



Synthesis and Characterization of Chitosan Nanoparticles and Evaluation of Antimicrobial Activity Antioxidant Activity



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Abstract

Nanoparticles derived from Chitosan have become a widely utilized material in biological experiments as an antimicrobial agent. Their application in food and health industries is also under rigorous investigation. The objective of this study was to synthesize chitosan nanoparticles (CNPs) from shrimp shell chitosan, characterize the particle size and their stability and to investigate the CNPs antimicrobial activity against selected strains of fungal and bacterial species. Characterization of the nanoparticles were done using surface plasmon resonance activity, zeta potential analysis and particles size distribution analysis. Disk diffusion method was employed to analyze the antimicrobial activity of the CNPs. The synthesized CNPs showed good stability with 52.3mV at 25.1 °C with a conductivity of 0.127mS/cm. The mean particle size was 32.2nm at 90° scattering angle under monodisperses form. Stable CNPs showed significant antimicrobial activity against *Streptomyces species*, *Pseudomonas fluorescense* *Staphylococcus aureus*, *Aspergillus niger* and *Aspergillus flavus*. Stable nanoparticles were synthesized from shrimp shell chitosan and they can be used as antimicrobial and antioxidant agents.

Keywords: Chitosan nanoparticles; Shrimp shell; Antimicrobial activity; Antioxidant activity

Introduction

Functional bioactive ingredients have received much attention in recent years from the scientific community, consumers and food manufacturers. Potential functional bioactive ingredients are vitamins, probiotics, bioactive peptides, antioxidants, etc. Scientific evidence to support the concept of health-promoting ingredients is growing steadily. Innovative functional foods that bring physiological benefits or reduce long-term risks of developing diseases are being developed by the scientific community Elliott & Ong [1]. However, due to the instability of functional ingredients under conditions encountered during processing (temperature, oxygen, light) or in the gut (pH, enzymes, presence of other nutrients), it is difficult to retain the health benefits of functional ingredients. Therefore, it is necessary to protect these molecules from harsh conditions by encapsulation or immobilization. Recently, various micro/nano carriers, particularly nanoparticles and nano fibers, have become available for enzyme immobilization Sawicka et al. [2]. Typically, smaller particles provide a larger surface area for the attachment of enzymes Jia et al. [3] and a shorter diffusional path for the substrates. Thus, nano-structured carriers have been utilized as carriers for enzyme immobilization Kim et al. [4]. Chitosan is a partially deacetylated polymer of N-acetyl glucosamine that can

be obtained through alkaline deacetylation of chitin. It consists of a β -(1,4)-linked-D-glucosamine residue with the amine groups randomly acetylated. The amine and -OH groups endow chitosan with many special properties, making it applicable in many areas and easily available for chemical reactions. Chitosan is safe, non-toxic and can interact with polyanions to form complexes and gels Se & Niranjana [5].

Nanomaterials are proposed to be the materials for the new millennium. Metal nanoparticles (1-100nm) have various functions that are not observed in bulk phase and have been studied extensively because of their exclusive catalytic, optical, electronic, magnetic, antimicrobial wound healing and anti-inflammatory properties. Nanomaterials exhibit unique and considerably changed physical, chemical and biological properties when compared to their bulk counterparts Thiruchenduran et al. [6], Chitin is a natural polysaccharide synthesized by a great number of living organisms and functions as a structural polysaccharide. Chitosan is the only pseudo natural cationic polymer which has many potential biomedical and other applications. Chitosan is natural, non-toxic, copolymer of glucosamine and N-acetylglucosamine prepared from chitin

by deacetylation, which in turn, is a major component of the shells of crustaceans. Recently, bio-catalysis and sustainable chemistry researchers are directed to develop new green methodologies that aim to reduce and prevent pollution at its source.

