Preparation and characterization of chitosan based metal nanoparticles and study of their antibacterial property

by

Gazi Jahirul Islam

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Philosophy (M.Phil) in Chemistry

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Khulna 9203, Bangladesh.
August, 2016

To whom it may concern

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Abstract

Chitosan is a natural polysaccharide and has significant biological and chemical properties such as biodegradability, biocompatibility, bioactivity, microbial activity and polycationicity. In this research, chitosan based nanoparticles have been prepared for bacteriological application. Chitosan has been prepared and characterized from locally available waste shrimp shell. Prepared chitosan have been used as the raw material for the production of chitosan based metal nanoparticles such as Chitosan based zinc nanoparticle (Chi-Zn NPs), Chitosan based copper nanoparticle (Chi-Cu NPs) and Chitosan based cadmium nanoparticle (Chi-Cd NPs). First chitin has been has been isolated from shrimp shell by demineralization and deproteinization followed by deacetylation to get chitosan. Purity and characteristics of chitosan isolated from shrimp shell has been compared with those of extra pure chitosan purchased from Sisco Research Laboratories (SRL) Private Limited, India. It was characterized by Fourier Transform Infra Red (FTIR) Spectroscopy, solubility, moisture and ash content. It has been found that moisture and ash content of prepared chitosan is 1.25%, 1.22% respectively while those for purchased extra pure chitosan is 1.24% and 1.19%. Both are readily soluble in 1% acetic acid and produce transparent viscous solution. FTIR result of the extracted chitosan is very much comparable to the standard one. It has been seen that there is an excellent agreement between extracted and purchased chitosan. Chitosan based metal nanoparticles have been synthesizes by solution casting method. But the techniques and conditions are different from previous studies [83, 84]. The synthesized NPs were characterized by using FTIR, TGA and SEM analysis. FTIR spectra of the NPs (except Chi-Cd) are very similar to those of the synthesized chitosan. This indicates the presence of chitosan polymer in the NPs matrices. The loss of mass in TG analysis also confirms the presence of chitosan polymer in the Chi-Zn NPs. SEM photograph clearly showed the synthesized materials are nanomaterials within the range of 1-100 nm. Produced NPs exhibited enhanced antibacterial activities. Zone of inhibition against *Pseudomonas aeruginosa, Salmonella bovismorbificans*, *Salmonella typhi* and *Escherichia coli* bacteria of Chi-Zn, Chi-Cu, Chi-Cu(II) and Chi-Cd NPs are 38, 31, 30 and 39 mm; 32, 36, 37 and 32 mm; 30, 35, 36 and 33 mm and 35, 31, 31 and 38 mm respectively, while those for standard drug kanamycin are 22, 22, 20 and 20 mm respectively.

Symbol and Abbreviations

DMAc	:	Dimethylacetamide
DMSO	:	Dimethyl sulphoxide
NPs	:	Nanoparticles
Chi	:	Chitosan
MCC	:	Microcrystalline chitin
HPLC	:	High-Performance Liquid Chromatography
LEDs	:	Light-emitting devices
NLO	:	Organic nonlinear optical
PRINT	:	Particle replication in non-wetting templates
TPC	:	Total plate count
MAP	:	Modified atmosphere packaging
BHA	:	Butylated hydroxyanisole
BHT	:	butylated hydroxytoluene
THQ	:	*tert*-butylhydoquinone
EDS	:	Energy dispersive x-ray spectrometer
SEM	:	Scanning electron microscopy
TGA	:	Thermogravimetric analysis
SRL	:	Sisco Research Laboratories

Contents

List of Tables

Page

CHAPTER IV

List of Figures

Page

CHAPTER I

CHAPTER III

CHAPTER IV

CHAPTER I

Introduction

1.1 General Introduction

Over the last one decade, interest in the naturally available class of polymers known as polysaccharides (Starch, cellulose, and chitin) has been increasing rapidly, because it is a renewable resource with a wide range of uses in nature, functioning as energy storage, transport, signaling, and structural components [1]. Chitosan, the deacetylated form of chitin, is a basic polysaccharide that is easily prepared from the shells of crabs, shrimp and prawns. Furthermore, it is an environmentally benign compound.

Chitosan shows biocompatibility and biodegradability characteristics suitable to create an environment biologically inert and flexible for sensing and manipulating macromolecules and microorganisms in devices. In particular, chitosan matrices provide a better environment for doping, blending and grafting of acids, oxides and salts to improve the conductance level comparable with synthetic ion-conducting polymers. Recently, active researches have been conducted on the use of Chitosan and it derivatives as waste water treatment materials, biosensors, artificial muscles, actuators, environmentally sensitive membranes, and components in high-energy batteries [2, 3] like electrolyte etc.

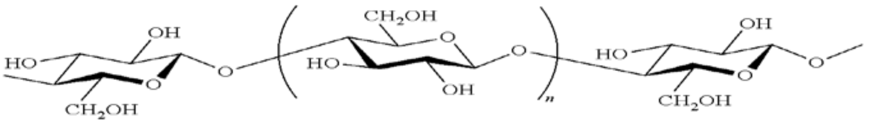
Therefore, the knowledge on physical and chemical behavior of this biopolymer and its nanocomposites are of scientific and technological interest for various purposes. The aims of this thesis are to prepare chitosan from shrimp shell and synthesize chitosan metal (Zn, Cu and Cd) nanoparticals and study the various physical, chemical and antimicrobial properties of those nanoparticals.

1.2 Chitin and Chitosan

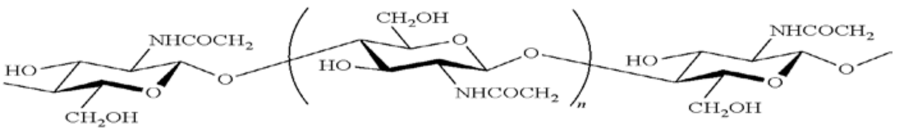
Chitosan is a fiber-like substance derived from chitin, a homopolymer of ß-(1J4)-linked *N*-cetyl-D-glucosamine. Chitin is widely distributed in marine invertebrates, insects, fungi, and yeast [4]. However, chitin is not present in higher plants and higher animals. Generally, the shell of selected crustacean consists of 30-40% protein, 30-50% calcium carbonate and calcium phosphate, and 20-30% chitin [5]. Chitin is widely available from a variety of source among which, the principal source is shellfish waste such as shrimps, crabs, and crawfish [6]. It also exists naturally in a few species of fungi.

In terms of its structure, chitin is associated with proteins and, therefore, high in protein contents. Chitin fibrils are embedded in a matrix of calcium carbonate and phosphate that also contains protein. The matrix is proteinaceous, where the protein is hardened by a tanning process [7]. Studies of [8] demonstrated that chitin represents 14-27% and 13-15% of the dry weight of shrimp and crab processing wastes, respectively. With regard to their chemical structure, chitin and chitosan have a similarity in chemical structure. Chitin is made up of a linear chain of acetylglucosamine groups while chitosan is obtained by removing enough acetyl groups (CH_3-CO) from chitin, the molecule and the resultant product is found to be soluble in most diluted acids. The actual difference between chitin and chitosan is the acetyl content of the polymer. Chitosan having a free amino group is the most useful derivative of chitin [9]. Chitosan is a non toxic, biodegradable polymer of high molecular weight, and is very much similar to cellulose, a plant fiber as shown in the following structure. After cellulose, chitin is the second most abundant natural biopolymer found in nature [10].

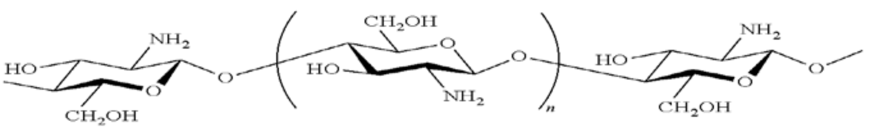
However, unlike plant fiber, chitosan possesses unique properties including the ability to form films, optical structural characteristics. Chitosan also possesses a positive ionic charge, which gives it an ability to chemically bind with negatively charged fats, lipids and bile acids [11]. Over the last several years, chitinous polymers, especially chitosan, have received much attention as one of the promising renewable polymeric materials for their extensive applications in the pharmaceutical and biomedical industries for enzyme immobilization and purification, in chemical plants for wastewater treatment, and in food industries for food formulations as binding, gelling, thickening and stabilizing agent [5, 12].



Cellulose



Chitin



Chitosan

1.3 Chemistry and physical properties of chitosan

Chemically, chitosan is a high molecular weight, linear, polycationic heteropolysaccharide consisting of two monosaccharides: N-acetyl-glucosamine and D-glucosamine. They are linked by β-(1→4) glycosidic bonds. The relative amount of these two monosaccharides in chitosan vary considerably, yielding chitosans of different degrees of deacetylation varying from 75% to 95%, molecular weight in the range of 50-2000, different viscosities and pKa values [23]. In addition, chitosan has three functional moieties on its backbone; the amino group on the C2, the primary and secondary hydroxyl groups on the C3 and C6 positions, respectively. These functional groups play important roles in different functionalities of chitosan. The amino group is the most important among the others, especially in acidic conditions, due to the protonation phenomenon, rendering it able to interact with negatively charged molecules (or sites). Additionally, chitosan polymer interacts with the metal cations through the amino groups, hydroxyl ions and coordination bonds. Most of the naturally occurring polysaccharides e.g., cellulose, dextrin, pectin, alginic acid, agar, agarose, and carragenas are natural and acidic in nature, whereas chitin and chitosan are examples of highly basic polysaccharides. Their properties include solubility in various media, solution, viscosity, polyelectrolyte behavior, formation, ability to form films, metal chelations, optical and structural characteristics [17].

Although the β(1→4)-anhydroglucosidic bond of chitin is also present in cellulose the characteristic properties of chitin/chitosan are not shared by cellulose[18]. Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone and chloroalcohols in conjunction with aqueous solutions of mineral acids [13] and dimethylacetamide (DMAc) containing 5 percent lithium chloride (LiCl) [19]. Recently the dissolution of chitosan in *N*- methyl morpholine-*N*-oxide (NMMO)/H O has been reported by Dutta *et al.* [20, 21]. The hydrolysis of chitin with concentrated acids under drastic conditions produces relatively the pure amino sugar, D- glucosamine.

Depending on the extent of deacetylation, chitin contains 5 to 8 percent (w/v) nitrogen, which is mostly in the form of primary aliphatic amino groups as found in chitosan. Chitosan undergoes the reactions typical of amines, of which *N*-acylation and Schiff reactions are the most important. Chitosan glucans are easily obtained under mild conditions but it is difficult to obtain cellulose glucans.

N-acylation with acid anhydrides or acyl halides introduces amido groups at the chitosan nitrogen. Acetic anhydride affords fully acetylated chitins. Linear aliphatic *N*-acyl groups higher than propionyl permit rapid acetylation of the hydroxyl groups in chitosan [15, 16]. Highly benzoylated chitin is soluble in benzyl alcohol, dimethyl sulphoxide (DMSO), formic acid, and dichloroacetic acid. The *N*-hexanoyl, *N*-decanoyl and *N*-dodecanoyl derivatives have been obtained in methanesulphonic acid [13].

Chitosan forms aldimines and ketimines with aldehydes and ketones, respectively, at room temperature. Reaction with ketoacids followed by reduction with sodium borohydride produces glucans carrying proteic and non-proteic amino acid groups. *N*-carboxy-methyl chitosan is obtained from glyoxylic acid. Examples of non-proteic amino acid glucans derived from chitosan are the *N*-carboxybenzyl chitosans obtained from *o*- and *p*-phthalaldehydic acids [22].

Chitosan and simple aldehydes produce *N*-alkyl chitosan upon hydrogenation. The presence of the more or less bulky substituent weakens the hydrogen bonds of chitosan; therefore, *N*-alkyl chitosans swell in water inspite of the hydrophobicity of alkyl chains. They retain the film forming property of chitosan [13, 14]. Chitosan is more versatile in comparision to chtin due to the presence of amino groups at the C-2 positions.

1.4 Chemical Properties of Chitosan

The chemical properties of chitosan are as follows:

- Linear polymine,
- Reactive amino groups,
- Reactive hydroxyl groups available,
- Chelates many transitional metal ions.

1.5 Biological Properties of Chitosan

Following are the biological properties of chitosan.

- Biocompatible
- Binds to mammalian and microbial cells aggressively,
- Regenerative effect on connective gum tissue,
- Acclerates the formation of osteoblast responsible for bone formation,
- Hemostatic,

- Fungistatic,
- Spermicidal,
- Antitumor,
- Anticholesteremic,
- Accelerates bone formation,
- Central nervous system depressant,

1.6 Source of Chitosan

In nature, chitosan is found in the cell walls of fungi of the class *Zygomycetes*, in the green algae *Chlorella sp.*, yeast and protozoa as well as insect cuticles and especially in the exoskeleton of crustaceans. Shrimp shell is one of the main sources of chitosan (Figure 1.2 and 1.3). Chitosan is a deacetylated derivative of chitin, the second abundant polysaccharide in nature after cellulose. In 1811, the French scientist, Henri Braconnot first discovered chitin in mushroom. In 1820, chitin was isolated from insect cuticles [24]. In 1859, Rouget reported finding chitosan after boiling chitin in potassium hydroxide (KOH). This treatment rendered the material soluble in organic acids. Hoppe-Seyler named it chitosan in 1894 [25]. Chitin is a major component of the hard shell that crustaceans, such as shrimp and crabs, possess. Bangladesh has a very large industry of shrimp. So the raw material of chitosan is available in Khulna region, Bangladesh (Figure 1.1).

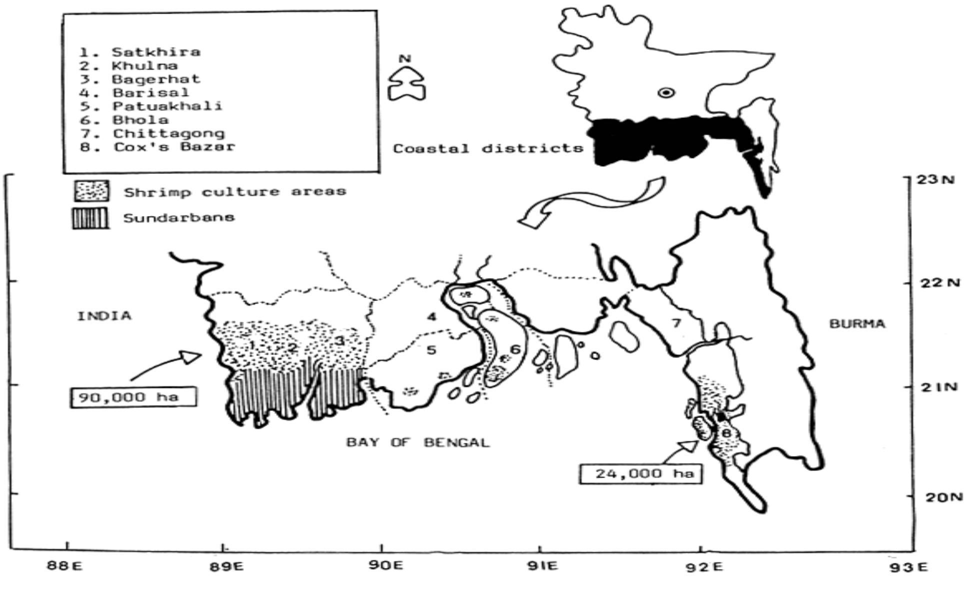


Figure 1.1: Regional shrimp industries in Bangladesh.

Figure 1.2: Shrimp

Figure 1.3: Shrimp shells.

1.7 Chitosan nanoparticles (Chi NPs)

Chitosan nanoparticles (Chi NPs) are having excellent physicochemical properties. They are environmentally friendly and bioactive. It is frequently used as a controlled-release drug carrier for gene transfer in artificial organs and for immune prophylaxis. In addition, chitosan nanoparticles have been used to improve the strength and wash ability of textiles and to confer anti-bacterial effects [26].

Chitosan nanoparticles are generally prepared by micro emulsion, self-assembling and ionotropic gelation methods [27]. Microemulsion method can produce nanoparticles with narrow size distribution by the expense of large quantities of organic solvent. Self-assembling is a simple method, but chitosan must be modified by introducing new chemical groups. Ionotropic gelation is a mild preparation method in aqueous environment, without the introduction of chemical groups into chitosan molecules [28]. The size of nanoparticles is affected by the concentration and molecular weight of chitosan, and nanoparticles concentration must be kept at low level to avoid flocculation [29]. Besides, the chitosan nanoparticles suspension is a thermodynamically unstable system and particle size changes during storage also [30]. Prepared chitosan nanoparticles by iontropic gelation, and then spraydried the nanoparticles suspension to produce a nanopowder to enhance the application and storage of nanoparticles. Ultrafine milling is also an effective method to produce chitosan nanopartices [31].

1.8 Applications of Chitosan and Chitosan based Nanoparticles

The interest in chitin originates from the study of the behaviour and chemical characteristics of lysozyme, an enzyme present in the human body fluids. It dissolves certain bacteria by cleaving the chitinous material of the cell walls [17]. A wide variety of medical applications for chitin and chitin derivatives have been reported over the last three decades [32, 33]. It has been suggested that chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in tissue culture [34].

