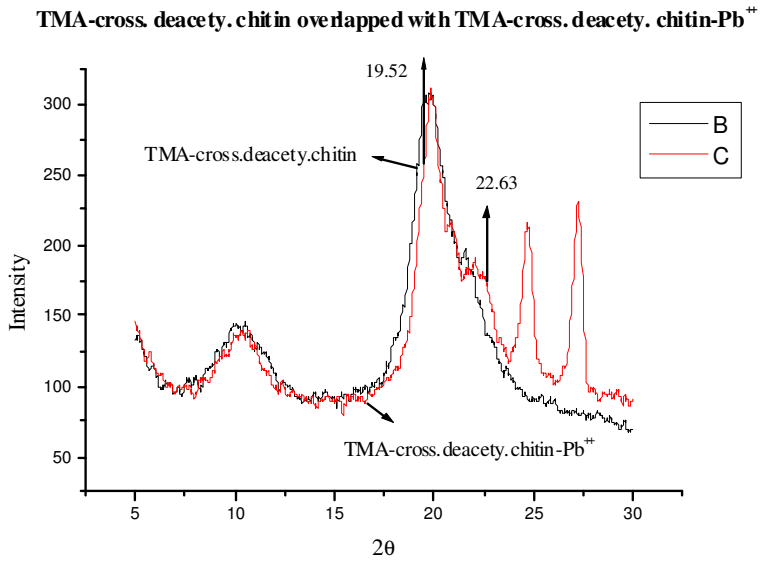


Figure 11: TMA-crosslinked deacetylated chitin overlapped with TMA-crosslinked deacetylated chitin-Pb^{++}

Trimellitic anhydride-crosslinked deacetylated chitin shows three characteristic peaks of crystalline region at 9.85, 19.70, 26.57^0 but Trimellitic anhydride-crosslinked deacetylated chitin-Pb complex show five characteristic peaks of crystalline region at 9.56, 19.90, 20.91, 24.70 and 27.19^0. And from literature $Pb(NO_3)_2$ gives a three characteristic peaks of crystalline region at 19.54, 38.10 and 22.66^0. $Pb(NO_3)_2$ characteristic peaks slightly shifted and chitosan characteristic peaks shows sharp in Trimellitic anhydride-crosslinked deacetylated chitin-Pb complex due to lower percentage of complex formation.

CHAPTER 4

"Metal complexes of crosslinked chitosans: Part II. An investigation of their hydrolysis to chitooligosaccharides using chitosanase"

Abstract

This paper investigates the behavior of crosslinked chitosans and metal-complexed crosslinked chitosans under similar hydrolytic conditions. Crosslinked chitosans with trimellitic anhydride, diisocyanatohexane, and dibromodecane as crosslinking agents under heterogenous reaction conditions, were used as metal complexing agents by equilibrating them with metal salts such as $ZnCl_2$, $MnSO_4$, $CuSO_4$, $CdSO_4$, $Pb(NO_3)_2$, and $HgCl_2$. Crosslinked chitosan without metal complexation had the same hydrolytic behavior as uncrosslinked chitosan. However, when the crosslinked chitosans were complexed with metals, their hydrolytic rates and extent of hydrolysis was significantly reduced. Thus while for chitosan about 840 µg/ml reducing sugar was produced in 4 hours time, and 780 µg/ml was produced for diisocyanatohexane crosslinked chitosan, only 400 µg/ml and 320 µg/ml was produced for cadmium sulfate with crosslinked chitosan and diisocyanatohexane crosslinked chitosan, respectively. Similar results are obtained for other crosslinking agents. Studies on preincubation of the metal with the enzyme show that of the metals studied, Mn has no effect on preincubatioin with the enzyme, Hg, Cd, Pb, and Cu completely deactivate the enzyme, while Zn reduces the enzyme activity by about 43.3%. Preincubation of the metal salts with the chitosan shows that Hg and Cu completely deactivate the molecule from enzyme hydrolysis, Cd and Zn inactivate it to the extent of 56.8% and 43.3% respectively, while Mn has no effect. Availability of the amino functions seems to be a key feature for the chitosanase to hydrolyze the chitosan polymer. This was also proved by the significant increase in the extent of hydrolysis for chitosan samples with 88% (final value 1120 µg/ml reducing sugar) and 85% deacetylation (final value 840 µg/ml reducing sugar). HPIC studies of the products show a variety of oligomers are produced in the chitosanase enzyme hydrolytic reaction.

4.1. Introduction

Chitosan is a partially deacetylated polymer of chitin. It comprises copolymers of glucosamine and N-acetyl glucosamine and the nature of chitosan differs on the

basis of preparation method. Metal complexed chitosans are known to exhibit antimicrobial and antitumour activity (Wang, Du, Liu, 2004; Qin, et al, 2002). Hydrolysis of chitosans by chitosanases is a major area of research (Sikorski, Sorbotten, Horn, Eijsink & Varum, 2006). Further, it is also known that hydrolyzed chitosans have enhanced antimicrobial properties (Se-Kwon Kim and Rajapakse, 2005; You-Jin Jeon, Park, Se-Kwon Kim, 2001; Kumar, Varadaraj, Gowda, Tharanathan, 2005). Alginate fibres modified with hydrolyzed chitosan showed antibacterial effects (Knill, Kennedy, Mistry, Miraftab, Smart, Groocock & Williams, 2004). Recently it has been shown that chitosan-metal complexes are far superior to chitosan and metal salts for studies related to *in vitro* antimicrobial activities (Wang, Du Fan Liu & Hu, 2005). Chitosan-copper complexes are known to have antitomour activities, especially at a ratio of 0.11 mol copper per one chitosan residue (Zheng, Yi, Wang, Zhang, & Du, 2006).

In a previous study on metal complexation with crosslinked chitosans, we have shown that when the crosslinked derivatives prepared were such that only the hydroxy groups were utilized in the crosslinking reaction, and the acetylamino groups of chitin were hydrolyzed only after the crosslinking was accomplished to ensure that all amino groups so produced would be available for metal complexation, and not partially used up in crosslinking, then the binding capacity of various crosslinked chitin and deacetylated derivatives for Cu, Cd, Hg, Zn, Mn, and Pb was in the region of 100, 140, 360, 88 , 5 and 60 mg/g (rounded off values) of polymer respectively, very close to the values obtained for uncrosslinked chitosan (Trimukhe & Varma, 2007). Morphological studies using WAXRD were in close agreement with the metal complexation data, showing complete loss of original chitosan peaks for the heavily complexed derivatives (Hg, Cu salts), and minor changes for the weakly complexed metals (Cd, Pb salts).

However, it is not known how the complexed metal affects the enzymatic hydrolysis profiles of these metal complexed chitosans and crosslinked chitosans

to produce chitooligosaccharides. The oligosaccharide products so obtained will be the object of further study on their antimicrobial properties. Therefore in this paper we present results of chitosanase enzyme hydrolysis study of a series of crosslinked chitosan derivatives and their metal complexes with $ZnCl_2$, $MnSO_4$, $CuSO_4$, $CdSO_4$, $Pb(NO_3)_2$, and $HgCl_2$. The results of this study are compared with un-crosslinked chitosan.

4.2. Material and methods

4.2.1. Chemicals

The chitosan used in this study was a commercial product of Meron Biopolymers, Cochin, Kerala, India. D-glucosamine was obtained from Sigma Chemical Co. (St. Louis, MO). All metal salts $ZnCl_2$, $MnSO_4$, $CuSO_4$, $CdSO_4$, $Pb(NO_3)_2$, and $HgCl_2$ were AR grade materials obtained from Merck and SD Fine Chemicals, India, and used without further purification.

4.2.2. Preparation of different crosslinked chitosan derivatives.

Detailed preparation methods of diisocyanatohexane (HDI) crosslinked with chitin, followed by deacetylation of the chitin; trimellitic anhydride (TMA) crosslinked with chitin, followed by deacetylation of the chitin; dibromodecane (DBD) crosslinked with chitin, followed by deacetylation of the chitin; diisocyanatohexane (HDI) crosslinked with chitosan, and their characterization are being published separately (Trimukhe & Varma, 2007).

4.2.2.1. Microorganism and culture conditions

The Streptomyces species producing chitosanase was isolated from soil by screening large number of isolates for chitosanase production. The screening was performed on the basis of zone of hydrolysis of acid swollen chitosan. The culture was maintained routinely on MGYP medium containing (g/l); maltose, 3; glucose, 10; yeast extract, 3; peptone, 5; agar, 15.

4.2.2.2. Enzyme production

For chitosanase production, the organism was first grown in the 50 ml of liquid medium containing (g/l); maltose, 3; glucose, 10; yeast extract, 3; peptone, 5; for 48 hrs at 30^0C and was used as an inoculum. This culture (4 ml) was inoculated in to 250 ml conical flasks containing 50 ml of enzyme production medium containing (g/l); chitosan, 5; peptone, 5; yeast extract, 5; K_2HPO_4, 1; NH_4Cl, 1.5; $MgSO_4.7H_2O$, 0.5. (pH 6.0) for 48 hours at 30^0C. The culture was harvested by centrifugation at 5000 rpm for 20 min and the extracellular broth was used as source of chitosanase enzyme. The Streptomyces species produced 25 ± 2.0 IU/mL (unpublished data).

4.2.2.3. Enzyme assay

Chitosan was dissolved in the 50mM acetate buffer of pH 5.0 to final concentration of 0.2%(w/v) and was used as substrate for the chitosanase assay. The reaction mixture contained 950 µl of substrate and 50 µl of suitably diluted enzyme. The reaction mixture was incubated at 50^0C for 30 minutes. All the chitosan derivatives were dissolved in the same buffer to final concentration of 0.2% (w/v) and used for the assay. To find out the effect of free metals on chitosan and enzyme, respectively, the various free metals were preincubated for five minutes with chitosan or enzyme at 30^0C followed by enzyme assay as mentioned above. The concentration of reducing sugar was determined by the method of Nelson (Nelson, 1944) using D-glucosamine calibration curve. One unit of enzyme was defined as the amount of enzyme that produced 1µmole of reducing sugar (D-glucosamine equivalent) per ml per minute under assay conditions.

4.2.2.4. Procedure for hydrolysis of chitosan derivatives by chitosanase

The hydrolysis mixture of 5 ml consisted of chitosan and chitosan derivatives were dissolved in the 50mM acetate buffer of pH 5.0 to final concentration of 0.2%(w/v) and 0.2 IU of chitosanase. The hydrolysis was carried out at 50^oC with

shaking at 150 rpm and the samples were removed periodically for the analysis of D-glucosamine (Nelson, 1944). The highest hydrolysis activity shown (for chitosan) was considered as 100% and relative activity for other derivatives was calculated.

4.2.2.5. *High Performance Ion Chromatography (HPIC)*

Dionex Ion Chromatograph equipped with GS-50 quaternary gradient form ED-50 electrochemical detector, Rheodyne injector and chromeleon software was used. The column was a CarboPack PA-10, 4X250 mm analytical column with a Carbopack PA-10 guard column. The mobile phase ratio was: A (water): B (200 mM of NaOH)/ (95ml): (5ml) at flow rate of 1ml/min and the Injection volume was 25 µl. Pressure drop was observed 2650 psi.

4.2.2.6. *Sample preparation*: 1 ml sample (1mg hydrolyzed chitosan) diluted to 5ml with deionized water.

4.2.2.7. *Procedure for % swelling of solvents in crosslinked chitin/chitosans:*

Approx. 100mg of dry deacetylated chitin or crosslinked deacetylated chitin was dispersed in various solvents and kept in contact for 24 h at room temperature. It was then filtered, wiped with absorbent filter paper and weighed.

Calculation:

$$\% \text{ Swelling} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Dry weight}} \times 100$$

4.3. Results and Discussion

Results of a previous investigation indicate that simple crosslinking of chitin with crosslinking agents like HDI, TMA, and DBD followed by deacetylation affords a

simple method of obtaining a resin with amino groups at the surface which give high degrees of complexation with heavy metals like Cu and Hg, comparable to the highest values reported in literature for chitosans, crosslinked chitosans, and chitosan beads (Trimukhe & Varma, 2007). These metal complexed crosslinked chitosans were investigated for their hydrolysis. Most published literature is based on crosslinking of chitosans directly with chemicals like glutaraldehyde, HDI, etc. which consumes the amino groups, which are so crucial for enhanced complexing of heavy metal ions. Using our methodology for crosslinking, we were able to preserve all the amino functions for use in metal complexation, thereby obtaining higher degrees of complexation as compared to systems (for e.g. chitosan instead of chitin as substrate) where some of the amino groups were consumed in the crosslinkuing reaction. These metal complexes have now been subjected for enzymatic hydrolysis, to ascertain the role of the complexed metal on the enzyme hydrolysis behavior.

One more point, regarding the type of crosslinked chitin/chitosan products that are obtained by our synthetic method, needs to be discussed. The heterogenous reaction conditions employed by us for crosslinking chitin are expected to give products with non-random crosslinking, which would occur only on the exposed surface of the chitin dispersed in a solvent medium. In future work we also plan to develop crosslinking reactions in solution, such as in DMAc-LiCl solvent systems, so as to obtain more random distribution of crosslinks. In such solution systems the degree of crosslinking would also be controlled more easily, so as to afford products with varying degrees of crosslinks distributed randomly. It may be mentioned that the intractable nature of chitin is such that solvent systems are very sensitive to added reagents. However, for the purpose of this research, we only intended to show the effect of first crosslinking chitin and then deacetylating it to obtain a product with all amino functions intact for use in complexation of metals. For materials such as chitin, studies on heterogenous crosslinking affords the possibility of developing commercial resins for applications due to the easy

reaction conditions amenable to large scale applications, which may not be possible with homogenous reactions of chitin.

