RESEARCH ARTICLE

Preparation of Chitosan Nanoparticles and their *In-vitro* Characterization

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ABSTRACT- Background- Chitosan is a natural, biocompatible, biodegradable, nontoxic and easily available polymer that can be used to prepare nanoparticles. Chitosan nanoparticles can be widely used in pharmaceutical industries as an antimicrobial agent or as drug delivery vehicle. The aim of the study was to prepare chitosan nanoparticles and characterize them.
Methods- Chitosan nanoparticles were prepared by ionic gelation method and characterized by UV-Vis spectroscopy, FTIR (Fourier transform infrared spectroscopy), DLS (Dynamic Light Scattering) and Scanning electron microscopy (SEM).
Results- The present study showed that chitosan nanoparticles were successfully prepared by ionic gelation method. The obtained chitosan nanoparticles were characterized and study revealed that they are stable spherical in shape. The size of chitosan nanoparticles (CSNPs) at selected concentration was 216 nm and zeta potential 50 mV was done by zeta sizer Nano S (Malvern, UK).
Conclusion- Chitosan nanoparticles were successfully prepared by ionic gelation method. These nanoparticles was highly effective in nanoparticles production.
Key-words- Chitosan, Chitosan nanoparticles, DLS, FTIR, SEM, UV-Vis spectroscopy

INTRODUCTION

Nanotechnology is the emerging science that deals with nm scale and nanoparticles are one of the building blocks in nanotechnology. Recently from last few years, nanotechnology and polymers together have captivated a tremendous interest in many areas including pharmaceutical industry and therapeutic innovation among others. Nanoparticles are the solid colloidal particles in nanometer range i.e. from 10–1000 nm [1]. Due to their small size and large surface area they exhibit unique physical and chemical properties. Nanoparticles can be prepared both from natural polymers such as protein, polysaccharide or synthetic polymer such polystyrene. The nanoparticles which are prepared from synthetic polymers involve heat, organic solvent or high shear force that can harm the drug stability. In contrast, nanoparticles prepared from natural polymers offer mild as well as simple preparation methods without the use of organic solvent and high shear force.

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Over the last few years chitosan nanoparticles, have gained considerable attention in present scenario due to their inherent biological properties. CSNPs are being used in a variety of different products and applications, ranging from pharmaceutical, drug delivery, tissue engineering, and food packaging to bio-sensing, enzymes immobilization, fuel cell manufacturing and waste-water treatment [2]. Chitosan [poly-(b-1/4)-2-amino-2-deoxy-D-glucopyranose] is a versatile biopolymer with film, fiber, and micro/nanoparticle forming properties; due to its abundance, low production cost, biodegradable, biocompatible, renewable and non toxic nature. It is chemically inert, non-toxic, natural polysaccharide possessing robust and broad antimicrobial activities due to its polycationic nature.

CSNPs can be easily prepared by Ionic gelation method [3]. It is a simple and mild method which is widely used for the preparation of CSNPs. It depends on the approach on ionic gelation where NPs are formed by means of electrostatic interactions between the positively charged CS chains and polyanions employed as cross-linkers like tripolyphosphate (TPP). Chitosan interacts with polyphosphate ions to form nanoparticles with different diameters depending on the mutual ratio among them. The characterization of chitosan nanoparticles can be done by various methods: Fourier Transform Infrared (FTIR) Spectroscopy is used for identification and

characterization of the functional groups on the surface of CSNP, Dynamic light scattering (DLS) is used for measuring the zeta size and zeta potential, Scanning electron microscopy (SEM) is used for the determination of their morphology and shape.

