Chitosan encapsulated ZnO nanoparticles for labeling applications

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Abstract. Chitosan (CS) encapsulated ZnO nanoparticles (NPs) were prepared in an aqueous phase at pH 7. Chitosan was initially attached with glycine to make it water-dispersible. The product was precipitated as micro gels by stirring with methanol and poly ethylene glycol mixture at pH~7. FTIR spectra showed a characteristic peak for the amide functional group, which had confirmed the substitution reaction. The peaks corresponding to the presence of glycine and PEG were also observed. ZnO NPs were dispersed in water after etching with acetic acid. The conjugate was obtained because of the electrostatic interaction between ZnO and chitosan in the solution. The photoluminescence spectrum exhibited the quenching of the characteristic excitonic peak of ZnO at 380 nm, but showed a new peak around 413 nm for the chitosan-ZnO conjugate.

1. Introduction

Chitosan is the second most naturally abundant polysaccharide, a linear chain of 2 amino-2 deoxy-D-glucopyranose. Because of its multi-functional, biodegradable, bio-absorptive and low level of toxicity, it has been useful in a wide range of applications [1-2]. In particular, it has been an attractive candidate for drug delivery applications over the past few years [3] and also useful in the formulations of the oral dispersive tablet ODT [4]. It is a completely or partially deacetylated product of chitin. Besides having reactive functional groups such as OH and NH2, the water dispersible nature of the chitosan is poor because of its rigid crystalline structure [5]. This poses a major problem towards its reactivity. Generally it is made soluble by the protonation of amino groups in an aqueous acidic medium. However, derivatives of chitosan such as formate, acetate, lactate, malate and glycolate, are water soluble due to the rise in polarity and the electrostatic repulsion between individual molecules[6]. Hence the derivatives were found to be highly reactive towards other biomolecules [7]. Recently, chitosan based organic-inorganic composites have gained attraction for novel biochemical applications. There are reports on conjugation of magnetic iron oxide nanoparticles with chitosan hydrogels for applications such as MRI contrasting agents, enzyme absorption and enzyme immobilization [8-9]. It is also interesting to conjugate chitosan with fluorescent semiconducting QDs or nanoparticles for the applications of biolabeling, wound dressing and drug delivery [10]. ZnO is already a well-known fluorescent material which has been gaining importance in biological applications, mainly because of its non-toxic nature. There are articles reporting the incorporation of ZnO QDs in chitosan [11-12].
However in this investigation, we explored the UV-blue fluorescent ZnO NPs prepared by an arc plasma method [13]. The surface of ZnO NPs was chemically treated to reduce their size and improve the surface functionality. On the other hand, the rigid nature of chitosan was modified by the substitution of amino groups with glycine, followed by crosslinking with PEG for the micro/nano gel preparatory process. In both cases, the water dispersible nature of the corresponding micro/nano particles was improved by the chemical modification. Finally ZnO NPs were incorporated into CS microgels by mixing the two solutions. The conjugate was investigated by UV-Vis absorbance, photoluminescence spectroscopy (PL) and transmission electron microscopy (TEM) with electron diffraction (ED) and energy dispersive spectra (EDS) to understand the different mechanism involved in the reaction.

2. Experiment

2.1 Materials and reagents
A commercially available low molecular weight chitosan (Sigma-Aldrich) was purchased. Analytical grade acetic acid, glycine, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), polyethylene glycol (PEG-10000), sodium hydroxide (NaOH), cellulose membrane and triply distilled water have been used. ZnO nanoparticles synthesized by the arc plasma method were used in all the experiments.