4.3.1. *Hydrolysis of chitosan derivatives by chitosanase.*

The hydrolysis profiles of all the chitosan samples and their metal complexes by estimation of the reducing sugars produced (Nelson, 1944) are presented in Figure 1. Data for Hg and Cu salt complexes cannot be obtained as they completely inhibit enzyme hydrolysis.

Thus fig.1a shows the profile for commercial chitosan (85% deacetylation), fig. 1b for laboratory produced deacetylated chitin (88% deactylation) (Trimukhe & Varma, 2007), fig.1c for HDI-crosslinked chitosan, fig.1d for HDI-crosslinked deactylated chitin as well as with complexed metal salts of Mn, Zn, Cd, and Pb, fig.1e for TMA-crosslinked deactylated chitin as well as with complexed metal salts of Mn, Zn, Cd, and Pb, and fig.1f with DBD- crosslinked deactylated chitin as well as with complexed metal salts of Mn, Zn, Cd, and Pb.

Solvent absorption studies of the heterogenously crosslinked chitin/ chitosan products showed no major changes in the absorption of different solvents before or after crosslinking (table 3). This shows that extent of crosslinking was small, and has had no major effect on solvent penetration, and therefore also no major effect for enzyme penetration for hydrolysis. Due to this reason the profile of hydrolysis also remains similar. The crosslinked deacetylated chitins do not dissolve in aqueous acids, thuis proving that crosslinking indeed occurred.

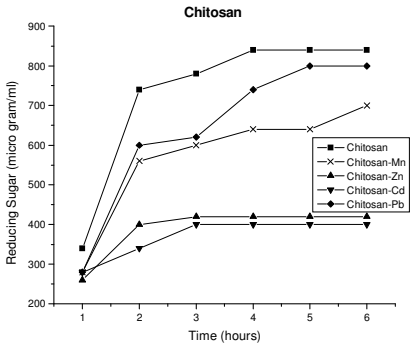


Fig 1a

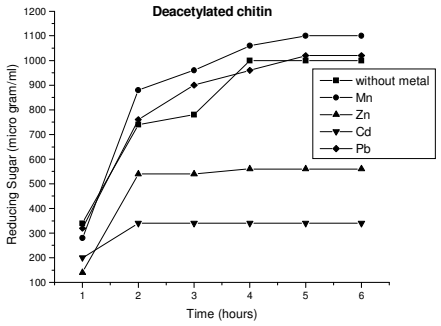


Fig 1b

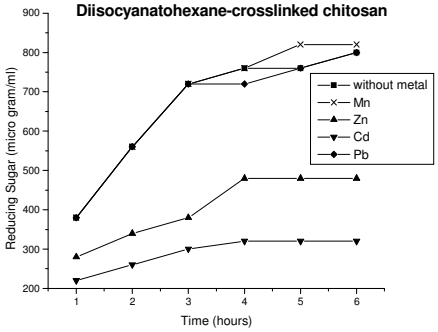


Fig. 1c

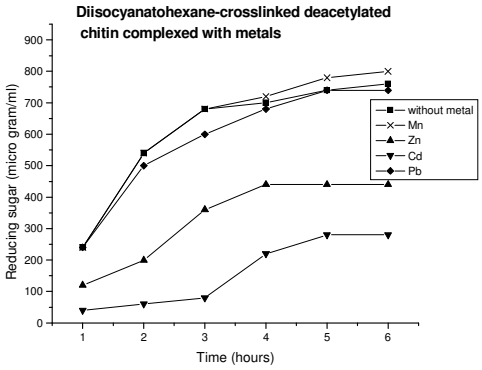


Fig. 1d

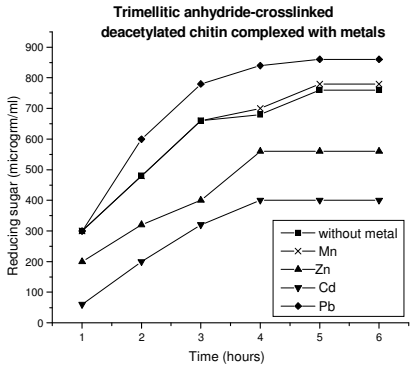


Fig. 1e

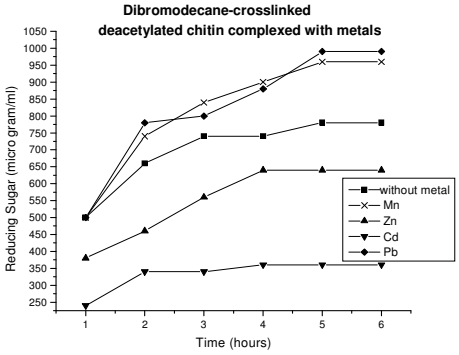


Fig. 1f

Figure 1 : Hydrolysis study of various crosslinked chitosans and chitosan with their metal complexes by estimation of reducing sugars produced, using Somyogi and Nelson Method (Nelson, 1944).
Fig.1a: chitosans and its metal complexes, Fig.1b : metal complexes of deacetylated chitin, Fig.1c: metal complexes of diisocyanatohexane crosslinked chitosan , Fig.1d: metal complexes of diisocyanatohexane crosslinked deacetylated chitin, Fig.1e: metal complexes of trimellitic anhydride crosslinked deacetylated chitin, Fig.1f : metal complexes of dibromodecane deacetylated chitin

Table 1. Swelling studies of deacetylated chitin and crosslinked deacetylated chitin in different solvents

Solvent used	% Swelling of deacetylated chitin and crosslinked deacetylated chitin			
	Deacetylated chitin	HDI-crosslinked deacetylated chitin	TMA-crosslinked deacetylated chitin	DBD-crosslinked deacetylated chitin
DMF	550.9	679.7	554.4	626.3
EtOH	580.1	570.2	492.5	567.1
H_2O	702.6	814.2	691.5	790.1
TCE	734.3	596.3	593.7	506.2
Hexane	788.1	704.2	673.8	651.7
Toluene	931.1	847.9	824.9	867.6

Table 2. Relative activities of the chitosanase enzyme towards hydrolysis of metal complexed chitosan derivatives

Metal linked	Derivative and relative activity (%)					
	Chitosan (Meron)	Deacetylated chitin	Diisocyanato hexane-crosslinked chitosan	Diisocyanato hexane-crosslinked deacetylated chitin	Dibromodecane-crosslinked deacetylated chitin	Trimellitic anhydride-crosslinked deacetylated chitin
- -	100	91.8	81.0	91.8	94.5	100
Hg^{++}	0.0	0.0	0.0	0.0	0.0	0.0
Mn^{++}	100	91.8	81.0	91.8	94.5	100
Cd^{++}	43.2	46.0	37.8	40.8	40.8	43.2
Pb^{++}	100	91.8	81.0	91.8	94.5	100
Cu^{++}	0.0	0.0	0.0	0.0	0.0	0.0
Zn^{++}	56.7	54.0	48.6	51.3	51.3	54.0

4.3.2. *Discussion on rates of hydrolysis.*

The graphs in Fig.1 show that chitosan derivatives show variation in the rate of hydrolysis. Chitosan, after its hydrolysis produces more amount of reducing sugar than its metal complexes. Chitosan complexes with Mn and Pb show steady increase in the amount of reducing sugar produced whereas chitosan complexes with Zn and Cd show no increase in the amount of reducing sugar produced after

3 hr of hydrolysis time.The amount of reducing sugar produced after 1 hr. is approximately equal for the chitosan, chitosan-Mn and chitosan-Pb. There is considerable increase in the amount of reducing sugar after 1 hr in case of dibromodecane-crosslinked deacetylated chitin-Mn and dibromodecane-crosslinked deacetylated chitin-Pb. A similar pattern of hydrolysis is observed in case of Diisocyanatohexane-crosslinked chitosan and deacetylated chitin. The derivative trimellitic anhydride-crosslinked deacetylated chitin shows fast hydrolysis of Pb complex (more reducing sugar produced). Approximately the same rate of hydrolysis of diisocyanatohexane-crosslinked deacetylated chitin, diisocyanatohexane-crosslinked deacetylated chitin-Mn and diisocyanatohexane-crosslinked deacetylated chitin-Pb is observed. The hydrolysis rate of chitosans complexed with Zn and Pb is less compared with other metal complexed derivatives (Pb, Mn,), as is to be expected from the results of loss of enzyme chitosanase activity caused by these metals (table 2, and discussions that follow). This shows that different sets of oligochitosans are produced by different metals complexed to chitosans.

4.3.3. *Discussion on extent of hydrolysis*

The profiles so exhibited all show the same trend – the samples with no metal bound show the maximum extent of hydrolysis at higher rates, while Cd, which is the strongest metal complex from amongst the investigated Mn, Zn, Cd, and Pb, has the lowest rates and extent of hydrolysis (final plateau value). Thus, for Cd-chitosan sample the ratio of chitosanase enzyme hydrolysis to chitosan is 1:2.1, for Pb-chitosan to chitosan it is 1:2, for Zn-chitosan to chitosan it is 1:1.23 and for Mn-chitosan to chitosan the ratio is 1:1.05. Thus, the trend of decreasing extent of hydrolysis is from weakest metal complex (Mn) to the strongest complex (Cd), with Pb and Zn being intermediate. The detailed studies on the complexation studies and morphological studies with these metal salts (Trimukhe & Varma, 2007) are in agreement with these results. The extremely high binding of Hg and Cu inhibits enzyme hydrolysis completely. WAXRD studies have clearly demonstrated that all the characteristic peaks of chitosan are completely destroyed

after complexation with Hg and Cu salts, while they are only partially affected by Pb and Cd salts (Trimukhe & Varma, 2007). Similar hydrolysis profiles are obtained with crosslinked chitosans and metal complexed crosslinked chitosans, though some minor changes can be observed. In most cases the weakly bound Mn and Pb have nearly the same final extent of hydrolysis as compared to the parent sample, generally within 5%. The deactylated chitin with 88 % degree of deacetylation had significantly higher extent of hydrolysis (final value 1120 µg/ml reducing sugar as compared to 840 µg/ml reducing sugar for commercial chitosan with 85% degree of deacetylation. It is apparent that greater the number of free amino groups, greater the extent of hydrolysis for un-crosslinked chitosans. However, mild crosslinking of the chitosans had no significant effect on the final extent of hydrolysis, as the enzyme is able to penetrate the crosslinked structure of the polymer, though the initial rates of hydrolysis of the un-crosslinked polymer is significantly greater as compared to the crosslinked polymers, as would be expected (fig.1a-e). Thus, it is seen that when chitin is crosslinked with a crosslinking agent and then deacetylated, the hydroxyl groups of chitin are used in the crosslinking reaction (as no amine functions are present) and amine groups are generated in the deacetylation reaction. As a result of this presence of amine groups on the surface of the crosslinked polymer, the hydrolytic enzyme activity of chitosanase is similar to standard unhydrolyzed chitosan. However, when chitosan is crosslinked, the amine groups also take part in the crosslinking reaction, their concentration is decreased, and the enzyme activity is reduced.

4.3.4. Relative activities of the chitosanase enzyme towards hydrolysis of metal complexed chitosan derivatives

The relative activities of the chitosanase enzyme towards hydrolysis for the chitosan derivatives linked to different metals are shown in the table 1. All chitosan derivatives linked with Hg and Cu did not show any activity. This is a reflection of the extent of metal binding to chitosan. Cu (100-106 mg/g binding) and Hg (348-361mg/g binding) are very strongly bound to the amino group

binding site, as is well known, and have recently been explored in detail in a previous publication from our group (Trimukhe & Varma, 2008). Apparently, the enzyme is inactivated due to the amino-bound metal. The relative activity for the chitosan derivatives linked to Zn (71-77 mg/g binding) and Cd (133 mg/g binding) is approximately 50 %, whereas Mn and Pb linked chitosan derivatives do not show any significant change in the activity as compared to the respective plain chitosan derivatives. Since Mn is weakly bound (4-7 mg/g binding) to the amine groups, the enzyme activity is not inhibited. Pb is bound to the chitosan (25-59 mg/g binding) to a larger extent, but apparently the amino functions are not used to the same extent in the complexation as for the other metals studied. The binding sites for Pb need to be investigated further.

4.3.5. *The effect of free metals on chitosan and chitosanase*

Table 2 shows data on the effect of free metals preincubated with chitosan and chitosanase. In order to find out whether the metals have any effect on the chitosanase activity in unbound form, the free metals were preincubated with chitosan and their chitosanase hydrolysis was studied. Similarly, it was pertinent to study if the free metal had any effect on the chitosanase enzyme activity, so the chitosanase preincubated with the metals and its activity was evaluated. When the free metal salts were preincubated with chitosan there was no change in the relative activity of enzyme compared with the respective metal derivative. However, the preincubation of free metals with enzyme caused total loss in the enzyme activity in case of Hg, Cu, Cd, and Pb. This shows that the enzyme can complex Hg, Cu, Pb, and Cd, and gets deactivated.

However, preincubation with Hg and Cu inhibited the enzyme when the chitosan was preincubated or when the enzyme was preincubated. This shows that the while the enzyme can complex Hg, Cu, Pb, and Cd, the chitosan complexes only Hg and Cu. Further, we speculate that Cu and Hg seem to be complexed very strongly with the amine functions of chitosans (as opposed to hydroxyl functions), and therefore preincubation with these metals inhibits completely the enzyme

activity towards hydrolysis. Mn had no effect on the enzyme activity, while for Zn it was reduced by about half. Free Mn had no effect on the chitosan, as it is a very weak complex (4-7 mg/g binding).