The poor solubility of chitin is the major limiting factor in its utilization and investigation of its properties and structure. Despite these limitations, various applications of chitin and modified chitins have been reported in the literature, e.g., as raw material for man-made fibres [13, 35, 36]. Fibres made of chitin and chitosan are useful as absorbable sutures and wound-dressing materials [17, 34, 37]. These chitin sutures resist attack in bile, urine and pancreatic juice, which are difficult with other absorbable sutures. It has been claimed that wound dressings made of chitin and chitosan fibres [34] accelerate the healing of wounds by about 75 per cent. Apart from their applications in the medical field, chitin and chitosan fibers have potential applications in wastewater treatment, where the removal of heavy metal ions by chitosan through chelation has received much attention [18, 36, 38]. Their use in the apparel industry, with a much larger scope, could be a long-term possibility [39-41].

1.8.1 Industrial Applications of Chitosan

Due to its physical and chemical properties, chitosan is being used in a vast array of widely different products and applications, ranging from pharmaceutical and cosmetic products to water treatment and plant protection. In different applications, different properties of chitosan are required. These properties change with, e.g., degree of acetylation and molecular weight as well.

1.8.1.1 Cosmetics

Chitosan and hair are complementary to each other owing to carry opposite electrical charges: chitosan positive and hair negative. A clear solution that contains chitosan forms a clear, elastic film on hair, thereby increasing its softness, smoothness, and mechanical strength. The material can also form a gel when added to mixtures of alcohol and water. Chitosan can be used in shampoos, rinses, permanent wave agents, hair colorants, styling lotions, hair sprays, and hair tonics. Several derivatives of chitosan and chitin have potential applications in hair care. They include glyceryl chitosan, an oligomer of hydrolyzed chitosan, n-hydroxypropyl chitosan, quaternary hydroxypropyl-substituted chitosan, polyoxyalkylene chitosan, chitosan oligosaccharides, chitin sulphate, and carboxymethyl chitin. Some derivatives of chitosan can form foam and create emulsifying action and chitin powder can be used directly in shampoo.

Both, chitin and chitosan can be used in toothpaste, mouthwashes and chewing gum. They freshen the breath and prevent the formation of plaque and tooth decay. Salts of chitosan, added to toothpaste, mask the unpleasant taste of silicon oxide and bind powders so that they maintain their granular shapes. Chitin can also be applied as a dental filler material and both chitin and chitosan absorb candida like thicans, a fungus that sticks to teeth, making them candidates to clean false teeth.

1.8.1.2 Water Engineering

Due to its polycationic nature, chitosan can be used as flocculating agent. It can also act as chelating agent, and heavy meatls trapper. Weltroswki [42, 43] used chitosan N-benzyl sulphonate derivatives as sorbents for removal of metal ions in acidic medium. In 1999, Bhavani and Dutta [44] repoted the removal of colour from dyehouse effluents using chitosan as an adsorbent. Considerable amounts of world production of chitin and chitosan and derivatives are used in wastewater treatment [45]. Chitosan molecules agglomerate largely anionic wastes in solution to form precipitates and floe, hence it act as a flocculent for recycling of food processing waste. Chitosan can compete effectively with synthetic resins in the capture of heavy metals from processing water. Chitin has been used to decontaminate plutonium containing wastewater, and water containing methyl-mercury acetate [39], a significant pollutant of wastewater from acetaldehyde production. Application of chitosan/ chitin mixture was found to remove arsenic from contaminated drinking water. Chitosan has also been found effective in the removal of petroleum and petroleum products from wastewater. The deacidifying ability of chitin is utilized in coffee industry and to clarify the beverages such as wine, beer, and fruit juices. Regenerated chitin, chitosan, and other chitinous membranes could be widely used for such processes as osmosis, reverse osmosis, micro-filtration, desalination, dialysis, and haemodialysis. Beds of flaked chitosan can also be used for purification of potable water [46].

1.8.1.3 Paper Industry

Biodegradable chitin and chitosan can strengthen recycled paper and increase the environmental friendliness of packaging and other products. Chitosan is already involved in the manufacture of paper because chitosan molecules greatly resemble those of cellulose the main constituent of plant walls. It also saves chemical additives and increases output. Lastly the paper produced with chitosan has a smoother surface and is more resistant to moisture. Among other things, chitosan is of great value in the production of toilet paper and for wrapping paper and cardboard. Hydroxymethyl chitin and other water soluble derivatives are useful end derivatives in paper making [13]. It can be used as a biodegradable packaging material for food wrap and other products.

1.8.1.4 Textile Industry

Derivatives of chitin have been produced and used to impart antistatic and soil repellent characteristics to the textiles. In textile industry, chitin can be used in printing and finishing preparations, while the chitosan is able to remove dyes from dye processing effluents. Besides these, chitin and chitosan both have made remarkable contribution to medical related textile sutures, threads, and fibres [34].

1.8.1.5 Food Processing

Use of chitosan in food industry is well known because it is not toxic for warm-blooded animals. Microcrystalline chitin (MCC) shows good emulsifying properties, superior thickening, and gelling agent for stabilizing foods. It is also used as a dietary fibre in baked foods. The use of MCC solved some of the problems such as, flavour, colour, and shelf-life, posed by other sources of fibre. It could be of special importance for manufacturing protein- fortified bread, even without such ingredients as emulsifiers and shortenings. Chitin and chitosan act as solid support for the entrapment of whole microbial, animal, or plant cell immobilization. Chitin has been used in immobilization of enzymes. It can be used as a non-absorbable carrier for highly concentrated food ingredients, e.g., (Food dyes and nutrients) [46].

1.8.1.6 Agriculture

Chitin treated seeds (wheat) were found to have growth accelerating and growth enhancing effects. Chitinous additions to the potting mixtures/soil resulted in significant reduction in root knot worm infestations and suppression of fungal pathogens [46].

1.8.1.7 Photography

In colour photography, chitosan has been used as a fixing agent for the acid dyes in gelatin and also acts as an aid to improve diffusion, an important step in developing photographs [13,17].

1.8.1.8 Chromatographic Separations

Chitin and chitosan find wide varieties of applications in chromatographic separations [47]. The presence of free -NH_2 groups, primary –OH groups and secondary –OH groups in chitosan makes it as an useful chromatographic support. Use of chitosan in thin layer chromatography for separation of nucleic acids have also been reported. Chitin and chitosan have used as sorbent material to solid phase extraction of phenol and chlorophenols by using High-Performance Liquid Chromatography (HPLC) [48].

1.8.1.9 Solid State Batteries

Due to insolubility of chitosan in water, it cannot take part alone in fabrication of solid state proton- conducting polymer batteries. Therefore, chitosan is dissolved in acetic acid to produce ionic conductivity. The conductivity is due to the existence of proton in the acetic acid solution. The transport of these protons is considered to occur through many microvoids in polymer. Small dielectric constants from piezoelectric studies attributed the presence of many microvoids in this polymer structure. The choice of a more suitable electrode material may produce a better battery system [49].

1.8.1.10 Chitosan Gel for LED and NLO Applications

Recently, dyes containing chitosan gels have been used as potential components in lasers and other light-emitting devices (LEDs). The process, called doping, utilizes dyes such as, porphyrin compounds that resemble the heme groups in blood. Research on porphyrins and other dyes, such as, fluorinated coumarin and rhodamine B for transparent thin films, nickel porphyrins to investigate any new properties of films are on the line. One of the authors (PKD) at the laboratory the chitosan containing azomethine chromophore as a pendant group for NLO applications has been reported (Unpublished results).

1.8.2 Biomedical Applications of Chitosan

The design of artificial kidney systems has made possible repetitive hemodialysis and the sustaining life of chronic kidney failure patients. Chitosan membranes have been proposed as an artificial kidney membrane because of their suitable permeability and high tensile strength. The most important part of artificial kidney is the semipermeable membrane and so far made from commercial regenerated cellulose and cuprophane. Since the primary action of the cellulose membrane is that of a sieve, there is little selectivity in the separation of two closely related molecules [20]. These novel membranes need to be developed for better control of transport, ease of formability and inherent blood compatibility.

A series of membranes prepared from chitin and its derivatives improved dialysis properties [50]. One of the most serious problems of using these artificial membranes is surface induced thrombosis, where heparization of blood is needed to prevent clotting, and people who are liable to internal hemorrhage can be dialysed only at great risk. Hence, these are the most challenging problem still to be resolved in the development of membranes which are inherently blood compatible. From these point of views, chitosan is hemostatic, i.e., causes clots.

1.8.2.1 Burn Treatment

Chitosan is a promising candidate for burn treatment. This is true since chitosan can form tough, water-absorbent, biocompatible films. These films can be formed directly on the burn by application of an aqueous solution of chitosan acetate. Another advantage of this type of chitosan treatment is that it allows excellent oxygen permeability. This is important to prevent oxygen-deprivation of injured tissues. Additionally, chitosan films have the ability to absorb water and are naturally degraded by body enzymes. This fact means that the chitosan needs not be removed. In most injuries (and specially burns), removing the wound dressing can cause damage to the injury site [52].

1.8.2.2 Artificial Skin

The effect of treatment with chitosan and saline solution on healing and fibroplasia of wounds made by scalpel insersions in skin and subcutaneous tissue in the abdominal surface of dogs have been reported [13]. The design for artificial skin, applicable to long-term chronic use focuses on a nonantigenic membrane which performs as a biodegradable template for the synthesis of neodermal tissue [51]. It appears that chitosan polysaccharides having structural characteristics similar to glycosamino glycans can be considered for developing such substratum for skin replacement [53-55]. Nowadays the investigation on brain-scal damage, plastic skin surgery are being made by the use of chitosan.

1.8.2.3 Opthalmology

Chitosan has replaced the synthetic polymers in opthalmological applications. Chitosan possesses all the characteristics required for an ideal contact lens; optical clarity, mechanical stability, sufficient optical correction, gas permeability, partially towards oxygen, wettability, and immunologically compatibility. Contact lenses are made from partially depolymerized and purified squid pen chitosan by spin casting technology, and these contact lenses are clear, tough, and possess other required physical properties such as, modulus, tensile strength, tear strength, elongation, water content, and oxygen permeability. Antimicrobial and wound healing properties of chitosan along with excellent film forming capability make chitosan suitable for development of ocular bandage lens [56].

1.8.2.4 Drug Delivery Systems

The applicability of natural polysaccharides such as, agar, konjac, and pectin in the design of dosage forms for sustained release has been reported [57, 58]. Despite the medical applications of chitin/chitosan described above, they are still utilized in the pharmaceutical field [59]. It is already known that compounds having a molecular weight lower than 2900 pass through membranes derived from chitosan [20]. Since chitin and chitosan do not cause any biological hazard and are inexpensive, these polymers might be suitable for use in the preparation of dosage forms of commercial drugs [60-62].

The most significant merit would be to create a desired environment with optimal response, minimum side effect and prolonged efficacy. This is a relatively new technology and requires an interdisciplinary scientific approach. Chitin/chitosan controlled delivery systems are at developing stage and being used for a wide variety of reagents in several environments [63, 64].

Chitosan and chitosan based nanoparticles possess enormous applications in pharmaceuticals, food, agriculture, cosmetics and medicines. Shrimp shell is one of the main sources of chitosan biopolymer. As agricultural product more that 96% shrimps of Bangladesh are cultivated in Khulna region. Most of the shrimp shells are used as garbage and is thrown to the environment. Therefore, a research project on chitosan extraction from waste shrimp shell and preparation of chitosan based metal nanoparticles has been undertaken in this thesis.

CHAPTER II

Literature Review

Chitosan nanoparticles were first described in 1994, when Ohya and coworkers [65] proposed the intravenous delivery of 5-fluorouracil, an anticancer drug, carried by chitosan nanoparticles obtained by emulsification and cross-linking. Since then, these systems have been extensively studied for drug delivery purposes and the original formulation was either used for other applications, such as the incorporation of active substances in toothpastes [66], or modified by the application of different preparat ion methods [67-69]. In addition, many researchers have developed new formulations of chitosan nanoparticles that included secondary matrix-forming materials [70-73]. In particular, varied methods have been developed, mainly involving emulsification, different types of coacervation, or even slight modifications of these. More specifically, the methods include emulsion-droplet coalescence [74], emulsion solvent diffusion [69], reverse micellar method [75], ionic gelation, polyelectrolyte complexation [67, 72] and desolvation [76], whose particularities will be described in the following sections. All these methods comprise bottom-up fabrication processes, which involve the assembly of molecules in solution to form defined structures [77], in this case, nanoparticles. Delivery systems resulting from bottom-up technologies usually display size polydispersity [78], which in some cases limits nanoparticle usefulness. In fact, it is assumed that in a polydisperse system, larger nanoparticles might have higher drug loading capacity, while smaller nanoparticles are expected to have higher efficiency at delivering drugs to tissues or cells. This means that, even if the drug carrier has high encapsulation efficiency, the efficacy of the delivery may be poor [79], compromising the therapeutic efficacy. Interestingly, a recent technological development related to a top-down process termed particle replication in non-wetting templates (PRINT),which is a modified soft lithography technique, has demonstrated independent control over nanoparticle size, as well as other parameters that include shape, modulus (stiffness) and surface chemistry [78, 80]. Nevertheless, although this technology appears very promising in drug delivery, so far, no application was reported for chitosan, even though the authors indicate that a wide range of materials can be used, including biodegradable and biocompatible polymers [78].

Among various semiconductor nanoparticles, nanosized zinc oxide (ZnO) particles are the most frequently studied because of their interest in fundamental study and also their applied aspects such as in solar energy conversion, varistors, luminescence, photocatalysis, electrostatic dissipative coating, transparent UV protection films, and chemical sensors. Previously, ZnO nanoparticles have been prepared by techniques including the sol-gel method [81, 82], precipitation [83], hydrothermal synthesis [84], and spray pyrolysis [85]. Since chitosan has tremendous ability to form metal complexes with zinc metal [86, 87], because of its amine groups and hydroxyl groups currently, chitosan-ZnO complex attracted great interests for its potential use as UV protector and medicament.

On the other hand, Cu NPs are attractive because in addition to their catalytic, optical and electrically conducting properties they are known to have significant antibacterial and antifungal properties [88-92]. For example, Esteban-Cubillo and coworkers have demonstrated bactericidal properties of Cu NPs prepared in the matrix of sepiolite [95]. On the other hand, the antimicrobial activity of acrylated Cu NPs is similar to that of conventional copper based biocides [94]. Additionally, Cioffi, et al. 2005 [93] have reported antifungal and antifouling properties of a Cu NP-polymer composite. M. Taner et al. recently reported synthesis of Ag-Cu nanoalloys and their bactericidal behavior against Escherichia coli (E. coli) [96]. But Ag known to accumulate in the human body over time, can lead to toxicity such as argyria [97].

Several methods have recently been developed for the syntheses of Cu NPs that include thermal reduction [98], sono-chemical reduction [99], metal vapor synthesis [100], chemical reduction [91], vacuum vapor deposition [92], radiation method [101], micro-emulsion technique [102-104] and laser ablationv [105]. Interestingly, most of these methods utilize oxygen-free environment for the synthesis as the incipient Cu NPs get readily oxidized in the atmosphere [91, 93, 99, 104]. For example, the synthesis of Cu NPs in aqueous solution using sodium dodecyl sulfate as the capping agent where they used the inert environment of a glove box with nitrogen flow to prevent the oxidation of the generated Cu NPs [104]. Further, Joshi et al. 1998 [106] reported the synthesis of Cu NPs by gamma radiolysis, in an aqueous system under nitrogen atmosphere.

The modern era of chitosan research was heralded by publications in the 1990_s that described the antimicrobial potentials of chitosan and its derivatives, exhibiting a wide spectrum of activities against human pathogens and food-borne microorganisms [107-

110]. The first study reporting antibacterial properties was reported by [111]. They reported that chitosan showed a broad range of activities and a high inactivation rate against both Gram-positive and Gram-negative bacteria [111]. However, although several studies have been published in this area, the exact mechanism of the antimicrobial activity of chitosan remains ambiguous.

Six major mechanisms have been proposed in the literature, as follows [107, 109, 110]: (1) the interaction between the positively charged chitosan amine groups and the negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents; (2) the activation of several defense processes in the host tissue by the chitosan molecule acting as a water-binding agent and inhibiting various enzymes by blocking their active centers; (3) the action of chitosan as a chelating agent, selectively binding metals and then inhibiting the production of toxins and microbial growth; (4) the formation, generally by high molecular weight chitosan, of an impervious polymeric layer on the surface of the cell, thereby altering cell permeability and blocking the entry of nutrients into the cell; (5) the penetration of mainly low-molecular weight chitosan into the cystosol of the microorganism to bind DNA, resulting in interference with the synthesis of mRNA and proteins; and (6) the adsorption and flocculation of electronegative substances in the cell by chitosan, distributing the physiological activities of the microorganisms, causing their death.. However, it is very important to mention that chitosan is soluble only in acidic media and therefore, the effect of pH on microorganisms must be considered together with the effect of chitosan. Thus, the synergetic effect of chitosan/pH together is probably the most evident explanation of the antimicrobial effect of chitosan.

More recently, showed that chitosan and its derivatives is more powerful antibacterial agent against Gram-negative bacteria than against Gram-positive microorganism [107]. Conversely, they demonstrated that Gram-positive bacteria are more sensitive to the antibacterial effect of chitosan than Gram-negative bacteria [110]. Therefore, the interpretation of the sensitivity of bacteria to chitosan is quite difficult.

