Research Article

Physicochemical and Functional Characterization of Chitosan Prepared From Shrimp Shells and Investigation of Its Antibacterial, Antioxidant and Tetanus Toxoid Entrapment Efficiency

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ABSTRACT

The objective of this research was to extract chitosan from shrimp shells involving demineralization, deprotenization, decolorization and deacetylation processes and to investigate its physicochemical, antioxidant, antibacterial and pharmaceutical properties in comparison with commercially available acetic acid soluble chitosan (C_1) and water soluble chitosan (C_2). Prepared chitosan (C_3) showed 75% DD by FT-IR spectra indicating its solubility in 1% acetic acid while higher degree of deacetylation 85% was observed for C_2. Similarly, the viscosity 602 cps and viscosity-average molecular weight 1249 kDa were also significantly higher for C_2 compared to C_1 and C_3. Among the micrometric properties bulkiness and turbidity was significantly higher for C_3 and tapping density (0.47 g/ml), Carr's index (25%) and Hausner's ratio (1.33) were higher for C_1 suggesting its overall good flow properties. Similarly FBC and WBC were significantly higher for C_3. Significant differences ($P<0.05$) were observed in all three chitosan samples for free radical scavenging activity being higher for C_3, suggesting it as a good antioxidant source. Also, C_3 showed higher antimicrobial activity against both gram positive and gram negative bacteria. Weight loss was up to 51% for C_3 by thermo gravimetric analysis revealing its relatively lower thermal stability as compared to both C_1, C_2 having weight loss up to 41%. Placebo microspheres showed smooth uniform surfaces, whereas, rough surfaces were obtained for tetanus toxoid encapsulated microspheres as confirmed by SEM for all the chitosan samples. The particle size of formulated chitosan microsphere was ranging between 5–50 µm and their tetanus toxoid entrapment efficiency obtained were 89%, 81% and 84% respectively for C_1, C_2 and C_3. Thus, overall results for chitosan prepared from shrimp shells suggested that it has good micrometric properties and functional properties which can be used as an antimicrobial, antioxidant and a pharmaceutical molecule for mucosal vaccine development.

Keywords: Antioxidant agent, Antimicrobial agent, Chitosan, Glutaraldehyde saturated toluene, Microspheres.

INTRODUCTION

Chitosan a poly-β-(1-4)-2-amino-2-deoxy-D-glucopyranose is a deacetylated product of chitin β-(1-4)-2-acetamido-2-deoxy-D-glucan.[1] which is a structural polysaccharide found in crustacean, insects and some fungi. Chitosan can be characterized in terms of its quality, intrinsic properties such as purity, molecular weight, viscosity, degree of deacetylation (DD) and physical forms. Furthermore, the quality and properties of chitosan product may vary widely because many factors in the manufacturing process can influence the characteristics of the final product. Native chitin molecular weight is larger than one million daltons and commercial chitosan have the molecular weight range of 50-2000 kDa, depending on the processing conditions and grades of the product.[2,3] Chitin with a degree of deacetylation of 75% or above is generally known as chitosan.[4] An increase in either temperature or strength of sodium hydroxide solution can enhance the removal of acetyl groups from chitin, resulting in a range of chitosan molecules with different properties and hence its applications.[5] Usually 1—3% aqueous acetic acid solutions are used to solubilize chitosan. Drawback with chitin and chitosan is that it is difficult to dissolve them in water and in neutral pH. So, water soluble derivatives of chitosan and chitosan have been synthesized by various researchers by chemical modification. These chemical modifications result in the formation of hydrophilic chitin or chitosan which have more affinity to water or organic solvents.[6]

Chitosan is among the most promising biomaterials in the world and have attracted considerable interest in the field of dietary supplements, water treatment, food preservation, agriculture, cosmetics, pulp and paper and has wide medicinal application.[7] Antimicrobial activities of chitosan relied on numerous intrinsic and extrinsic factors such as pH, micro organism species, presence or absence of metal cations, pKa, and molecular weight (Mw) and degree of deacetylation (DD) of chitosan.[8] The broad spectrum antibacterial activity of chitosan was first proposed by Allan and Hardwiger.[9] along with great commercial potential. Antimicrobial activity of chitosan has been demonstrated against many bacteria, filamentous fungi and yeasts, but lower toxicity was reported toward mammalian cells.[10–13] Antioxidant activity is one of the well known functions of chitosan and its derivatives.[14] Many studies have shown that chitosan inhibit the reactive oxygen species (ROS) and prevent the lipid oxidation in biological systems. Several mechanisms about the antioxidant action of chitosan have been proposed.[15,16] The interaction of chitosan with metal ions could involve several complex actions including adsorption, ion-exchange and chelation.[17] The hydroxyl

group's (—OH) and amino group's ($—NH_2$) in chitosan are the key functional groups for its antioxidant activity.[10,15,17]

As a natural cationic polyelectrolyte material, chitosan has received attention since decade for microspheres or nanospheres.[18] Chitosan microspheres are one of the most widely studied drug delivery systems for the controlled release of drugs such as antibiotics, anticancer agents, proteins, peptide drugs and vaccines.[19] In addition, a chitosan microsphere has a special feature of adhering to the mucosal surface and transiently opening the tight junction between epithelial cells.[20] and also has good coagulation ability and immune stimulating activity.[21] Chitosan microspheres plays important role in drug delivery as it is biocompatible with living tissues since it does not cause allergic reactions. It breaks down slowly to harmless products (amino sugars), which are completely absorbed by the human body.[22] Chitosan has showed special quality of gelling upon contact with anions thus allowing the formation of beads under very mild conditions.[23] Chitosan microspheres have also shown a pH dependent swelling behavior which makes them appropriate for the delivery of drugs or vaccines in the gastric cavity.