MATERIALS AND METHODS

Preparation of chitosan nanoparticles- Chitosan nanoparticles (CS) were prepared by ionic gelation method [3] in the Department of Biotechnology, Defense Research Development Establishment (DRDE), Gwalior in the duration of 2014. The CS nanoparticles were obtained by inducing gelation of a CS solution with Sodium tripolyphosphate (TPP). Ionotropic gelation takes place due to the interaction between positively charged amino groups and negatively charged TPP. For this purpose chitosan was dissolved in 1% acetic acid aqueous solutions under magnetic stirring at room temperature for 20–24 hr until a clear solution was obtained. Different concentration of chitosan ranging from 0.05–0.5% w/v was prepared. Surfactant tween 80 [0.5% (v/v)], was added to chitosan solutions in order to prevent particle aggregation and then chitosan solutions were raised to pH 4.6–4.8 with 1N NaOH. Sodium tripolyphosphate solution of 0.1% was a prepared by dissolving 10mg of TPP in 10ml of deionised water and diluted to obtained different concentrations: 0.25, 0.50, 0.75, 1, 1.5, and 2 mg/ml. All solutions were filtered through 0.22 micron filter (Millipore). TPP solution was added dropwise with a syringe to chitosan solution under magnetic stirring at 800 rpm at room temperature in the ratio 2.5: 1(v/v) (chitosan: TPP). Samples were visually observed and categorized into three different categories viz: clear solution, opalescent suspension, and aggregates. The zone of the opalescent suspension, correspond to very small particles. The resulting chitosan particle suspension was centrifuged at 12000 g for 30 min. The pellet resuspended in water. The chitosan nanoparticles suspension was then freeze-dried before further use or analysis.

Characterization of chitosan nanoparticles- The prepared chitosan nanoparticles were characterized by the following method-

Ultraviolet–visible Spectroscopy (UV-Vis)- To verify the formation of nanoparticles the solution was scanned in the range of 200–600 nm in a spectrophotometer (Implen GmBH) using a quartz curette with water as the reference.

Scanning Electron Microscopy (SEM)- The size and the morphology of dried chitosan nanoparticles were examined in Quanta 400 ESEM/EDAX (FEI). Vacuum dried small amount of prepared chitosan nanoparticles samples were kept on an SEM stub using double-sided adhesive tape at 50 mA for 6 min through a sputter. Afterwards, the stub containing the sample was placed in the scanning electron microscopy (SEM) Chamber. The photomicrograph was taken at an acceleration voltage of 20 KV.

Dynamic Light Scattering (DLS)- The average particle size of nanoparticles measured as described by Agnihotri *et al.* [4] Particle size distribution and zeta potential of chitosan nanoparticles were measured through DLS with Zetasizer Nano S (Malvern, UK). The analysis was carried out at a scattering angle of 90° at a temperature of 25°C using nanoparticles dispersed in de-ionized distilled water (2 mg of sample was dissolved in 5 ml of deionized water and then sonication is done in sonics vibra cell sonicator). Particle size distribution of the nanoparticles is reported as a polydispersity index (PDI).

Fourier Transform Infrared (FTIR) Spectra- FTIR analysis of different chitosan nanoparticles sample was performed with a2 technologies portable attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (ATRS-FTIR). Sample spectra were recorded in the middle infrared range from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4cm in the absorbance mode for 10 scans at room temperature [5]. FTIR spectra of chitosan nanoparticles were obtained by placing 1 mg of sample on the sensor of the instrument and spectrum was then compared with the spectrum of chitosan and TPP standard.

RESULTS

Preparation of chitosan nanoparticles- The chitosan nanoparticles were prepared within 2 hrs by ionic gelation method [3]. The chitosan molecules were gelled on contact with poly-anions due to the formation of inter and intra-molecular cross linkages mediated by poly anions [6]. The chitosan nanoparticles were prepared upon addition of negatively charged tripolyphosphate (TPP) solution to positively charged chitosan solution immediately under magnetic stirring at room temperature [7,8]. Preliminary experiments were done in order to determine the optimum ratio that results in nanoparticles with small size and narrow size distribution. The zone of particle formation was investigated and the mean size and size distribution of each batch of chitosan nanoparticle suspension were analyzed using the Zetasizer analysis (Table 1).

As seen from table a clear solution was observed when both CS and TPP concentration were small, whereas aggregates were formed spontaneously when they were too large. The zone of opalescent suspension, which would represent a suspension of colloidal particles, was found when CS and the TPP concentration were appropriate. The same result was summarized in (Table 2).

