Synthesis and Characterization of High Molecular Weight Chitosan, and Antioxidant Activity of Its Chitosan Oligosaccharide Encapsulation

Hari Sharan Adhikari¹, Aditya Garai², Bishnu Prasad Marasini³, Rameshwar Adhikari¹, Paras Nath Yadav*⁵

¹Department of Applied Sciences, Pashchimanchal Campus, Institute of Engineering, Tribhuvan University, Pokhara, Nepal
²Department of Inorganic and Physical Chemistry, Indian Institute of Science, Bangalore, India
³Department of Biotechnology, National College, Tribhuvan University, Naya Bazar, Kathmandu, Nepal
⁴Research Center for Applied Science and Technology, Tribhuvan University, Kathmandu, Nepal
⁵Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal

*Corresponding E-mail: pnyadav219@gmail.com

(Received: September 26, 2020; Revised: January 4, 2021; & Accepted: January 4, 2021)

Abstract
High molecular weight nanocrystalline chitosan (HMWNC) with $M_w$ of 350 kDa and 66.92% DDA was prepared by alkaline N-deacetylation of chitin that was abundantly obtained after demineralization and deproteinization of pulverized crab shells, and commercially available chitosan oligosaccharide (COS) with average $M_w< 3000$ Da and 87% DDA was encapsulated with HMWNC. The encapsulation of COS as a pharmaceutical ingredient into HMWC was hypothesized to enhance the bioavailability of COS in target cells. The HMWNC encapsulated chitosan oligosaccharide (COS-HMWNC) showed in vitro antioxidant activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals ($IC_{50} = 512.6±14.4 \mu g/mL$) while others showed less than 50% inhibition at 1000 $\mu$g/mL. The lower the molecular weight of chitosan, the higher was the antioxidant activity. The study showed that the encapsulation of COS molecules in HMWNC could be used as a simple and effective way of enhancing the antioxidant activity of COS.

Keywords: Antioxidant activity, chitin, chitosan oligosaccharide, high molecular weight nanocrystalline chitosan, encapsulation

Introduction
Chitin, the most abundant natural polymer after cellulose, is a homopolymer of 2-acetamido-2-deoxy-β-D-glucose monomers linked through β-(1→4) linkages [1]. The crustacean shells as the major commercial source of chitin consist of 15-40% chitin, 20-40% protein, and 20-50% calcium carbonate and magnesium carbonate minerals in close association with minor constituents like lipids and pigments, astaxanthin, and other minerals [2]. Chitin is obtained from the exoskeleton of crustaceans after demineralization with hydrochloric acid and deproteinization with alkali. Chitosan is a partially deacetylated derivative of chitin and a copolymer of N-acetyl and deacetyl α-(1,4)glucosamine (C₆H₁₁O₄N) units. Chitosan is a cationic polysaccharide due to the protonation of an amino group [3] in the backbone (figure 1) and a semi-synthetic natural product with multipurpose applications [1-4]. Chitosan is an adsorptive material [5-7], a pharmaceutical excipient [8], a permeation enhancer [9], and a hemostatic agent [10] used in the targeted delivery of drugs [11]. It shows antimicrobial [12], anticancer [13], antifungal [14], antioxidant [15] and anti-obesity [16] activities. The functional properties of chitosan such as biocompatibility and bioactivity [17] are greatly influenced by its degree of deacetylation (DDA) and molecular weight ($M_w$) [18]. High molecular weight chitosan (HMWC) has been reported as a natural antioxidant material with a high potential for biomedical applications [19]. Nano chitosan, prepared as biocompatible polymeric nanoparticles, undergoes prolonged circulation in blood with more extravasation and passive targeting...
[20]. So, it is a suitable drug delivery candidate [21-22] with a controlled delivery to the target cells in a sustained release manner by enhanced permeation and retention (EPR) effect [23]. The nanocrystalline chitosan, with a high hydrophilic and nanoparticulate creating behavior, has a great potential for the development of new complexes for regenerative medicine and tissue engineering [24].