In the present study, chitosan was extracted from shrimp shells and its physicochemical, antioxidant, antibacterial and tetanus toxoid entrapment properties were analyzed in comparison with commercially available acetic acid soluble chitosan and water soluble chitosan.

MATERIALS AND METHODS

Low molecular weight chitosan soluble in 1% acetic acid (C_1), water soluble chitosan (C_2), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was obtained from Sigma-Aldrich (USA). Tetanus toxoid (TT, 3000 Lf/ml) was obtained from Serum Institute of India, Ltd., Pune. Butylated hydroxyanisole (BHA) was obtained from Sisco research laboratories (SRL). Glutaraldehyde, Span-80, Nutrient broth (NB) and Nutrient agar (NA) as well as all other chemicals and reagents used for various analyses were of analytical grade and were purchased from HiMedia laboratories (India). Detergent compatible protein assay (DC) kit was obtained from Bio-Rad (USA).

Microorganism collection

Microorganism for the assessment of chitosan antibacterial activity was obtained from National Collection of Industrial Microorganisms(NCIM), Pune.They were *Escherichia coli* (NCIM, 2931) and *Klebsiella pneumoniae* (NCIM, 2957) as a gram negative strains and *Staphylococcus aureus* (NCIM, 2079), *Sarcina lutea* (NCIM, 2493), *Staphylococcus epidermidis* (NCIM, 2493) and *Bacillus subtilis* (NCIM, 2063) used were Gram positive in nature.

Preparation of chitosan

Shrimp shells were collected from Mumbai, Local market. Shrimp shells were scraped free of loose tissue, washed with cold water and dried in oven at 60 °C. Dried shells were grind in mixer, sieved to particle size 500 µm followed by 250 µm and were stored in air tight containers at ambient temperature until further processed. No et al.[24] modified method was used for preparation of chitosan which included demineralization, deproteinization, decoloration, and deacetylation processes. Initially, the shrimp shells powder was demineralized with 1N HCl, with solid to solvent ratio of 1:15 (w/v) with constant stirring for 30 min at ambient temperature followed by vacuum filtration. The residue was washed for 30 min with tap water and then oven dried for overnight. Further, demineralized powder was deproteinized with 3.5% NaOH solution in the ratio 1:10 (w/v) for 2 h, at 65 ^{o}C with constant stirring. The mixture was vacuum filtered and the residue was washed as above and oven dried for 2 h. Decoloration of the residue was carried out using acetone wash for 10 min and dried for 2 h at ambient temperature, followed by bleaching with 1% sodium hypochlorite solution for 5 min at ambient temperature with solid to solvent ratio 1:10 (w/v). The residue obtained was then washed with tap water and vacuum dried for 2 h. Further, removal of acetyl groups from chitin was achieved by refluxing for 12 h at 110 ^{o}C using 50% sodium hydroxide with solid to solvent ratio 1:15 (w/v). The resulting chitosan (C_3) obtained was washed and neutralized with tap water followed by rinsing with hot distilled water at 90 ^{o}C, filtered and oven dried at 60^{o}C for 24 h and stored in airtight containers till further use.

Physicochemical characterization of chitosan

Moisture

Moisture content of all the C_1, C_2, and C_3 chitosan samples was determined by using the gravimetric method reported by Black et al.[25] Briefly, the samples were dried to constant weight in oven at 105 °C and moisture content was calculated as follows:

$$\text{Moisture (\%)} = \frac{W1 - W2 \times 100}{W_1}$$

Where, W_1 = weight (g) of sample before drying.

W_2 = weight (g) of sample after drying.

Ash

The ash content was determined using laboratory muffle furnace (Fourtech, Mumbai, India) as per AOAC method.[26] In brief, 1 g of each sample was taken in pre weighed crucible with lid and placed in muffle furnace and was maintained at 575 ± 10 °C for 6 h. After cooling, the crucibles were removed from the furnace and were placed in the desiccators. The above process of heating and cooling was repeated until constant weight was obtained. The ash with crucible and lid was weighed

when sample turns to gray. The percent ash was calculated as follows:

$$\text{Ash (\%)} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \times 100$$

Viscosity

Viscosity of the chitosan was determined with a Brookfield viscometer (Model DV-IIþ Brookfield) as described by Fernandez.[27] with few modifications. Chitosan solution was prepared using 1% acetic acid at 1% concentration on a dry weight basis. Measurement was made in triplicates using spindle number 2 at 50 rpm at 28 ^{0}C for each solution and the values were reported in centipoises (cPs) units.

Viscosity-average molecular weight (Mv)

The molecular weight in Dalton for all chitosan samples was measured by dissolving 1% chitosan (w/v) each in 1% glacial acetic acid. Intrinsic viscosity (ŋ) was measured using Ubbelohde glass capillary viscometer as reported by No et al.[28] and molecular weight of chitosan was determined using Mark-Houwink equation relating intrinsic viscosity with empirical viscometric constants; K = 1.81*10^{3}cm^{3}/g and a = 0.93 for chitosan as follows; ŋ = KM^{a}.

Degree of deacetylation (DD)

To investigate the degree of deacetylation in the sample, Fourier transform infrared spectrometry (FT–IR) was employed, using (Shimadzu FT–IR–8400) instrument available at our department. DD determined using the equation proposed by Domszy and Roberts et al.[29] as given below;

$$DD = 100 - [(A_{1655}/A_{3450}) \times 100/1.33]$$

Where, A_{1655} were the absorbance at 1655 cm^{-1} of the amide–I measure of N-acetyl group; A_{3450} were the absorbance at 3450 cm^{-1} of hydroxyl bond as integral standard to correct differences in different chitosan powder; 1.33 denoted the value of ratio A_{1655}/A_{3450} for fully N-acetylated chitosan. It was assumed that the value of this ratio is zero for fully deacetylated chitosan having rectilinear relationship between the N-acetyl group content and the absorbance of the amide-I band.