Bio-catalysis is a promising technique based on the use of natural renewable biological materials, such as enzymes and polymers, that provide cleaner methodologies with high selectivity and energy-efficient operation under mild conditions in contrast to the traditional chemical catalyst. Chitosan (2-acetamido-2-deoxy- α -D-glucose-(N-acetylglucosamine)) is a partially deacetylated polymer of chitin and is usually prepared from chitin by reflux with a strong alkaline solution. Some time ago, the utility of chitosan as an efficient eco-friendly basic biocatalyst for Michael additions was reported and it could already be shown that chitosan can catalyze the addition of bi-functional active methylene's to arylidene malonitriles and enamines. Pyrroles are known to be widely important heterocycles, key structural units of chlorophyll and hemoglobin ensuring the photosynthesis in plants and oxygen exchange in animals. A great variety of natural compounds includes the pyrrole scaffold fulfilling several diverse major biological functions. No wonder that many potent drugs represent various pyrrole derivatives. A lot of pyrroles have been recently shown to be COX-2 iso enzyme inhibitors, DNA minor groove recognition reagent DB884, bio antioxidants. Different pyrroles are known to possess anti-inflammatory, antiviral, anti-proliferative, antibacterial, antidepressant, antipsychotic, antihyperglycemic activities. In recent years, ultrasonic chemistry has received an increasing attention ultrasound irradiation, by cavitation collapse, is able to activate numerous organic reactions. Many organic reactions could be carried out in higher yields, shorter reaction time and milder reaction conditions under ultrasound irradiation than that of conventional methods. Compared to conventional heating which provides thermal energy in the macro system, ultrasound irradiation is able to activate many organic reactions by providing the activation energy in micro environment. Therefore, we developed an environmentally benign methodology for the synthesis of diastereoselective dihydropyrrole derivatives via three component reaction of 2-arylidene malonitrile, hydantoin, and aromatic amines in the presence of Cs-NPs under ultrasonic irradiation Chitosan is the second abundant polysaccharide found on earth and it is extracted majorly from the shrimp and crab processing waste. Nanoparticles synthesized from chitosan have been widely studied as a bio-compatible alternative for metal nanoparticles in biological applications such as drug delivery. The aim of this study was to synthesize, characterize and evaluate the antimicrobial activity of the CNPs.

Materials and Methods

Shrimp shell Chitosan powder was purchased from Sigma Aldrich, India. Metaphosphoric acid flakes and concentrated glacial acetic acid was also purchased from Sigma Aldrich, India.

Preparation of nanoparticles

The 1% (w/v) chitosan solution was neutralized with 1N

sodium hydroxide to a pH of 7. The precipitated chitosan particles were then washed several times with double distilled water and centrifuged at 5000rpm for 30 mins. The precipitate was dissolved in 0.1M acetic acid solution under 400rpm for 24hours on a hotplate at 80°C. The solution was centrifuged at 5000rpm for 30mins and the supernatant was collected. 400ml double distilled water was added and stirred for 30mins at 400rpm. Centrifugation at 5000rpm for 30mins was conducted several times until a clear solution was obtained.

Characterization studies chitosan nanoparticles

Surface plasmon resonance detection (SPR) (UV)

Optical SPR activity of chitosan nanoparticles were observed at 200nm-400nm spectrum using UV-2450, SHIMADZU Spectrophotometer.

FT-IR analysis

The nanoparticles were harvested and characterized by FT-IR. The FT-IR spectrum was taken in the mid IR region of 400-4000 cm^{-1} . The spectrum was recorded using ATR (attenuated total reflectance) technique. The sample was directly placed in the KBr crystal and the spectrum was recorded in the transmittance mode.

Particle size and zeta potential analysis

The charge, size and distribution of the nanoparticles were measured using dynamic light scattering technique (HORIBA, SZ-100).

High Resolution Scanning electron microscopy

The structural morphological characteristics of the bacterial sample were observed under scanning electron microscope (HR-SEM) Hitachi's SU6600 at magnification ranging from 10X to 600,000X operated at accelerating voltage of 30kv.

Antimicrobial activity

Antimicrobial activity of CNPs was determined based on colony forming units (CFU) by in-vitro assays (disk diffusion). The disc was dipped in CNPs solution and then the agar plates were incubated at 37 °C for 2 and 4 days for fungi and bacteria, respectively. The zone size was determined by measuring the diameter of the inhibition zone in mm Yamac & Bilgili [7]. The positive controls were Itraconazole and Ketoconazole 30 μ g 5mm discs for fungi species and tetracycline, ampicillin, penicillin and CMZ 30 μ g 5mm disc for bacterial species. Distilled water was used as negative control for the antimicrobial assay.