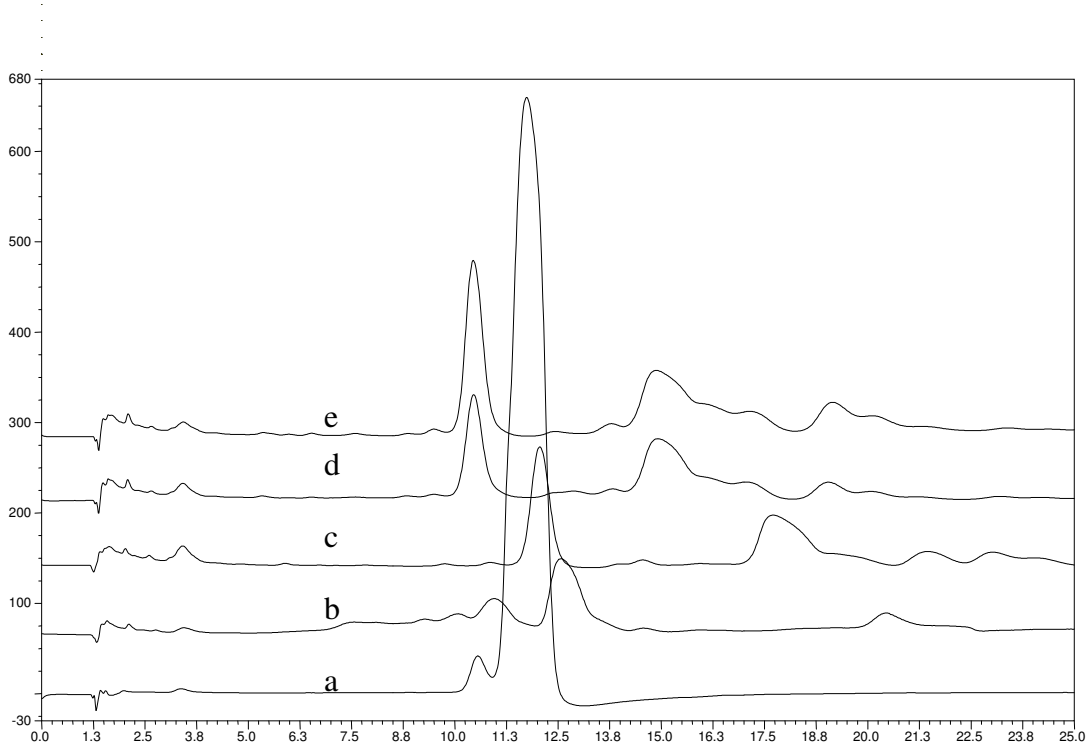


Figure 2: HPIC spectra of the products of enzyme hydrolysis
Curve a : glucosamine as standard peak
Curve b : chitosan as standard peak
Curve c : chitosan hydrolysed for 6 hrs
Curve d : chitosan Cd^{++} hydrolysed for 6 hrs
Curve e : chitosan Pb^{++} hydrolysed for 6 hrs

4.3.6. *Products of chitosanase enzyme hydrolysis*

Figure 2 shows HPIC spectra of the products of chitosanase enzyme hydrolysis. Unhydrolyzed chitosan has one major and one minor peak upstream of the glucosamine monomer standard peak (curves a and b). However, when this chitosan is hydrolyzed, it produces a glucosamine peak and another three peaks (one major, two minor) downstream, showing oligomers produced. However, the

Cd and Pb complexed chitosans produce a different set of oligomers after hydrolysis (curves d and e), with peaks on either side of the hydrolyzed chitosan peak (curve c), showing a greater spread of oligomers being produced. Curves could not be generated for Hg and Cu complexed chitosans as they completely inhibit hydrolsis.

4.3.7. Conclusions

Chitosanase hydrolysis of metal complexed chitosans as well as crosslinked chitosans and their metal complexes shows that the enzyme hydrolysis rates and extents are inhibited by metal complexation. Several factors play a role in the hydrolytc behavior of these complexes. The morphology of the metal complexes also plays an important role on the hydrolysis, as shown by complete enzyme inhibition by Cu and Hg which complex with the amino functions of chitosan, and partial inhibition by Pd and Cd which make available some amino functions as evidenced by low levels of metal complexation. Availability of the amino functions on chitosans thus seems to be a key feature for the chitosanase enzyme to hydrolyze the chitosan polymer. This was also proved by the significant increase in the extent of hydrolysis for chitosan samples with 88% as compared to the sample with 85% deacetylation. The chitooligosaccharide products of enzyme hydrolysis also have a different profile for the metal complexed chitosan as compared to the normal chitosan, showing the possibility of varying the oligosaccharide product range by hydrolyzing metal complexes of chitosans.

4.4. References:

Devlieghere, F., Vermeulen, A., Debevere, J. (2004). J. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiology, 21, 703–714.

Jeon, Y.J., Park, P.J., Kim, S.K. (2001).). Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydrate Polymers, 44, 71-76.

Jha, N., Leela, I., Prabhakar Rao, A.V.S. (1988). Removal of cadmium using chitosan J. Environ. Eng, 114, 962.

Khor, E., Lim, L.Y. (2003). Implantable applications of chitin and chitosan. Biomaterials, 24, 2339–2349.

Kim, S.K.,Rajapakse, N. (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydrate Polymers, 62(4), 357-368.

Knill, C.J., Kennedy, J.F., Mistry, J., Miraftab, M., Smart, G., Groocock, M.R., Williams, H.J. (2004). Alginate fibres modified with unhydrolysed and hydrolysed chitosans for wound dressings. Carbohydrate Polymers, 55, 65-76.

Kumar, A.B.V., Varadaraj, M.C., Gowda, L.R., Tharanathan, R.N. (2005). Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papin and pronase, and their bactericidal action against Bacillus cereus and Escherichia coli. Biochemical Journal, 391(2), 167-175.

Kurita, K. (1986). `Chemical Modifications of Chitin and Chitosan ' in Chitin in Nature and Technology, (Muzzarelli, R.A.A., Jeuniaux, C., Gooday, G.W., eds), 287-293. Plenum Press, New York, USA.

Majeti, N.V., Ravi Kumar, (2000). A review of chitin and chitosan applications. Reactive & Functional Polymers, 46, 1–27.

Miyazaki, S., Ishii, K., T. Nadai, T. (1981). The use of chitin and chitosan as drug carriers. Chem. Pharm. Bull, 29.

Nelson, N.A., (1944). A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem, 153, 235 - 380.

Qin, C., Du, Y., Xiao, L., Li, Z., Gao, X. (2002). Enzymic preparation of water-soluble chitosan and their antitumor activity. International Journal of Biological Macromolecules. 31, 111-117.

Senel, S., Kas, H.S., Squier, C.A. (2000). Application of chitosan in dental drug delivery and therapy, in: R.A.A. Muzzarelli (Ed.), Chitosan Per os: From Dietary Supplement to Drug Carrier, Tec, Grottammare, 241– 256.

Shahidi, F., Arachchiand, J.K.V., Jeon, Y.J. (1999). Food applications of chitin and chitosans. Review article. Trends in Food Science & Technology, 10, 37-51.

Sikorski, P., Sorbotten, A., Horn, S.J., Eijsink, V.G.H., Varum, K.M. (2006). Serratia marcescens Chitinases with Tunnel-Shaped Substrate-Binding Grooves Show Endo Activity and Different Degrees of Processivity during Enzymatic Hydrolysis of Chitosan. Biochemistry, 45, 9566-9574.

Singla, A.K., Chawla, M. (2001). Chitosan: some pharmaceutical and biological aspects-an update. Journal of Pharmacy and Pharmacology, 53, 1047– 1067.

Trimukhe, K.D. and Varma, A.J. (2007). Complexation of heavy metals by crosslinked chitin and its deacetylated derivatives, Carbohydr. Polym., 71, 66-73.

Varma, A.J., Deshpande, S.V., Kennedy, J.F. (2004). Metal complexation by chitosan and its derivatives: a review. Carbohydrate Polymers, 55, 77–93.

Varum, K.M., Holme, H.K., Izume, M., Stokke, B.T., Smidsrod, O. (1996). Determination of enzymatic hydrolysis specificity of partially N-acetylated chitosans. Biochimica et Biophysica Acta, 1291, 5-15.

Wang, X., Du, Y., Liu, H. (2004). Preparation, characterization and antimicrobial activity of chitosan-Zn complex. Carbohydrate Polymers, 56, 21–26.

Wang, X., Du, Y., Fan, L., Liu, H., and Hu, Y. (2005). Chitosan-metal complexes as antimicrobial agent : Synthesis, characterization and structure-activity study. Polym. Bull., 55, 105-113.

Zheng, Y., Yi, Y., Qi, Y., Wang, Y., Zhang, W., Du, M. (2006). Preparation of chitosan-copper complexes and their antitumor activity. Bioorganic & Medicinal Chemistry Letters, 16, 4127-4129.

4.5. Appendix 5: HPIC of hydrolyzed chitosans with progress of hydrolysis reaction

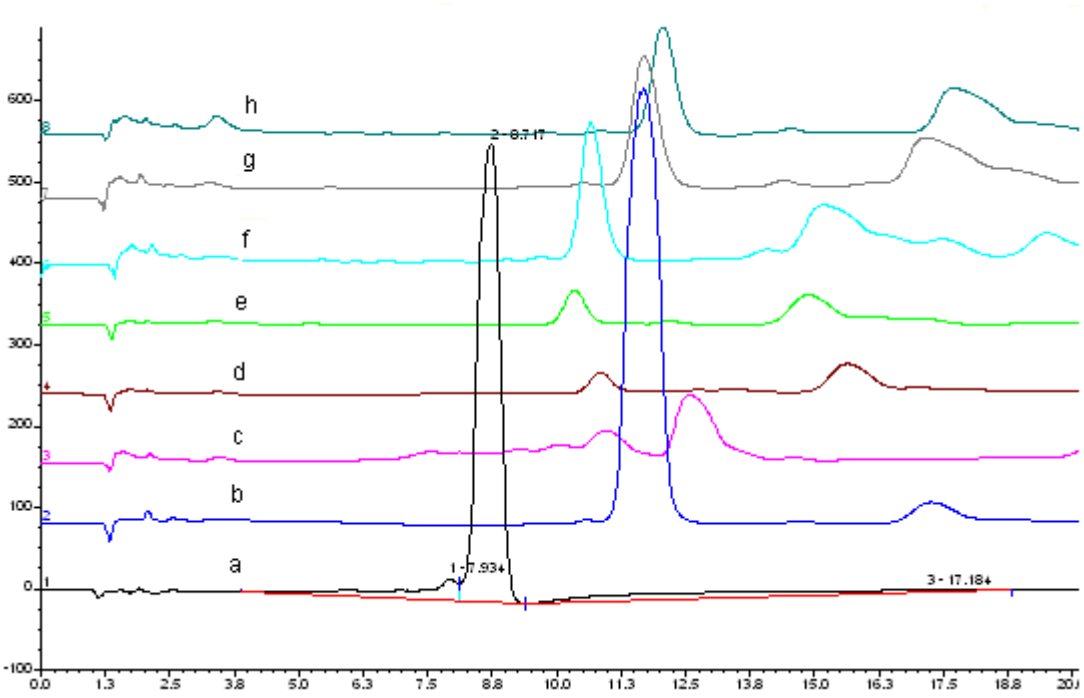


Figure 3: HPIC spectra of the products of enzyme hydrolysis of chitosan for various times
Curve a: glucosamine as standard peak
Curve b: chitosan hydrolysed 1hr spiked with glucosamine
Curve c: chitosan (unhydrolysed)
Curve d: chitosan hydrolysed for 1 hrs
Curve e: chitosan hydrolysed for 2 hrs
Curve f: chitosan hydrolysed for 4 hrs
Curve g: chitosan hydrolysed for 5 hrs
Curve h: chitosan hydrolysed for 6 hrs

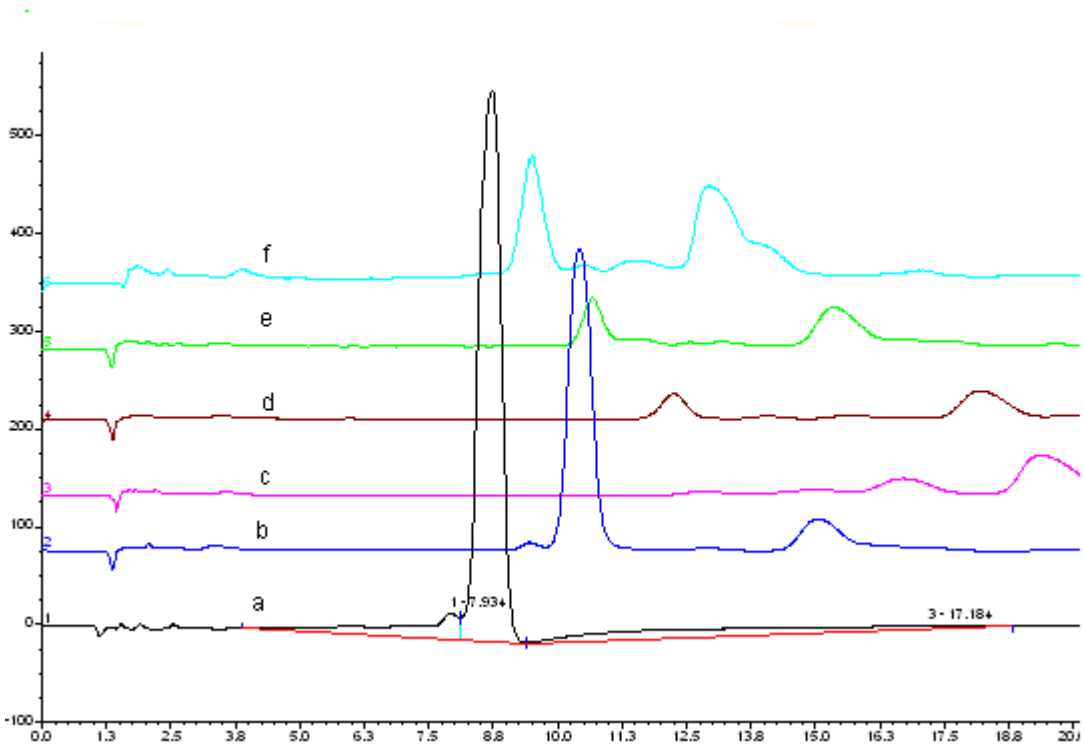


Figure 4: HPIC spectra of the products of enzyme hydrolysis of chitosan-Zn^{++} for various times
Curve a: glucosamine as standard peak
Curve b: chitosan hydrolysed-Zn 1hr spiked with glucosamine
Curve c: chitosan-Zn^{++} (unhydrolysed)
Curve d: chitosan-Zn^{++} hydrolysed for 1 hrs
Curve e: chitosan-Zn^{++} hydrolysed for 2 hrs
Curve f: chitosan-Zn^{++} hydrolysed for 4 hrs