Evaluation of micrometric properties

Chitosan samples were characterized for various micrometric properties like bulk density, bulkiness, tapped density, Carr's index and Hausner's ratio**.** Bulk density of chitosan was determined by using modified method of Wang and Kinsella.[30] In brief, 1 g of chitosan sample earlier passed through 250 mesh size was placed in a 15 ml tapered graduated centrifuge tube and was vibrated on a vortex mixer for 1 min and placed by gently tapping the tube on the bench top for 10 times. The volume of a sample was recorded in ml and the bulk density was calculated using Eq. (1). The reciprocal of bulk density was measured as bulkiness of chitosan as given in Eq. (2). Tapped density was determined by placing a graduated cylinder, containing a known amount of powder and was operated for fixed number of taps (50). The tapped density was computed by using the weight of powder in cylinder and its tapped volume.[31] as given in Eq. (3). Carr's index, an important parameter to study compressibility behavior of powder blend was calculated from the results of bulk density and tapped density using Eq. (4). Hausner's ratio, a measure of flow ability of drug was calculated using Eq. (5) as follows;

$$\text{Bulk density} = \frac{\text{Weight of powder (g)}}{\text{Volume of powder (ml)}} \quad \ldots\ldots\ldots (1)$$

$$\text{Bulkiness} = 1/\text{Bulk density} \quad \ldots\ldots\ldots\ldots\ldots (2)$$

$$\text{Tapped density} = \frac{\text{Weight of powder (g)}}{\text{Tapped volume of powder (ml)}} \quad \ldots\ldots\ldots (3)$$

$$\text{Carr's index} = \frac{\text{Tapped density-Bulk density}}{\text{Tapped density}} \times 100 \quad \ldots\ldots\ldots\ldots (4)$$

$$\text{Hausner's Ratio} = \frac{\text{Tapped density}}{\text{Bulk density}} \quad \ldots\ldots\ldots\ldots\ldots (5)$$

Turbidity

Turbidity of chitosan sample was determined using Jackson turbidity unit and the values obtained were expressed as nephelometric turbidity unit (NTU).

Functional properties

Water binding capacity (WBC)

C_1 and C_2 chitosan sample was measured using a modified method of Wang and Kinsella.[30] Water absorption was initially carried out by weighing a centrifuge tube containing 0.5 g of sample each. 10 ml of water was added by mixing on a vortex mixture for 1 min to disperse the sample. The content was left at ambient temperature for 30 min with intermittent shaking every 10 min and was centrifuged at 2000 rpm for 25 min. The supernatant was decanted and the tube was weighed again. WBC was calculated as follows:

$$\text{WBC (\%)} = \frac{\text{Water bound (g)}}{\text{Sample weight (g)}} \times 100$$

Fat binding capacity (FBC)

Chitosan sample was measured using a modified method of Wang and Kinsella.[30] Fat absorption was initially carried out by weighing a centrifuge tube containing 0.5 g of sample. To this 10 ml of oil was added by mixing on a vortex mixture for 1 min to disperse the sample. The content was left at ambient temperature for 30 min with intermittent shaking every 10 min and was centrifuged at 2000 rpm for 30 min. After that supernatant was decanted the tubes were weighed again. FBC was calculated as follows:

$$\text{FBC (\%)} = \frac{\text{Fat bound (g)}}{\text{Sample weight (g)}} \times 100$$

Antioxidant activity

DPPH scavenging assay

DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of all chitosan samples was measured by DPPH assay according to reported literature.[31] with minor modification. In brief, sample (0.5 mg/ml) each, was taken in 50% methanolic solution containing 1 ml of 1 mM 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in 0.5% acetic acid. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm against blank as 50% methanol using a spectrophotometer (Shimadzu, Kyoto, Japan). Butylated hydroxyanisole (BHA) was used as a standard for comparison. Scavenging activity of chitosan was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{(Abs control – Abs sample)}}{\text{Abs control}} \times 100$$

Where, Abs control was the absorbance of DPPH and methanol; Abs sample was the absorbance of DPPH radical and chitosan samples each of C_1, C_2 and C_3.

ABTS scavenging assay

The scavenging activity of chitosan samples against ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radicals was determined by following the method described by Re et al.[32] The ABTS radical cations were pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium per sulfate and incubating for 12–16 h in the dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the ABTS solution was equilibrated to O.D. 0.70 (± 0.02) by diluting with water. Then, 1 ml of this solution was mixed with 50 µl of the test sample (0.5 mg/ml) and the absorbance was measured at 734 nm after 5 min. The percentage inhibition of ABTS by the chitosan samples was calculated and was compared with BHA (0.05 mg/ml). ABTS scavenging activity of chitosan was calculated as follows:

$$\text{ABTS scavenging activity (\%)} = \frac{\text{(Abs control – Abs sample)}}{\text{(Abs control)}} \times 100$$

Where, Abs control is the absorbance of ABTS radical and methanol, Abs sample is the absorbance of ABTS radical and chitosan samples each of C_1, C_2 and C_3.

Nitric oxide scavenging assay

The method by Bakthavatchala et al.[33] was adopted to determine the nitric oxide radical scavenging activity of chitosan sample. In brief, sodium nitroprusside (10 mM aqueous 2 ml) was mixed with chitosan sample (0.5 mg/ml) and was incubated at 37°C. After 150 min, 0.5 ml incubated solution was mixed with 0.5 ml griess reagent prepared using 1 ml sulphanilic acid (0.33%) in 20% glacial acetic acid and mixed at room temperature for 5 min. Further, 1 ml of N-(1-napthyl) ethylene diamine dichloride (0.1% w/v) was added and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The percent of scavenged nitric oxide with respect to control was calculated, scavenging activity of chitosan was calculated as follows:

$$\text{NO scavenging activity (\%)} = \frac{\text{(Abs control – Abs sample)}}{\text{Abs Control}} \times 100$$

Where, Abs control is the absorbance of methanol and Abs sample is the absorbance of nitric oxide radical and chitosan samples each of C_1, C_2 and C_3.