Antioxidant activity of the nanoparticles

Percent discolouration = $(1 - (\text{Absorbance of the sample} / \text{Absorbance of the control})) \times 100$

Antioxidant activity of the Chitosan NPs was evaluated using DPPH radical scavenging activity. A dose of 0.1ml of the colloidal Chitosan NPs was added to 1.0ml ethanol solution of 0.1mM DPPH radical. The mixture was shaken vigorously for 2 min and incubated at 25°C in the dark for 90min. The absorbance of the sample

was measured using UV-2450, Shimadzu Spectrophotometer at 520nm against ethanol blank. A negative control was taken after adding DPPH solution to 0.1ml of deionized water. The percent of DPPH discoloration of the sample was calculated according to the following equation Xu & Chang [8]. Free radical scavenging activity was expressed as an equivalent of mmol Trolox standard. Linearity range of the calibration curve was 0.1 to 10 μ mol/ml (Correlation

coefficient $R^2=0.9$).

Results and discussion

Surface plasmon resonance (UV) analysis

A characteristic absorbance peak was observed at 260nm showing the optical SPR activity of the synthesized nanoparticles (Figure 1).

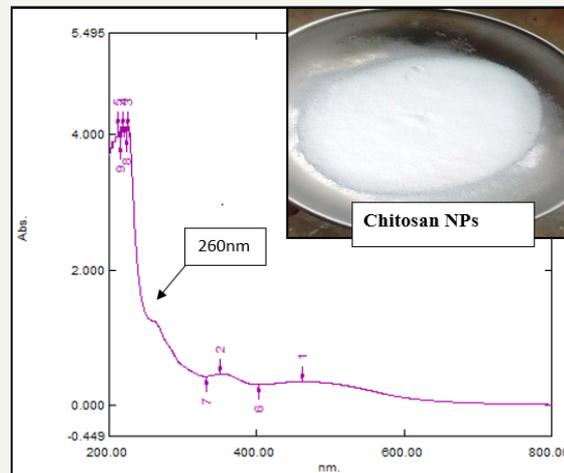


Figure 1: UV- visible spectra of Chitosan nanoparticles synthesized from Shrimp cells.

Fourier transformance infrared spectroscopic analysis (FT-IR)

The functional groups from the FT-IR spectra of Chitosan NPs sample, change in wave number of the functional groups was observed due to the reduction and stabilization. Characteristic of Chitosan was shown by a broad absorption band in the range 3206 cm^{-1} which is attributed to O-H stretching vibrations. The stretching vibrations of methylene C-H at 2308 cm^{-1} , absorption

peak at 1678 cm^{-1} correspond to the NH_2 . The spectra of Chitosan showed the different vibration that occurs after deacetylation process, which was not the emergence of vibration C=O at 1363 cm^{-1} region, which indicates the vibration of C=O has been reduced on Chitosan, The vibrations bands at 1032 cm^{-1} showed C-O-C vibration inside chitin ring and produced many peaks caused by the presence of hydroxide from chitin which contains a single bond C-O (Figure 2).