CHAPTER 5

"Environment friendly crosslinked chitosan as a matrix for selective adsorption and purification of lipase of Aspergillus niger"

Abstract

Chitosan and its derivatives have been used as affinity matrices for purification of lipase from *Aspergillus niger* NCIM 1207. Ten derivatives of chitosan and crosslinked chitosan were evaluated for adsorption of lipase out of which trimellitic anhydride-crosslinked deacetylated chitin adsorbed lipase selectively, which was eluted easily with 0.1M phosphate buffer, pH 7.0. Approximately 500 units of lipase were adsorbed per gram of trimellitic anhydride-crosslinked deacetylated chitin and 70% of the activity was eluted with increase in specific activity (63.17). These results suggested that trimellitic anhydride-crosslinked deacetylated chitin is an excellent, easy to prepare, and inexpensive substrate to get approximately 5.19 fold purification of the crude lipase with 70% yield. Further 9.43 fold purification occurs on eluting through sephacryl-100. This appears to be an inexpensive method for large scale purification of lipases for industrial applications. Crosslinked chitosan is a biodegradable polymer that hydrolyzes easily. This also possibly represents the first commercial application of an environment-friendly and abundantly available biopolymer chitosan for such an industrial production of lipases.

5.1. Introduction

Lipases (EC 3.1.1.3) catalyze novel reactions both in aqueous and non-aqueous media, especially for synthesis of esters. These enzymes have a number of unique characteristics, including substrate specificity, stereo-specificity, regio-selectivity. Lipases have been widely used for biotechnological applications in the dairy industry, oil processing, production of surfactants, and preparation of enantiomerically pure pharmaceuticals [1]. Polysaccharides such as agarose, cellulose and dextran constituted classical soft gel matrices with little non-specific interaction and high compatibility with several enzymes. However, the poor mechanical strength hinders their application in high operating pressures. Synthetic polymers such as acrylates, polyamides, derivatized polystyrenes are more resistant to pressures than polysaccharides, but are less suitable for

immobilization of enzymes due to their lower compatibility and higher non-specific interaction with the target substrate.

On the other hand, chitosan, obtained from chitin, an ingredient in the shells of crustaceans, is a natural polymer that exists widely in nature. Chitosan has been considered as a potential chromatographic matrix, which possesses free amino and hydroxyl groups on its polysaccharide chain, and is biocompatible, biodegradable, and has good mechanical properties. The amino and hydroxyl functional groups provide active reaction sites for the easy coupling of various enzymes. Like other polysaccharides, pure chitosan gels exhibits insufficient mechanical strength. However, crosslinking the chitosan can possibly afford gels having the requisite mechanical strength for use as an affinity chromatography matrix. Approaches to prepare modifed chitosan may find applications in biomedical and bioseparation fields, including nano- and macro scale separations and classical affinity and non-affinity based chromatography techniques. The chemically modified chitosan has been used for sorption and enzyme immobilization [2]. Chitosan has also been used as microaffininy ligand for purification of chitinases by affinity precipitation and aqueous two phase extraction [3]. The trypsin inhibitor was purified using chitosan coated silica gel by affinity chromatography [4]. The commonly used method of lipase purification involves hydrophobic interaction chromatography followed by size exclusion chromatography [5]. Not much information is available on purification of lipases using chitosan or modified chitosan derivatives. We have prepared a series of different crosslinked chitosan derivatives and evaluated them for lipase adsorption and elution. The lipase used was highly acidic, and was obtained from *Aspergillus niger* NCIM 1207 which is active and stable at extremely acidic pH [6]. In this study, we have shown that one of the modified chitosans (trimellitic anhydride-crosslinked deacetylated chitin) interacted selectively and specifically with the acidic lipase leading to its five-fold purification with 70% recovery. Further purification of >95% occurs by loading and eluting this purified enzyme onto sephacryl-100. This appears to be a simple and quick two step method of

obtaining significantly pure lipases for industrial applications. Chitosan is an inexpensive, abundant, naturally available biodegradable polymer obtained from chitin, a product of the fish processing industry. It hydrolyzes easily even in its crosslinked form when disposed off, and is thus an environment-friendly material [7]. It is known to be biocompatible, and has excellent physical and mechanical properties. Its application for industrial production of lipases, which has high potential for use in production of various industrial chemicals, would be of great interest to industrial chemists.

5.2. Experimental

5.2.1. *Chemicals*

Malt extract, yeast extract, peptone was obtained from Hi-Media, India. Chitosan was obtained from the Meron Biopolymers, Cochin, Kerala, India. The reference proteins, α -Lactalbumin (14.2 Kdal), Carbonic unhydrase (29 Kdal), Albunin(45 Kdal), BSA (66 Kdal), Phosphorylase b (97.4 Kdal). β - Galactosidase (116 Kdal) and p-Nitrophenylpalmitate (pNPP) were obtained from Sigma-Aldrich Co., St. Louis, MO USA. All the other chemicals were of Analar grade and obtained from local sources. Polyacrylic acid (PAA) (Average molecular weight is 4,50,000) was obtained from Aldrich Chemical Co. and polyethyleneimine was obtained from Polysciences.

5.2.2. *Microorganisms and growth media*

The culture was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. This culture was maintained on Potato dextrose Agar (PDA) and subcultures once in every three months. PDA contained (g/l) extract from 200 g of potatoes, glucose, 20.0 g; yeast extract, 1.0 g; and agar, 20.0 g. Synthetic oil based medium (SOB) with 1% olive oil described by was used for lipase production. [6]

5.2.3. *Enzyme production and enzyme assay*

The procedure for enzyme production and assay was followed as reported earlier [6]. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 umole of p-nitrophenol from pNPP per minute per milliliter of the culture filtrate under assay conditions. The protein concentration of enzyme sample was estimated by Folin Lowry [8] using Bovine Serum Albumin as standard.

5.2.4. *Preparation of modified chitosans*

The detailed procedures for synthesis of crosslinked chitosans and chitin, and deactylated chitin after crosslinking by diisocyanatohexane (HDI), trimellitic anhydride (TMA), and dibromodecane (DBD) are being published separately [9]. The procedure for polyacrylic acid (PAA), polyethyleneimine (PEI) and glutaraldehyde crosslinked chitin and their deactylations are detailed below:

5.2.4.1. *Synthesis of Derivative of HDI-crosslinked chitosan with PAA and HDI-crosslinked deacetylated chitin:*

4g (0.025M) dry HDI-crosslinked chitosan or HDI-crosslinked deacetylated chitin was dispersed in dry and distilled DMF (200mL) taken in round bottom flask equipped with a reflux condenser and calcium guard tube. It was stirred vigorously at room temp. 400mg dimethylaminopyridine (DMAP) was added in the reaction flask and polyacrylic acid (in various moles i.e. 0.056M, and 0.007M resp.) diluted in 50ml dry distilled DMF and added dropwise in the reaction flask over a period of 30 min. After completion of addition reaction mixture was reflux for 6 hours. The reaction mixture was filtered and the solid was separated and washed with DMF till unreacted PAA removed and finally washed with acetone and dried it.

5.2.4.2. *Synthesis of Derivative of TMA-crosslinked deacetylated chitin with PEI*

4g (0.025 M) dry TMA-crosslinked deacetylated chitin was dispersed in dry and distilled DMF (70mL) taken in round bottom flask equipped with calcium guard tube. It was stirred vigorously at room temp. 500mg Polyethyleneimine was

added in the reaction flask. The reaction flask cool to 0°C in ice water then added 100mg dicyclohexylcarbodimide and 25mg dimethyl amino pyridine. Kept the reaction flask at 0°C for 1hr with vigorously stirring. Then at room temp. continued for 18 hrs. The reaction mixture was filtered and the solid was separated and washed with DMF, CH_2CL_2, H_2O and finally wash with chloroform and dried it.

5.2.4.3. *Synthesis of Derivative of HDI-crosslinked deacetylated chitin with glutaraldehyde:*

30 mL glutaraldehyde (25% aqueous solution) taken in round bottom flask equipped with calcium guard tube and added 1g HDI-crosslinked deacetylated chitin. Then reaction flask was stirred vigorously at room temp. for 68 hr. Rection was monitored by TLC. Then stopped the reaction. The reaction mixture was filtered and the solid was separated and washed with methanol and dried it.

5.2.4.4. *Adsorption and elution of lipase*

The modified chitosan material was washed with 0.1 M phosphate buffer followed by washing with distilled water. This washed preparation was used for adsorption of lipase. The adsorption experiments were carried out in 250 ml flask containing 50 ml crude enzyme solution, 0.5 g chemically modified chitosan. The mixture was stirred at 160 rpm at room temperature for 1 h and supernatant was decanted. The enzyme bound chitosan was washed twice with distilled water to remove the traces of crude enzyme solution. The supernatant was analyzed for lipase activity and protein. The enzyme bound chitosan was treated with 0.1M phosphate buffer pH 7.0 and it was kept shaking at 160 rpm for 4 h at room temperature. The chitosan was separated by filtration and the eluant was analyzed for lipase activity and protein

5.2.4.5. *Electrophoresis*

SDS-PAGE was performed using 10% Polyacrylamide gel according to [10a]. Electrophoresis was carried out at a constant current of 20 mA. Proteins were stained by silver stain method [10b].

5.3. Results and discussion:

5.3.1. **Evaluation of chitosan derivatives for adsorption of lipase**

Ten derivatives of chitosan were evaluated for adsorption and elution profiles of lipase. Table 1 shows the results of adsorption and subsequent elution of crude lipase from the surface of different chitin and chitosan derivatives. As expected, chitin (sample no. 1), with practically no amino functional groups present, adsorbed only a very small amont of the lipase (13.96%), whereas chitosan (sample no.2), with 85% deacetylation of the acetylamino groups of chitin, adsorbed nearly quantitative amount of the lipase loaded on it (96.44%). Derivative of HDI-crosslinked deacetylated chitin with PAA (1: 2.24 mole/mole) and derivative of HDI-crosslinked deacetylated chitin with PAA (1: 0.28 mole/mole) (sample nos. 3 and 4) also did not adsorb any lipase. This again shows that this lipase needs amino functional groups and not carboxyl groups for adsorption. This was further proved by adding amino groups to such a compound by preparing a derivative of TMA-crosslinked deacetylated chitin reacted with polyethyleneimine (PEI) (sample no. 7), where the adsorption increased to 86.55%, whereas removal of amino groups in by reaction of HDI-crosslinked deacetylated chitin with glutaraldehyde (wherein the aldehyde groups react with the amino groups) (sample no. 5) results in very low adsorption of lipase (17.08%). The sample HDI-crosslinked chitosan (sample no. 8) does not adsorb lipase quantitatively (74.32%), due to removal of some amino groups in the crosslinking reaction. However, in the sample where HDI is crosslinked with chitin and then deacetylated (sample no. 10), thus using only hydroxyl groups for crosslinking, and then deacetylating this product to produce a product where all the amino groups are now available for adsorption, we indeed obtained nearly quantitative adsorption (96.11%). The same was the case with dibromodecane and

trimellitic anhydride crosslinked chitin, followed by its deacetylation (sample nos. 6 and 9, respectively). Since the trimellitic anhydride (TMA) sample (sample no.9) adsorbed 100% of the lipase and also gave maximum elution (70%), we selected this sample for further studies in purification of the crude lipase by adsorption and elution. Further studies were performed on parameters such as adsorption & elution efficiencies and reusability of the chitosan matrix for adsorption as well as elution.

5.3.2. **Parameters**:

5.3.2.1. Adsorption efficiency of TMA-crosslinked deacetylated chitin

To find out adsorption efficincy of TMA-crosslinked deacetylated chitin, 1g of TMA-crosslinked deacetylated chitin was taken in 250ml Erlenmeyer flask. Enzyme was added in the range of 250 IU –1000 IU. 500 IU can easily adsorbed on 1g TMA-crosslinked deacetylated chitin (Table 2).