Antibacterial properties

The activity of all the chitosan samples was tested against six bacterial strains as mentioned above. The Gram negative strains tested included *E. coli*, *K. pneumonia* and the Gram positive strains used were *S. aureus, B. subtilus, S. epidermidis* and *S. lutea.* The nutrient broth (NB) media was selected as growth medium for revival of all microbial cultures. The pH of medium was adjusted to 6.5–7.0 pH and it was sterilized by autoclaving it for 20 min at 121 °C temperature and 15 psi pressure. Bacterial cultures 1% (v/v) were inoculated aseptically under laminar air flow cabinet in nutrient broth (NB) media. Furthermore, cultures were incubated at 37 °C, 140 rpm in shaking incubator for 24 h. After completion of incubation period the turbidometric measurement for cell saturation using spectrophotometer at 600 nm was done to ensure the bacterial culture growth. Chitosan taken for assessing the antibacterial activity was 250µg/well. The 100 µl volumes of microorganisms were spread plated on nutrient agar (NA) medium from their respective revived cultures as reported by Tareq et al.[34]

Thermo gravimetric analysis (TGA)

TGA and differential thermo gravimetric (DTA) of all chitosan samples were simultaneously carried out using, thermal analyzer (SHIMADZU-DTG-60H). Approximate 5 mg sample was taken for analyzing devolatilization characteristics at the temperature range 30 °C – 700 °C with the rate of 10 °C/min under nitrogen atmosphere.

Chitosan microspheres preparation, characterization and drug entrapment

Placebo microsphere

Chitosan placebo microspheres were prepared following the techniques reported by Jameela et al.[35] with some modification. Briefly, 1.5% chitosan was dissolved in 1% acetic acid containing 1% NaCl stirred well for 1 h to form chitosan gel and kept overnight for stabilization. Glutaraldehyde saturated toluene (GST) was used as a cross linking agent in the preparation of microspheres. 100 ml of Glutaraldehyde and 100 ml of toluene was taken in a beaker and it was stirred at 1200 rpm for 1 h and kept overnight for stabilization. The upper toluene layer saturated with Glutaraldehyde was separated out using separating funnel and was used as a GST. For preparation of chitosan microspheres, 4 ml of 1.5% chitosan gel was dissolved in 1.0 ml of 0.01 N HCl aqueous solutions and stirred well for 5 min. To this mixture 50 ml

toluene containing 10% span-80 was added and stirred well for 1.30 h. Furthermore, 10 ml of GST having 10% span-80 was added drop by drop using syringe at the rate of 1 ml/min with continuous stirring resulting in to formation of microspheres. Formulated microspheres were centrifuged at 2000 rpm followed by thrice washing with toluene and acetone and were further air dried and stored in air tight container at room temperature. These microspheres were spread on the clean glass slide using glass rod and observed under the optical microscope and were further characterized using scanning electron microscope.

Tetanus toxoid (TT) entrapped chitosan microspheres

TT entrapped chitosan microspheres were prepared following the techniques reported by Jameela et al.[35] with some modification. Briefly, 1% (w/v) aqueous solution of chitosan was prepared in 1% (w/v) acetic acid containing 1% (w/v) sodium chloride. The resulting solution was stirred on a magnetic stirrer to form a gel and kept overnight for stabilization. A dispersion phase was prepared by mixing 50 ml of toluene and 5 ml of span-80 stirred at 1200 rpm for 10 min. To this dispersion phase, 4 ml of chitosan gel with 1ml of 0.01% hydrochloric acid and 2 ml of TT (1500 Lf) solution containing 1.5% (w/v) trehalose were added. At the end of second hour, 5 ml of glutaraldehyde saturated toluene (GST) was introduced drop wise while stirring was continued up to 2 h followed by addition of another 5 ml of GST. The stirring was further continued for 2 h, after which the suspension of microspheres were centrifuged and separated. The pellet obtained was washed five times with 5 ml volumes of toluene followed by three times washing with 5 ml volumes of acetone. Further, air dried and stored in air tight container at room temperature. These microspheres were spread on the clean glass slide using glass rod and observed under the optical microscope and were further characterized using scanning electron microscope.

Infrared spectroscopy

To investigate the functional groups in the placebo microspheres as well drug loaded microspheres, FT-IR spectra of the samples were measured by using Fourier transform infrared spectroscopy (Shimadzu FT-IR-8400), available at our department. Samples were well mixed with IR grade KBr in the ratio of 1:9 respectively. All IR spectra were measured at 4 cm^{-1} resolution using IR range of 4000-400 cm^{-1} and scanning speed 15 scan/min in transmission mode.

Surface morphology

The scanning electron microscope (SEM) (JEOL, JSM-6360A, Japan) was used to examine surface morphology and features of the resultant tetanus toxoid encapsulated microspheres and placebo microspheres. The sample powder was sprinkled as a thin layer on an adhesive tape placed on the brass sample holder. The adhered sample was then coated with gold powder using the sputtering device and then transferred into the JEOL sample chamber for the analysis.