Table 1: Average size of chitosan nanoparticles prepared at different concentration

CS (mg/ml)	TPP (mg/ml)	Avg. particle size (nm)	Visual identification	Poly dispersity index (PDI)
0.5	0.25	–	Clear solution	–
0.5	0.5	168.4 ± 15	Opalescent solution	0.266
0.5	0.75	>1000	Aggregates	*
0.5	1	>1000	Aggregates	*
1	0.25	–	Clear solution	–
1	0.5	177.3 ± 10	Opalescent solution	0.209
1	0.75	184 ± 8	Opalescent solution	0.223
1	1	>1000	Aggregates	*
1.5	0.5	204 ± 4	Opalescent solution	0.371
1 .5	0.75	238.2 ± 7	Opalescent solution	0.157
1.5	1	>1000	Aggregates	*
1.5	1.5	>1000	Aggregates	*
2	0.5	–	Opalescent solution	–
2	0.75	231.7 ± 13	Opalescent solution	0.356
2	1	216.9 ±10	Opalescent solution	0.297
2	1.5	>1000	Aggregates	*
2.5	0.5	–	Clear solution	–
2.5	0.75	423 ± 6	Opalescent solution	0.445
2 .5	1	241 ± 9	Opalescent solution	0.371
2.5	1.5	>1000	Aggregates	*
3	0.5	–	Clear solution	–
3	0.75	319.2 ± 4	Opalescent solution	0.361
3	1	291.9 ± 2	Opalescent solution	0.142
3	1.5	>1000	Aggregates	*
4	0.5	–	Clear solution	–
4	0.75	605 ± 12	Opalescent solution	0.762
4	1	682 ± 7	Opalescent solution	0.658
4	1.5	670 ± 5	Opalescent solution	0.688
5	1	>1000	Aggregates	*
5	1.5	>1000	Aggregates	*

Chitosan, TPP- Sodium tri polyphosphate, * PDI >1.00, ±= Standard deviation, - = not estimated
Chitosan: TPP (2.5: 1; v/v) Tween 80 (0.5% v/v), measurements are performed two times

Table 2: Condition for formation of the chitosan nanoparticles

Concentration of Cs (mg/ml)	Concentration of TPP (mg/ml)				
	0.25	**0.5**	**0.75**	**1.0**	**1.5**
0.5	x	√	↓	↓	↓
1.0	x	√	√	√	↓
2.0	x	x	√	√	↓
3.0	x	x	√	√	↓
4.0	x	x	√	√	√
5.0	x	x	↓	↓	↓

x= Clear solution, √= Opalescent solution, ↓= Aggregate

Chitosan concentration was highly effective in nanoparticles production, and for nanoparticles formation, the chitosan concentration should be less than or equal to 4 mg/ml for selected TPP concentrations and at fixed chitosan concentration, mean diameter of nanoparticles increases with the elevation of TPP concentration. In the range of minimum criteria for nanoparticles formation, the concentration of CS can be up to 4 mg/ml, while the maximum TPP final concentration was only 1.5 mg/ml (Table 2). But according to dynamic light scattering guidelines (DLS) guidelines PDI (poly-dispersity index) value was favorable (<0.5). Therefore, CS concentration of ≤3 mg/ml was recommended. It can be noted that particle size is dependent on both CS and TPP concentration, the minimum size (168 nm) was obtained for the lowest CS and TPP concentration (0.5 mg/ml) and maximum size (682 nm) having CS (4 mg/ml) and TPP (1 mg/ml). Our results showed that by increasing the chitosan concentration from 0.5–4 mg/ml at a constant TPP concentration 1 mg/ml, the size of nanoparticles increases. For further study, we had chosen CS concentration 2 mg/ml and TPP 1 mg/ml for the above mentioned condition i.e. CS/TPP ratio was 5:1.

Characterization of chitosan nanoparticles- To verify the validity of prepared chitosan nanoparticles, whereas characterized by SEM, FTIR, DLS and UV Spectroscopy.

UV-Analysis- The absorption peak for CSNPs was obtained at 226 nm (Fig. 1).