The antioxidant defense mechanism involves scavenging and minimizing the formation of free radicals and reactive oxygen species (ROS). The systemic ineffectiveness in antioxidant defense mechanism causes oxidative stress and cellular damage [25] that leads to the progress of chronic and age-related diseases like cancer and immune system decline [26-27]. Chitosan is a sustainable natural antioxidant and its antioxidant activity is owing to the abstraction of a proton from free radicals by an amino group in the C2 position and hydroxy groups in the C3 and C6 positions of the pyranose ring [13,28]. Low molecular weight chitosan shows more scavenging of free radicals [29-30] due to its higher mobility that minimizes the possibility of both intermolecular and intramolecular hydrogen bond formation and the amino groups remain rather free to abstract proton from free radicals [29].

Chitosan has been used in the target delivery of the anticancer therapeutic agent, and nano chitosan loaded with the therapeutic agent is more stable with enhanced permeability and bioactivity [31]. Due to in vivo renal degradation, chitosan is less toxic to healthy cells [32]. Nano chitosan is biocompatible and gets internalized by the cells [33].

Encapsulation is the technique of loading a therapeutic agent inside a suitable carrier. The carrier brings about the increase in stability, bioavailability, and delivery of the therapeutic agent in a sustained release manner that prolongs its cellular uptake and presence in blood [34-35]. Cellular uptake of the encapsulated agent is essentially dependent on the charge of the Carrier [36]. The cationic carrier makes its way towards the negatively charged cell membranes [37].

The work comprised of extraction of chitin from crab shells and alkaline N-deacetylation of chitin to chitosan, preparation of high molecular weight nanocrystalline chitosan (HMWNC), physicochemical and structural characterization of HMWNC, encapsulation of COS in HMWNC and correlative study of antioxidant activity of HMWNC, COS and the encapsulated COS-HMWNC. The purpose of this work lies in bioconversion of hazardous fishery wastages of crab shells into chitosan as a value-added biomaterial of functional antioxidant activity and a controlled delivery agent of drugs.

Materials and Methods

Materials
Crab shells as fishery wastages were collected from the local market of Kathmandu, Nepal. Glacial acetic acid (Merck 99-100%), hydrochloric acid (Merck 99%), sodium hydroxide (Merck, 99%), sodium acetate (Merck), ethanol (Sigma-Aldrich, 99.80%), methanol (Sigma-Aldrich, 99.80%), chitosan oligosaccharide (Sigma-Aldrich, 87%) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, 95%) were used as received.

Measurements
The FT-IR spectrum in the powdered state was measured in the 4000-400 cm\(^{-1}\) regions with ATR-GeXPm experimentation using BRUKER 1 003 3610 FT-IR spectrophotometer. The solid-state \(^{13}\)C-NMR spectrum was measured using BRUKER AC-800 Delta 2 NMR spectrometer with cross-polarization at a field strength 400 MHz, scans 276, and contact time of 3.5 mins. Powder X-Ray Diffraction (XRD) measurement was performed at scanning scope of 2θ at 0 to 60 degrees with an exposure time of 400 S using a D8 advance BRUKER diffractometer with Cu target (\(\lambda= 0.1541 \text{ nm}\)) at 40 kV. The elemental analysis was performed with a Thermo Finnigan FLASH EA 112CHNS microanalyzer with carrier gas He (140 mL/min) using CHNS/NCS column PQS SS 2M 6X5 mm in the oven at 75°C.

Experiments

Synthesis of nanocrystalline chitosan
Corresponding to the protocol of the previous works [38-39], the crab shell powder was demineralized
and deproteinized into chitin, and crab shell chitosan was synthesized by alkaline deacetylation of chitin. Nanocrystalline chitosan was prepared with minor modification in the method given by Pighinelli et al. [24]. Crab shell chitosan (0.5 g) was dissolved in 100 mL of 1% acetic acid solution under stirring at 60 °C for 36 hours, and 5 mL of glycerol as a plasticizer was added. Sodium hydroxide solution was added under stirring till neutrality. The solution was allowed to stand at 5 °C for twenty-four hours, filtered and washed with 2 L of deionized water, and the residue of nanocrystalline chitosan was dried at room temperature.

Determination of degree of deacetylation (DDA) and molecular weight (M<sub>w</sub>)

The DDA of nanocrystalline chitosan was determined with Fourier Transform- Infrared (FT-IR) spectroscopic method using the equation:

\[
DDA = 100 - \left( \frac{A_{1658}}{A_{3364}} \right) \times 100 / 1.33
\]

where \( A_{1658} \) cm\(^{-1} \) and \( A_{3364} \) cm\(^{-1} \) are absolute heights of N-H stretch of amide (A<sub>NH</sub>) and hydroxyl absorption bands (A<sub>OH</sub>) respectively [40]. The absolute absorption heights were measured after the adoption of baselines corresponding to the amide and hydroxyl absorption peaks (figure 2).