Entrapment efficiency of microspheres

Entrapment efficiency of chitosan microspheres using TT was evaluated using following the technique reported by Dodane et al.[36] The amount of TT entrapped in microspheres was determined by digesting 20 mg each of microspheres prepared by using C_1, C_2 and C_3 chitosan each in 100 ml of methanol hydrochloric acid mixture 99:1 (v/v) and the solution was sonicated for 20 min. After subsequent dilution the absorbance was determined spectrophotometrically at 750 nm wavelength. TT present in the aqueous phase was determined by using detergent compatible (DC) protein assay against a supernatant of blank chitosan microspheres. The Percent efficiency was calculated as follows:

$$\text{TT entrapped (\%)} = \frac{\text{Measured amount of TT}}{\text{Theoretical amount of TT loaded}} \times 100$$

Statistical analysis

For the purpose of statistical comparison of data obtained from this study, one way analysis of variance (ANOVA) was employed using Microsoft excel 2007, using ($P<0.05$) as a significance level.

RESULTS AND DISCUSSION

Results obtained for Physicochemical, micrometric and functional characterization for various chitosan samples were as given in Table 1.

Physicochemical Properties

Moisture

The moisture contents of all chitosan samples were not significantly different, ranging from 1.6% to 2.1% being higher for water soluble chitosan (C_2). The mirror differences observed could be attributed to the extent of the drying process and exposure to the atmosphere. Chitosan is hygroscopic in nature; therefore, it is likely that chitosan samples were affected by small moisture absorption during storage.[27] Lower the moisture content of chitosan, the better the shelf stability and hence the quality. According to Li et al.[37], commercial chitosan products contain less than 10% moisture content.

Ash

Ash content is an important parameter that affects chitosan solubility, viscosity and also other important characteristics.[38] The ash contents were not significantly varying in commercially available chitosan C_1 and C_2 as well as laboratory prepared chitosan C_3 and were obtained in the range of 3.35% to 4.35%, indicating the effectiveness of the demineralization step in removing minerals.

Viscosity

The viscosity values obtained were significantly different for all the chitosan samples ranging from 228 to 602 cPs, being higher for water soluble chitosan (C_2). Similarly, Bough et al.[39] reported that viscosity of chitosan varied considerably from 60 to 5110 cPs depending on the species and the preparation method used. Whereas, No et al.[40] reported viscosity values ranging from 26 to 360 cPs for chitosan samples prepared from crab shells.

Viscosity-average molecular (Mv) weight

The viscosity-average molecular (Mv) weight obtained for all the chitosan samples were significantly different ranging from 329 to 1249 kDa Table 1, being relatively lower for laboratory prepared chitosan (C_3). Native chitin molecular weight is larger than one million daltons and commercial chitosan have the molecular weight range of 50 kDa—2000 kDa,

depending on the processing conditions and grades of the product.[37, 3] It has been reported that low molecular weight chitosan is suitable for preparation of micro and nanoparticles as compared to higher molecular weight chitosan to elicit immune response in the host.[41]

Solubility

The prepared chitosan from shrimp shells waste was found to be soluble in 1% acetic acid solution and partially soluble in water.

Degree of Deacetylation (DD)

DD was calculated by using equation proposed by Domszy and Roberts.[28] Depending on the source and preparation procedure, DD may range from 30% to 95%.[42] Degree of deacetylation of the laboratory prepared chitosan (C_3) was found to be 75%, which was relatively similar to those of commercially available chitosan C_1 and C_2 being 75-85% respectively.

Micromeritic properties

Micrometric values obtained for bulk density, bulkiness, tapping density, Carr's Index, Hunsner's ratio and turbidity were tabulated in Table 1. Micromeritic properties are one of the most important aspects for the designing, formulating and maintaining quality of any pharmaceutical product. The bulk density values obtained for C_3 were significantly lower than C_2 and C_1, indicating its relatively more porosity. Cho et al.[43] reported that lower bulk density may indicate that the chitosan is more porous and may have been subjected to a lower alkali concentration treatment for deproteinization. The Hausner's ratio and Carr's index are both measures of the flow properties of powders. A Hausner's ratio < 1.25 indicates a powder that is free flowing, whereas > 1.25 indicates poor flow ability, while smaller the Carr's index better the flow properties of the material. For example, 5–15 indicates excellent, 12-16% good, 18-21% fair and 23-28% poor flow.[31] The Hausner's ratio obtained for C_2 and C_3 were lower than 1.25 indicating their free flowing property, while C_1 was showing slightly higher value than 1.25. Carr's index indicated good flow properties for all the chitosan except C_1, which was between 23-28% as given in Table 1. Overall, Micromeritic properties suggest the non aggregated nature of all chitosan samples indicating there suitability as a potential candidate for preparing floating micro particulate drug delivery system. Reported values for chitosan turbidity are ~70 NTU correlating well with the turbidity value obtained for C_3 (95.13 NTU), C_1 (83.99 NTU) and C_2 (61 NTU) turbidity.

Functional Properties

Water binding capacity (WBC)

Among the functional properties analyzed, WBC for acetic acid soluble chitosan; C_1 and C_3 were significantly different and were respectively as 669% and 804% Table 1. These results were supported by similar observations made by Rout et al.[44] for chitosan samples, wherein, WBC ranged from 581% to 1150% with an average of 702%. Also, Cho et al.[44] reported the WBC ranging from 458% to 805% for five commercial chitosan from shrimp and crab shells.

Fat binding capacity (FBC)

The results obtained for FBC of chitosan samples were significantly different ranged from 284% to 589% (Table 1), being higher for laboratory prepared chitosan (C_3). Fat binding capacity signifies how the chitosan can easily bind or absorb fat. Average fat binding capacity reported by Rout et al.[44] for crawfish chitosan and commercial crab chitosan using soybean oil were 706% and 587% respectively.