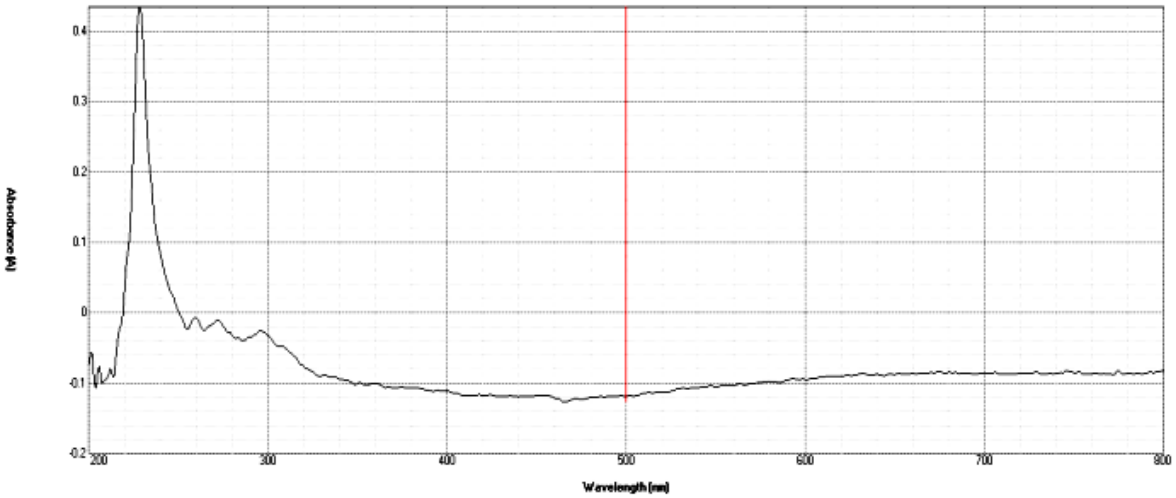

Fig. 1: UV absorption spectra of CSNPs

Stability studies of CSNP- The stability of CSNPs was also determined by measuring its absorption spectrum after 8 weeks. No significant changes in the absorbance were observed during the storage, indicating that the CSNPs did not agglomerate and they were stable during this period.

SEM Analysis- The morphology of CSNPs was observed and the results are shown in Fig. 2. CSNPs revealed a very homogenous morphology and they are spherical in shape.

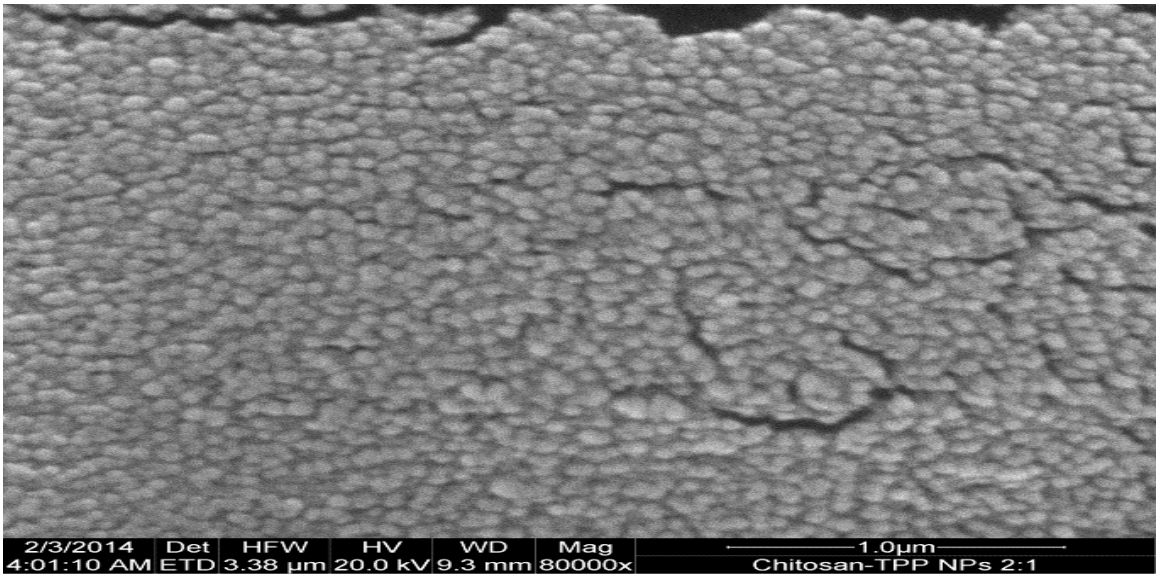

Fig. 2: SEM analysis of CSNPs

Dynamic Light Scattering (DLS) Analysis- DLS was used to measure hydrodynamic diameter in the nanometer range. The size of CSNPs at selected concentration was 216 nm and zeta potential 50 mV (Fig. 4).