5.3.2.2. Elution of enzyme by phosphate buffer of different molar concentration

1 g TMA-crosslinked deacetylated chitin was taken in 250ml Erlenmeyer flask. Enzyme (500IU) was added. After completion of reaction, elution of enzyme was carried out with 10 mM; 50 mM and 100mM phosphate buffer pH 7. Maximum elution (70.8 %) was obtained by using 100 mM phosphate buffer (Table 3).

5.3.2.3. Reusability of TMA-crosslinked deacetylated chitin

The reusability of matrix is important parameter that can determine the economic viability of any biosynthetic process [11]. To determine reusability of TMA-crosslinked deacetylated chitin enzyme was adsorbed and eluted using 100 mM phosphate buffer pH 7.After elution of enzyme, matrix was treated with 1M Phosphate buffer for 2 h and then washed twice with distilled water .The treated matrix was reuse for adsorption of lipase for 8 times. It was found that each time adsorption efficiency was 100% and % elution was 70%.

5.3.3. Purification of lipase by using TMA-crosslinked deacetylated chitin

500 IU of lipase was adsorbed on 1g of TMA-crosslinked deacetylated chitin. This adsorbed enzyme eluted (80%) by using 100mM phosphate buffer pH 7 was concentrated and applied on SDS PAGE.

SDS PAGE analysis (Fig. 1) Lane B shows that crude enzyme preparation shows 12 bands while TMA-crosslinked deacetylated chitin eluted enzyme shows five band in which major band of lipase found at 31 Kdal (Lane D). After adsorption of lipase on TMA-crosslinked deacetylated chitin supernatant was left having unadsorbed proteins excluding lipase was concentrated and loaded on gel which shows maximum band (Lane C). This unadsorbed fraction does not show lipase band. From 100% protein loaded only 17% of it bound to TMA-crosslinked deacetylated chitin was selectively lipase and 83% was remain in supernatant was other proteins. Adsorption of other proteins on TMA-crosslinked deacetylated chitin was restricted so that lipase will selectively bind and during elution only lipase was eluted, as a result of this SDS PAGE of TMA-crosslinked deacetylated chitin eluted fraction shows five bands. The adsorption of lipase on chitosan derivatives and elution was lead to get partially purified enzyme.

There are reports available in which chitosan has been used as matrix for immobilization of enzyme [12-14] but there were no reports about this adsorption that lead to increase in a specific activity of enzyme. This paper deals with selective adsorption of lipase on TMA-crosslinked deacetylated chitin because for adsorption 41.15 mg protein was loaded from which only 17%(7.16 mg) of protein was adsorped and 83%(33.99 mg) of protein was retained in supernatant. This indicated that only lipase was adsorbed on the modified chitin which was eluted with high specific activity (Table 4). Chitosan was chemically modified such that only lipase will adsorped at the same time adsorption of unwanted protein was prohibited. .

Till today several attempts have been made for immobilization of enzyme on chitosan. Monteiro reported the use of chitosan as sorbent for transition metal ions [15]. Rorrer had reported use of chitosan beads for removal of cadmium ion from wastewater [16]. Gamzazade had studied lipoprotein sorption by sulfo-derivatives of chitosan but no reports are available on chitosan adsorption that lead to increase in specific activity of enzyme [17]. In this study 500 IU of lipase was adsorped and 350 IU was eluted with 63.17 specific activity it means that there is specific adsorption as well as elution of lipase. Chitosan and its derivatives can be used for enzyme adsorption. Chitosan derivatives TMA-crosslinked deacetylated chitin can be used as affinity matrix for lipase from *Aspergillus niger* NCIM 1207. Saxena et al. reported affinity chromatography has been used as purification step in 27% of scheme [5]. Usually affinity chromatography method can be applied at an early stage but as affinity matrices are expensive, inexpensive materials are needed for purification of enzymes, particularly for industrial applications. Chitosan appears economically attractive since chitin is the second most abundant biopolymer in the nature next to cellulose, and is biocompatible, biodegradable, and has structural strength [18, 1].

5.4. Conclusion

Ten derivatives of chitosan was screened for lipase adsorption from which TMA-crosslinked deacetylated chitin was selected as ideal support for adsorption as it has several advantages that 500 IU can easily adsorped on 1g of support, easy elution and reusability. This adsorption on chitosan derivative TMA-crosslinked deacetylated chitin lead to partial purification of lipase from *Aspergillus niger* NCIM 1207.

5.5. References:

[1] P. K. Ghosh, R. K. Saxena, R. Gupta, R. P. Yadav, and S. Davidson, Sci. Prog., 1996, 79(pt. 2), 119-57.

[2] R. S. Juang, F. C. Wu, and R. L. Tseng, Advances in Environmental Research, 2002, 6(2), 171-177.

[3] S. Teotia, R. Lata, and M. N. Gupta, Journal of Chromatography, A, 2004, 1052(1-2), 85-91.

[4] F. Xi, and J. Wu, Reactive and Functional Polymers, 2006, 66(6), 682-688.

[5] R. K. Saxena, A. Sheoran, B. Giri, and W. S.Davidson, Journal of Microbiological methods, 2003, 52, 1-18.

[6] N. D. Mahadik, U. S. Puntambekar, B. B. Bastawde, and J. M. Khire, Biochemistry, 2002, 38, 715-721.

[7] K. D. Trimukhe, S. Bachate, D. V. Gokhale, and A. J. Varma, International Journal of Biological Macromolecules, 2007, 41, 491-496.

[8] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., 1995, 193, 265-275.

[9] K. D. Trimukhe and A. J. Varma, Carbohydrate Polymers, 2008, 66-73.

[10a] U. K. Laemmli, Nature (London), 1970, 227, 680-685.

[10b] R.C. Switzer, C.R. Merril and S.A. Shifrin. S.A. (1979). Highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem., 98, 231-237.

[11] S. Hari Krishna, S. Divakar, S. G. Prapulla, and N. G. Karanth, J. Biotech., 2001, 409(11), 241-6.

[12] D. Magnin, S. Dumitriu, P. Magny, and E. Chornet, Biotechnol Prog., 2001, 17, 734-741.

[13] E. B. Pereira, H. F. Castro, F. F. Moraes, and G. M. Zanin, Appl. Biochem. Biotechnol., 2001, 91, 739-52.

[14] T. Tan, F. Wang and H. ZHAng, J. Mol. Catal. B Enzym., 2002, 18, 325-31.

[15] Jr. O. A. C. Monteiro, and I. C. Airold, Journal Colloid Interface Science, 1999, 212, 212-219.

[16] G. L. Rorrer, T. Y. Hsien, and J. D. Way, Ind. Eng. Chem. Res., 1993, 32, 2170- 2178.

[17] A. I. Gamzazade, S. M. Nasibov, and S. V. Rogonzhin, Carbohydrate polymer, 1997, 34, 381.

[18] K. Kifune, In Advances in Chitin and Chitosan, C. J. Brine, P. A. Sanford, J. P. Zikakis, J. P., Eds., Elsevier: Essex, England, 1992, 9.

Table1: Adsortion and elution efficiencies of crude lipase on different chitosan derivatives

S.No	Chitosan derivatives	% Adsorption	% Elution[a]
1	Chitin	13.96	ND
2	Chitosan	96.44	50.86
3	Derivative of HDI-crosslinked deacetylated chitin with PAA (1: 2.24 mole/mole)	0.0	ND
4	Derivative of HDI-crosslinked deacetylated chitin with PAA (1: 0.28 mole/mole)	0.0	ND
5	Derivative of HDI-crosslinked deacetylated chitin with glutaraldehyde	17.08	ND
6	DBD-crosslinked deacetylated chitin	88.06	51.70
7	Derivative of TMA-crosslinked deacetylated chitin with PEI	86.55	68.52
8	HDI-crosslinked chitosan	74.32	ND
9	TMA-crosslinked deacetylated chitin	100.0	70.0
10	HDI-crosslinked deacetylated chitin	96.11	65.89

ND: Not determined (These derivatives showed less than 80% adsorption and hence they were not processed for elution).

Table 2: Adsorption efficiency of TMA-crosslinked deacetylated chitin

Activity loaded (IU)	Adsorption (%)
250	100.0
500	100.0
750	95.0
1000	90.0

Table: 3 Elution of enzyme by phosphate buffer of different molar concentration

Phosphate buffer (mM)	% Adsorption	% Elution
10.0	100.0	2.09
50.0	100.0	28.38
100.0	100.0	70.8

Table 4: Purification of lipase from *Aspergillus niger* NCIM 1207

Steps	Activity (IU)	Protein (mg)	Specific activity (IU mg^{-1})	Yield (%)	Fold purification
Culture filtrate	500	41.15	12.15	100.0	1.0
TMA-crosslinked deacetylated chitin	350	5.54	63.17	70.0	5.19
Sephacryl - 100 elution	242.88	2.11	114.62	48.4	9.43

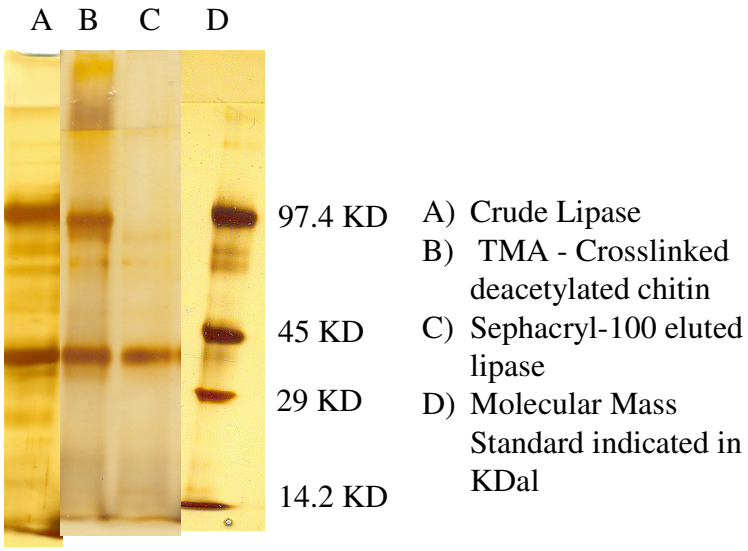


Figure 1: Electrophoretic pattern of proteins obtained at each step of lipase purification

CHAPTER 6

"Thermal properties of chitosans, crosslinked chitosans and metal complexed chitosans"

Abstract:

A series of heavy metal complexes (Cu, Cd, Pb, Hg, Zn, Mg) of chitosan and crosslinked chitosans using diisocyanatohexane (HDI), trimellitic anhydride (TMA) and dibromodecane (DBD) as crosslinking agents were prepared, and their thermal properties evaluated by thermogravimetric studies under nitrogen atmosphere in the temperature range 50-600 ^{0}C. Even though all the chitosans and crosslinked chitosans without any metal ions bound to them exhibited two stage decomposition curves, in all cases the second stage was not very pronounced, and the onset of this second stage started from nearly the same position as the final degradation temperature of the first stage degradation Therefore for all practical purposes the decomposition is can be considered single stage, with an onset of degradation temperature in the range 244 – 250 ^{0}C and range for first step final degradation temperature 332 – 350 ^{0}C and final degradation temperature range 548 – 560 ^{0}C). In contrast, all the metal complexes of chitosans had distinct two-stage decomposition curves. The metal complexes with Cu, Cd and Hg ions, which exhibit the highest complexing ability to the chitosans (Hg 354-364 mg/g chitosan; Cu 100-112 mg/g chitosan, and Cd 121 – 160 mg/g chitosan), were found to have the lowest onset of degradation temperatures (range 194 – 210 ^{0}C) and the lowest final degradation temperatures (generally less than 294 - 304 ^{0}C for Hg, 296 - 338 ^{0}C for Cu complexes, and 305 – 368 ^{0}C for Cd complexes). Mn ion which has the lowest binding abilities to chitosans (Mn 5 -7 mg/g chitosan) showed the reverse behaviour, these complexes exhibited the least effects on the onset (240 – 248 ^{0}C) and final degradation temperatures (range 300 – 368 ^{0}C). Zn (binding ability 74 -87 mg/g chitosan) and Pb (binding ability 39 – 62 mg/g chitosan) ions have a binding ability intermediate to Cu/Cd/Hg and Mn extremes, and therefore the effects on onset and final degradation temperatures are intermediate to these values. These studies have shown that a study of thermogravimetry of chitosan metal complexes can be a valuable tool in evaluating the binding abilities of various metal complexes.