Antioxidant activity

In-vitro antioxidant activity for all chitosan was determined in terms of free radicals scavenging using DPPH, ABTS and NO radicals (Figure 1) DPPH is usually used as a substrate to determine the antioxidant activity of a proton donating substance. Moreover, it has been reported that free radicals of DPPH can react with the free amino (—NH_2) groups of chitosan to form stable macromolecule and the amino groups can form ammonium (—NH_3^+) groups by absorbing a hydrogen ion from the solution.[45] From the graph, it was observed that laboratory prepared chitosan (C_3) was showing more percent inhibition as compared to both commercial chitosan standard BHA (0.5 mg/ml) was used for comparing, Also, C_3 showed highest DPPH scavenging activity up to 34% followed by C_1 (32%) and lowest was observed for C_2 (10%). Similarly, C_3 showed highest ABTS scavenging activity up to 24% followed by C_1 (22%) and lowest scavenging activity was observed for C_2 (11%).

In case of nitric oxide radicals, C_1 showed highest scavenging activity up to 61% followed by C3 (59%) and significantly lower scavenging activity was observed for C2. Excess, nitric oxide radicals contributes in inflammation, cancer and also in other pathological conditions.[46]

Table 1: Physicochemical, Micrometric and Functional characterization of Chitosan

Physicochemical Properties	C_1	C_2	C_3
Moisture content (%)	1.6 ± 0.40^a	2.1 ± 0.35^a	1.7 ± 0.35^a
Ash content (%)	3.35 ± 0.2^a	4.35 ± 0.7^a	3.35 ± 0.2^a
Viscosity (cPs)	261 ± 2.9^a	602 ± 2.6^b	228 ± 2.9^c
Molecular Weight (kDa)	659 ± 1.1^a	1249 ± 1.7^b	329 ± 1.8^c
Solubility	1% Acetic acid	Distilled water	1% Acetic Acid
Degree of deacetylation (%)	78%	85%	75%
Micrometric Properties			
Bulk Density (g/ml)	0.35 ± 0.01^a	0.34 ± 0.00^a	0.22 ± 0.02^b
Bulkiness	2.83 ± 0.2^a	2.88 ± 0.0^a	4.58 ± 0.5^b
Tapping Density (g/ml)	0.47 ± 0.0^a	0.41 ± 0.5^b	0.27 ± 0.3^c
Carr's Index (%)	25 ± 0.06^a	15 ± 0.03^b	$18 \pm 0.07^{a,b}$
Haunsner's ratio	1.33 ± 0.10^a	1.18 ± 0.04^a	1.23 ± 0.10^a
Turbidity (NTU)	81.7 ± 1.5^a	61.7 ± 2.9^b	95.0 ± 1.0^c
Functional Properties			
WBC %	669 ± 15.3^a	—	804 ± 34.9^b
FBC %	472 ± 15.8^a	284 ± 21.5^b	589 ± 27.7^c

Note: Mean ± standard deviation (SD) of triplicates determinations. Means with different super scripts a, b and c in each column are significantly different ($P < 0.05$) whereas C_1; acetic acid soluble chitosan, C_2; water soluble chitosan, C_3; Laboratory prepared acetic acid soluble chitosan.

Table 2: Antibacterial Activity of Chitosan against Gram Negative and Gram Positive bacteria

Chitosan samples	*Gram negative (mm)*		*Gram positive (mm)*			
	E.coli	*P.klebsheila*	*S.aureus*	*S.leutia*	*S.epidermidis*	*B.subtilus*
C_1	7.1 ± 0.7^a	5.3 ± 0.5^a	5.5 ± 0.5^a	5.5 ± 0.5^a	4.1 ± 0.7^a	5.5 ± 0.2^a
C_2	9.5 ± 0.9^b	2.8 ± 0.3^b	3.5 ± 0.3^b	3.5 ± 0.2^b	2.7 ± 0.3^b	4.7 ± 0.5^b
C_3	9.1 ± 0.5^b	6.8 ± 0.5^a	5.8 ± 0.3^a	5.8 ± 0.5^a	5 ± 0.5^a	6 ± 0.5^a

Note: Mean ± standard deviation (SD) of triplicates determinations. Means with different superscripts a, b and c in each column are significantly different ($P < 0.05$) whereas C_1; acetic acid soluble chitosan, C_2; water soluble chitosan, C_3; Laboratory prepared acetic acid soluble chitosan.

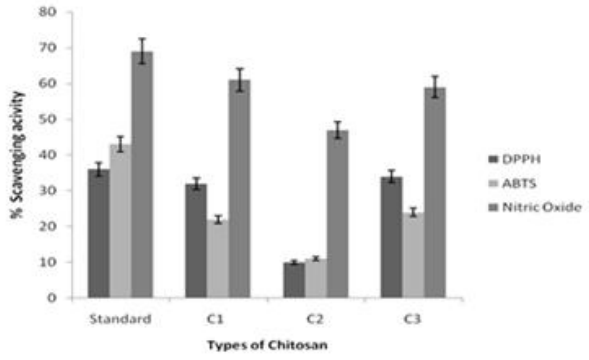


Figure 1: Standard; Butylated hydroxyanisole (BHA) (0.05 mg/ml), C_1- acetic acid soluble chitosan (0.5 mg/ml), C_2- water soluble chitosan (0.5 mg/ml), C_3- Laboratory prepared acetic acid soluble chitosan (0.5 mg/ml).