Encapsulation of chitosan oligosaccharide

Encapsulation of COS in HMWNC was carried out with a facile synthetic modification in the method of encapsulation of oregano essential oil in chitosan nanoparticles given by Hosseini et al. [42]. The lyophilized solution of nanocrystalline chitosan (1 mg) was added dropwise to 1 mL of chitosan oligosaccharide solution (1% (w/v) in 1% acetic acid solution) with constant stirring for half an hour, ultrasonicated in an ice bath for 5 min, the opalescent solution was centrifuged for 30 min, and the residue obtained upon filtration was dried at 60 °C for 36 hours. The supernatant solution over the centrifuged mass of chitosan encapsulation was isolated and both were stored at 4 °C.

Antioxidant assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was done according to the protocol of Brand William with slight modifications [43]. Test compounds were allowed to react with stable 1, 1-diphenyl-2-picrylhydrazyl free radical for half an hour at 37 °C. After incubation, the decrease in absorption was measured at 517 nm using a multi-plate reader (EPOCH2, microplate reader, Biotek). Now the percentage of DPPH free radical scavenging activity was calculated by using the following formula:

\[
\text{Percentage scavenging} = \frac{(A_o-A_s)}{A_o} \times 100
\]

where, \( A_0 \) = absorbance of the DPPH, \( A_s \) = absorbance of the DPPH free radical solution containing the sample extract. Quercetin (Himedia) was used as a standard control. All experiments were performed in triplicate. The standard graph was plotted to take the concentration on the X-axis versus the percentage of scavenging activity on the Y-axis.

Results and Discussion

General

Exoskeletons and shells of crustaceans contain chitin with minerals (mainly CaCO\(_3\)) and protein. The appearance of brilliant white crystals of chitin after demineralization and deproteinization shows that crab shells are a major source of chitin. The crab shell chitosan was obtained as a high molecular weight chitosan with viscosity average M<sub>w</sub> of 350 kDa and 66.92% DDA. The chitosan yield (31.4%) was close to the reported chitosan yield of 32.2% from crab shell wastes upon 2 h deacetylation with 40% sodium hydroxide solution in a solid/solvent ratio of...
1:10 (w/v) [44], but higher than crab chitosan yield of 16.7% reported upon 30 min deacetylation with 45% sodium hydroxide solution in a solid/solvent ratio of 1:10 (w/v) [45]. COS was commercially available as a yellow crystalline solid (figure 3a) soluble in water, and HMWNC was synthesized from crab shell as an off-white solid powder (figure 3b) insoluble in water and partially soluble in dimethyl sulfoxide (DMSO).

Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectroscopy in the mid spectral region of 4000-400 cm\(^{-1}\) provides a convenient technique for a qualitative structural elucidation and determination of DDA in chitosan [46-49]. The overall FT-IR spectrum of chitosan resembles the spectrum of cellulose with additional characteristic bands attributed to acetamido and amino group vibrations [50]. The physicochemical and biological properties of chitosan; like crystallinity, hydrophobicity, degradation, and cell response are greatly dependent on DDA [51]. The FT-IR spectroscopic method of determination of DDA is limited by the possible inaccuracy caused by interferences due to humidity and impurities in the sample [49]. Normally, the increase in DDA causes a decrease in \(M_w\), which brings about the variations in physicochemical and functional properties [44]. The DDA is affected by extraction conditions viz temperature, chitin/alkali concentration, reaction time, the particle size of chitin, and also the native source of chitin in nature [52-55].

The broadband in the FT-IR spectrum of crab shell chitosan (figure 4) corresponds to ν(O-H) stretch at 3364 cm\(^{-1}\), ν(\(\text{N-H}\)) stretch at 3273 cm\(^{-1}\), and intramolecular hydrogen bonds [56]. The weak ν(aliphatic C-H)symmetric stretch at 3058 cm\(^{-1}\) and ν(aliphatic C-H) asymmetric stretch at 2873 cm\(^{-1}\) are characteristic peaks of polysaccharides. The presence of residual N-acetyl groups is confirmed by a sharp characteristic ν(C=Oamide I) stretch at 1658 cm\(^{-1}\), ν(C=O amide II) stretch at 1560 cm\(^{-1}\) and the stretching vibration of ν(C-N amide III) band at 1313 cm\(^{-1}\)[56]. The broad peak at 1377 cm\(^{-1}\) is attributed to symmetrical deformation vibration of C-H bonds of the methyl group. The medium absorption band at 1064 cm\(^{-1}\) and the sharp band at 1027 cm\(^{-1}\) correspond to C-O stretching [56]. The band at 894 cm\(^{-1}\) corresponds to the C-O-C symmetric skeletal stretching vibration of chitosan [56-57]. The lowering of ν(N-H amide) angular bending vibration to 1560 cm\(^{-1}\) in crab shell chitosan is indicative of a strong hydrogen bonding in nanocrystalline form [58].

