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RESEARCH ARTICLE

Synthesis and characterization of dye coated fluorescent chitosan nanoparticles

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Abstract

Synthesis and characterization of fluorescent chitosan nanoparticles was carried out. Chitin was prepared from prawn shells using deproteination, demineralization, and decolourization processes. Deproteination process was carried out to remove the protein present in the prawn shells and demineralization process was carried out to remove the minerals present in the prawn shells and finally decolourization process was done to remove the colour of the prawn shells. Chitosan was prepared using deactylation of chitin with sodium hydroxide. Fluorescent chitosan nanoparticles were prepared by interaction between amine groups of chitosan with rhodamine 6G. The X-ray diffractometer showed the amorphous nature of the chitosan nanoparticles and Transmission electron microscopy (TEM) results revealed spherical shape of the nanoparticles (50 nm). Fourier transform infrared spectroscopy (FTIR) results confirmed the reaction occurred between dye and chitosan nanoparticles. UV-spectrofluorometer showed the excitation (525 nm) and emission (557 nm) spectra of the fluorescent chitosan nanoparticles.

Keywords: Chitosan, chitin, prawn shells, fluorescent chitosan nanoparticles, rhodamine 6G.

Introduction

Nanotechnology is encompassing science, engineering and applications of submicron materials connecting unique physical, chemical and biological properties of nanoscale substances in basically new and constructive ways (Sekhon, 2012). Nanoparticle is described as a small thing that performs as an entire unit in terms of its transport and properties (Singh *et al.,* 2011). Currently nanotechnology is often used for variety of applications in fiber, textiles, agriculture, electronics, forensic and medical therapeutics (Poovi *et al.,* 2011). Chitosan is a cationic polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine component derived by partially deacetylation of chitin, which is the second most profuse biopolymer of the world and usually found in the exoskeleton or cuticles of many invertebrates and in the cell wall of most fungi and some algae (Douglas *et al.,* 2006; Du *et al.,* 2008). Three kinds of functional groups such as an amino group, primary and secondary hydroxyl groups are present in chitosan. The backbone of glucosamine of the deacetylated chitosan has high density of amine group, which allowing the strong electrostatic interactions with proteins and genes (Qi and Xu, 2004a; Gan and Wang, 2007).

Chitosan is extensively used in pharmaceutical research and in industry as a carrier for drug delivery and as a biomedical material due to its biodegradable, non-toxic and bio-compatibility properties (Pourdounighi *et al.,* 2010). Chitosan can be shaped into various forms such as gel, fibers, sponges, films, beads, microsheres, and nanoparticles. There are several methods that have been used for the preparation of chitosan nanoparticles such as ionic gelation, microemulsion, emulsification solvent diffusion and polyelectrolyte complex, ionic gelation and self assemble polyelectrolytes are widely used for the preparation of chitosan nanoparticles. These methods are easy to carry out without the use of organic solvent or high shear force. Due to its large surface area there has been a growing interest in the investigation of chitosan nanoparticles (Liu *et al.,* 2007; Tiyaboonchai, 2003).There is a great demand to develop highly sensitivity, nonisotopic analysis system for biological applications (Santra *et al.,* 2001).

Among the various non-isotopic analysis methods, fluorescent probes is a significant non-isotopic analysis technique in biomedical and biotechnological fields, however, the conventional organic fluorescent labels often suffer the problems of photobleaching, instability and sensitivity to environmental conditions such as pH variations (He *et al.,* 2001; Huo, 2007). To overcome these problems, a number of nanoparticles such as dye doped silica, quantum dots and metal nanoparticles have been explored as signaling probes for bio-analysis, dye doped nanoparticles has high signal amplification, excellent photostability and easy surface modification properties (Zhao *et al.,* 2003; Smith *et al.,* 2006). In recent times, polymer nanoparticles having directional receiver molecules are expected to be novel diagnostic agents and targeting drug carriers to target specific cells and organs, in addition bioconjugated fluorescent polymers have been widely used in various biological sensing applications (Konno *et al.,* 2004; Disney *et al.,* 2004). Among the various polymers, chitosan is biodegradable, biocompatible polymer, which displays low immunogenicity and minimal toxicity (Qi and Xu, 2004a; Gan and Wang, 2007).

The primary amine groups of chitosan make it possible for easier coupling of biomolecules such as DNA, antibodies, proteins, enzyme as well as fluorescent dyes (Douglas *et al.,* 2006; Jiayin and Jianmin, 2006). At present, chitosan nanoparticles have been extensively developed and explored for various applications. Early reports proved that chitosan nanoparticles are a suitable matrix for coupling biomolecular and fluorescent molecule concurrently. Tallury *et al.* (2009) prepared the Fluorescein isothiocyanate labeled chitosan nanoparticles using water in oil microemulsion method and used for cell imaging. Katas and Wen (2011) stated that the fluorescent chitosan dextransulfate nanoparticles could be used for various medical applications. The present work investigated the preparation of chitosan from prawn shell using four different processes and chitosan nanoparticles were characterized using X-ray diffractometer, FTIR spectrophotometer, TEM and finally measured using fluorescence microscope.