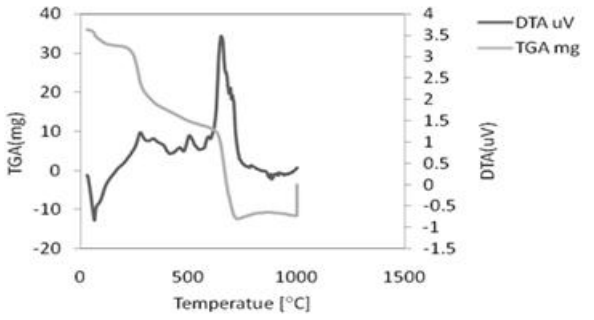
Antimicrobial activity

It was observed that all types of chitosan inhibited the growth of representative strains of Gram-positive and Gram-negative bacteria as represented in (Table 2). All chitosan samples C_1, C_2 and C_3 inhibited Gram-negative bacteria *E. coli* and *K.* pneumoniae strain that are major water borne food pathogens. Laboratory prepared chitosan (C_3) showed better antibacterial activity than commercial chitosan C_1 and C_2. Similarly, C_3 chitosan showed maximum antibacterial activities against Gram-positive species. C_2 showed significantly lower antibacterial activity against all bacterial strains except *E. coli*. Usually, bacterial growth inhibition mechanism is thought to be due to the amino group catatonically charged combines with anionic components such as N-acetylmuramic acid, sialic acid and neuraminic acid, on the cell surface which may suppress bacterial growth by impairing the exchanges with the medium, chelating transition metal ions and inhibiting enzymes.[47, 48]

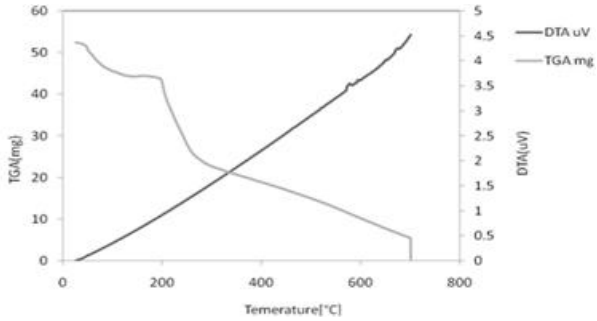
Thermo gravimetric Analysis

Thermo gravimetric analysis is very helpful to understand the degradation temperature, moisture content and percentage of inorganic and organic components in material. Two stage thermal degradation as represented

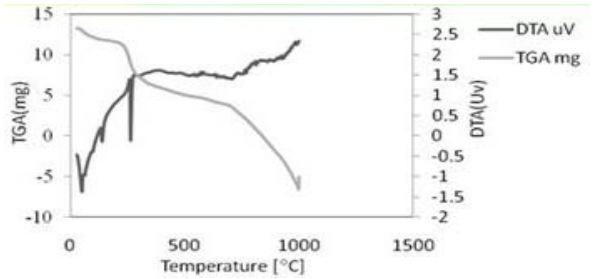
in Figure 2(a) (b) and (c) was observed in the TGA curves of all chitosan samples, which was similar to the other reported literature.[49–51] The weight loss observed in first stage was about 9% between 46 to 129 °C for C_1, 15% between 27 to183 °C for C_2 and 9.5% between 36.90 to 119.72 °C for C_3 chitosan, which could be attributed to moisture vaporization. Similarly, the second stage weight loss begins at approximately 203 °C, 178 °C and 170 °C respectively for C_1, C_2 and C_3 with corresponding weight loss of 41% in both C_1, C_2 and 51% in C_3 chitosan. Second stage was attributed to deacetylation, depolymerization and decomposition of chitosan samples.[49–51] Furthermore, the total weight remaining after 500 °C was nearly 49%, 43% and 38.5% respectively for C_1, C_2 and C_3 chitosan. Overall, C_3 chitosan showed comparatively more weight loss with progress in temperature indicating its less thermal stability as compared to commercial one which might be due to introduction of weak linkage into the polymer chain depending upon process condition and impurities.[52]



(a)



(b)



(c)

Figure 2: Thermogravimetric graphs of (a) Acetic acid soluble commercial chitosan (C_1) (b) Water soluble commercial chitosan (C_2) (c) Laboratory prepared acetic acid soluble chitosan (C_3).

Chitosan microspheres

The placebo microspheres and TT encapsulated microspheres prepared using C_1, C_2 and C_3 were characterized as follows;

Fourier transforms infrared spectroscopy

Fourier transforms infrared spectroscopy was used to determine the functional groups and corresponding specific transmittance peak in each of chitosan its microspheres and tetanus toxoid encapsulated microspheres using C_1, C_2 and C_3.

Generally, chitosan shows bands at 3000–3500 cm^{-1} that corresponds to O–H stretching vibrations of hydroxyls and N–H stretching vibrations of free amino groups and band at 1400–1650 cm^{-1} is attributing to the C=O stretching (amide I) of O=C–NHR, as reported by Chatterjee et al.[53] Similarly, Radhakumary[54], reported unique chitosan characteristic peaks around 1363 cm^{-1} attributing to the CH_3 bending vibration and 1645 cm^{-1} assigned to amide group (O=C–NHR).

Our results as represented in Figure 3 respectively for C_1, C_2 and C_3 chitosan powder, also indicated similar broad transmittance peak in the range 3000 cm^{-1} to 3500 cm^{-1}, especially at 3352.67 cm^{-1}, 3315.96 cm^{-1} and 3356.13 cm^{-1} respectively for C_1A, C_2A and C_3A attributed to OH and NH stretching vibration in all chitosan powders. Also, weak peak at 1375 cm^{-1}, 1375.96 cm^{-1} and 1365 cm^{-1} respectively for C_1A, C_2A and for C_3A was attributing to CH bending vibration of CH_3 methyl group, which was less intense confirming the deacetylation which plays important role in solubility of chitosan.

After placebo microspheres formation using C_1 Figure 3 (a) B it was observed that, a weak transmittance peak at 2900 cm^{-1} shifts to 2934 cm^{-1} which corresponds to CH_3 stretching vibration and also less intense peak at 1366 cm^{-1} shifts to 1349 cm^{-1} attributing to CH_3 bending vibration. The new sharp peak at 1622 cm^{-1} represents stretching vibrations of C=N is Schiff's base formed by the reaction of Glutaraldehyde and chitosan resulting in formation of microspheres.