The microbial counts in their study were for lactic acid bacteria, *Enterobacteria, Pseudomonas* and *Staphylococcus*. The initial total plate count (TPC) of fresh lingcod was 3.67 log CFU/g, which then rapidly increased to 6.16 and 8.36 log CFU/g on day 6 and day 14, respectively [112]. When chitosan coatings were used, the results showed a 0.15-0.64 reduction in TPC. Moreover, the TPCs of chitosan-coated samples stored under vacuum or modified-atmosphere packaging were significantly lower than those of the control sample during the subsequent cold storage. The combination of chitosan coating and vacuum or modified atmosphere packaging (MAP) resulted in 2.22-4.25 reductions in TPC for the first 14 days of cold storage. The TPCs of chitosan-coated and MPA samples were lower than 10^5 CFU/g even after 21 days of cold storage. This result indicated a significant delay of microbial spoilage. Qi, Zhang and Lan-Lan [114] reported that because non-fermenting Gram-negative bacteria are dominant in the initial microbial flora of fish and shellfish sourced from cold seawater, controlling the growth of these Gram-negative bacteria may be important for the preservation of oysters. They demonstrated that combined treatment with chitosan and ozonated water had better antibacterial effect than either treatment alone. When only aerobic plate count was measured, the authors showed that the product shelf life with the combination of chitosan with ozonated water was at least 20 days, whereas it was only 8 days for the control sample, 10 days for the ozonated samples, and 14 days for the chitosan-treated samples. The fish oil incorporated in chitosan coatings lowered significantly the total and psychrotrophic counts in frozen lingcod fillets over three months cold storage [113]. Chitosan coatings enriched with cinnamon oil decreased effectively total viable counts and psychrotropic bacteria in rainbow trout (*Oncorhynchus mykiss*) during 16-day cold storage [117].

Along with the growing consumer demand for seafood devoid of synthetic antioxidants, chitosan has been a booming antioxidant agent in fish and shellfish. The antioxidant activity of chitosans of different viscosities (360, 50 and 14 cP) in cooked, comminuted flesh of herring (*Clupea harengus*) was investigated [115]. The oxidative stability of fish flesh during cold storage at 4 ˚C with the addition of chitosan at concentrations of 50, 100 and 200 ppm was compared with that of fish treated with conventional antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and *tert*-butylhydoquinone (THQ) (all at 200 ppm). A study indicated that the antioxidant capacity of chitosan added to fish muscle depends on its molecular weight (MW) and concentration in the product [115]. Similarly, the antioxidative effects of chitosan in salmon depended on its molecular weight (tested at MW = 30, 90 and 120 kDa) and concentration (evaluated at 0.2%, 0.5% and 1% w/w) [116]. The combination of chitosan with modified atmosphere packaging enhanced the lipid stability of lingcod (*O. elongates*) within 21 days of cold storage [112, 113].

Additionally, chitosan coatings enriched with cinnamon oil could suitably delay lipid oxidation in the refrigerated rainbow trout during 16 days of storage and markedly reduced the TBARS and PV values as compared with the control product [117]. Mao and Wu [118] showed that lipid oxidation of kamabako gel from grass carp (*Ctenopharyngodon* idellus) significantly decreased when a 1% chitosan solution was added.

Edible bio-based films have been investigated for their abilities to avoid moisture or water absorption by the seafood matrix, oxygen penetration to the food matrix, aroma loss and solute transport out of the product [119]. Based on this consideration, one of the most perspective active bio-film is the one based on chitosan. More recently, two review studies have reported the application of chitosan as bioactive film in the food industry [120]. Chitosan film, like many other polysaccharide based films, tend to exhibit resistance to fat diffusion and selective permeability to gases [121].

The use of chitosan as a coagulating agent for removing suspended solids from various processing streams has been widely investigated including cheese whey and dairy wash water, in the processing of poultry and seafood products [122-126]. Chitosan at a concentration of 10 mg/L reduced up to 98% the total suspended solids in shrimp processing wastewater [127]. Protein recoveries from surimi wash water (SWW) using 150 mg/L chitosan-alginate complex per liter SWW at mixing ratio of 0.2 resulted in 78-94 % adsorption after 24 h. This result was higher than the one obtained by using 50 mg/L, which yielded 81-90 % protein adsorption in the same treatment time [123].

Objectives of the Research

Chitosan, a natural and linear biopolyaminosaccharide, has received much attention as a functional biopolymer with applications in pharmaceuticals, food, cosmetics and medicines. Shrimp shell is one of the main sources of chitosan biopolymer. In Khulna region different types of shrimps, such as *Macrobrachium rosenbergii*, Black Tiger shrimp etc are cultivated and shell of these shrimps are directly thrown to the environment as garbage. Therefore, in this research project waste shrimp shells are used as the raw material for the production of chitosan and the produced chitosan has been used for the preparation of chitosan based metal nanoparticles. The preparation and characterization of chitosan NPs would be very important and would have many applications in various commercial and medical purposes as discussed earlier sections. So the specific objectives of present research are as follows:

(a) Extraction of Chitin from shrimp shell.
(b) Preparation of Chitosan from extracted Chitin.
(c) Characterization of prepared chitosan.
(d) Preparation of chitosan based nanoparticles of various metals (Zn, Cu and Cd etc.).
(e) Characterization of prepared chitosan based metal nanoparticles.
(f) Investigation of antibacterial activity against some bacteria of prepared chitosan-metal NPs.

CHAPTER III

Experimental

Materials and Methods

3.1 Instruments

Electric balance (AND-HR-200)
Electric oven (YCO-010 Series)
IR (IR Prestige-21, Shimadzu)
Furnace (Thermo Scientific Thermolyne), FB1415M)
Sonicator (POWERSONIC 603)
Scanning electron microscopy (SEM) (JEOL JSM-6490LA)
Thermal gravimetric analysis (TGA-50, Shimadzu)

3.2 Raw Materials, Chemicals and Reagents

Shrimp shell
Hydrochloric acid (E-Merck, Germany)
Acetic acid
Zinc oxide
Copper (ll) sulphate pentahydrate
Hydrazine hydrate solution
Sodium hydroxide, (E-Merck, Germany)
Cadmium sulphate

3.3 Glass apparatus and others

Glass rod
Funnel
Beaker (different size)
Round bottle flask
Condenser
Measuring cylinder
Pipette
Spatula
Oil bath
Thermometer

Some apparatus used in the Experiment

Figure 3.1: 4 digit electric balance

Figure 3.2: Oven

Figure 3.3: FTIR

Figure 3.4: Digital heater with magnetic stirrer

Figure 3.5: Scanning electron microscopy **(**SEM)

Figure 3.6: Thermal gravimetric analysis (TGA)

3.4 Preparation of Chitosan

Dried shrimp shell waste was obtained from a commercial shrimp shell processor of Khulna. Upon receipt, shells were then further sun dried for a period of 24 hours or longer until completely dried. To obtain a uniform size product, the dried shell was ground into course particles through a centrifugal grinding mill. Dried ground shell was placed in opaque plastic bottles and stored at ambient temperature until used .The production of chitosan from shrimp shell waste was carried with a modified method.

3.4.1 Steps of methods: This process mainly involved the following steps.

3.4.1.1 Demineralization of shells

Demineralization is simply replacing all cations with hydrogen ions (H^+) and all anions with hydroxide ions (OH^-). Demineralization process is the modern industrial water softening process.

In this step, the shells were suspended in 7% HCl at room temperature in the ratio of 1:12 (V/W). After 45 hours, the shells were quite squashy and were rinsed with water to remove acid and calcium chloride.

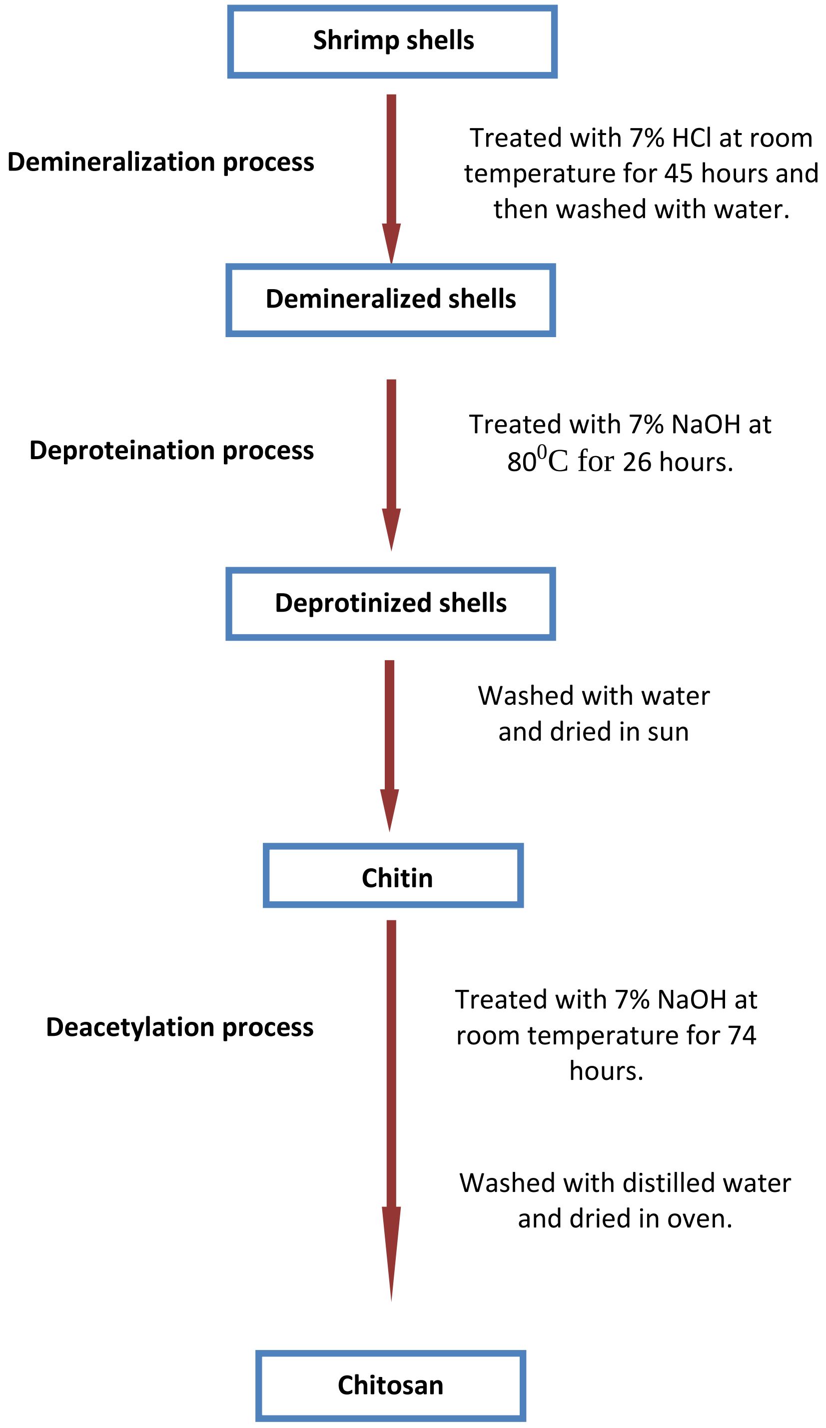
3.4.1.2 Deproteinization of shells

Deproteinization is the process of removing protein from a biological sample, either by precipitation of protein form solution or by hydrolysis. To remove protein from a substance, the demineralized shells were then treated with 7% NaOH at 80^0C for 26 hours with a solvent to solid ratio of 1:12 (V/W) .The residue was then collected and washed to neutrality in running tap water. Then it was dried in sun and the product is chitin.

3.4.1.3 The preparation of Chitosan by deacetylation of chitin

Removal of acetyl groups from the chitin was achieved by using 70% NaOH solution with a solvent to solid ratio of 1:12 (V/W) at room temperature for 74 hours. The mixture was stirred frequently for homogenous reaction. The resulting chitosan were washed in running tap water and rinsed with distilled water .Then filtered and dried in the oven. The shrimp shell chitosan thus obtained was white to off white flakes.

3.4.1.4 Flow diagram of the preparation of Chitosan



3.5 Preparation of Chitosan Nanoparticles

3.5.1 Chitosan-Zn NPs

3.5.1.1 Materials

(i) 1% acetic acid solution
(ii) Chitosan
(iii) ZnO
(iv) 1 M NaOH solution

3.5.1.2 Method

0.3 gm ZnO powder was dissolved in 80 mL of 1% acetic acid where it changed to zinc cations. 1 gm of chitosan was dissolved in 100 mL 1% acetic acid. 20 mL ZnO solution and 20 mL chitosan solution was mixed and sonicated for 10 min. The solution was then heated in water bath at (50–80◦C) for about 1 hr with magnetic stirring. Then 1M NaOH was added to the mixture drop by drop until the NPs formation completed. It was then filtered and washed with distilled water for several times and dried in oven. Here we added NaOH after heat and NPs formation began immediately after addition of small amount of NaOH and formation completed more quickly. This process is different from other available methods [82-84].

3.5.2 Chitosan-Cu metal NPs

3.5.2.1 Materials

(i) Chitosan (prepared)
(ii) Copper (II) sulphate pentahydrate ($CuSO4.5H_2O$)
(iii) Acetic acid
(iv) Hydrazine hydrate solution
(v) Sodium hydroxide solution (NaOH)

3.5.2.2 Method

In the following schematic presentation (Figure3.5.2.1) showed how chi-Cu-Nps has been prepared. This preparation is carried out according to Mallick S. *et al.* 2012 [105]. In this experiment 1.08 g of copper (II) sulphate pentahydrate was added to 100 mL of 1% acetic acid solution and 1.0 g of chitosan was dissolved in 100 mL 1% acetic acid solution. Then 50 mL of both solutions ware mixed and sonicated for 10 min and transferred to a 250 mL round bottom flask placed in an oil bath with vigorous stirring and refluxed for 20 min at ~100 °C, resulting in a light blue colored solution shown in Figure 3.5.2.2(a). To this about 1.0 mL of 0.6 M NaOH solution was added, upon which the color of the solution turned light brown. After about 20 min, nearly 1.0 mL of hydrazine hydrate solution was added to the above solution with constant stirring. A yellow solution was formed, which was turned into reddish brown as in Figure 3.5.2.2(b). The reaction was allowed to continue for an additional 10~20 min. The flask was then taken out from the oil bath and cooled to room temperature. The solution along with the precipitate was centrifuged at 5300 rpm and pellet washed with distilled water several times to remove hydrazine.