Solid-state \(^{13}\)C-NMR spectroscopy

The structure with non-deacetylated chitin and chitosan units with a numbering of carbon atoms in the pyranose ring is shown in figure 5. Solid State \(^{13}\)CNMR spectrum of crab shell chitosan (figure 6), used to characterize chitosan structure, showing the chemical shift (δ) signals at 23.07 ppm (methyl carbon of acetamido moiety), 57.50 ppm (C\(_2\)), 60.92 ppm (C\(_6\)), 75.70 ppm (C\(_3\), C\(_5\)), 83.45 ppm (C\(_4\)), 104.57 ppm (C\(_1\) of pyranose ring carbons), and 174.23 ppm (C=O), was indicative of incomplete deacetylation [59]. The signals were in close agreement with the reported chitosan chemical shift (δ) signals at 24-25 ppm (carbon atom of the methyl moieties of the acetamido groups), 57-60 ppm (C\(_2\)), 60-63 ppm (C\(_6\)), 76-78 ppm (C\(_3\), C\(_5\)), 84 ppm (C\(_4\)), 106 ppm (C\(_1\) of pyranose ring carbons), and 178 ppm (attributed to C=O indicative of incomplete deacetylation) [60-61].

X-ray diffraction analysis

Powder X-ray diffractogram of crab shell chitosan (figure 7) showed two crystalline reflection peaks at 9.5° and 19.6°, in close agreement with the reported peaks at 10° and 20° for chitosan oligosaccharide [44]. The particle size of chitosan (D) was 76 nm, as
estimated with the help of Debye-Scherrer formula [62]. It was indicative of the aggregation of chitosan as a nano-based drug carrier system within the submicron range of <500 nm particle size [63].

Figure 5: Structure with non-deacetylated chitin and chitosan units with numbering of carbon atoms in the pyranose ring

The degree of crystallinity was 32.84%, calculated in terms of crystallinity index, with the help of formula

\[
\text{crystallinity index} = \left( \frac{I_c - I_{am}}{I_c} \right) \times 100
\]

where \(I_c\) (arbitrary unit) is maximum intensity of the crystalline peak at \(\sim 20^\circ\) and \(I_{am}\) (arbitrary unit) is the intensity of amorphous diffraction at \(12.6^\circ\) for chitosan chain [64]. It showed substantial order of particles in crystalline form. The crystallinity of chitosan, though partially affected by the factors like a spatial hindrance, hydrophobic force, and \(\pi-\pi\) stacking [65], has been reported to be mainly dependent on DDA [66-70].

Figure 6: Solid-state \(^{13}\)C-NMR spectrum of crab shell chitosan

Elemental microanalysis

For chitosan with 100% DDA, corresponding to the monomer structure of unit formula weight 161.15, calculated percentages of elements are C, 52.16; H, 9.38; N, 8.69. Elemental microanalysis of crab shell chitosan (66.92% DDA) with the percentages as C, 41.10; H, 6.00; N, 6.50 were in agreement with the reported values of elemental microanalysis of chitosan oligosaccharide (87% DDA) as C, 40.05; H, 6.41; N, 7.29 [65]. The values of the calculated C/N ratio of chitosan with 100% deacetylation, crab shell chitosan (66.92% DDA and \(M_w\) 350 kDa), and commercial chitosan oligosaccharide (87% DDA, average \(M_w < 3000\) Da) are 6.00:1, 6.32:1, and 5.49:1 respectively. The alkali concentration, reaction conditions, and presence of impurities have been reported to cause the lowering of the C/N ratio [44,71].