Similarly, difference in spectra of microspheres using C_2 Figure 3 (b) B it was observed that CH_3 stretching vibration at transmittance 2908 cm^{-1} shifts to 2925 cm^{-1} and peak at 1355 cm^{-1} shifts to 1389 cm^{-1}, which corresponds to CH_3 bending vibration. The new sharp peak at 1634 cm^{-1} represents stretching vibrations of C=N is Schiff's base.

Similarly, difference in spectra of microspheres using C_3 Figure 3 (c) B, it was observed that, CH_3 stretching vibration at transmittance 2922 cm^{-1} shifts to 2945 cm^{-1}, and peak shift at 1355 cm^{-1} to 1403 cm^{-1} could be due to CH_3 and CH_2 bending vibrations. Also new sharp peak at 1648 cm^{-1} represents stretching vibrations of C=N is Schiff's base. There were no significant difference between FT–IR spectra of placebo microspheres and tetanus toxoid encapsulated microsphere for all chitosan

showing very less or no interaction between chitosan polymer and tetanus toxoid.

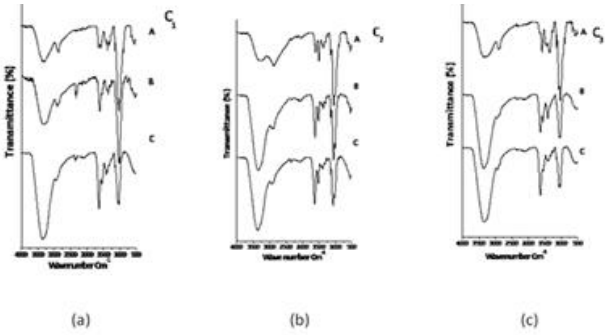


Figure 3: (a) FT-IR of C_1 (A) Chitosan powder (B) Chitosan Microspheres. (C) Tetanus toxoid loaded microspheres (b) FT-IR of C_2 (A) Chitosan powder (B) Chitosan microspheres (C) Tetanus toxoid loaded microspheres (c) FT-IR of C_3 (A) Chitosan powder (B) Chitosan Microspheres (C) Tetanus toxoid loaded microspheres.

Surface morphology

Placebo as well as tetanus toxoid (1500 Lf/ml) entrapped microsphere prepared using glutaraldehyde saturated toluene as a cross linker were observed for surface morphology using scanning electron microscopy. The size of individual microsphere was in the range of 3–10 µm and few microspheres were up to 50 µm sizes for all C_1, C_2 and C_3 chitosan samples. Smooth uniform surface was observed for placebo microspheres Figure 4 (a) (c) and (e) and slight increase in mean diameter was observed for tetanus toxoid entrapped microspheres Figure 4 (b), (d) and (e) with rough surface, which may be due to increased viscosity of drug polymer dispersion constituting the internal phase of emulsion, which leads to large droplets and formulation of larger microspheres.[55] Further, toxoid encapsulated microspheres showed a porous structure, resembling a sponge, which might be due to diffusion out of water from emulsion droplets to the continuous phase during chitosan solidification process.[56] Whereas, the cube like structure of placebo and encapsulated microspheres of C_2 chitosan may be because of differences in solubility of uncrossed polymer.

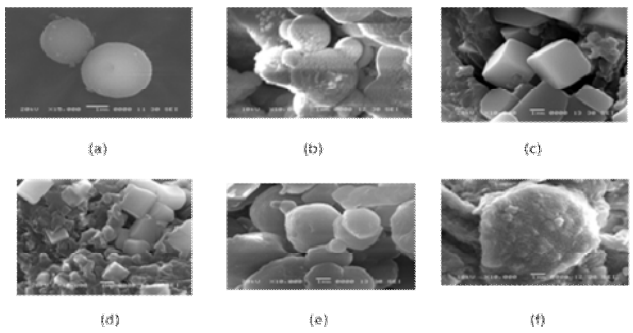


Figure 4: (a) C_1 placebo microspheres (b) C_1 Tetanus toxoid microspheres (c) C_2 placebo microspheres (d) C_2Tetanus toxoid microspheres (e) C_3 Placebo microspheres (f) C_3 Tetanus toxoid microspheres.

Microsphere entrapment efficiency

The major factors generally affect the proteins release from chitosan microspheres are the chemical structure of the protein, and its interaction with chitosan molecules in the release medium.[57] The protein entrapment efficiency of microspheres for all chitosan were significantly different and, indicated high entrapment efficiency for C_1 microspheres (89%) followed by C_3 (84%) and C_2 (81%). Thus, attempt to prepare chitosan microspheres by using Glutaraldehyde saturated toluene cross linker for encapsulating tetanus toxoid was achieved with good entrapment efficiency for all the chitosan.

CONCLUSION

Present study indicated that chitosan was prepared successfully from shrimp shells at laboratory scale with 75% DD as determined by FT–IR and could be solubilized in 1% acetic acid suitable for drug entrapment. Its physico-chemical properties and micrometric properties were comparable to both commercial chitosan C_1 and C_2, indicating its suitability for commercial use. Prepared chitosan (C_3) was found to be more effective against Gram negative bacteria as compared to Gram positive. It also showed high antioxidant activity, which might be effective in preventing or reducing various stress-induced diseases. Further, it indicated good drug entrapment efficiency for tetanus toxoid comparable to those of commercial chitosan.

Overall, efforts in this research indicated that the prepared chitosan having good structural morphology, functional potential and specified interactions for microspheres preparation, microsphere for mucosal vaccination. Results also suggested that prepared chitosan macromolecules can be used commercially in food supplements and drug preparation as well as in turn may reduce the pollution created by sea food waste products.

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