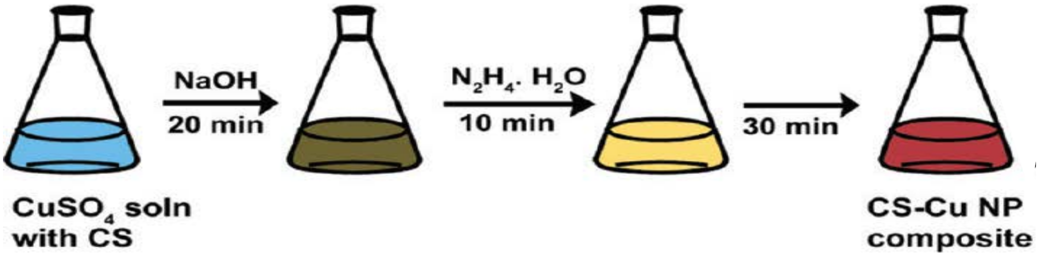


Figure 3.5.2.1 Schematic presentation of chi-Cu-Nps synthesis

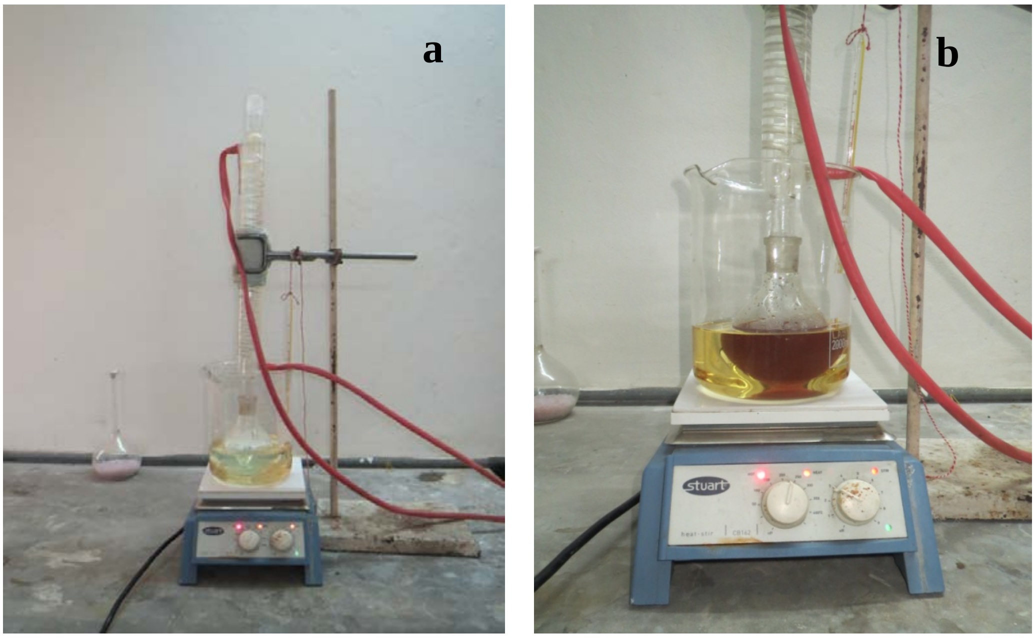


Figure 3.5.2.2: Preparation of Chitosan-Cu metal NPs

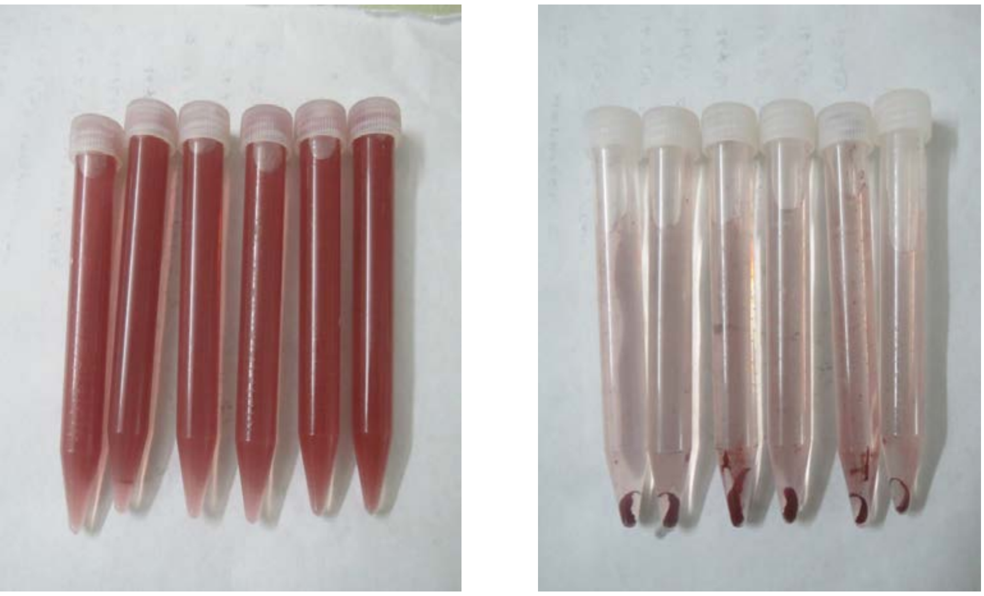


Figure 3.5.2.3: Separation of prepared Chitosan-Cu metal NPs

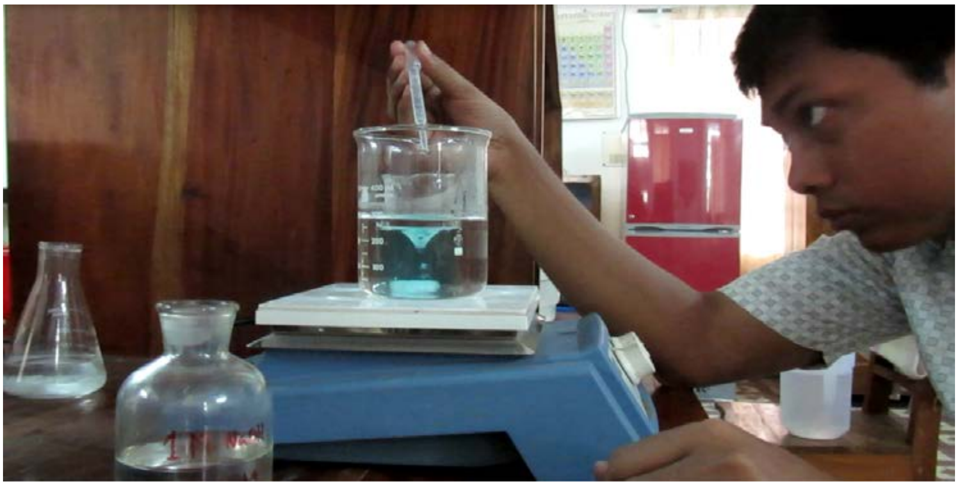
3.5.3 Chitosan-Cu (II) NPs

3.5.3.1 Materials

(i) Chitosan (prepared)
(ii) Copper (ll) sulphate pentahydrate ($CuSO_4.5H_2O$)
(iii) Acetic acid
(iv) NaOH solution

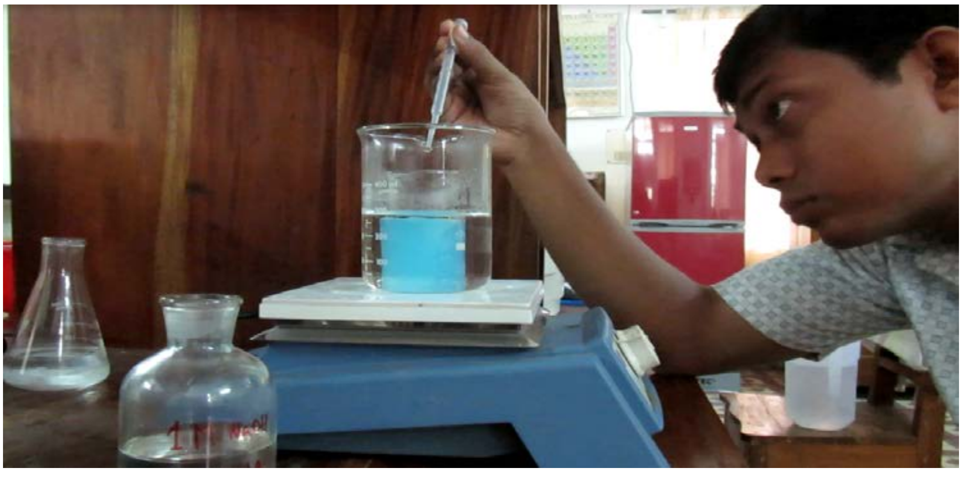
3.5.3.2 Method

0.8 g Copper (ll) sulphate ($CuSO_4.5H_2O$) was added in 100 mL of 1% acetic acid and 1 g of chitosan was dissolved in another 100 mL of 1% acetic acid solution. 20 mL of each solutions ware mixed and sonicated for five minutes. The solution was then heated in water bath at (50–80˚C) for about 20 min with magnetic stirring. Then 1.0M NaOH solution was added to the mixture slowly and drop by drop with special care until the blue NPs formation completed. Successive steps of the preparation of Chi-Cu(II) NPs has been shown in the following Figure 3.5.3.1 . It was then filtered and washed with distilled water for several times then dried up with continuous stirring. This synthesis process is different from other available methods [91-94, 96, 100, 102, 103, 106].



This photograph was taken after 20 minute

This photograph was taken after 30 minute



This photograph was taken after 40 minute

Figure 3.5.3.1: Schematic diagram of preparation of Chitosan-Cu (II) NPs

3.5.4 Chitosan-Cd NPs

3.5.4.1 Materials

1. Chitosan (prepared)
2. Cadmium sulphate ($CdSO_4.8/3H_2O$)
3. Acetic acid
4. NaOH solution

3.5.4.2 Method

3.8 gm Cadmium sulphate was added in 100 mL of 1% acetic acid and sonicated until dissolved. 1 gm of chitosan was dissolved in another 100 mL of 1% acetic acid solution. 20 mL of each solutions ware mixed and sonicated for few min. The solution was then heated in water bath at (50–80°C) for about 20 min with magnetic stirring. Then 1M NaOH solution was added to the mixture drop by drop until the off-white NPs formation completed. It was then filtered and washed with distilled water for several times then dried up. Condition of this method of preparation of chitosan based Cd-NPs.

3.6 Moisture Content

Moisture content of the prepared chitosan was determined by the gravimetric method. 2.0 g of chitosan was weight and then it was heated at 102 ºC for 40 minutes in an oven. Then it was kept in a desiccator for 10 minutes and cooled to normal temperature. Immediately it was weighted and % of moisture content was calculated by the following formula.

$$\text{\% of moisture content} = \frac{(\text{Wet weight, g} - \text{dry weight, g}) \times 100}{\text{Wet weight, g}}$$

3.7 Ash Content

Ash content measurement was done at the Quality Control Department of ACI Pharmaceuticals Ltd., Narayngang, Bangladesh. To determine the ash value of chitosan, 2.0g of chitosan sample was placed into previously ignited, cooled and tarred crucible. The samples were heated in a muffle furnace preheated to 650 ºC for 4 hr. The crucibles were allowed to cool in the furnace to less than 200 ºC and then placed into desiccators with a vented top.

$$\text{\% of Ash content} = \frac{(\text{Weight of residue, g}) \text{ X } 100}{\text{Sample weight, g}}$$

3.8 Solubility Test

Solubility of chitosan was observed in different types of mineral acids, organic acids and other types of chemical solvents.

3.9 FTIR Test

Infrared spectra were recorded on FTIR spectrophotometer (IR Prestige-21, Shimadzu) in the region 4500-400 cm^{-1} using KBr pellets at the Quality Control Department of ACI Pharmaceuticals Ltd., Narayngang, Bangladesh.

The samples were put in an agate mortar, thoroughly powdered with potassium bromide and then transferred in a mini-disc holder and a disc was made by automatic hydraulic press. The KBr disc was mounted in the sample cavity of the machine. The spectra were calibrated against 1601.8 cm^{-1} peak of the polystyrene film. The tentative band assignments of the important IR bands of the various complexes and the concerning ligands have been made empirically on the basis of some standard books.

3.10 SEM test

Scanning electron microscopy (SEM) is a method for high-resolution imaging of surfaces. The SEM uses electrons for imaging, much as a light microscope uses visible light. The advantages of SEM over light microscopy include much higher magnification (>100,000X) and greater depth of field up to 100 times that of light microscopy. Qualitative and quantitative chemical analysis information is also obtained using an energy dispersive x-ray spectrometer (EDS) with the SEM [128].

The SEM generates a beam of incident electrons in an electron column above the sample chamber. The electrons are produced by a thermal emission source, such as a heated tungsten filament or by a field emission cathode. The energy of the incident electrons can be as low as 100 eV or as high as 30 keV depending on the evaluation objectives. The electrons are focused into a small beam by a series of electromagnetic lenses in the SEM column. Scanning coils near the end of the column direct and position the focused beam onto the sample surface. The electron beam is scanned in a raster pattern over the surface for imaging. The beam can also be focused at a single point or scanned along a line for x-ray analysis. The beam can be focused to a final probe diameter as small as about 10 Å [129].

To create an SEM image, the incident electron beam is scanned in a raster pattern across the sample's surface. The emitted electrons are detected for each position in the scanned area by an electron detector. The intensity of the emitted electron signal is displayed as brightness on a display monitor and/or in a digital image file. By sychromizing the position in the image scan to that of the scan of the incident electron beam, the display represents the morphology of the sample surface area. Magnification of the image is the ratio of the image display size to the sample area scanned by the electron beam. The incident electrons cause electrons to be emitted from the sample due to elastic and inelastic scattering events within the sample’s surface and near-surface material. High-energy electrons that are ejected by an elastic collision of an incident electron, typically with a sample atom’s nucleus, are referred to as backscattered electrons. The energy of backscattered electrons will be comparable to that of the incident electrons. Emitted lower-energy electrons resulting from inelastic scattering are called secondary electrons. Secondary electrons can be formed by collisions with the nucleus where substantial energy loss occurs or by the ejection of loosely bound electrons from the sample atoms. The energy of secondary electrons is typically 50 eV or less [128, 129].

Two electron detector types are predominantly used for SEM imaging. Scintillator type detectors (Everhart-Thornley) are used for secondary electron imaging. This detector is charged with a positive voltage to attract electrons to the detector for improved signal to noise ratio. Detectors for backscattered electrons can be scintillator types or a solid-state detector [130, 131].

The SEM column and sample chamber are at a moderate vacuum to allow the electrons to travel freely from the electron beam source to the sample and then to the detectors. High-resolution imaging is done with the chamber at higher vacuum, typically from 10.5 to 10.7 Torr. Imaging of nonconductive, volatile, and vacuum-sensitive samples can be performed at higher pressures [132].

3.11 TGA test

Thermogravimetric analysis or **thermal gravimetric analysis** (TGA) is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate), or as a function of time (with constant temperature and/or constant mass loss), in an atmosphere of nitrogen, helium, air, other gas, or in vacuum [133]. TGA can provide information about physical phenomena, such as second-order phase transitions, including vaporization, sublimation, absorption, adsorption, and desorption. Likewise, TGA can provide information about chemical phenomena including chemisorptions, desolvation (especially dehydration), decomposition, and solid-gas reactions (e.g., oxidation or reduction) [133].

TGA is commonly used to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition, oxidation, or loss of volatiles (such as moisture). Common applications of TGA are (1) materials characterization through analysis of characteristic decomposition patterns, (2) studies of degradation mechanisms and reaction kinetics, (3) determination of organic content in a sample, and (4) determination of inorganic (e.g. ash) content in a sample, which may be useful for corroborating predicted material structures or simply used as a chemical analysis. It is an especially useful technique for the study of polymeric materials, including thermoplastics, thermosets, elastomers, composites, plastic films, fibers, coatings and paints.

Instrumental apparatus

Thermogravimetric analysis (TGA) relies on a high degree of precision in three measurements: mass change, temperature, and temperature change. Therefore, the basic instrumental requirements for TGA are a precision balance with a pan loaded with the sample, and a programmable furnace. The furnace can be programmed either for a constant heating rate, or for heating to acquire a constant mass loss with time.

Methods

The TGA instrument continuously weighs a sample as it is heated to temperatures of up to 2000 °C for coupling with FTIR and Mass spectrometry gas analysis. As the temperature increases, various components of the sample are decomposed and the weight percentage of each resulting mass change can be measured. Results are plotted with temperature on the X-axis and mass loss on the Y-axis. The data can be adjusted using curve smoothing and first derivatives are often also plotted to determine points of inflection for more in-depth interpretations.

Sample weight can range from 1 mg to 150 mg. Sample weights of more than 10 mg are preferred, but excellent results are sometimes obtainable on 1 mg of material. Weight change sensitivity of 0.01 mg. Samples can be analyzed in the form of powder or small pieces so the interior sample temperature remains close to the measured gas temperature.

3.12 Antibacterial Activity

The bacteria (test organisms) were collected from microbiology laboratory, Fish Inspection & Quality Control, Department of Fisheries, Khulna, Bangladesh. All steps of the work were carried out at this laboratory.

The complexes were screened for antibacterial activity against *Pseudomonas aeruginosa* (Gram negative), *Salmonella bovismorbificans* (Gram-negative), *Salmonella typhi* (Gram-negative), *Escherichia coli* (Gram-negative). The activities were carried out with the help of disc diffusion technique [134, 135]. Each disc contained 30 μg of compound and it was placed on bacteria inoculated plates. The growth inhibition results were compared with standard antibiotics, Kanamycin.

Culture media

The instant nutrient agar medium was weighed (28 grams), dispersed in one liter of distilled water and allowed soaking for 10 minutes shown in Figure 3.12.1. Swirled to mix and autoclaved at 15 lb/(inch)^2 pressure at 121°C. After this, the medium was cooled at 47°C.

Figure 3.12.1: Preparation of Culture media

Preparation of fresh culture

50 mL of medium was poured in a test tube as in Figure 3.12.2. The test microorganisms of pure culture were streaked on the nutrient media with the help of sterile loop in an aseptic and incubated at 37°C for 24 hours. The culture thus obtained was considered fresh culture. Fresh culture of this type was always used throughout the sensitivity testing.

Figure 3.12.2: Preparation of fresh culture

Preparation of plates

The medium was poured into sterile petridishes in an aseptic condition on a level horizontal surface so as to give a uniform depth of approximately 4 mm shown in Figure 3.12.3. Then the medium had been allowed to cool to room temperature in order to solidify the medium.

Figure 3.12.3: Preparation of plates

Preparation of discs

Sterile filter paper discs were taken and the test material of known concentration was applied on the discs with the help of a micropipette as shown in Figure 3.12.4. The solvents from the discs were evaporated by hot air blower. In the similar way control discs (containing only the solvents) were also prepared.

Figure 3.12.4: Preparation of discs

Placement of the discs and incubation

The solidified agar plates were seeded with 70 μL of fresh culture with the help of a micropipette and spread the micro organisms with the help of a sterile spreader in an aseptic condition.

The prepared discs of samples were placed gently on the freshly seeded solidified agar plates with a sterile forceps. Standard discs and control discs were also placed on the test plates to compare their effect with tested samples (Figure 3.12.5).

Figure 3.12.5: Placement of the discs

Then the plates were kept in a refrigerator at 4°C for 24 hours in order that the materials had sufficient time to diffuse to a considerable area of the plates (Figure 3.12.6). After this, the plates were incubated at 37°C for 16 hours.

Figure 3.12.6: Discs and incubation

Calculation of the zone of inhibition

After incubation, the diameter of the zone of inhibition were observed and measured in mm by a transparent scale show in Figure 3.12.7.