6.1. Introduction

A key property of functional is their ability to complex with a variety of metal ions in solution. A very large number of publications have dwelt on the complexation ability of chitosan and its crosslinked derivatives with complex transition metals, organic species like dyes, and enzymes (Varma, Deshpande, & Kennedy, 2004; Trimukhe & Varma, 2008; Juang, Wu, & Tseng, 2002; Merrifield, Davids, MacRae, & Amirbahman, 2004; Tan, Wang, Peng, & Tang, 1999; Li, Chen, & Liu, 2003; Taboada, Cabrera, & Cardenas, 2003; Rhazi, Desbrieres, Tolaimate, Rinaudo, Vottero, Alagui, El Meray, 2002; Domard & Piron, 2000; Dobetti & Delben, 1992; Schmuhl, Krieg, & Keizer, 2001; Bassi, Prasher, & Simpson, 2000). Recently we reported (Trimukhe & Varma, 2008) our detailed investigation into the heavy metal ion binding (Hg, Cu, Cd, Pb, Zn, Mn) to a series of crosslinked chitosans, using trimelitic anhydride, diisocyanatohexane, and dibromodecane as crosslinking agents. This is the first study wherein chitin was first crosslinked and then deacetylated to give crosslinked chitosans retaining all the amino groups, which are crucial functional groups for specific heavy metal ion complexation. We also investigated the morphologies of these metal complexes by a study of SEM and WAXRD (Trimukhe and Varma, 2007). Morphological information from SEM studies indicate that metal ions like Hg which are very strongly bound to the chitosan and crosslinked chitosans, even to the extent of 364 mg/g chitosan, are not seen as distinct moieties on the surface of the polymer, whereas with decreasing extent of binding, in the order Hg > Cu > Cd > Zn > Pb > Mn, we observed increasing presence of metal on the surface. These results agreed with the morphological information obtained with WAXRD studies, as well as with the metal complexation data of these metals with these same chitosan polymer / crosslinked polymer systems. However, thermal properties of these metal complexes are not reported. Thermal stability data of metal complexes is important to confirm the presence of metal ions present either as inclusion complexes or as adsorbed species on the surface of the polymer. Metals ions present as inclusion complexes

are expected to have a greater effect on the thermal properties of the polymer, in addition to specific effects of different metal ions. Therefore this paper investigates the thermal degradation of chitosan and crosslinked chitosan metal complexes under nitrogen atmosphere in the temperature range 50-600 ^{0}C, and attempts to correlate the thermal degradation behavior of metal complexes of chitosans with the binding abilities of metal ions with chitosans (expressed as binding mg (metal ion) / g (chitosan)) for a series of heavy metals. This appears to be the first such reported correlation between the thermal degradation of metal complexes of crosslinked chitosans and their saturated heavy metal ion complexation values.

6.2. Experimental Section:

6.2.1. Materials

The chitin and chitosan used in this study are commercial products of Meron Biopolymers, Cochin, Kerala, India. D-glucosamine was obtained from Sigma Chemical Co. (St. Louis, MO). Diisocyanatohexane (HDI) was obtained from Aldrich Chemical Co., trimellitic anhydride (TMA) and dibromodecane (DBD) was obtained from Merck. Dimethylformamide, toluene and sodium hydroxide pellets were AR grade chemicals, obtained from SD fine chemicals, Mumbai. Sodium hydride was obtained from Merck. 4-Dimethylaminopyridine was purchased from Lancaster Company. All metal salts were AR grade materials and used without further purification. The salts $ZnCl_2$, $MnSO_4$, $CdSO_4$, $Pb(NO_3)_2$ were obtained from Loba Chemie, Mumbai, $CuSO_4$ was from SD Fine Chemicals, Mumbai, and $HgCl_2$ was from Merck.

6.2.2. Synthetic procedures

Preparation of different crosslinked chitosan with different metal ions.

Detailed preparation methods crosslinked chitin using diisocyanatohexane (HDI), dibromodecane (DBD) and trimellitic anhydride (TMA) and of deacetylation of chitin have recently been published (Trimukhe & Varma, 2008). Metal ion complexation studies with Cu^{++}, Cd^{++}, Hg^{++}, Zn^{++}, Pb^{++}, Mn^{++} salts has also been published (Trimukhe & Varma, 2008), along with their morphological properties (Trimukhe & Varma, 2007).

6.2.3. Thermogravimetric Analysis (TGA).

Thermogravimetry (TG), differential thermogravimetry (DTG) and differential thermal analysis (DTA) were carried out using a Seiko Instruments TG/DTA 32 instrument equipped with a SSC 5100 Disk Station and SP-530 Plotter. The studies were carried out in a nitrogen atmosphere at a heating rate of 10 ^{o}C, in the range 50-600 ^{0}C.

6.3. Results and Discussion

There are several reports in literature of changes occurring in the thermal degradation behavior of chitosans on crosslinking or on their chemical modification. For example, in one study (Neto, et al., 2005) addition of PEO grafts led to a slight increase in thermal stability (from 297.3 to 300.7 ^{0}C), whereas crosslinking the chitosan decreased the thermal stability to a small extent (from 297.3 to 288.8 ^{0}C). In another report, crosslinking was seen to improve the heat stability of the chitosan (Beppu et al., 2004). They also found that histidine modified chitosans could bind Cu ions, with the result that two new peaks were observed in the TGA and DTG curves at about 230 ^{0}C and 510 ^{0}C . Hong et al. found that the thermal degradation can be correlated to the rate of heating the sample, and that the degradation of chitosan is single stage decomposition (Hong et al., 2007). Modification of chitosan with cyclic oxegenated compounds (Tirkistani, 1998a) as well as with Schiff's bases led to a decrease in thermal

stability (Tirkistani, 1998b). Similarly, lactic acid side chains on chitosan decreased its thermal stability (Qu et al, 2000).

Thus, it is clear that chemical modifications as well as metal complexations generally lead to a decrease in thermal stability of chiotosans. However, the present report on the correlations of thermal degradation properties of a series of metal complexes of a series of crosslinked chitosans (and chitosan itself as a control standard) shows that the decrease in the observed thermal stability is intimately related to the specific type of metal complex. Table 1 shows the TGA results for all samples of chitosans / crosslinked chitosans and their metal complexes, while table 2 shows the metal binding data. Figures 1-6 show the TGA patterns (single stage and two stages) for all the samples studied. We have ignored the initial water loss stage below 100 ^{0}C in our discussions. All the chitosans and crosslinked chitosans without any metal ions bound to them exhibited, for all practical purposes, single stage degradations (the second stage nearly merging with the first stage, i.e., the start temperature of the second stage degradation is the same as the end temperature of the first stage) with the onset of degradation temperature being observed to be in the range 244 – 250 ^{0}C, the range for first step final degradation temperature was 332 – 350 ^{0}C and for the final degradation temperature the range 548 – 560 ^{0}C (sample nos. 1-6 in Table 1). In contrast, all the metal complexes of chitosans had distinct two-stage decomposition curves. The metal complexes with Cu, Cd and Hg ions, which exhibit the highest complexing ability to the chitosans (Hg 354-364 mg/g chitosan; Cu 100-112 mg/g chitosan, and Cd 121 – 160 mg/g chitosan) (table 1, sample nos. 7-24), were found to have the lowest onset of degradation temperatures (range 194 – 210 ^{0}C) and the lowest final degradation temperatures (generally less than 294 - 304 ^{0}C for Hg, 296 - 338 ^{0}C for Cu complexes, and 305 – 368 ^{0}C for Cd complexes), as can be seen from the data in table 1 and figs 1-6.

Mn ion which has the lowest binding abilities to chitosans (Mn 5 -7 mg/g

chitosan) (table 2) showed the reverse behaviour, these complexes exhibited the least effects on the onset (240 – 248 ^{0}C) and final degradation temperatures (range 300-368 ^{0}C (table 1 sample nos. 31036)).

Zn (binding ability 74 -87 mg/g chitosan) and Pb ions (binding ability 39 – 62 mg/g chitosan) (table 2) have a binding ability intermediate to Cu/Cd/Hg on the one hand and Mn on the other extreme (see sample nos. 25-30 and 37-42 in table 1), and therefore the effects on onset and final degradation temperatures are intermediate to these values, i.e onset temperatures 205 – 250 ^{0}C and final first stage temperatures between 302-350 ^{0}C. Above 550 ^{0}C most of the samples were completely degraded, while the TGA curves above 550 ^{0}C for some samples had a tailing effect and were not flat (figs. 2-6). These samples (samples 9,14,17,21, 25, 2629, 31, 33 in table 1) had final decomposition temperatures in excess of 600 ^{0}C, though not much inference can be drawn about this aspect with the present data, and more work needs to be done to understand this aspect. This investigation will continue with other supporting experiments, but is beyond the scope of the present investigation.

Thus, the thermal behavior of homologous series of metals with chitosans can throw useful new light on the strength and type of metal complex, and whether the metal complex specifically uses the amino functional groups or not. It is well known that the amino group of chitosan has a stabilizing effect on the molecule, proved by the higher thermal stability of chitosan over chitin (Tirkistani, 1998b), therefore those metals which specifically tie up the amino functional groups, such as Cu and Hg ions, cause the maximum thermal instability. Metals which are bound only peripherally on the chitosan / crosslinked chitosan will have minimal effect on the thermal degradation behavior.

6.4. References:

Bassi, R., Prasher, S. O., & Simpson, B. K. (2000). Removal of selected metal ions from aqueous solutions using chitosan flakes. Sep. Sci. Technol., 35(4), 547-560.

Beppu, M. M., Arruda, E. J., Vieira, R. S. & Santos, N. N. (2004). Adsorption of Cu(II) on porous chitosan membranes functionalized with histidine. Journal of Membrane Science, 240, 227-235.

Dobetti, L., & Delben, F. (1992). Binding of metal cations by N-carboxymethyl chitosans in water. Carbohydrate Polymers, 18(4), 273-82.

Domard, A., & Piron, E. (2000). Recent approach of metal binding by chitosan and derivatives. Adc. Chitin science, 4(EUCHIS '99), 295-301.

Hong, P. Z., Li, S. D., Ou, C. Y., Li, C. P., Yang, L. & Zhang, C. H. (2007). Thermogravimetric analysis of chitosan. Journal of Applied Polymer Science, 105, 547-551.

Juang, R. S., Wu, F. C., Tseng, R. L. (2002). Use of chemically modified chitosan beads for sorption and enzyme immobilization. Advances in Environmental Research, 6, 171-177.

Li, H. B., Chen, Y. Y., & Liu, S. L. (2003). Synthesis, characterization, and metal ions adsorption properties of chitosan-calixarenes (I). Journal of Applied Polymer Science, 89, 1139-1144.

Merrifield, J. D., Davids, W. M., MacRae, J. D., & Amirbahman, A. (2004). Uptake of mercury by thiol-grafted chitosan gel beads. Water Research, 38, 3132-3138.

Neto, C. G. T., Giacometti, J. A., Job, A. E., Ferreira, F. C., Fonseca, J. L. C. & Pereira, M. R. (2005). Thermal analysis of chitosan based networks. Carbohydrate Polymers, 62, 97-103.

Qu, X., Wirsen, A. & Albertsson, A. C. (2000). Effect of lactic/glycolic acid side chains on the thermal degradation kinetics of chitosan derivatives. Polymer, 41, 4841-4847.

Rhazi, M., Desbrieres, J., Tolaimate, A., Rinaudo, M., Vottero, P., Alagui, A., & EI Meray, M. (2002). Influence of the nature of the metal ions on the complexation with chitosan. Application to the treatment of liquid waste. European Polymer Journal, 38(8), (English). 1523-1530.

Schmuhl, R., Krieg, H. M., & Keizer, K. (2001). Adsorption of Cu(II) and Cr(VI) ions by chitosan: Kinetics and equilibrium studies. Water SA, 27(1), 1-7.

Taboada, E., Cabrera, G., & Cardenas, G. (2003). Retention capacity of chitosan for copper and mercury ions. Journal of the Chilean Chemical Society, 48(1), 7-12.

Tan, S., Wang, Y., Peng, C., & Tang, Y. (1999). Synthesis and adsorption properties for metal ions of crosslinked chitosan acetate crown ether. Journal of Applied Polymer Science, 71, 2069-2074.

Tirkistani, F. A. A. (1998a). Thermal analysis of chitosan modified by cyclic oxygenated compounds. Polymer Degradation and stability, 61, 161-164.

Tirkistani, F. A. A. (1998b). Thermal analysis of some chitosan Schiff bases. Polymer Degradation and stability, 60, 67- 70.

Varma, A. J., Deshpande, S. V., & Kennedy, J. F. (2004). Metal complexation by chitosan and its derivatives: a review. Carbohydrate Polymers, 55, 77-93.

Trimukhe, K.D. and Varma, A.J. (2008). Complexation of Heavy Metals by Crosslinked Chitin and its Deacetylated Derivatives. Carbohydrate Polymers, 71, 66-73.