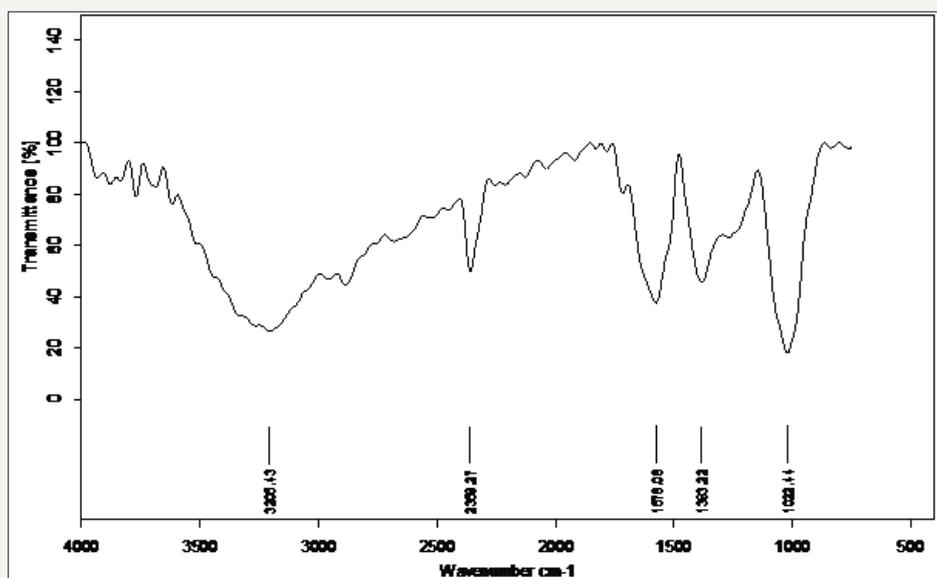


Figure 2: FT-IR spectrum of Chitosan nanoparticles synthesized from Shrimp cells.

Zeta potential and particle size (DLS) analysis

The mean zeta potential of the synthesized CNPs were showing good stability with 52.3mV at 25.1°C with a conductivity of 0.127mS/cm (Figure 3a). The mean particle size distribution of the CNPs was 32.2nm at 90° scattering angle under monodisperse form (Figure 3b).

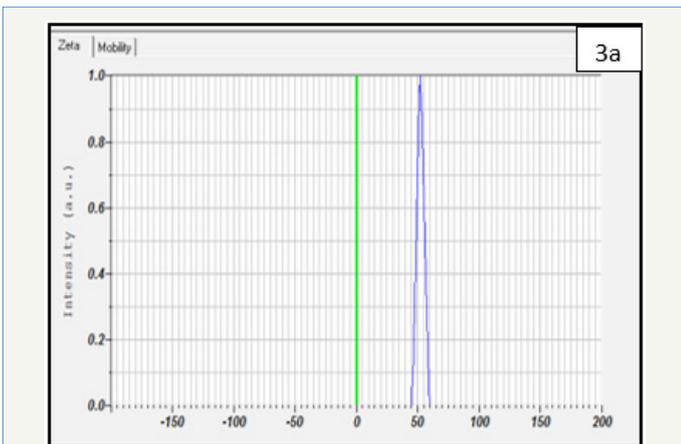


Figure 3a: Showing Zeta potential analysis and of Chitosan nanoparticles synthesized from Shrimp cells.

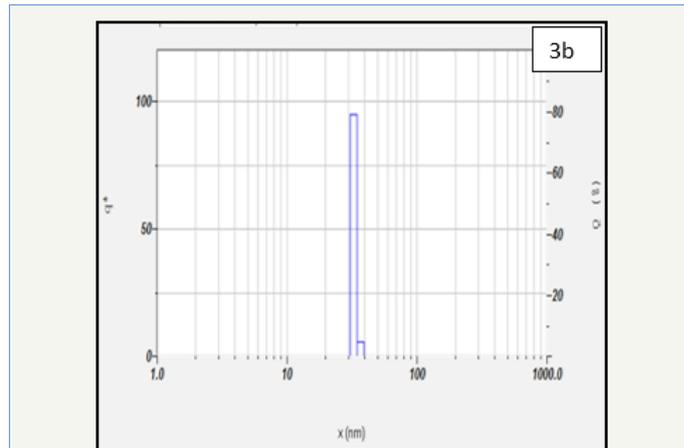


Figure 3b: Showing particle size distribution.

High Resolution Scanning electron microscopy

The formed Chitosan NPs appears slightly aggregated due to the absence of strong surface protecting ligands and found to be irregular in shape. The scanning electron micrographs of Chitosan revealed that it has a rough surface and it showed prominent sheath-like layers (Figure 4).