Materials and methods

Chemicals: Sodium hydroxide, hydrochloric acid, oxalic acid, potassium permanganate and rhodamine 6G, Dimethyl sulfoxide were purchased from Himedia, Mumbai, India.

Preparation of chitosan: The prawn shells were obtained from Tuticorin coastal area, TN, India. Then, the prawn shells were washed several times with tap water and dried in sun shadow. Chitosan was prepared using modified procedure of Elder *et al.* (2004). The deproteination of Prawn shells (10 g) was carried out using 5% sodium hydroxide solution at 90-100°C for 1 h. During this reaction, the proteins were removed and then the shells were washed several times to remove excess of sodium hydroxide. Hydrochloric acid (5%) was used to remove minerals from prawn shells and this reaction was carried out at room temperature for 1 h and then, the shells were washed thoroughly and decolorized with 1% potassium permanganate (30 min) and 1% oxalic acid (30 min to 2 h) at room temperature. Then 50 % sodium hydroxide was used for the deacetylation process and this reaction was carried out for 24-48 h at 90-100°C.

Preparation of chitosan nanoparticles: Ionic gelation method was employed for the preparation of chitosan nanoparticles. 3 g of chitosan was mixed with 1% acetic acid (v/v) and mixed well using magnetic stirrer. The chitosan nanoparticles were formed by adding tripolyphosphate (0.25% w/v) drop by drop under magnetic stirring. Then, the solution was centrifuged at 10,000 rpm for 10 min to remove the residual sodium tripoly phosphate and the particles were freeze-dried.

Preparation of fluorescent chitosan nanoparticles: Freeze-dried chitosan nanoparticles (5 mg) was re-dispersed in 5 mL DMSO solution followed by the addition of 0.5 mL NaOH (0.1 M) and rhodamine 6G dissolved in methanol at 10.0 mg mL^{-1}. The reaction was allowed to proceed for 4 h in the dark at room temperature (Jiayin and Jianmin, 2006). The prepared dye labeled chitosan nanoparticles were centrifuged and washed several times with methanol until the free rhodamine 6G could not be detected in the supernatant.

Nanoparticle characterization: The prepared chitosan and chitosan nanoparticles were characterized using X ray diffractometer (Bruker D8 Advance using CuKα radiation at the 40 kev in the range of 10-80). Scanning electron microscope (Hitachi, Model: S-3400N) was used to examine the shape of the nanoparticles. Fourier infrared spectrophotometer (Thermo Nicolet Model: 6700) analysis was carried out to identify the functional groups of chitosan and fluorescent chitosan nanoparticles. Spectrofluorometer (Fluorolog: FL3-11) was used to record the fluorescence excitation, and emission of fluorescent chitosan nanoparticles. Transmission electron microscope (Philips CM200) was used to measure the size of the chitosan nanoparticles. The fluorescence image was recorded in fluorescence microscope (Nikon fluorescent Microscopy).

Results and discussion

Chitin was prepared from prawn shells using deproteination, demineralization, and decolourization processes. The obtained white powder after the decolorization process was chitin and it was deacetylated with NaOH, chitosan was obtained as a result of deacetylation process. Chitosan nanoparticles was formed by ionic interaction between tripolyphosphate and $–NH_3^+$ of chitosan molecule (in acidic solution, NH_2 of chitosan molecule is protonized, to be $–NH_3^+$). After freeze drying process, the chitosan nanoparticles were obtained and fluorescent chitosan nanoparticles were obtained by the interaction between the amino group of chitosan nanoparticles and rhodamine 6G. Figure 1 shows the XRD pattern of chitosan nanoparticles prepared via ionic gelation method. No peak is found in the X-ray diffractograms of chitosan nanoparticles.

Fig. 1. XRD pattern of chitosan nanoparticles.