2.2 Preparation of chitosan-glycine derivative
A required amount of CS (0.41g) was dissolved in 2% aqueous acetic acid solution and stirred for 3 h to a complete dissolution. The solution was re-precipitated by a slow addition of NaOH at pH 7-8 for size reduction and improvement in water dispersibility. This step was repeated twice. The solution was again dissolved in the same quantity of acetic acid, stirred for 2 h and maintained at the pH of 5-6. In a separate container, an equimolar mixture of glycine and EDC (0.14 M) were stirred in water for 5 min. The glycine solution was then mixed with chitosan and stirred for 6 h. It was then kept without stirring for 2 days and later neutralized with NaOH in the pH range of 6.5–7. No precipitation was found, which confirmed the formation of the chitosan-glycine derivative. It was precipitated by an excess methanol after the addition of PEG, stirred for a few minutes and rested for 2 days. The precipitate was dialyzed repeatedly against distilled water in a cellulose membrane for about 2 days. The small sized particles were collected by microcentrifugation, discarding the sediment at the bottom. Finally the precipitate was freeze dried for further experiments.

2.3. Surface treatment of ZnO nanoparticles
Etching was carried out by a 1% aqueous acetic acid saturated solution of ZnO for the size reduction. The small sized particles were immediately separated and purified by a repeated centrifugation and re-dispersed in water. The centrifugation was repeated until the pH was brought to 7.

2.4. Encapsulation of ZnO with chitosan derivative
The dispersion of ZnO NPs in water was added with a measured quantity of PEGylated Chitosan-glycine derivative and stirred in a beaker for 24 h. The final product was collected from a microcentrifuge using a fixed angle rotor (9201 g) after 5 min. The supernatant containing small size particles was discarded. The sediment was redispersed in distilled water and purified by a repeated centrifugation (20630g). The dispersion of ZnO-chitosan conjugate was finally collected for characterization.

2.5. Characterization
2.5.1. FTIR spectroscopy. The KBr pellets of pure chitosan and their derivatives were recorded using a Jasco FTIR/460. All spectra were acquired from 64 scans between 4000 to 400 cm$^{-1}$ at a resolution of 4 cm$^{-1}$.
2.5.2. UV-Vis spectroscopy. The absorbance of the pure ZnO dispersed in water and chitosan encapsulated ZnO were measured using a Shimadzu UV 2450 UV-Vis spectrophotometer with a
double beam monochromator of minimum spectral width of 0.1 nm. The measurement range is from 190 to 1100 nm.

2.5.3. Photoluminescence spectroscopy. The photoluminescence of pure ZnO and chitosan-ZnO conjugate were observed using a He-Cd laser of excitation wavelength 325 nm. The signals were detected with a charge coupled device connected to an Oriel MS 257 Monochromator.

2.5.4. Transmission Electron Microscope. The pure and conjugated samples were observed under TEM (JEOL JEM 2010) attached to an Oxford (LINK) ISIS) energy dispersive spectra.

2.5.5 Particle size analyzer. The particle size was measured by a Horiba LB550 particle size analyzer based on dynamic light scattering. The measurement range is from 1 to 6000 nm.

3. Results

3.1. Results of modification of chitosan

The whole scheme of chitosan modified with glycine followed by crosslinking with PEG is shown in figure 1. This reaction was enhanced by the addition of EDC crosslinker. The solubility of the polymer was improved after the substitution of glycine while the pH was maintained at 7. The OH groups of the PEG interact either with free amino groups of chitosan or that of glycine. The FTIR spectra of chitosan (CS) and the derivative (CS-Gly-PEG) are shown figure 2a and 2b respectively. We have observed large and intense bands around 3200-3400 cm\(^{-1}\) correspond to hydrogen bonded OH stretching overlapped with NH stretching bands for both the samples. C-H stretching was found at 2880 cm\(^{-1}\). The pure sample showed the characteristic peaks correspond to amide I bond at 1657 cm\(^{-1}\) (C=O stretching), amide II bond at 1594 cm\(^{-1}\) (N-H bending) and amide III bond at 1377 cm\(^{-1}\) (C-N stretching coupled with N-H deformation) [14]. CS-Gly-PEG showed enhanced intensity of 1657 cm\(^{-1}\) related to amide bond formed between glycine and chitosan [14] and weak intensity of 1594 cm\(^{-1}\) peak confirming the substitution. The derivative showed additional peaks with respect to PEG at 1280 cm\(^{-1}\) and 1109 cm\(^{-1}\) [15]. Also, the intensity of the peak at ~2880 cm\(^{-1}\) increased due to the addition of PEG.