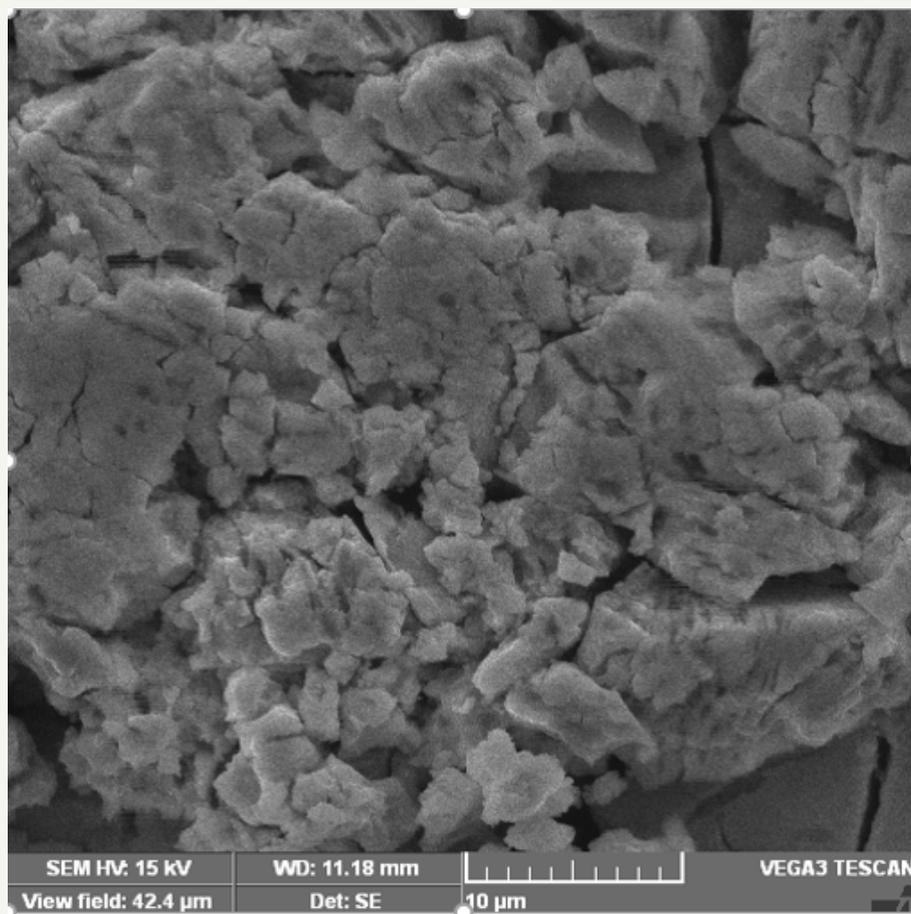


Figure 4: SEM analysis of Chitosan nanoparticles synthesized from Shrimp cells.

Antimicrobial activity

CNPs showed significant ($p < 0.05$) inhibitory effect against *Aspergillus niger* and *Aspergillus flavus* species when compared to IZ and KZ positive controls. A significant ($p < 0.05$) antibacterial activity against *Streptomyces* species, *Pseudomonas fluorescens* and *Staphylococcus aureus* has been identified. CNPs showed significantly high ($p < 0.05$) inhibitory effect on *Pseudomonas fluorescens* compared to other species and positive controls CAN, PCN and CMZ (Table 1&2). The antimicrobial activity is

probably derived, through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged nanoparticles. It reflects that Chitosan nanoparticles have an excellent anti-bacterial and anti-fungal effect and potential in reducing bacterial, fungal growth for practical applications, Chitosan nanoparticles promises the up scalable and non-toxic method of production of a variety of nanoparticles. Applications of Chitosan nanoparticles based on these findings may lead to valuable discoveries in various fields such as medical devices and antimicrobial systems.

Table 1: Inhibitory effect of ZnNPs (170ppm) on selected fungal strains

Fungal Strains	Inhibition Zone mm	IZ Equivalent %	KZ Equivalent %
<i>Aspergillus niger</i>	5.05 (± 0.24)	361.2141	216.7519
<i>Sclerotium sp</i>	0.31 (± 0.15)	40.68867	36.68711
<i>Rhizopus rolfsii</i>	0.51 (± 0.10)	43.25183	40.65554
<i>Aspergillus flavus</i>	1.69 (± 0.1)	148.5	113

170 ppm CIBC loaded Chitosan NPs showed significant ($p < 0.05$) inhibitory effect on *Aspergillus niger* and *Aspergillus flavus* species and several folds inhibitory activity greater than IZ (Itraconazole) and KZ (Ketoconazole, Antibiotic discs) positive controls

Table 2: Inhibitory effect of ZnNPs (170ppm) on selected bacterial strains.