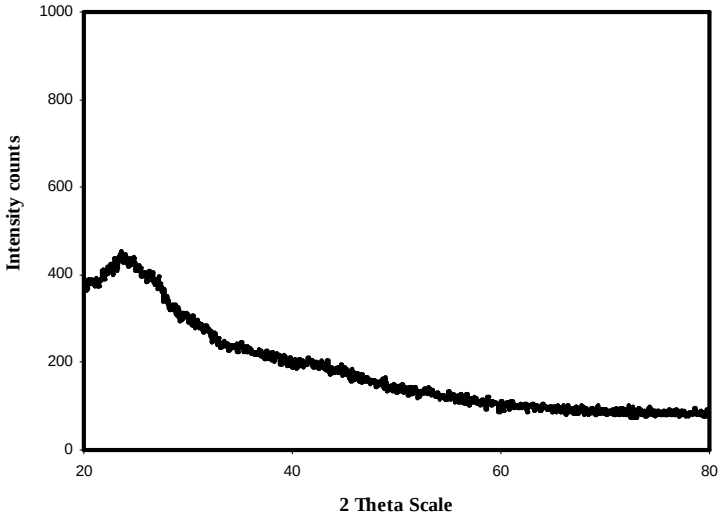


Fig. 2. FTIR spectrum of chitosan nanoparticles and fluorescent chitosan nanoparticles.

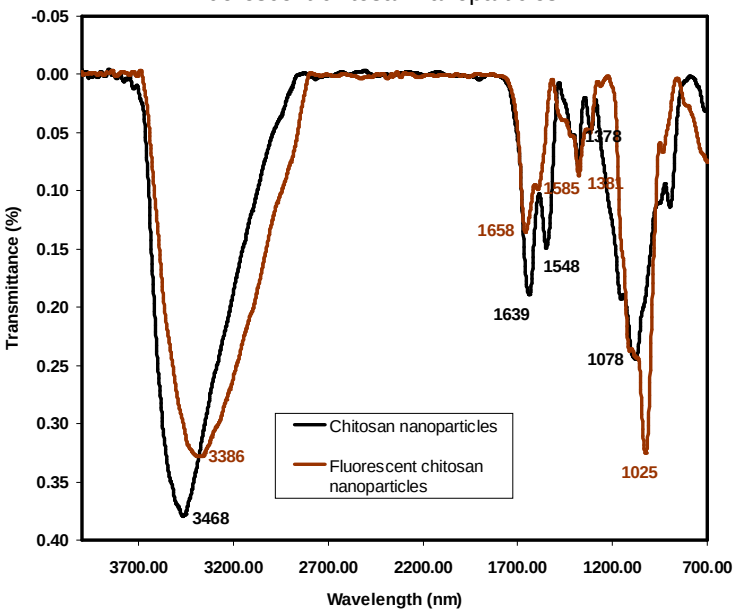


Fig. 5. UV-spectrofluorometer image of fluorescent chitosan nanoparticles.

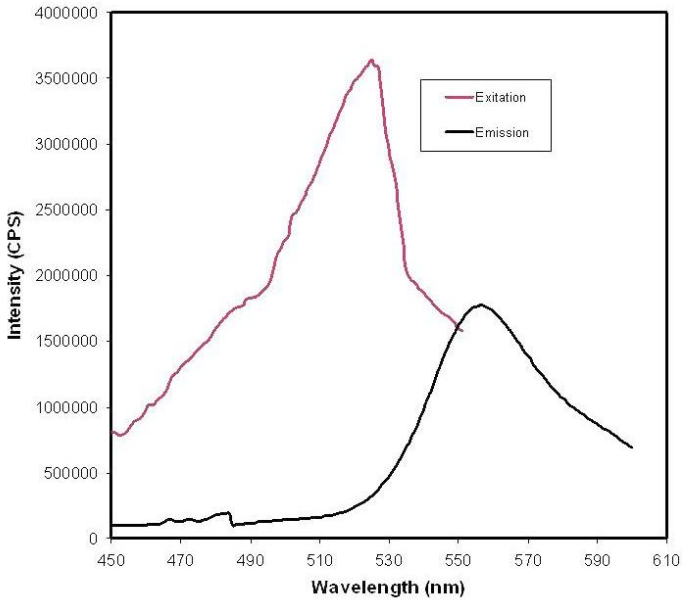


Fig. 3. TEM image of chitosan nanoparticles.