Figure 3.12.7: Calculation of the zone of inhibition

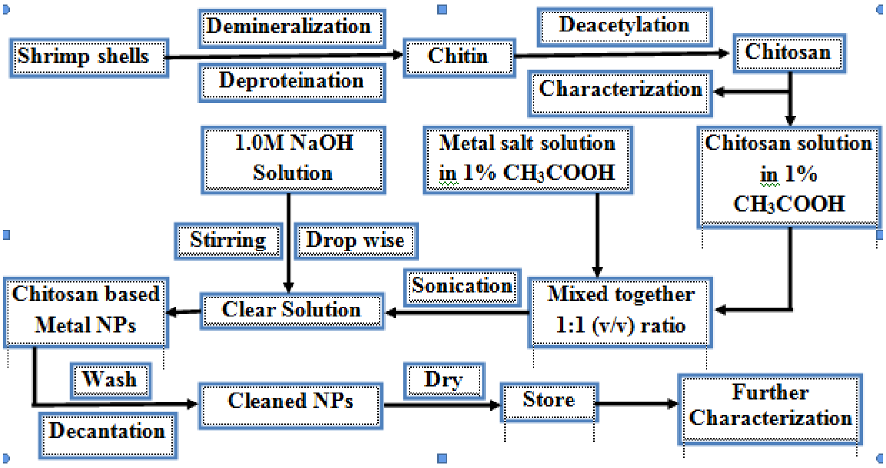
CHAPTER IV

Result and Discussion

4.1 General

Over the last few decades, biomaterials have been extensively developed and applied in medical devices. Among these materials, bioabsorbable polymers have attracted special attention for orthopedic applications where a transient existence of an implant can provide better results, when compared with permanent implants. Chitosan, a natural biopolymer, has generated enormous interest due to its various advantages such as biocompatibility, biodegradability and osteoconductive properties. In this thesis a novel process for the production of chitosan-based products has been introduced as potential medical applications for the future generations of bioabsorbable implants. Chitosan-based nanoparticles, with different properties, were successfully prepared, showing high performance towards antibacterial activities.

Chitosan is made from shrimp shell by a chemical process involving demineralization (DM), deproteinization (DP) and deacetylation (DA). The effects of altering or excluding the processing or using chitosan for the production of metal nanoparticles and their characteristics analyses have been demonstrated. Solution cast scheme was employed for the production of nanoparticles. Outline of the research can be stated as follows:



Color of chitosan obtained from shrimp shell is milk like white. Chitosan yield was with average of 53.46 % from chitin while the chitin yield was 40 % from shrimp shell.

Figure 4.1.1: Chitin

Figure 4.1.2: Chitosan

4.2 Characterization of Chitosan

4.2.1 Moisture content

The results of physico-chemical and functional properties of the prepared chitosan are given in Table 4.2.1. The prepared chitosan from chitin as well as shrimp shell was compared with the extra pure one. Usually chitosan from shrimp shells contain moisture in the range 1.00-1.30% depending on the season, relative humidity and intensity of sun light. It has been found that moisture content of prepared chitosan is

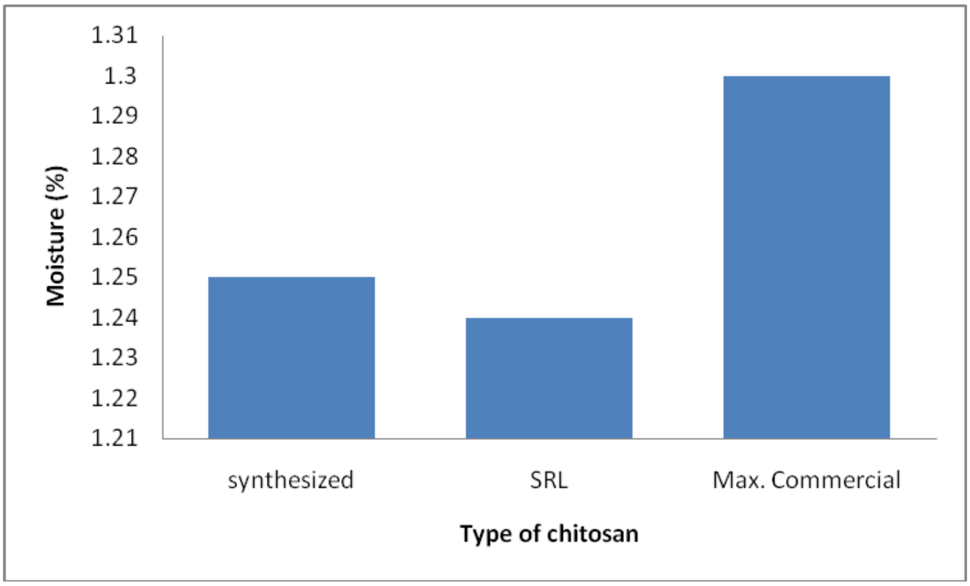


Figure 4.2.1: Moisture content of the chitosan

1.25% while that for purchased extra pure is 1.24% (Figure 4.2.1) and has a good agreement.

4.2.2 Ash content

Ash content exhibits the effectiveness of the demineralization step in removing minerals. From the Table 4.2.1 it is seen that the ash content for the synthesized and pure chitosan is 1.22 % and 1.19 %, while ~1.27 % ash content has been reported in the commercially available chitosan as shown in Figure 4.2.2.

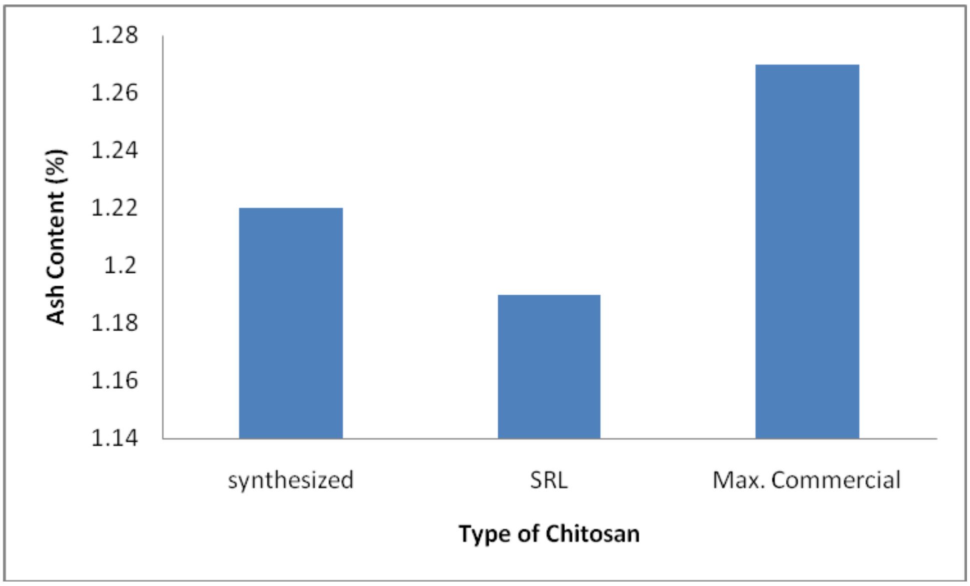


Figure 4..2.2: Ash content of the chitosan

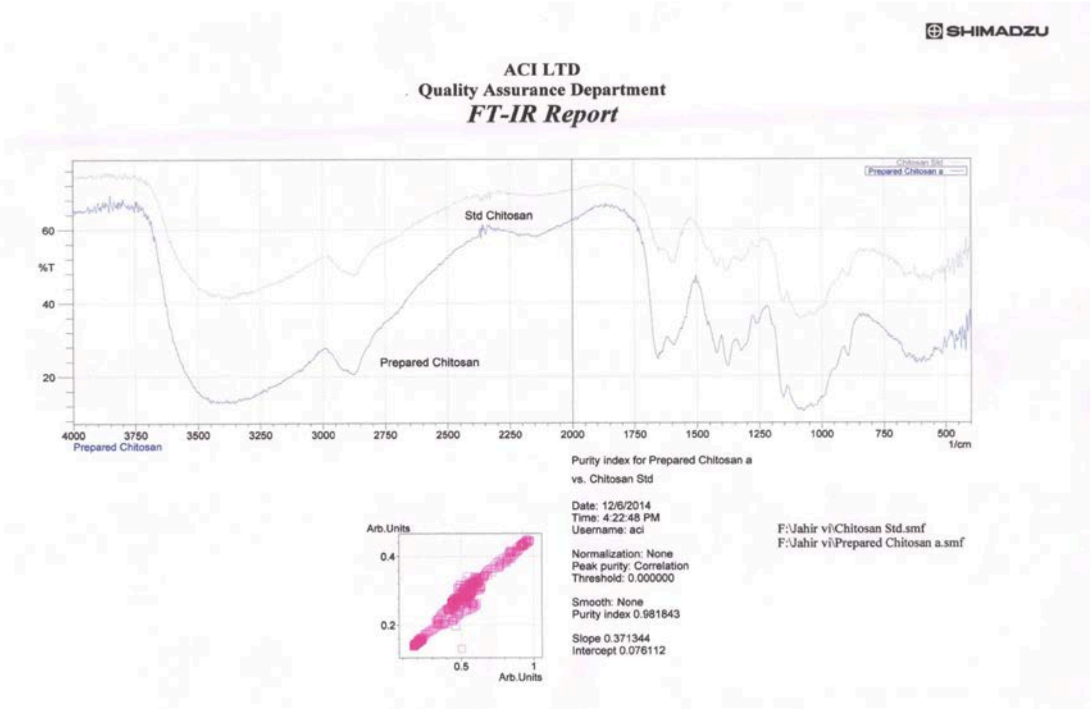
Table 4.2.1: Moisture, Ash content and Solubility of chitosan

Obs. No.	**Test Parameters**	**Synthesized %**	**Standard (SRL) %**	**Commercial %**
1	Moisture	1.25	1.24	1.00 – 1.30
2	Ash	1.22	1.19	~1.27
3	Solubility	Soluble in 1% CH_3COOH	Soluble in 1% CH_3COOH	Soluble in 1% CH_3COOH

Comparing the results of moisture, ash content and solubility it can be stated that chitosan has been extracted from the waste shrimp shell and is pure.

4.2.2 FTIR Spectroscopy

Figure 4.2.2.1 shown FTIR spectra of synthesized and standard chitosan. In the figure upper spectrum is for standard chitosan and lower spectrum corresponds to synthesized chitosan. From the figure it is seen that the broad absorption band at 3450-3400 cm^{-1} may be explained due to OH and amine N-H symmetrical stretching vibrations. A peak at 2950-2800 cm^{-1} was due to symmetric $-CH_2-$ stretching vibration attributed to pyranose ring [136]. A peak at 1156 cm^{-1} was assigned to the structure of saccharide. The sharp peak at 1350 cm^{-1} was assigned to $-CH_3$ in amide group [137]. The broad peak at 1021 and 1098 cm^{-1} indicated the C-O stretching vibration in chitosan and peaks at 1675 and 1600 cm^{-1} were due to -C=O stretching (amide I) and $–NH_2$ stretching (amide II). The absorption bands at 1200 cm^{-1} was assigned to the anti-symmetric stretching of C-O-C bridge, and 1100-1020 cm^{-1} were assigned to the skeletal vibrations involving the C-O stretching [138]. Results have also been tabulated in Table 4.2.2.1. Therefore it is seen that both synthesized and extra pure chitosan exhibit almost similar spectra. This result clearly states that chitosan has been extracted from the waste shrimp shell successfully.



ACI LTD
Quality Assurance Department
FT-IR Report

Purity index for Prepared Chitosan a
vs. Chitosan Std

Date: 12/6/2014
Time: 4:22:48 PM
Username: aci

Normalization: None
Peak purity: Correlation
Threshold: 0.000000

Smooth: None
Purity index 0.981843

Slope 0.371344
Intercept 0.076112

F:\Jahir vi\Chitosan Std.smf
F:\Jahir vi\Prepared Chitosan a.smf

Figure 4.2.2.1: FTIR Spectrum of Prepared and Standard Chitosan

Table 4.2.2.1: FTIR studies of pure chitosan

Wave length, (cm^{-1})	**mode & resolution**	**Tentative band**
3450-3400	symmetric stretching, very broad	-OH and amine N-H
2950-2800	symmetric stretching, broad	$-CH_2$ (pyranose ring)
1675	symmetric stretching, medium	-C=O (amide I)
1600	symmetric stretching, medium	-NH (amide II)
1350	bending, sharp	$-CH_2$ in amide group
1200	anti-symmetric stretching	C-O-C bridge
1100-1020	vibrations, broad	-C-O

4.2.3. Chitosan Based Metal Nanoparticle

Chitosan Based Metal NPs were synthesized by modified solution cast method. Four different NPs (Chi-Zn, Chi-Cu, Chi-Cu(II) and Chi-Cd NPs) of different size and shapes were produced. Figure 4.2.3.1-4.2.3.4 shows four different NPs. Chi-Zn NPs and Chi-Cd NPs are off white colored. Chi-Cu NPs is deep brown color and Chi-Cu(II) NPs is faded blue color. All the NPs possess different colors from the respective raw materials used during synthesis. This indicates that NPs were synthesized through chemical process. Chi-Zn, Chi-Cu(II) and Chi-Cd NPs were synthesized via similar experimental steps, while Chi-Cu need reduction at distillation process in presence of hydrazine. Produced NPs were characterized by FTIR, TGA and SEM analyses. FTIR spectra and data's have been presented in Figure 4.2.3.1-4.2.3.4 and Table 4.2.3.1-4.2.3.4. It is seen from the FTIR that all the spectra of the NPs (except Chi-Cd) are very similar to those of the synthesized chitosan. This indicates the presence of chitosan polymer in the NPs matrices.

Figure 4.2.3.1: Chi-Zn NPs

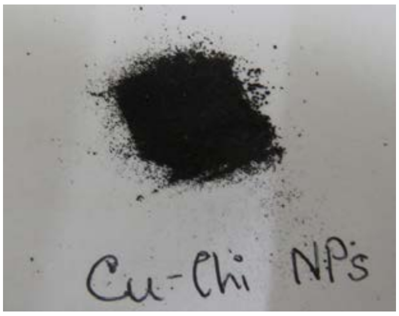


Figure 4.2.3.2: Chi-Cu NPs

Figure 4.2.3.3: Chi-Cu(II) NPs

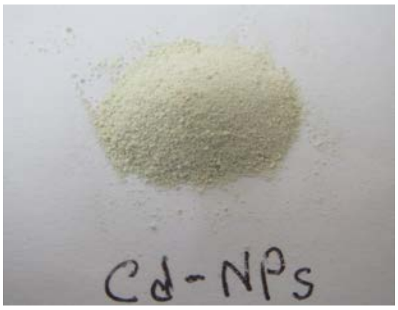
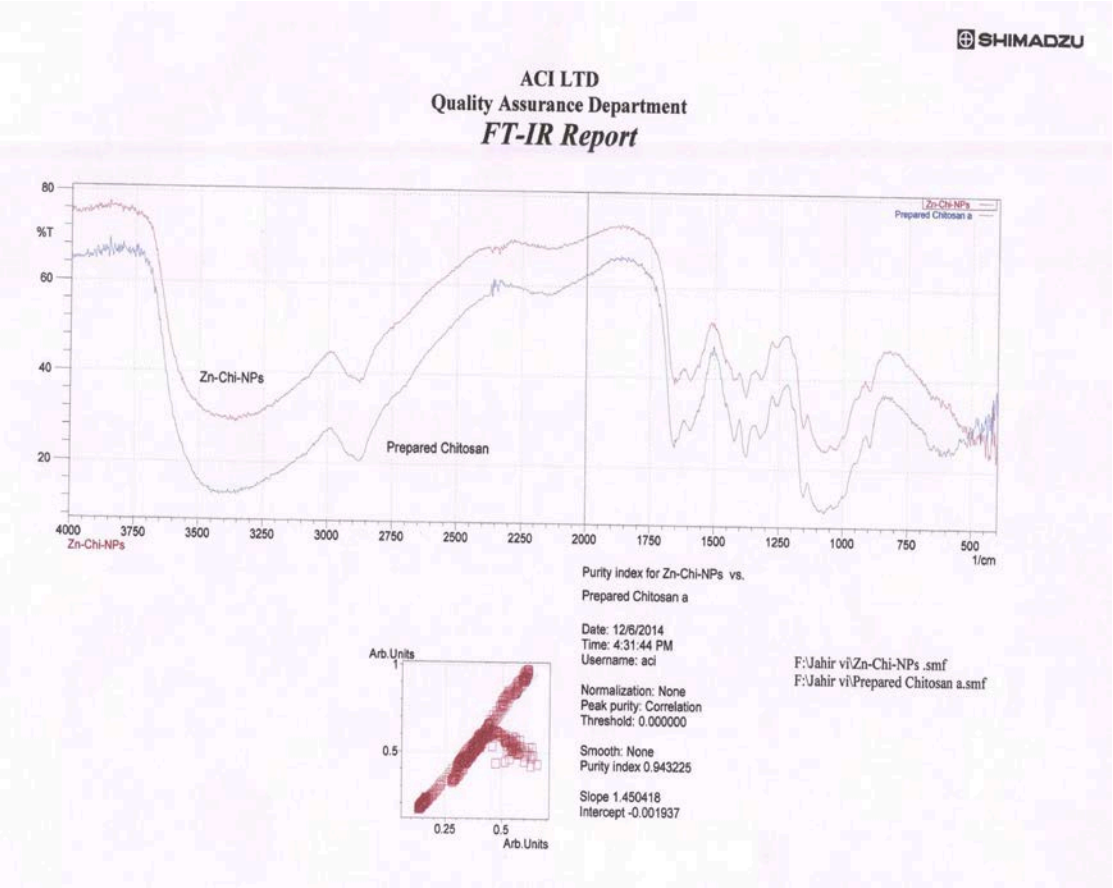