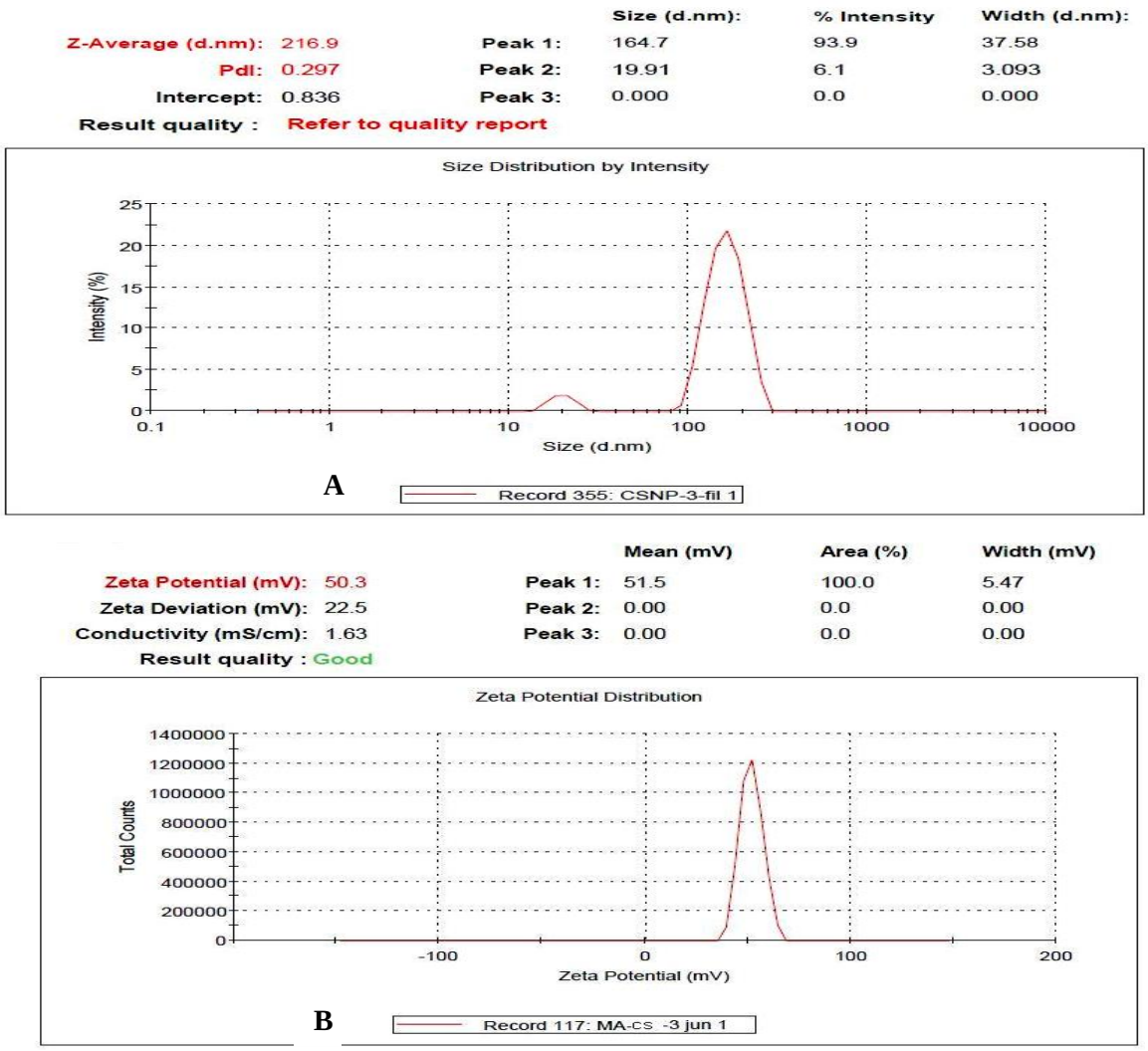

Fig. 3: DLS analysis of CSNPs

FTIR Analysis- The spectrum of CS, TPP, and CSNPs were shown in Fig. 4. In CS spectrum, the peak of OH group at 3424–3269 cm^{-1} and the band 1651 cm^{-1} (C=O stretching in amide group, amide I vibration), and 1592 cm^{-1} (N-H bending in amide group, amide II vibration, respectively was seen in pure CS. In the spectrum of TPP, the peak of PO_4^{-2} group was seen at 1138 and 888 cm^{-1}. In the spectrum of chitosan nanoparticles, the peaks of both CS and TPP are seen in Fig. 4.