Encapsulation of chitosan oligosaccharide

The entrapment of lyophilized solution of crab shell chitosan viz. HMWNC in COS solution (1% w/v in 1% acetic acid solution) showed a substantial loading of COS inside the high molecular weight chitosan carrier. The purpose of encapsulation lies in the formation of the ultrathin and uniform membrane to improve the bioactivity with the mass transfer of COS to the cells and aqueous phase bioadhesivity of protonated chitosan to undergo permeation through the negative cell surfaces in vitro. This process could bring about the transportation of chitosan through the epithelial surfaces to enhance its cellular bioavailability [28]. The encapsulated COS with low \(M_w\) and more DDA could undergo more protonation and attraction to negatively charged cell surfaces to show higher cytotoxicity [5,72]. Conventional nanocarriers are eliminated from the systemic
circulation by the process of phagocytosis [73]. But, high molecular weight nanocrystalline chitosan, being a permeation enhancer and biocompatible carrier could prevent phagocytosis, and increase the targeting ability of encapsulated COS. Moreover, it is a positively charged material to be easily taken up by the negatively charged membranes [36] of the target cells. The smart designing of drug carriers is crucial towards an increase in cellular uptake, interaction and bioavailability, and targeted delivery of therapeutic materials [74].

Antioxidant activity

Antioxidant profiles (figure 8) and the variation of antioxidant IC$_{50}$ values (table 1) of HMWNC, COS, and COS-HMWNC showed that encapsulation was worthwhile towards the antioxidant enhancement.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition (%)</th>
<th>IC$_{50}$±SEM (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMWNC</td>
<td>&gt;50</td>
<td>NA</td>
</tr>
<tr>
<td>COS</td>
<td>&gt;50</td>
<td>NA</td>
</tr>
<tr>
<td>COS-HMWNC</td>
<td>65</td>
<td>512.6±14.4</td>
</tr>
<tr>
<td>Quercetin$^b$</td>
<td>92</td>
<td>2.3±0.1</td>
</tr>
</tbody>
</table>

SEM = Standard error of the mean at n=3
NA = not available /not tested because of less than 50% inhibition.

Quercetin$^b$ was used as a standard for radical scavenging activity

Figure 8: Antioxidant profiles of COS and COS-HMWNC

The encapsulation COS-HMWNC showed a functional antioxidant activity (IC$_{50}$ = 512.6 μg/mL), in agreement with the reported IC$_{50}$ of natural antioxidant chitosan at 500 μg/mL [75], with scavenging of free radicals (% RSC 65), whereas COS and HMWNC showed less than 50% RSC. The results of inhibition percentages showed that there was an increase in antioxidant activity with the increase in DDA, decrease in M$_w$, and encapsulation of COS with HMWNC as a carrier. The results are indicative of the assumption of encapsulated antioxidant material in minimum size with an increase in bioavailability and control release [76-78].

Conclusions

High molecular weight nanocrystalline chitosan was obtained as a semi-synthetic natural product from fishery wastages of crab shells. Encapsulation of commercially available chitosan oligosaccharide with this product was found significant towards the enhancement of antioxidant activity. The cellular uptake of antioxidant chitosan in vitro was found to increase with the increase in DDA and decrease in M$_w$. The antioxidant activity is attributed to cellular uptake of the compound and its action towards scavenging of free radicals inside the cell. On this basis, cellular uptake for the net antioxidant function can be generally stated to be more in the samples with more DDA and less M$_w$. The study leaves the area of further investigation on the development of sustainable and functional antioxidants with cellular specificity and controlled release of nano encapsulated chitosan.

Acknowledgments

We acknowledge Nepal Academy of Science and Technology (NAST), and Indian National Science Academy (INSA) for a research fellowship at Indian Institute of Science (IISc), Bangalore, India, and NAST in addition for Ph. D. fellowship to Hari Sharan Adhikari. We are thankful to Professor A. R. Chakravarty (IISc, Bangalore, India) and Dr. Yuba Raj Pokhrel (South Asian University, New Delhi, India) for their support to our work.

References


25. J. E. Kinsella, E. Frankel, B. German and J. Kanner, Possible mechanisms for the protective role of antioxidants in wine and plant foods: physiological mechanisms by which flavonoids, phenolics, and other phytochemicals in wine and plant foods prevent or ameliorate some common chronic diseases are discussed, *Food Technology (USA)*, 1993, 47(4), 85–89.


46. J. Brugnerottoa, J. Lizardi, F. M. Goycoolea, W.