Trimukhe, K.D. and Varma, A.J. (2008). A morphological study of heavy metal complexes of chitosan and crosslinked chitosans by SEM and WAXRD. Carbohydrate Polymers, 71, .698-702

Table 1: Thermal analysis of chitosan, deacetylated chitin, HDI-crosslinked chitosan, HDI-crosslinked deacetylated chitin, TMA-crosslinked deacetylated chitin, DBD-crosslinked deacetylated chitin and their metal ions in nitrogen atmosphere in the range 50- 600 ^{0}C

S. No.	Sample name	First stage ^{o}C		Second stage ^{o}C		Percentage weight loss at ^{o}C				
		Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
1	Chitosan	244	332	---	548	11	42	69	86	97
2	Deacetylated chitin	246	350	---	550	10	39	68	89	100
3	HDI-crosslinked chitosan	246	350	406	550	11	38	63	87	98
4	HDI-crosslinked deacetylated chitin	250	350	---	550	13	37	67	87	95
5	TMA-crosslinked deacetylated chitin	248	340	---	552	16	44	69	87	97
6	DBD-crosslinked deacetylated chitin	248	346	---	560	12	39	65	84	96
7	Chitosan-Cu^{++}	205	322	396	450	15	45	56	88	88
8	Deacetylated chitin-Cu^{++}	236	338	436	500	18	44	60	89	92
9	HDI-crosslinked chitosan-Cu^{++}	200	296	400	*	17	65	78	100	100
10	HDI-crosslinked deacetylated chitin-Cu^{++}	212	308	400	450	17	44	59	90	90
11	TMA -crosslinked deacetylated chitin-Cu^{++}	208	306	400	450	23	55	67	97	98
12	DBD-crosslinked deacetylated chitin-Cu^{++}	208	308	400	450	27	55	66	96	97
13	Chitosan-Cd^{++}	248	322	498	550	14	33	50	60	85
14	Deacetylated chitin-Cd^{++}	252	368	498	*	11	27	59	67	85
15	HDI-crosslinked chitosan-Cd^{++}	250	308	498	552	24	44	60	71	94
16	HDI-crosslinked deacetylated chitin-Cd^{++}	200	350	500	552	29	48	64	76	98
17	TMA -crosslinked deacetylated chitin-Cd^{++}	248	305	500	*	24	46	59	68	93
18	DBD-crosslinked deacetylated chitin-Cd^{++}	250	316	500	550	24	43	58	66	92
19	Chitosan-Hg^{++}	204	304	430	550	13	61	74	89	99
20	Deacetylated chitin-Hg^{++}	200	302	---	560	10	61	75	88	97
21	HDI-crosslinked chitosan-Hg^{++}	194	300	498	*	12	59	72	83	91
22	HDI-crosslinked deacetylated chitin-Hg^{++}	200	300	---	560	11	60	77	90	99
23	TMA-crosslinked deacetylated chitin-Hg^{++}	210	294	---	600	08	59	72	86	95
24	DBD-crosslinked deacetylated chitin-Hg^{++}	206	300	---	600	12	61	73	87	95
25	Chitosan-Zn^{++}	250	306	500	*	18	43	57	66	86
26	Deacetylated chitin-Zn^{++}	250	350	480	*	12	32	51	66	83
27	HDI-crosslinked chitosan-Zn^{++}	248	316	500	552	17	39	56	66	90
28	HDI-crosslinked deacetylated chitin-Zn^{++}	252	320	500	556	16	37	54	64	86
29	TMA-crosslinked deacetylated chitin-Zn^{++}	250	294	538	*	18	40	54	64	74
30	DBD-crosslinked deacetylated chitin-Zn^{++}	250	312	494	552	17	38	52	66	90
31	Chitosan-Mn^{++}	248	350	502	*	11	41	64	73	91
32	Deacetylated chitin-Mn^{++}	248	346	---	550	13	41	66	93	99
33	HDI-crosslinked chitosan-Mn^{++}	248	340	406	*	13	41	67	92	100
34	HDI-crosslinked deacetylated chitin-Mn^{++}	248	350	---	548	12	40	66	95	100
35	TMA-crosslinked deacetylated chitin-Mn^{++}	248	300	---	550	14	45	64	91	100
36	DBD-crosslinked deacetylated chitin-Mn^{++}	240	340	---	550	13	39	64	90	98

37	Chitosan-Pb^{++}	248	302	448	508	12	42	55	93	97
38	Deacetylated chitin-Pb^{++}	232	308	454	508	13	43	59	96	99
39	HDI-crosslinked chitosan-Pb^{++}	205	312	448	512	10	41	54	87	93
40	HDI-crosslinked deacetylated chitin-Pb^{++}	218	312	448	502	11	40	58	93	95
41	TMA-crosslinked deacetylated chitin-Pb^{++}	226	300	450	512	12	42	56	92	97
42	DBD-crosslinked deacetylated chitin-Pb^{++}	228	312	448	502	13	42	57	94	96

* > 600 ^{0}C

Table 2: Metal complexation data for different chitosans and crosslinked chitosans
(Average values of three different analytical methods - Atomic Absorption spectrophotometer method, UV-Vis spectrometry, and titrymetric methods, as in Trimukhe & Varma, 2008)

Sample Name.	Cu^{++} mg/g	Cd^{++} mg/g	Hg^{++} mg/g	Zn^{++} mg/g	Mn^{++} mg/g	Pb^{++} mg/g
Chitosan	103	135	354	86	6	43
Deacetylated Chitin	112	121	364	81	5	50
HDI-crosslinked deacetylated Chitin	108	145	359	81	7	46
DBD-crosslinked deacetylated Chitin	108	160	362	83	05	39
TMA-crosslinked deacetylated Chitin	109	134	361	87	7	61
HDI-crosslinked Chitosan	100	133	362	74	06	62

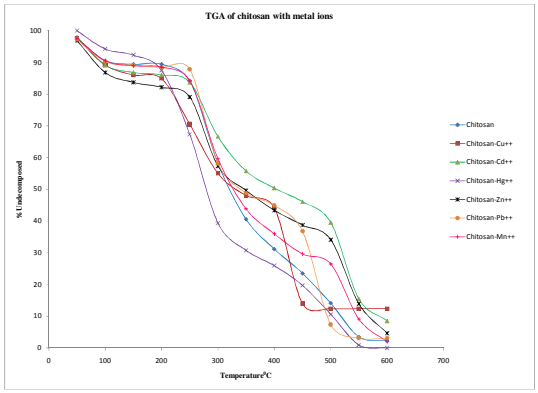


Figure 1: TGA of chitosan with metal ions

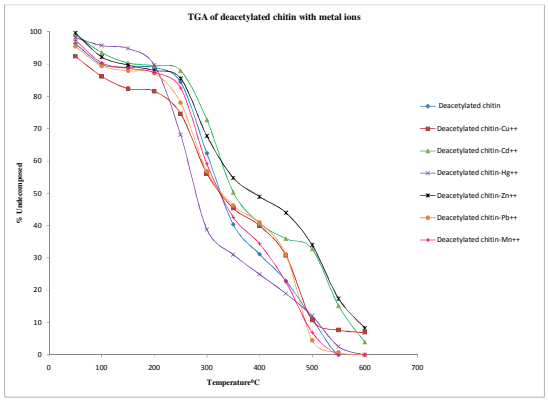


Figure 2: TGA of deacetylated chitin with metal ions

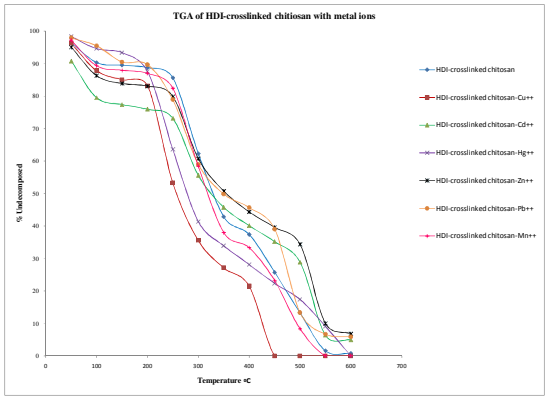


Figure 3: TGA of HDI crosslinked chitosan with metal ions

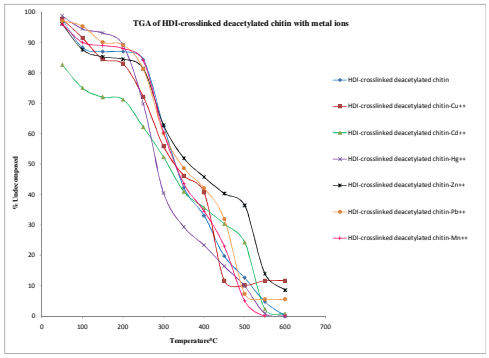


Figure 4: TGA of HDI-crosslinked deacetylated chitin with metal ions

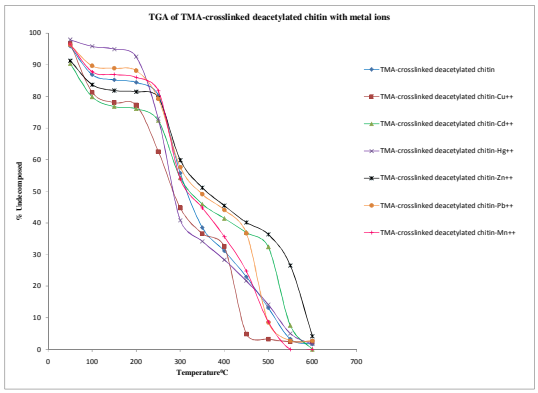


Figure **5:** TGA of TMA-crosslinked deacetylated chitin with metal ions

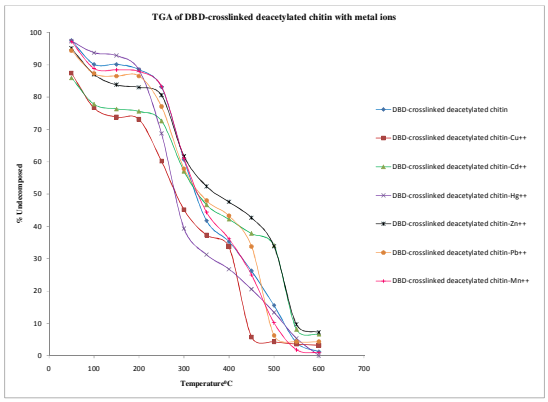


Figure 6: TGA of DBD-crosslinked deacetylated chitin with metal ions

6.5. Appendix 6: TGA of chitosan and crosslinked chitosans with metal ions

Table 3: Thermal analysis of chitosan, deacetylated chitin, HDI-crosslinked chitosan, HDI-crosslinked deacetylated chitin, TMA-crosslinked deacetylated chitin, DBD-crosslinked deacetylated chitin at nitrogen atmosphere in the range 50- 600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
Chitosan	244	332	---	548	11	42	69	86	97
Deacetylated chitin	246	350	---	550	10	39	68	89	100
HDI-crosslinked chitosan	246	350	406	550	11	38	63	87	98
HDI-crosslinked deacetylated chitin	250	350	---	550	13	37	67	87	95
TMA-crosslinked deacetylated chitin	248	340	---	552	16	44	69	87	97
DBD-crosslinked deacetylated chitin	248	346	---	560	12	39	65	84	96

Table 4: Thermal analysis of chitosan with metal ions in nitrogen atmosphere in the range 50-600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
Chitosan	244	332	---	548	11	42	69	86	97
Chitosan-Cu^{++}	205	322	396	450	15	45	56	88	88
Chitosan-Cd^{++}	248	322	498	550	14	33	50	60	85
Chitosan-Hg^{++}	204	304	430	550	13	61	74	89	99
Chitosan-Zn^{++}	250	306	500	*	18	43	57	66	86
Chitosan-Mn^{++}	248	350	502	*	11	41	64	73	91
Chitosan-Pb^{++}	248	302	448	508	12	42	55	93	97

Table 5: Thermal analysis of deacetylated chitin with metal ions in nitrogen atmosphere in the range 50-600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
Deacetylated chitin	246	350	---	550	10	39	68	89	100
Deacetylated chitin-Cu^{++}	236	338	436	500	18	44	60	89	92
Deacetylated chitin-Cd^{++}	252	368	498	*	11	27	59	67	85
Deacetylated chitin-Hg^{++}	200	302	---	560	10	61	75	88	97
Deacetylated chitin-Zn^{++}	250	350	480	*	12	32	51	66	83
Deacetylated chitin-Mn^{++}	248	346	---	550	13	41	66	93	99
Deacetylated chitin-Pb^{++}	232	308	454	508	13	43	59	96	99

Table 6: Thermal analysis of HDI-crosslinked chitosan with metal ions in nitrogen atmosphere in the range 50- 600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
HDI-Crosslinked chitosan	246	350	406	550	11	38	63	87	98
HDI-Crosslinked chitosan-Cu^{++}	200	296	400	*	17	65	78	100	100
HDI-Crosslinked chitosan-Cd^{++}	250	308	498	552	24	44	60	71	94
HDI-Crosslinked chitosan-Hg^{++}	194	300	498	*	12	59	72	83	91
HDI-Crosslinked chitosan-Zn^{++}	248	316	500	552	17	39	56	66	90
HDI-Crosslinked chitosan-Mn^{++}	248	340	406	*	13	41	67	92	100
HDI-Crosslinked chitosan-Pb^{++}	205	312	448	512	10	41	54	87	93

Table 7: Thermal analysis of HDI-crosslinked deacetylated chitin with metal ions in nitrogen atmosphere in the range 50- 600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
HDI-Crosslinked deacetylated chitin	250	350	---	550	13	37	67	87	95
HDI-Crosslinked deacetylated chitin-Cu^{++}	212	308	400	450	17	44	59	90	90
HDI-Crosslinked deacetylated chitin-Cd^{++}	200	350	500	552	29	48	64	76	98
HDI-Crosslinked deacetylated chitin-Hg^{++}	200	300	---	560	11	60	77	90	99
HDI-Crosslinked deacetylated chitin-Zn^{++}	252	320	500	556	16	37	54	64	86
HDI-Crosslinked deacetylated chitin-Mn^{++}	248	350	---	548	12	40	66	95	100
HDI-Crosslinked deacetylated chitin-Pb^{++}	218	312	448	502	11	40	58	93	95

Table 8: Thermal analysis of TMA-crosslinked deacetylated chitin with metal ions in nitrogen atmosphere in the range 50- 600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
TMA-crosslinked deacetylated chitin	248	340	---	552	16	44	69	87	97
TMA-crosslinked deacetylated chitin-Cu^{++}	208	306	400	450	23	55	67	97	98
TMA-crosslinked deacetylated chitin-Cd^{++}	248	305	500	*	24	46	59	68	93
TMA-crosslinked deacetylated chitin-Hg^{++}	210	294	---	600	08	59	72	86	95
TMA-crosslinked deacetylated chitin-Zn^{++}	250	294	538	*	18	40	54	64	74
TMA-crosslinked deacetylated chitin-Mn^{++}	248	300	---	550	14	45	64	91	100
TMA-crosslinked deacetylated chitin-Pb^{++}	226	300	450	512	12	42	56	92	97

Table 9: Thermal analysis of DBD-crosslinked deacetylated chitin with metal ions in nitrogen atmosphere in the range 50- 600 ^{0}C