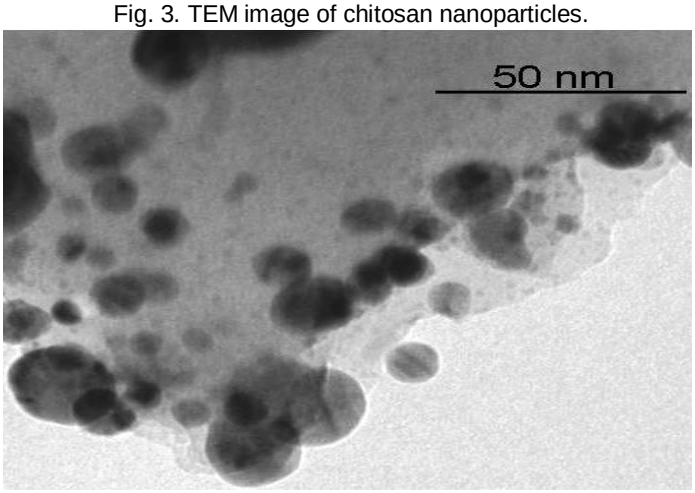
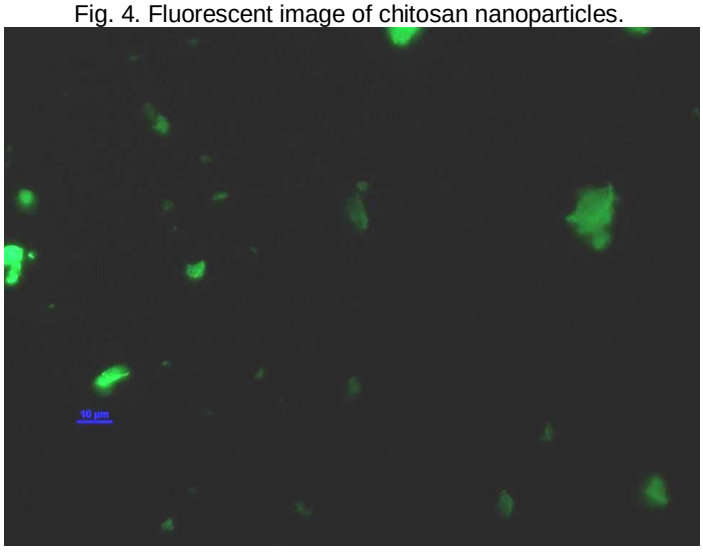


Fig. 4. Fluorescent image of chitosan nanoparticles.



This illustrates that the synthesized chitosan nanoparticles are amorphous in nature. Early studies have reported that the drying process have an effect on the crystallinity of chitosan (Qi and Xu, 2004a). Qi *et al.* (2004b) prepared chitosan nanoparticles and used for bactericidal activity and he reported that the prepared chitosan nanoparticles were amorphous nature. The present findings showed the synthesis of chitosan nanoparticles. Figure 2 shows the FTIR spectrum of chitosan and fluorescent chitosan nanoparticles. FTIR spectrum shows the surface functional group of the nanoparticles. The black line shows FITR graph for chitosan nanoparticles. The band at 3468 cm^{-1} reveals the O-H stretching vibrations. The peak at 1639cm^{-1} corresponds to the primary amine group of chitosan nanoparticles. The band at 1548 $^{cm-1}$ shows N-H bend vibrations. The peak at 1078 cm^{-1} indicates C-O symmetric stretching vibrations. The N-O Stretching vibrations occurred around 1381 cm^{-1} (Liu *et al.,* 2007; Pourdounighi *et al.,* 2010). In fluorescent chitosan nanoparticles, the new sharp peak occurs at 1025 cm^{-1} indicates C-N stretching of alkaline amine and the peak at 1078 cm^{-1} of chitosan nanoparticles disappears. Thus, FTIR results confirmed the reaction occurred between rhodamine 6G and chitosan nanoparticles. Rhodamine dye gets attached to the chitosan nanoparticles by the interaction between the amino groups of chitosan nanoparticles forming fluorescent chitosan nanoparticles. Figure 3 shows the TEM image of the chitosan nanoparticles. The chitosan nanoparticles are spherical in shape (scale bar at 50 nm). The fluorescence image of fluorescent chitosan nanoparticles emit green light in blue filter (Fig. 4). This result confirmed that rhodamine 6G molecules are added to the chitosan nanoparticles.

The fluorescence excitation and emission spectra of rhodamine 6G labeled chitosan nanoparticles were examined by spectrofluorometer. Figure 5 shows the fluorescence excitation and emission spectra fluorescent chitosan nanoparticles. The fluorescent excitation of dye labeled chitosan nanoparticles were found at 525 nm and emission occurred at 557 nm. The dye doped chitosan nanoparticles can be used for cell imaging, DNA protection process, thus, there are numerous unique advantages concerning this novel fluorescent label method based on the fluorescent nanoparticles by combination of nanotechnology, biotechnology and fluorescent label technology (He *et al.,* 2001).

Conclusion

Chitosan was prepared from prawn shells by 4 sequential processes. The amorphous chitosan nanoparticles were obtained using ionic gelation method. The biocompatible fluorescent chitosan nanoparticles were prepared by reacting amino group of chitosan nanoparticles with rhodamine 6G. The FTIR spectrum of chitosan nanoparticles and fluorescent chitosan nanoparticles confirmed the reaction between the chitosan nanoparticles and rhodamine 6G. The prepared fluorescent chitosan nanoprobe could be used for cell imaging applications in near future.

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