Bacterial Strain	Chitosan Nano Particles	TCN Equivalent %	ACN Equivalent %	PCN Equivalent %	CMZ Equivalent %
<i>Bacillus subtilis</i>	0.15 (± 0.002)	4.22	4.69	13.39	3.65
<i>Streptomyces species</i>	0.62 (± 0.001)	14.69	20.85	18.13	72.3
<i>Pseudomonas fluorescens</i>	2.55 (± 0.022)	84.7	179.69	249.38	232.39
<i>Staphylococcus aureus</i>	2.21 (± 0.003)	23.33	68.59	86.35	62.57

A significant ($p < 0.05$) antibacterial activity against *Streptomyces* species, *Pseudomonas fluorescens*, and *Staphylococcus aureus* has been identified. Chitosan NPs showed more inhibitory effect on *Pseudomonas fluorescens* compared to other species and positive controls CAN (Canamycin), PCN (Penicillin) and CMZ (Cefmetazole) and equivalent antibacterial activity with TCN (Tetracycline).

Antioxidant activity

The in vitro antioxidant activity Chitosan was evaluated and compared as radical scavengers against 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), hydroxyl radical (OH), and superoxide radical (O_2^-) using established methods, and the effect of the molecular weight, the concentration, the newly generated hydroxyl group, the extra introduced positive charge of quaternary ammonium salt group, etc, on the antioxidant activity of these high molecular weight Chitosan is discussed. The data obtained in vitro models exhibited good antioxidant potency and suggested the possibility that high molecular weight Chitosan based films could be effectively employed as natural antioxidant materials for application in the field of food and medicine. Here in the concentrations of Chitosan NPs were (170ppm, 100ppm and 50ppm) Percent discoloration was 5.25 (± 0.03) and μ /mol Trolox equivalent activity was 0.240 at 170ppm, Percent discoloration was 1.08 (± 0.07) and μ /mol Trolox equivalent activity was 0.060 at 100ppm, Percent discoloration was 0.74 (± 0.04) and μ /mol Trolox

equivalent activity was 0.037 at 50ppm (Table 3). Antioxidant activities according to the DPPH assay and ROS determination tests. Furthermore, undesirable inflammatory reactions were not induced. Due to Chitosan favorable biological properties such as non-toxicity, biocompatibility, biodegradability and antibacterial ability, they are also promising candidates for drug delivery carriers and cell proliferation enhancers. However, most of these studies are still at the laboratory level. Additional studies are necessary before their industrial application. We hope that more Chitosan-based nano carriers can be developed and applied in the biochemical and food engineering fields soon Sekar Praveen kumar et al. [9]

Conclusion

From the results it can be concluded that stable chitosan nanoparticles having a mean particles size of 32.2nm can be derived from shrimp shell chitosan. The results also showed significant antimicrobial activity of CNPs against *Streptomyces* species, *Pseudomonas fluorescens* *Staphylococcus aureus*, *Aspergillus*

niger and *Aspergillus flavus*. Further studies are recommended for other pathological toxicological microbial strains. The findings in this study may lead to the development of CNPs-based new antimicrobial systems for medical applications, Hence, the current

investigations showed the superiority of Chitosan NPs are very good for antioxidant activity for and suggested an efficient system for delivering Chitosan with antioxidant activities.

Table 3: Antioxidant activity of Chitosan NPs.

Chitosan NP Concentration	Percent Discolouration	μ /mol Trolox Equivalent Activity
170 ppm	5.25 (\pm 0.03)	0.24
100ppm	1.08 (\pm 0.07)	0.06
50ppm	0.74 (\pm 0.04)	0.037

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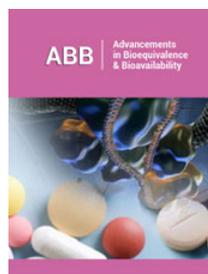
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