Figure 4.2.3.4: Chi-Cd NPs



ACI LTD
Quality Assurance Department
FT-IR Report

Purity index for Zn-Chi-NPs vs.
Prepared Chitosan a

Date: 12/6/2014
Time: 4:31:44 PM
Username: aci

Normalization: None
Peak purity: Correlation
Threshold: 0.000000

Smooth: None
Purity index 0.943225

Slope 1.450418
Intercept -0.001937

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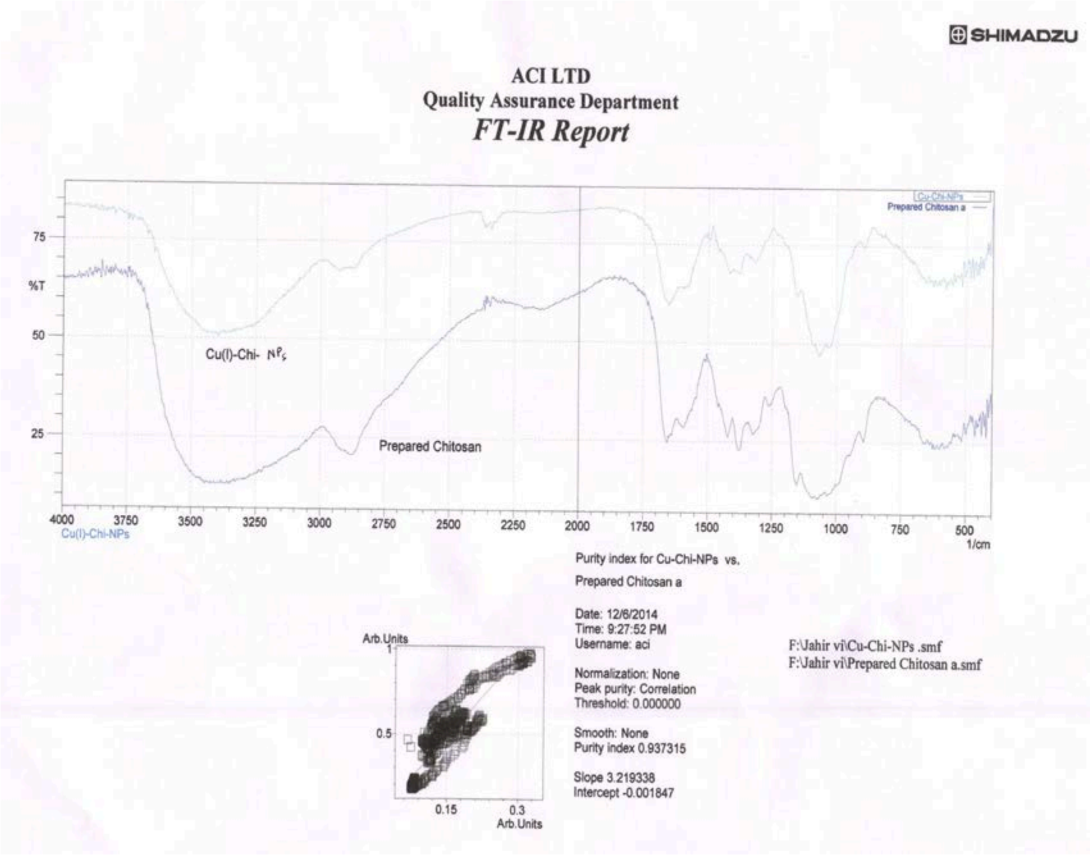
F:\Jahir vi\Prepared Chitosan a.smf

Figure 4.2.3.1: FTIR Spectrum of Prepared Chitosan and Chi-Zn NPs

Table 4.2.3.1: FTIR studies of Chitosan-Zn NPs.

Wave length, (cm^{-1})	mode & resolution	Tentative band
3450-3400	symmetric stretching, very broad	-OH and amine N-H
2950-2800	symmetric stretching, broad	$-CH_2$ (pyranose ring)
1675	symmetric stretching, medium	-C=O (amide I)
1600	symmetric stretching, medium	-NH (amide II)
1350	bending, sharp	$-CH_2$ in amide group
1200	anti-symmetric stretching	C-O-C bridge
1100-1020	vibrations, broad	-C-O



ACI LTD
Quality Assurance Department
FT-IR Report

Purity index for Cu-Chi-NPs vs.
Prepared Chitosan a

Date: 12/6/2014
Time: 9:27:52 PM
Username: aci

Normalization: None
Peak purity: Correlation
Threshold: 0.000000

Smooth: None
Purity index 0.937315

Slope 3.219338
Intercept -0.001847

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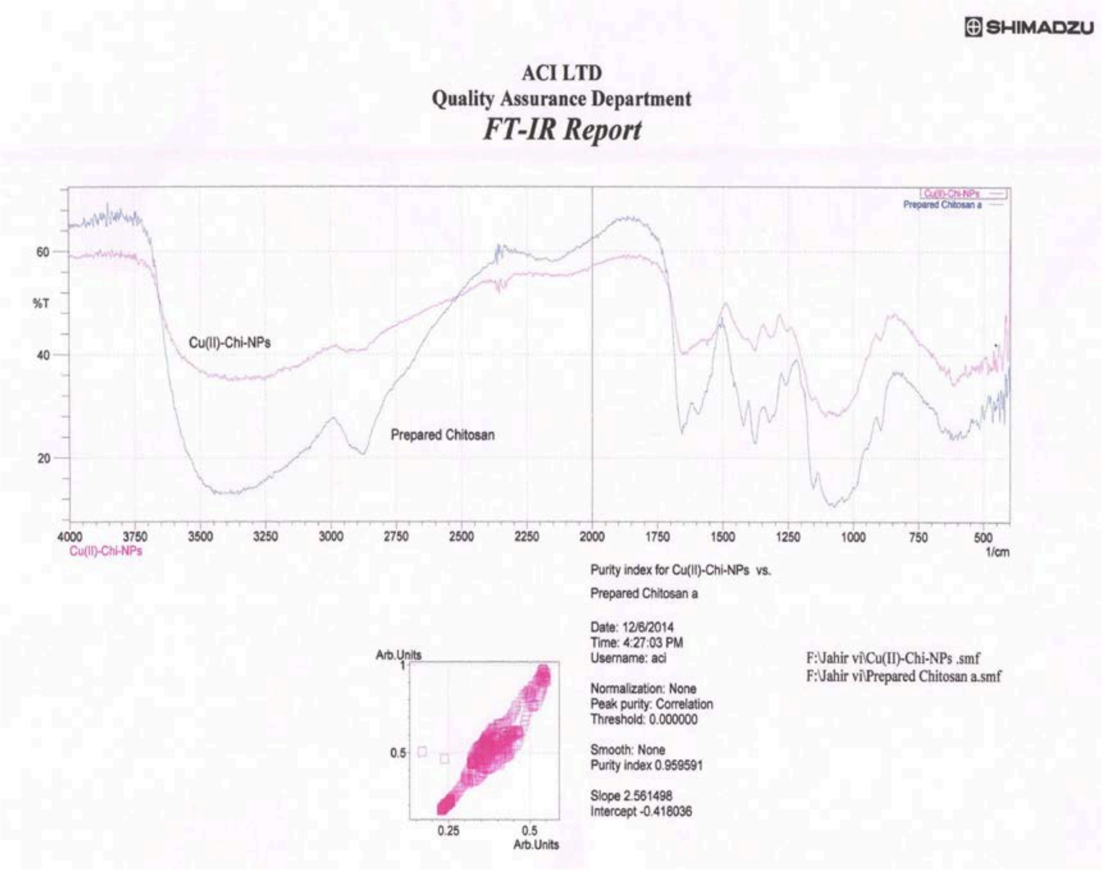
F:\Jahir vi\Prepared Chitosan a.smf

Figure 4.2.3.2: FTIR Spectrum of Prepared Chitosan and Chi-Cu metal NPs

Table 4.2.3.2: FTIR studies of Chi-Cu metal NPs.

Wave length, (cm^{-1})	**mode & resolution**	**Tentative band**
3450-3400	symmetric stretching, broad	-OH and amine N-H
2950-2800	symmetric stretching, broad	-CH_2 (pyranose ring)
1675	symmetric stretching, medium	-C=O (amide I)
1600	symmetric stretching, medium	-NH (amide II)
1350	bending, sharp	-CH_2 in amide group
1200	anti-symmetric stretching	C-O-C bridge
1100-1020	vibrations, broad	-C-O


ACI LTD
Quality Assurance Department
FT-IR Report

Purity index for Cu(II)-Chi-NPs vs.
Prepared Chitosan a

Date: 12/6/2014
Time: 4:27:03 PM
Username: aci

Normalization: None
Peak purity: Correlation
Threshold: 0.000000

Smooth: None
Purity index 0.959591

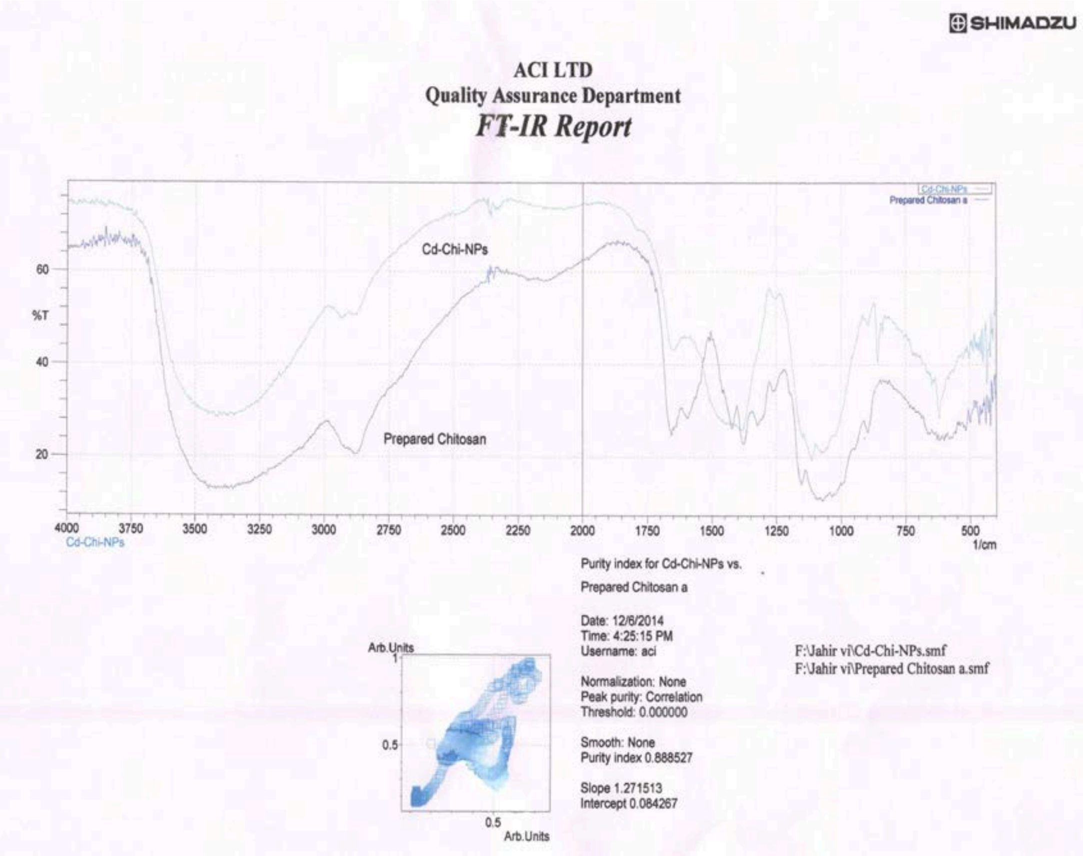
Slope 2.561498
Intercept -0.418036

F:\Jahir vi\Cu(II)-Chi-NPs .smf
F:\Jahir vi\Prepared Chitosan a.smf

Figure 4.2.3.3: FTIR Spectrum of Prepared Chitosan and Chi-Cu(II) NPs

Table 4.2.3.3: FTIR studies of Chi-Cu (II) NPs.

Wave length, (cm^{-1})	mode & resolution	Tentative band
3450-3400	symmetric stretching, broad	-OH and amine N-H
2950-2800	symmetric stretching, broad	-CH_2 (pyranose ring)
1675	symmetric stretching, medium	-C=O (amide I)
1600	symmetric stretching, medium	-NH (amide II)
1350	bending, sharp	-CH_2 in amide group
1200	anti-symmetric stretching	C-O-C bridge
1100-1020	vibrations, broad	-C-O



SHIMADZU

ACI LTD
Quality Assurance Department
FT-IR Report

Purity index for Cd-Chi-NPs vs.
Prepared Chitosan a

Date: 12/6/2014
Time: 4:25:15 PM
Username: aci

Normalization: None
Peak purity: Correlation
Threshold: 0.000000

Smooth: None
Purity index 0.888527

Slope 1.271513
Intercept 0.084267

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F:\Jahir vi\Prepared Chitosan a.smf

Figure 4.2.3.4: FTIR Spectrum of Prepared Chitosan and Chi-Cd NPs

Table 4.2.3.4: FTIR studies of Chi-Cd NPs.

Wave length, (cm^{-1})	mode & resolution	Tentative band
3450-3400	symmetric stretching, very broad	-OH and amine N-H
2950-2800	symmetric stretching, broad	-CH_2 (pyranose ring)
1675	symmetric stretching, medium	-C=O (amide I)
1600	symmetric stretching, medium	-NH_2 (amide II)
1350	bending, sharp	-CH_2 in amide group
1200	anti-symmetric stretching	C-O-C bridge
1100-1020	vibrations, broad	-C-O

4.2.4 Thermal gravimetric analysis (TGA)

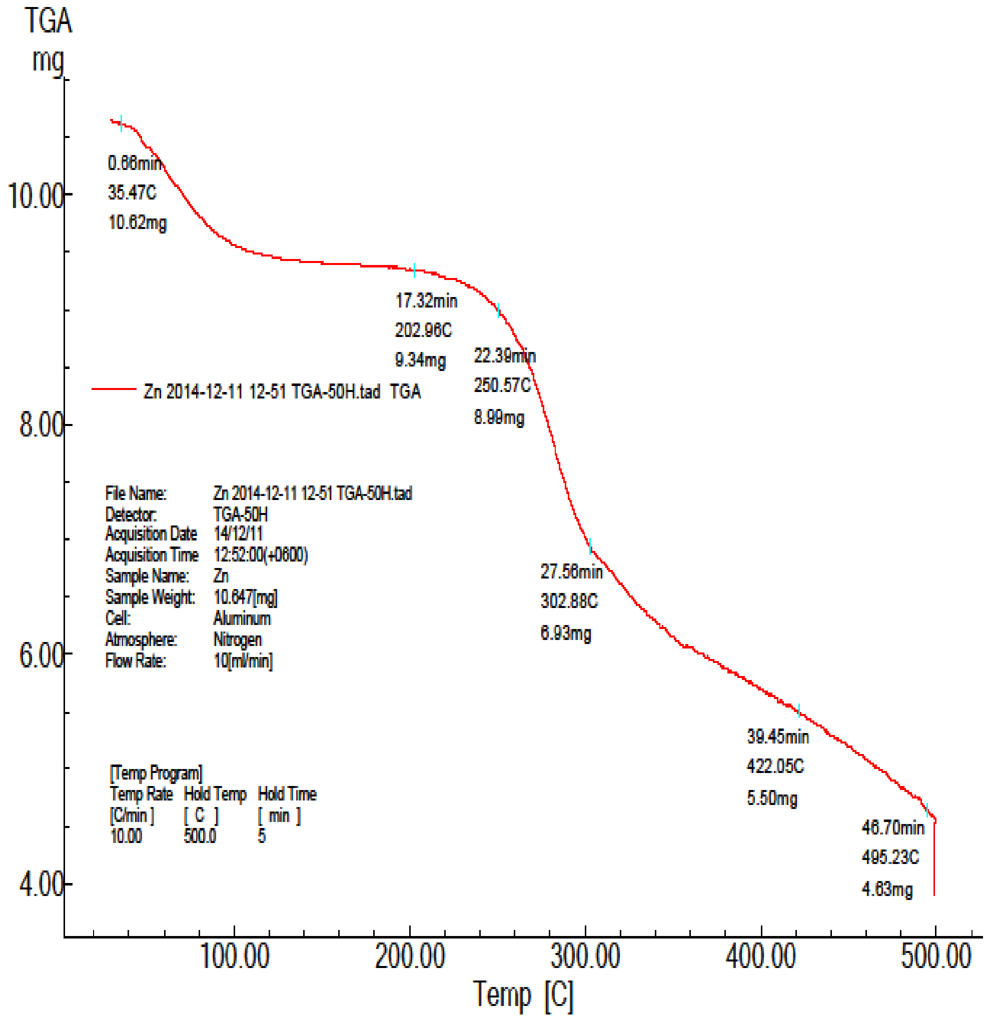
The thermal analyses were obtained by TA Isntruments-2100 thermal analysis system. Sample in the range of 16-30 mg, but accurate weighted case to case were taken into an aluminum made crucible of which the volume was 100 µL. Then it was inserted in the furnace. The crucible was hermetically sealed and a pinhole was punched into the crucible lid.