**Figure 1.** (a) Reaction between chitosan and glycine in the presence of EDAC. (b) Reaction of CS-Gly with PEG

TEM photograph of the chitosan derivative (CS-gly-PEG) with electron diffraction pattern is shown in the inset of figure 3. The shape and size of the polymer changed with a change of setting-time after the addition of PEG. But generally a wide range of particle sizes were present as observed by the particle size analyzer, which showed the range to be around 1000 nm (figure 3). The small size particles can be collected by using the centrifugation as was done in all of our experiments.
3.2. Results of surface treated ZnO NPs

The ZnO nanoparticles (NPs) prepared in our laboratory were used throughout this investigation. They were prepared by using the arc plasma method. The detailed experimental investigations and properties of the NPs were discussed elsewhere [13]. In fact, as-prepared NPs were of different morphology (rods, spheres, wires) and sizes (10-500 nm). But they exhibited good quality in terms of a bright and fundamental UV exciton emission at 380 nm in addition with a very weak green emission. The absorbance is around 375 nm. Because of their improved emission efficiencies and low toxicity, the surface was chemically modified with organic chains to be functional in biological applications and reported in our previous article [16]. In the present investigation, we reduced the size and obtained uniform sized particles in order to facilitate the conjugation. It was achieved by the etching with 1% acetic acid. In this case, we used a saturated solution of ZnO in acetic acid. The initial pH was around 5. This leads to partial etching and reduction in size of the NPs. Indeed, Meulenkamp has previously investigated the etching of ZnO NPs on colloidal solutions [17]. The reaction mechanism is shown in the following scheme 1. But we used as-prepared NPs in our experiments. During the experiment, the first quantities of ZnO were dissolved completely to produce Zn$^{2+}$ ions in the solution. Later this process is gradually slowed down due to saturation by the addition of excess amount of NPs. The final product is completely an acetate sol of ZnO. The small sized particles were immediately separated using low speed centrifugation in the supernatant by discarding the large size particles at the bottom. Because there is always a possibility of further etching in the supernatant with the presence of free H$^+$ ions, the high speed centrifugation was repeated to purify and collect the small sized NPs in distilled water. We obtained the final dispersion of NPs at pH 7. It was stable over a long time for almost 3 months confirming no further etching or acidity in the dispersion.

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\text{ZnO} + \text{CH}_3\text{COOH} \rightarrow \text{ZnOH}^+ + \text{CH}_3\text{COOH}
\]

\[
\text{ZnOH}^+ + \text{CH}_3\text{COOH} \rightarrow \text{Zn}^{2+} + \text{CH}_3\text{COO}^- + \text{H}_2\text{O}
\]

The TEM photograph of the etched NPs is shown in figure 4. The particle size was measured by a particle size analyzer. Both confirmed the size is around 5-10 nm. The electron diffraction showed circular patterns corresponding to the smaller size as well as a decrease in crystalline nature of the
NPs. But we observe no change in the UV-Vis absorbance of this sample, except the reduction in the intensity analogous to a decrease in concentration.

4. Discussion

ZnO conjugated chitosan nanoparticles were obtained by stirring the two mixtures at room temperature for 24 h. Chitosan is a cationic polymer basically. The interaction between ZnO NPs dispersed in water and the polymer was considered to be electrostatic in nature as mentioned in our previous report although the polymer was PNIPAM in that case [18]. The schematic of the interaction is shown in figure 5. However in this report, we have deliberately carried out chemical modification on the surface of ZnO for a size reduction below 10 nm and to improve functionality of NPs. The absorbance properties did not change noticeably even after chemical treatment of the nanoparticles as shown in figure 6 which compares pure ZnO (6a) and chitosan-ZnO (6b). The absorbance peak was observed at 365 nm in both the cases.