Sample name	First stage ^{0}C		Second stage ^{0}C		Percentage weight loss at ^{0}C				
	Onset degrade	Final degrade	Onset degrade	Final degrade	200	300	400	500	550
DBD-Crosslinked deacetylated chitin	248	346	---	560	12	39	65	84	96
DBD-Crosslinked deacetylated chitin-Cu^{++}	208	308	400	450	27	55	66	96	97
DBD-Crosslinked deacetylated chitin-Cd^{++}	250	316	500	550	24	43	58	66	92
DBD-Crosslinked deacetylated chitin-Hg^{++}	206	300	---	600	12	61	73	87	95
DBD-Crosslinked deacetylated chitin-Zn^{++}	250	312	494	552	17	38	52	66	90
DBD-Crosslinked deacetylated chitin-Mn^{++}	240	340	---	550	13	39	64	90	98
DBD-Crosslinked deacetylated chitin-Pb^{++}	228	312	448	502	13	42	57	94	96

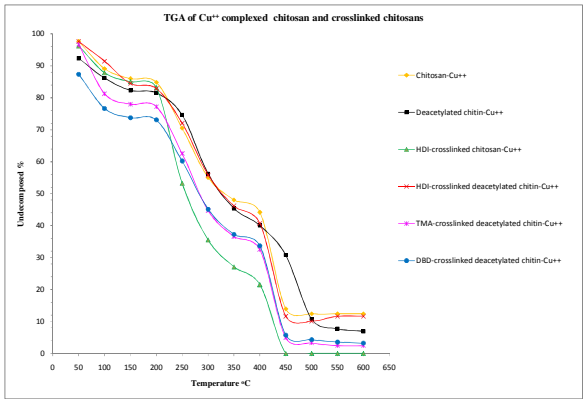


Figure 7: TGA of Cu^{++} complexed chitosan and crosslinked chitosans

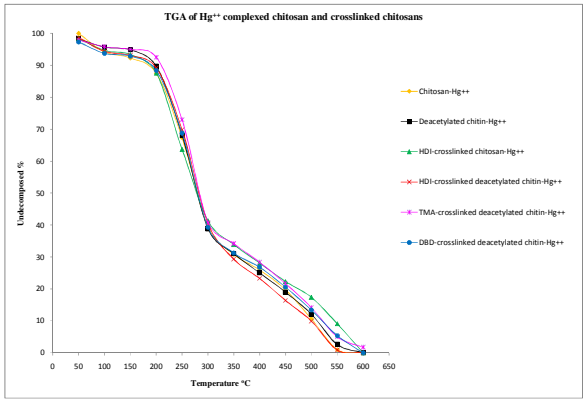


Figure 8: TGA of Hg^{++} complexed chitosan and crosslinked chitosans

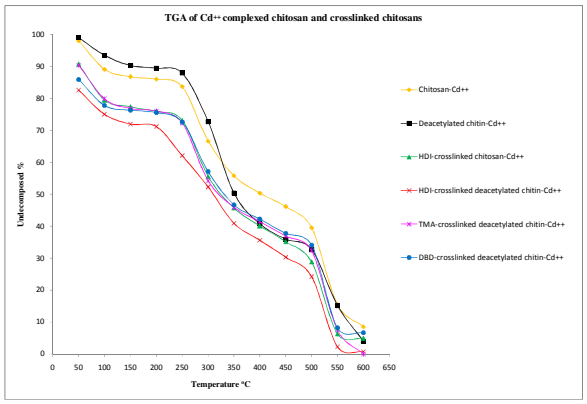


Figure 9: TGA of Cd^{++} complexed chitosan and crosslinked chitosans

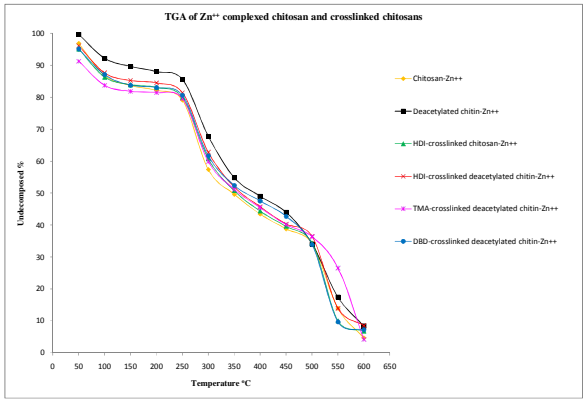


Figure 10: TGA of Zn^{++} complexed chitosan and crosslinked chitosans

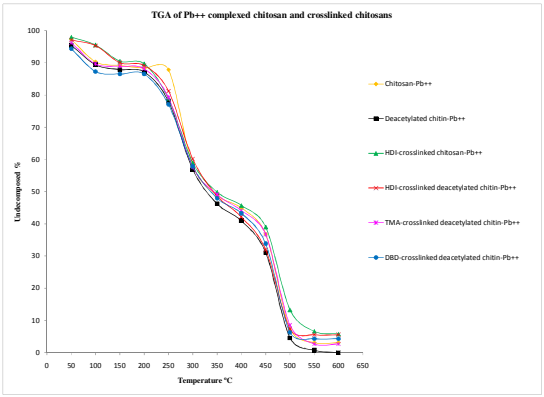


Figure 11: TGA of Pb^{++} complexed chitosan and crosslinked chitosans

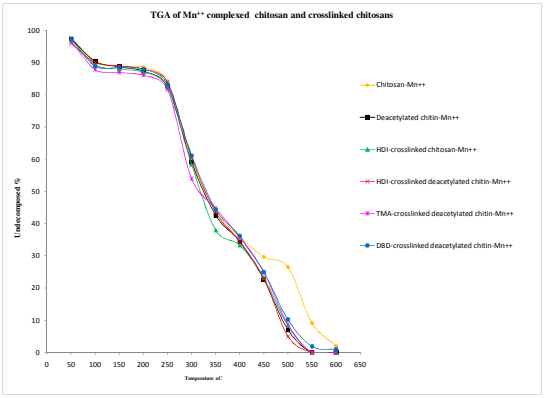


Figure 12: TGA of Mn^{++} complexed chitosan and crosslinked chitosans

CHAPTER 7

"Conclusions and suggestions for further work"

This thesis seeks to present a comprehensive documentation of the studies on crosslinked chitin/chitosan as it appears in published literature, as well as presenting a detailed investigation of a series of newly synthesized and characterized crosslinked chitosan derivatives incorporating special structural features such as availability of all amino functional groups on the exposed surface of the crosslinked chitosan. This was accomplished by first crosslinking chitin, using three different crosslinking agents, followed by deacetylation. These new polymers were then investigated for their heavy metal binding properties and specificities, the morphologies of the complexed polymers, applications in enzyme purification, enzyme mediated hydrolysis to produce chitooligosaccharides, and thermal properties of the metal complexed chitosans and crosslinked chitosans.

The results of our studies showed that availability of all amino groups of chitosan for complexation with metals can play a key role in enhancing the binding ability of the metal ions to crosslinked chitosans. Further, these crosslinked derivatives could be used in powder form, and the additional step of preparing beads was found to be not necessary for ease of separation of the crosslinked powder by filtration. The binding capacity of various crosslinked chitin and deacetylated derivatives for Cu, Cd, Hg, Zn, Mn, and Pb was in the region of 100, 140, 360, 88 , 5 and 60 mg/g (rounded off values) of polymer respectively, very close to the values obtained for uncrosslinked chitosan. For Cu ions, the Langmuir equation was found to be the best fit for HDI crosslinked deacetylated chitin and TMA crosslinked deacetylated chitin.

The morphologies of the metal complexed crosslinked chitosans are also dependent on the position of the metal ion i.e either as “inclusion” complex as for strongly bound Cu, Hg, and Cd ions, or complexation on the periphery as for weak complexes of Mn ion. These were proved by both SEM and WAXRD studies. In SEM studies, the presence of metal ions on the surface of the chitosans could be detected with decrease in metal ion binding, in the following sequence

Hg > Cu > Cd > Zn > Pb > Mn. Particularly in the case of Pb ions, the presence of these ions is clearly seen on the surface of the polymer by SEM. The number of ions of Mn complexed on the polymers were too few (5 mg/g of chitosan) to be visible. SEM of Hg and Cu complexes do not how the "holes" observed in the crosslinked polymers as they bind specifically to amino groups of chitosan, but for Cd, Zn, Mn, and Pb complexes, these "holes" are clearly visible. The morphological studies conducted using WAXRD are in close agreement with the metal complexation data, showing complete loss of original chitosan peaks for the heavily complexed derivatives, and minor changes for the weakly complexed metals.

The thermal properties of metal complexes of crosslinked chitosans also showed a corelationship with the strength of binding of individual metal ions. The metal complexes with Cu, Cd and Hg ions, which exhibit the highest complexing ability to the chitosans (Hg 354-364 mg/g chitosan; Cu 100-112 mg/g chitosan, and Cd 121 – 160 mg/g chitosan), were found to have the lowest onset of degradation temperatures (range 194 – 210 ^{0}C) and the lowest final degradation temperatures (generally less than 294 - 304 ^{0}C for Hg, 296 - 338 ^{0}C for Cu complexes, and 305 – 368 ^{0}C for Cd complexes). Mn ion which has the lowest binding abilities to chitosans (Mn 5 -7 mg/g chitosan) showed the reverse behaviour, these complexes exhibited the least effects on the onset (240 – 248 ^{0}C) and final degradation temperatures (range 300 – 368 ^{0}C). Zn (binding ability 74 - 87 mg/g chitosan) and Pb (binding ability 39 – 62 mg/g chitosan) ions have a binding ability intermediate to Cu/Cd/Hg and Mn extremes, and therefore the effects on onset and final degradation temperatures are intermediate to these values. These studies have shown that a study of thermogravimetry of chitosan metal complexes can be a valuable tool in evaluating the binding abilities of various metal complexes.

Chitosan and its crosslinked derivatives when used as affinity matrices for purification of lipase from *Aspergillus niger* NCIM 1207 showed that Trimellitic

anhydride-crosslinked deacetylated chitin adsorbed lipase selectively. These results suggested that trimellitic anhydride-crosslinked deacetylated chitin is an excellent substrate to get approximately 5.19 fold purification of the crude lipase with 70% yield. Further 9.43 fold purification was shown to occur on eluting through sephacryl-100. This appears to be an inexpensive method for large scale purification of lipases for industrial applications. Further work on purifications of a variety of different enzymes and specific uptake of specific metals from a mixture of metal ions would be a fruitful area of further research.

In another study metal-complexed crosslinked chitosans, the complexes were used as substrates for hydrolysis with chitosanase enzyme, and the products characterized for chitooligosaccharaides formation. Crosslinked chitosan without metal complexation had the same hydrolytic behavior as uncrosslinked chitosan. However, when the crosslinked chitosans were complexed with metals, their hydrolytic rates and extent of hydrolysis was significantly reduced. Thus while for chitosan about 840 µg/ml reducing sugar was produced in 4 hours time, and 780 µg/ml was produced for diisocyanatohexane crosslinked chitosan, only 400 µg/ml and 320 µg/ml was produced for cadmium sulfate with crosslinked chitosan and diisocyanatohexane crosslinked chitosan, respectively. Similar results are obtained for other crosslinking agents. Studies on preincubation of the metal with the enzyme show that of the metals studied, Mn has no effect on preincubatioin with the enzyme, Hg, Cd, Pb, and Cu completely deactivate the enzyme, while Zn reduces the enzyme activity by about 43.3%. Preincubation of the metal salts with the chitosan shows that Hg and Cu completely deactivate the molecule from enzyme hydrolysis, Cd and Zn inactivate it to the extent of 56.8% and 43.3% respectively, while Mn has no effect. Availability of the amino functions seems to be a key feature for the chitosanase to hydrolyze the chitosan polymer. This was also proved by the significant increase in the extent of hydrolysis for chitosan samples with 88% (final value 1120 µg/ml reducing sugar) and 85% deacetylation (final value 840 µg/ml reducing sugar). These are the first studies of this kind, showing the hydrolytic behavior of different samples of

crosslinked chitosans containing metals. High performance ion chromatography (HPIC) studies of the products show a variety of oligomers are produced in the chitosanase enzyme hydrolytic reaction. As is well known, chitooligosacchrides have many applications in antimicrobial formulations, and these studies can lead to new chitooligosacchride products.

All these studies showed that crosslinked chitosan constitute a group of polymers that are an important area for research and development, with several possibilities for developing uniquely tailored crosslinked polymer structures for specific applications.

List of Publications resulting from this dissertation:

1. Complexation of heavy metals by crosslinked chitin and its deacetylated derivatives.
   K.D.Trimukhe, A.J.Varma
   Carbohydrate Polymers, 71, 66-73, (2008).

2. A morphological study of heavy metal complexes of chitosan and crosslinkedChitosans SEM and WAXRD.
   K. D. Trimukhe, A. J. Varma
   Carbohydrate Polymers, 71, 698-702, (2008).

3. Metal complexes of crosslinked chitosans Part II. An investigation of their hydrolysis to chitooligosaccharides using cgitosanase.
   K. D. Trimukhe; S. Bachate; D. V. Gokhale; A. J. Varma
   International Journal of Biological Macromolecules, 41, 491-496, (2007).

4. Environment friendly crosslinked chitosan as a matrix for selective adsorption and purification of lipase of Aspergillus niger.
   K. D. Trimukhe; N. Mahadik; D. V. Gokhale; A. J. Varma
   (Submitted, December 2007).

5. Metal complexes of crosslinked chitosans: correlations between metal ion complexation values and thermal properties.
   K. D. Trimukhe; A. J. Varma (Submitted, December 2007).

6. Synthesis, applications and characterizations of of crosslinked chitosan and its derivative studies: A review
   K. D. Trimukhe; A. J. Varma (Manuscript in preparation).