4.2.4.1 TGA of Chi-Zn NPs

Thermal details of chi-Zn NPs are shown in Table 4.2.4.1 and Figure 4.2.4.1. It can be seen from Figure 4.2.4.1 that three consecutive weight loss steps were observed. The first weight loss was about 6.79 wt% at 55-190 ºC, which might be responsible for the loss of moisture content of adhering water molecules and crystalline water. The second weight loss was about 25.80 wt% in the range of 220 to 320 ºC, which was due to scission of the ether linkage in the chitosan backbone. In the third stage, the weight loss was about 33.19 wt% in the range of 320-500 ºC, which may be corresponds to the thermal decomposition of glucosamine residues that means the loss of organic part of the NPs [139]. But the trouble, in the machine experiments of weight loss could not be continued further. The TG analysis also confirms the presence of chitosan polymer in the Chi-Zn NPs.

Table 4.2.4.1: TGA studies of Chi-Zn NPs

Percentage of Decomposition (%)	**Decomposition Temperature (ºC)**
10	100
20	270
30	290
40	310
50	430
57	500



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Detector: TGA-50H
Acquisition Date 14/12/11
Acquisition Time 12:52:00(+0600)
Sample Name: Zn
Sample Weight: 10.647[mg]
Cell: Aluminum
Atmosphere: Nitrogen
Flow Rate: 10[ml/min]

[Temp Program]

Temp Rate [C/min]	Hold Temp [C]	Hold Time [min]
10.00	500.0	5

Figure 4.2.4.1: TGA thermogram of Chitosan-Zn NPs.

4.2.4.2 TGA of Chitosan-Cu metal NPs

TGA details of chitosan-Cu(0) NPs is shown in Table 4.2.4.2 and Figures 4.2.4.2. It can be seen from Figure 4.2.4.2 that three consecutive weight loss steps were observed in the examined material. The first weight loss was about 19.70 wt% at 40 – 180 ºC, which might be responsible for the loss of moisture content of adhering water molecules and crystalline water. The second weight loss was about 35.87 wt% in the range of 200 to 400 ºC, which was due to scission of the ether linkage in the Chi backbone. In the third stage, the weight loss was about 21.57 wt% in the range of 420–500 ºC, which may be corresponds to the thermal decomposition of glucosamine residues that means the loss of organic part of the NPs [139]. But the problem in the machine, experiments of weight loss could not be continued further. The TG analysis also confirms the presence of chitosan polymer in the Chi-Cu NPs.

Table 4.2.4.2: TGA studies of Chi-Cu metal NPs

Percentage **of** Decomposition (%)	Decomposition Temperature (ºC)
10	90
20	180
30	230
40	290
50	350
60	430
70	470
77	500

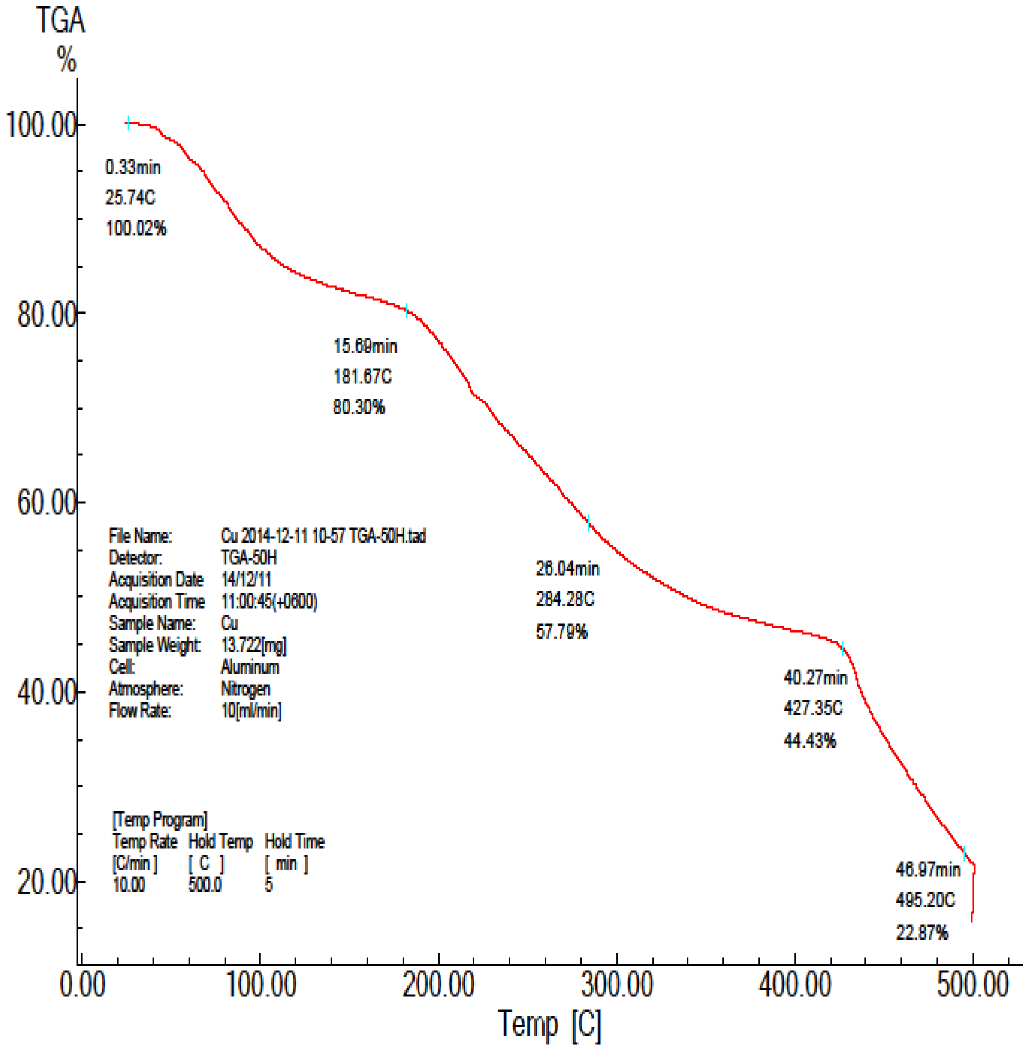


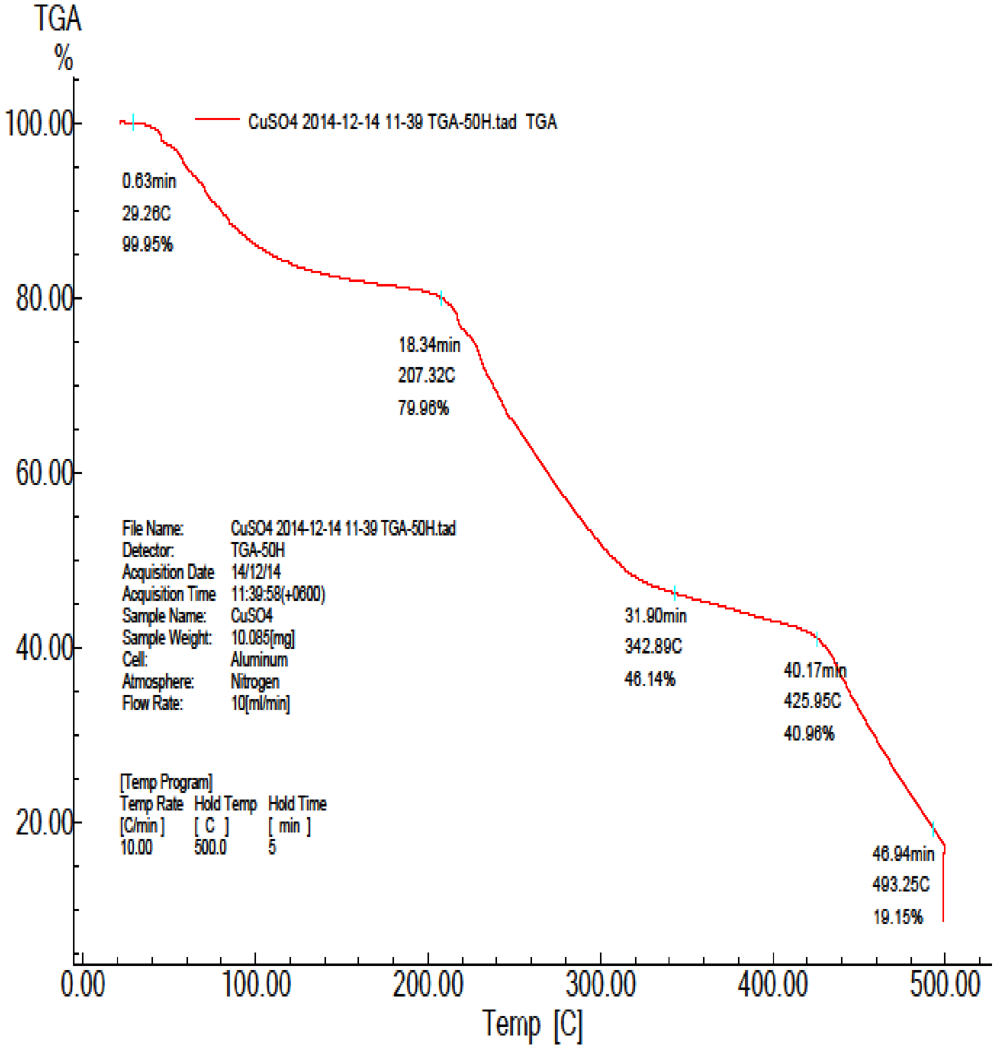
Figure 4.2.4.2: TGA thermogram of Chitosan-Cu metal NPs.

4.2.4.3 TGA of Chi-Cu(II) NPs

Thermal details of Chi-$CuSO_4$ NPs are shown in Table 4.2.4.3 and Figures 4.2.4.3. It can be seen from Figure 4.2.4.3 that three consecutive weight loss steps were observed in the Chi-$CuSO_4$ composite nano-material. The first weight loss was about 20.04 wt% at 45-200 ºC, which might be responsible for the loss of moisture content of adhering water molecules and crystalline water. The second weight loss was about 39.00 wt% in the range of 200 to 420 ºC, which was due to scission of the ether linkage in the chitosan backbone. In the third stage, the weight loss was about 21.81 wt% in the range of 420–500 ºC, which may be corresponds to the thermal decomposition of glucosamine residues that means the loss of organic part of the NPs [139]. But the difficulty, in the machine, experiments of weight loss could not be continued further. The TG analysis also confirms the presence of chitosan polymer in the Chi-Cu(II) NPs.

Table 4.2.4.3: TGA studies of Chi-Cu(II) NPs

Percentage of Decomposition (%)	Decomposition Temperature (ºC)
10	90
20	210
30	250
40	280
50	320
60	420
70	460
80	495



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Detector: TGA-50H
Acquisition Date 14/12/14
Acquisition Time 11:39:58(+0600)
Sample Name: CuSO4
Sample Weight: 10.085[mg]
Cell: Aluminum
Atmosphere: Nitrogen
Flow Rate: 10[ml/min]

[Temp Program]

Temp Rate [C/min]	Hold Temp [C]	Hold Time [min]
10.00	500.0	5

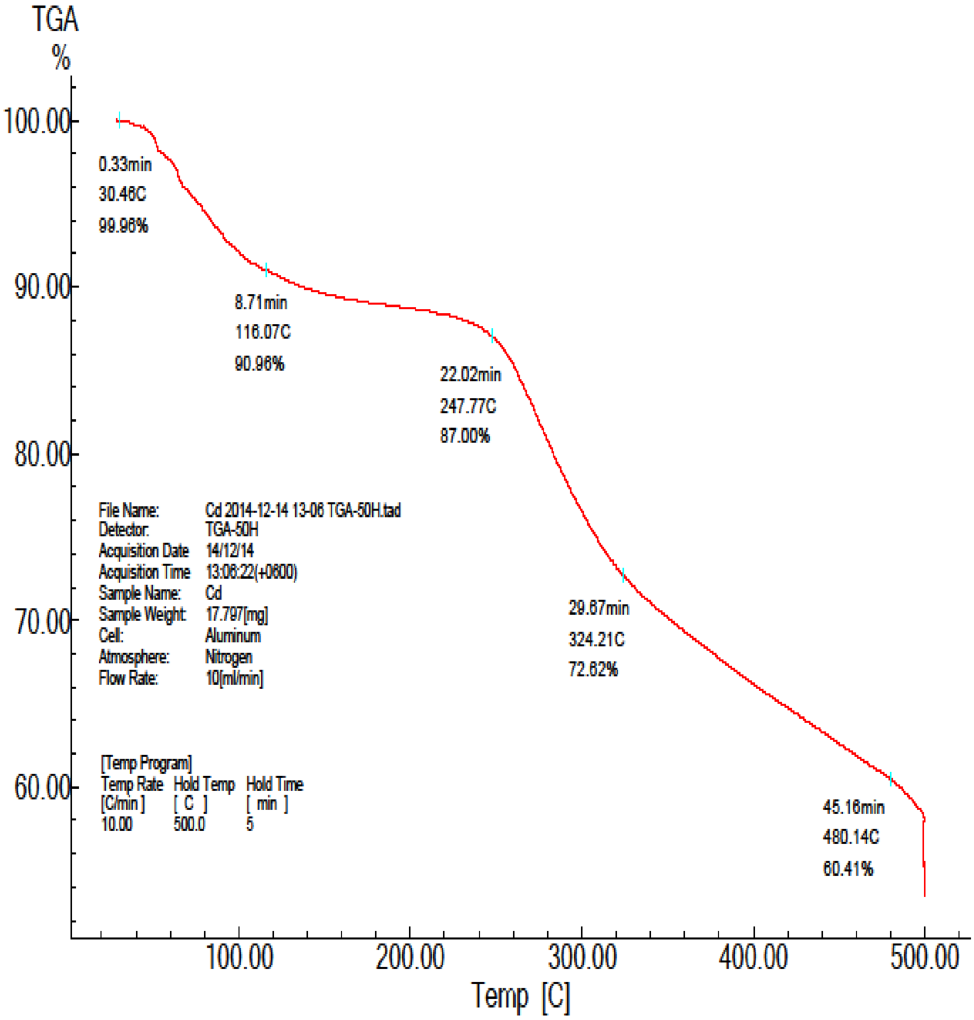
Figure 4.2.4.3: TGA thermogram of Chitosan-Cu(II) NPs.

4.2.4.4 TGA of Chi-Cd NPs

TGA details of Chi-Cd NPs are shown in Table 4.2.4.4 and Figures 4.2.4.4. It can be seen from Figure 4.2.4.4 that three consecutive weight loss steps were observed in the composite material. The first weight loss was about 15 wt% at 45 – 180 ºC, which might be responsible for the loss of moisture content of adhering water molecules and crystalline water. The second weight loss was about 13.38 wt% in the range of 220 to 320 ºC, which was due to scission of the ether linkage in the chitosan backbone. In the third stage, the weight loss was about 10.62 wt% in the range of 320–500 ºC, which may be corresponds to the thermal decomposition of glucosamine residues that means the loss of organic part of the NPs [139]. But the trouble in the machine experiments of weight loss could not be continued further. The TG analysis also confirms the presence of chitosan polymer in the Chi-Cd NPs.