![Figure 4. TEM photograph of acetic acid etched ZnO NPs and electron diffraction pattern in the inset](image)

![Figure 5. Interaction between cationic polymer (CS-gly-PEG) and ZnO NPs](image)

![Figure 6. UV-VIS spectra of (a) Pure ZnO NPs dispersed in water (b) ZnO NPs encapsulated by](image)

Figure 7a and 7b shows the TEM photograph after the encapsulation of nanoparticles by chitosan polymer. The incorporation of ZnO NPs was further confirmed by the EDS result in the inset of figure 7b. The signals with reference to Cu were from the metal grid used for TEM measurements. We
observed spherical shaped polymer NPs of different sizes all through the copper grid. This is possibly due to (1) strong electrostatic interaction caused an assembly of CS polymers due to the incorporation of small size ZnO NPs and (2) expulsion of PEG crosslinker during the conjugation between ZnO and polymer. The electrostatic interaction (1) is already represented in figure 5. Expulsion of PEG (2) is a significant result during encapsulation. Wu et al observed similar results during ammonium glycyrrhizinate incorporation in chitosan-PEG network [19]. They reported irregularly shaped chitosan NPs after coordinating PEG and uniformly shaped NPs before coordinating. In fact the interaction between PEG and CS is through weak intermolecular hydrogen bonding between OH groups of PEG and NH$_2$ groups of CS. Hence PEG is considered to be loose in the polymer network. Since we used high molecular weight (~10000) PEG for crosslinking, it may not be active as a crosslinker in the small sized CS polymer NPs especially after conjugation which caused strong electrostatic interaction to support the assembly of CS NPs. This is supported by the FTIR result as shown in Figure 2c. Despite finding the peaks (1280 and 1109 cm$^{-1}$) correspond to PEG in the derivative sample, they disappear after incorporation of ZnO. In addition, the evident rise in the intensity of C-H peak at 2800 cm$^{-1}$ after PEG crosslinking (Figure 2b), reduces again after ZnO incorporation (Figure 2c). The change could also be observed in the peak (3000-3400 cm$^{-1}$) related to OH and NH stretching associated with hydrogen bonding.

The peak becomes intense, narrow and blue shifted to 3443 from 3429 cm$^{-1}$ after the encapsulation of ZnO. It was due to the decrease in the extent of H-bonding and the interaction of polymers with NPs. After the addition of NPs into the network of CS, the main peaks at 3400 and 1650 cm$^{-1}$ shifted with respect to the type of interaction between NPs and polymers [14]. The intensity of the peak at 1640 cm$^{-1}$ increased drastically and also broadened after ZnO incorporation, which confirms the clear interaction between chitosan and ZnO. It may also be due to the presence of C-O from our native ZnO samples prepared by arc plasma method using carbon arc which always shows a peak in this region.

Photoluminescence of the pure ZnO and CS encapsulated ZnO are shown in figure 8. Pure ZnO exhibited the characteristic and intense exciton emission at 373 nm with a weak emission around 430 nm, which is usually assigned as zinc interstitials. The difference in emission of the pure ZnO and ZnO-chitosan under a UV lamp is depicted in the inset of figure 8. Pure chitosan also exhibits a weak emission around 440 nm. But after the encapsulation with the chitosan derivative, it showed a broad and intense emission centered around 413 nm. The encapsulation by chitosan usually quenches emission properties of QDs as reported in [20]. Quenching of emission peaks and corresponding energy transfer between MWCNT and modified chitosan was reported in detail [21]. They concluded that it is possibly due to photoinduced electron transfer or energy transfer at the interface. In our case,
the excitonic emission of ZnO is quenched after encapsulation of chitosan, possibly due to the hole capture by CS, and showed a new blue emission peak corresponding to the interaction between NPs and polymer. However the mechanism is not clear at this stage and it needs further investigation. The dispersion remained stable over 3 months because of the stabilization of small sized ZnO NPs by the encapsulation.

5. Conclusions

The ZnO-chitosan nanoparticle conjugate was obtained by an electrostatic interaction after their respective chemical modification. The stabilization of NPs by CS in an aqueous medium at neutral pH will be a ready to use dispersion with an improved functionality. CS significantly quenched the fundamental excitonic emission of ZnO and exhibited a new broad peak in the visible region. The cross linking by PEG was found to be ineffective after conjugation, however further investigation in this aspect will improve the characteristics of the polymer networks for encapsulating the NPs.

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