Table 4.2.4.4: TGA studies of Chi-Cd NPs

Percentage of Decomposition (%)	**Decomposition Temperature (ºC)**
10	110
20	280
30	340
40	500



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File Name:          Cd 2014-12-14 13-06 TGA-50H.tad
Detector:           TGA-50H
Acquisition Date    14/12/14
Acquisition Time    13:06:22(+0600)
Sample Name:        Cd
Sample Weight:      17.797[mg]
Cell:               Aluminum
Atmosphere:         Nitrogen
Flow Rate:          10[ml/min]

[Temp Program]
Temp Rate   Hold Temp   Hold Time
[C/min ]    [  C  ]     [  min  ]
10.00       500.0       5
```

Figure 4.2.4.4: TGA thermogram of Chitosan-Cd NPs.

4.2.5 Scanning Electron Microscope analysis

4.2.5.1 SEM analysis of Chi-Zn NPs

The size and shape of nanoparticle was determined by using Scanning Electron Microscope. The SEM image was taken at X5,000 & X10,000 magnification. The images of the nanoparticles were illustrated in Fig 4.2.5.1. The image shows that most of the morphologies of Chi-Zn NPs are flakes like shape with smooth surface and the size of the particles varies from 10-50 nm. These results agreed fairly well with those of Radyum Ikono (2012) [140] and Saptashi Ghosh (2014) [141].

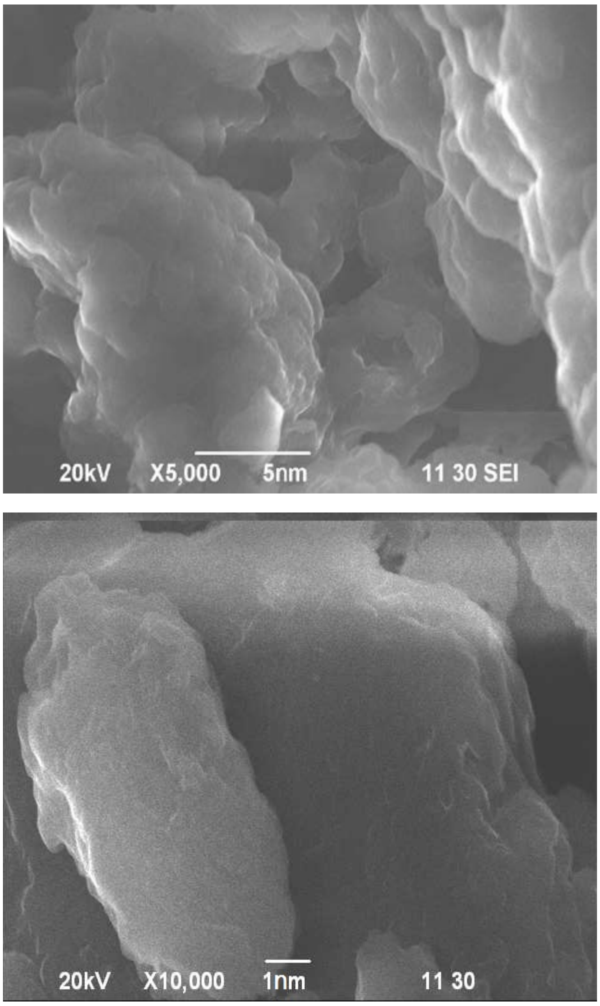

Figure 4.2.5.1: SEM of Chitosan-Zn NPs

4.2.5.2 SEM analysis of Chi-Cu metal NPs

The SEM image for Chi-Cu metal NPs was taken at X5,000 & X25,000 magnification. The images of the nanoparticles were illustrated in Fig 4.2.5.2 and it shows that most of the morphologies of Chi-Cu metal NPs are also flakes like shape as like CS-Zn NPs. But the surface was not smooth and the size of the particles varies from 10-50 nm.

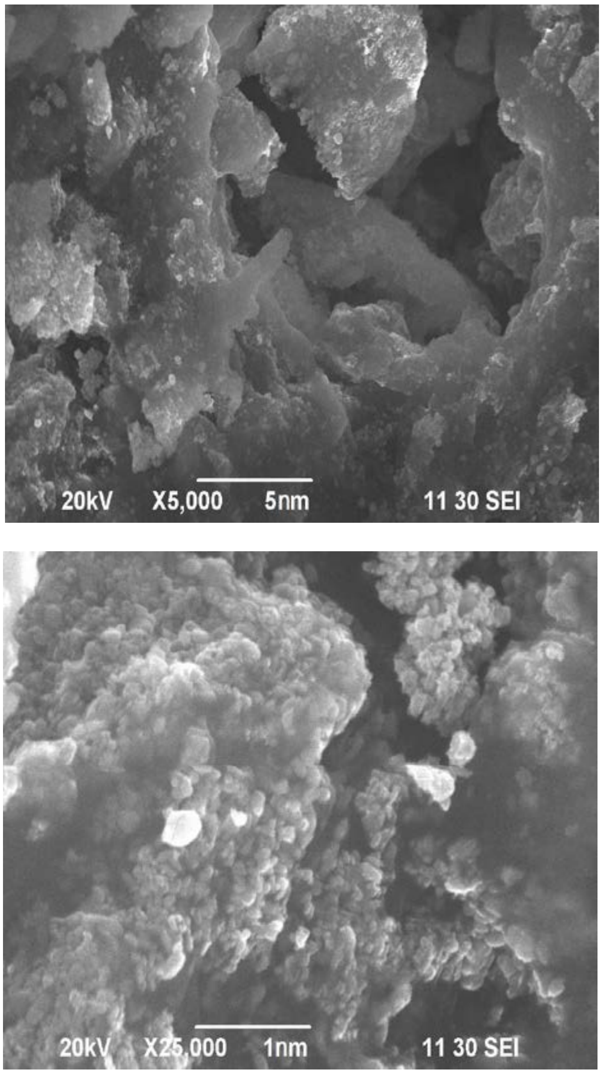

Figure 4.2.5.2: SEM of Chitosan-Cu metal NPs

4.2.5.3 SEM analysis of Chi-Cu (II) NPs

The SEM image for Chi-Cu (II) NPs was taken at X5,000 & X25,000 magnification. These SEM image is looking not so good. Here we can't differentiate the particular NPs. But from the Fig 4.2.5.3, roughly we can say that the morphologies of Chi-Cu (II) NPs are also flakes like shape and the sizes of the particles within 100 nm.

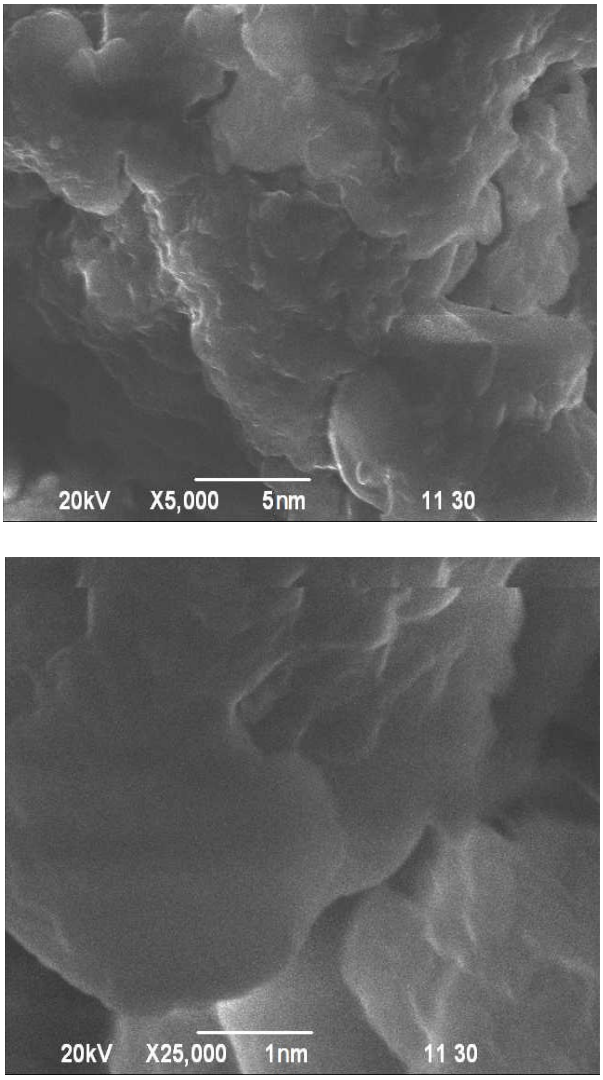

Figure 4.2.5.3: SEM of Chitosan-Cu (II) NPs

4.2.5.4 SEM analysis of Chi-Cd NPs

The SEM image for Chi-Cd NPs was taken at X10,000 & X25,000 magnification. The images of the nanoparticles were illustrated in Fig 4.2.5.4 and it shows that most of the morphologies of Chi-Cd NPs are spherical in shape and the size of the particles varies from 1-5 nm.

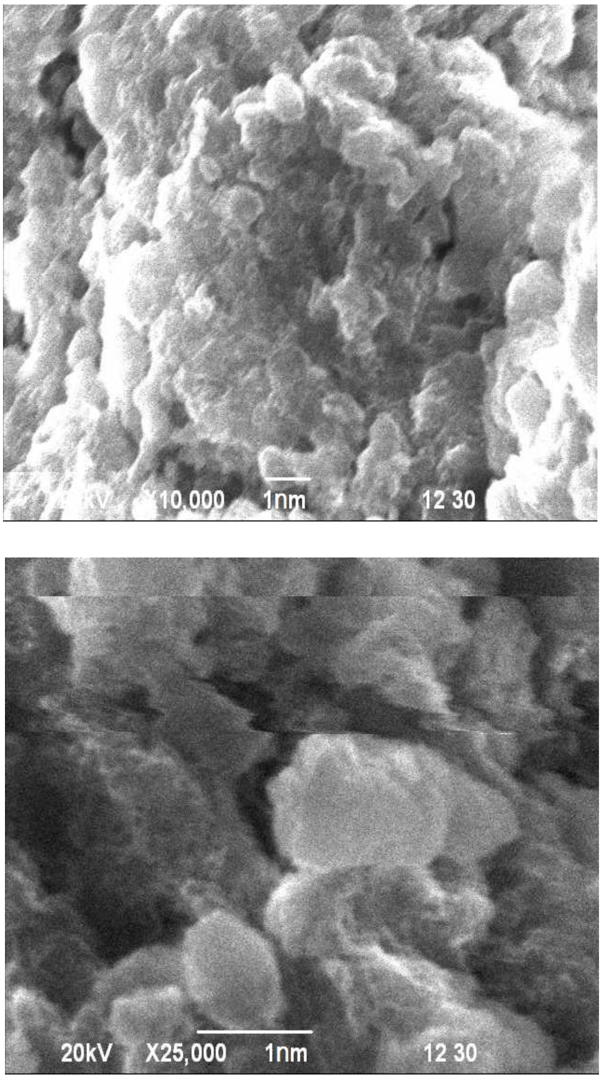

Figure 4.2.5.4: SEM of Chitosan-Cd NPs

4.2.6 Antibacterial Analysis

Biological studies were observed in the antibacterial activities of the targeted chitosan-metal NPs against Gram-negative (*Pseudomonas aeruginosa*, *Salmonella bovis morbificans*, *Salmonella typhi*, *Escherichia coli).* Result from the agar disc diffusion tests for antibacterial activities of target compounds are presented in Table 4.2.6.1 and illustrated in Figure 2.4.6.2 – 2.4.6.5 respectively. The diameters of zone of inhibition (in mm) of the standard drug kanamycin against bacteria *Pseudomonas aeruginosa* (Gram-negative), *Salmonella bovismorbificans* (Gram-negative), *Salmonella typhi* (Gram-negative), *Escherichia coli* (Gram-negative) were found to be 22, 22, 20 and 20 mm respectively & the graphical comparision of zone of inhibition of the chitosan-metal NPs with standard against the microorganisms are shown in Figure 4.2.6.1.

Under identical conditions Table 4.2.6.1 shows that Chi-Zn NPs (NPs 1) has 38, 31, 30 and 39 mm, Chi-Cu NPs (NPs 2) has 32, 36, 37 and 32 mm, Chitosan-$CuSO_4$ NPs (NPs 3) has 30, 35, 36 and 33 mm and Chi-Cd NPs (NPs 4) has 35, 31, 31 and 38 mm against *Pseudomonas aeruginosa, Salmonella bovis morbificans*, *Salmonella typhi*, *Escherichia coli* respectively. Whereas zone of inhibition of the chitosan against *Escherichia coli* are found 16 mm [142] & 19 mm [143] and against *Pseudomonas aeruginosa* is 12 mm [143].

From this antibacterial activity experiment we found that all the four prepared NPs are very much active. Even, all the complexes are more active than the standard drug *kanamycin*. Among the four complexes, Chi-Zn NPs shown the highest inhibition zones, nearly double than drug *kanamycin* against bacteria Pseudomonas aeruginosa and *Escherichia coli*. Chi-Cu metal NPs have the maximum inhibition zone in case of *Salmonella bovismorbificans* and *Salmonella typhi.*

Although the exact mechanism of the antimicrobial activity of chitosan remains ambiguous, six major mechanisms have been proposed in the literature, as follows [107, 109, 110]: (1) the interaction between the positively charged chitosan amine groups and the negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents; (2) the activation of several defense processes in the host tissue by the chitosan molecule acting as a water-binding agent and inhibiting various enzymes by blocking their active centers; (3) the action of chitosan as a chelating agent, selectively binding metals and then inhibiting the production of toxins and microbial growth; (4) the formation, generally by high molecular weight chitosan, of an impervious polymeric layer on the surface of the cell, thereby altering cell permeability and blocking the entry of nutrients into the cell; (5) the penetration of mainly low-molecular weight chitosan into the cystosol of the microorganism to bind DNA, resulting in interference with the synthesis of mRNA and proteins; and (6) the adsorption and flocculation of electronegative substances in the cell by chitosan, distributing the physiological activities of the microorganisms, causing their death.

Table 4.2.6.1: Results of the antibacterial activity of the different NPs

Bacteria code	Name of the bacteria	Diameter of inhibition zone of bacteria in different NPs* (mm)				
		1	2	3	4	Kanamycin 30µg/disc
A	*Psudomonas aeruginosa* (-ve)	38	32	30	35	22
B	*Salmonella bovis morbificans*(-ve)	31	36	35	31	22
C	*Salmonella typhi* (-ve)	30	37	36	31	20
D	*Escherichia coli* (-ve)	39	32	33	38	20

(1) Chi-Zn NPs*, (2) Chi-Cu NPs*, (3) Chi-Cu(II) NPs*, (4) Chi-Cd NPs*

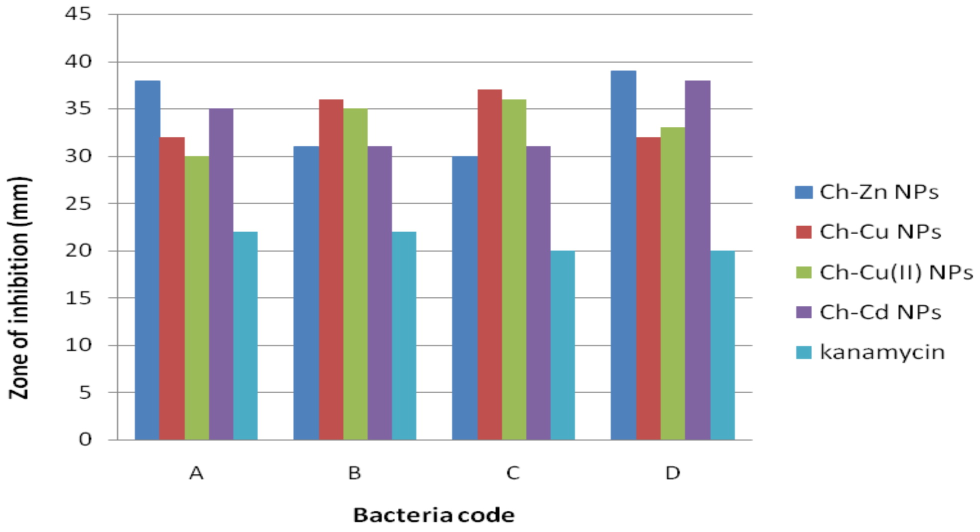

Figure 4.2.6.1: Graphical comparision of zone of inhibition of the NPs 1-4 with standard (Kanamycin)

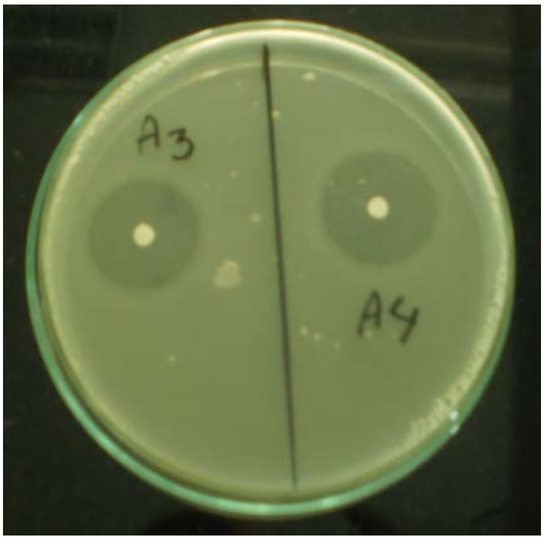

Figure 2.4.6.2: Photographic representation of zone of inhibition of NPs **3** and **4** respectively against the bacteria *Psudomonas aeruginosa.*

Figure 2.4.6.3: Photographic representation of zone of inhibition of NPs **1** and 2 respectively against the bacteria *Salmonella bovis morbificans.*

Figure 2.4.6.4: **P**hotographic representation of zone of inhibition of NPs **1** and **2** respectively against the bacteria *Salmonella typhi*.

Figure 2.4.6.5: Photographic representation of zone of inhibition of NPs **3** and **4** respectively against the bacteria *Escherichia coli*.

CHAPTER V

Conclusion

This research was devoted to extraction of chitosan from shrimp shell and preparation of chitosan based nanoparticles of various metals (Zn, Cu and Cd) for their biological application. Investigation of antibacterial activity of prepared chitosan-metal NPs against some bacteria was also a major part of our research. So, we have tried and successfully have prepared chitosan from shrimp shell and chitosan-metal NPs in our lab. We have characterized chitosan by FITR, moisture content, ash content, solubility test and NPs by using FTIR, TGA and SEM analysis. The findings can be concluded as-

- Chemical treatments, demineralization, deproteinization and deacetylation was adopted on getting chitosan from waste shrimp shell.
- Purity of the chitosan was confirmed by FITR, moisture content, ash content and solubility test and comparing the results with extra pure one.
- Chitosan based metal NPs (Chi-Zn NPs, Chi-Cu NPs, Chi-Cu(II) NPs and Chi-Cd NPs) synthesized by modified solution cast method.
- SEM analysis confirmed the sizes of NPs. Chi-Zn NPs and chi-Cu NPs varied from 10-50 nm and looks like flake. Chi-Cu(II) NPs are also flake like shape and the sizes of the particles within 100 nm. Chi-Cd NPs are spherical and the size of the particles varied from 1-5 nm.
- Prepared NPs showed enhanced antibacterial activity than standard drug kanamycin. Among the four complexes, Chi-Zn NPs shown the highest inhibition zones, nearly double than the standard drug kanamycin against bacteria Pseudomonas aeruginosa and Escherichia coli. Chi-Cu metal NPs have the maximum inhibition zone in case of Salmonella bovismorbificans and Salmonella typhi.

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