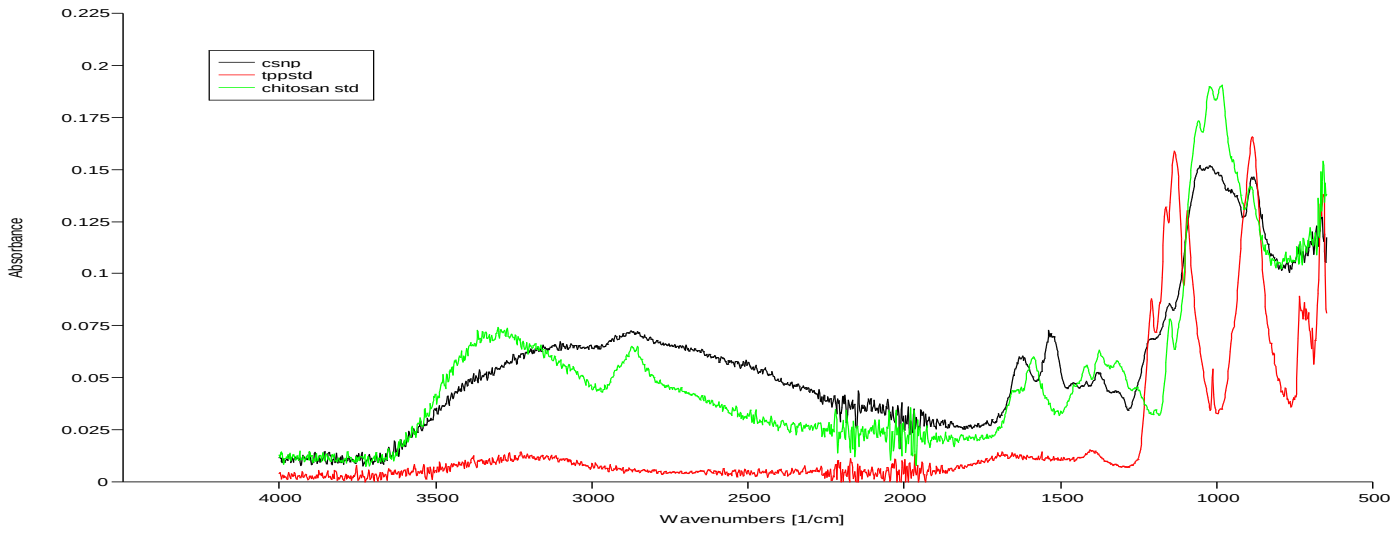

Fig. 4: FTIR Analysis of CSNPs

Table 3: Characterization of prepared chitosan nanoparticles

S. No	Methods	CSNPs
1	**UV Spectroscopy**	Peak obtained at 226 nm
2	**SEM**	Homogenous and Spherical in shape
3	**DLS**	Size 216 nm and zeta potential at 50 mV
4	**FTIR**	Peak of OH group of chitosan becomes wider and peak of PO_4^{-2} group was seen at 1138 and 888 cm^{-1}

DISCUSSION

The chitosan nanoparticles were prepared by ionic gelation method. For the success of e size chitosan with nano-sized scale, the concentration of chitosan and TPP should be optimized [9]. The characteristics have been found to affect the biological performance of CS/TPP nanoparticles [10]. Chitosan has amino groups that can undergo proto-nation at low pH due to which its solubility enhances and it becomes soluble in acidic solution. TPP is a cross-linking agent i.e. a multivalent anion that possesses negative charge. The formation of CSNPs takes place due to the attraction between positively charged chitosan and negatively charged TPP [11,12]. The size of the chitosan nanoparticles depends largely on the concentration of chitosan and TPP solution. It was seen that the size of nanoparticles increases as the concentration of CS and TPP increases up to a particular concentration after that aggregation was found. The increase of the particle size due to the increase in the CS concentration could be attributed to the dense spatial distance among chitosan molecules at a higher concentration which resulted in the formation of larger particles. On the contrary, the smaller particle size was obtained with the lower chitosan concentration through decreased viscosity during ionic gelation. The lower concentration of chitosan provided a nice dispersion of chitosan molecules which allowed efficient electronic interactions between the cationic chitosan and negatively charged TPP. The aggregation was also found when the TPP concentration exceed the CS concentration, which might be due to the fact that more chitosan chains were cross-linked in the presence of a high concentration of TPP or adding an excess of TPP to a nanoparticle dispersion culminates in a clear flocculation of the nanoparticles, which have a tendency to aggregate once all their surface charges have been nullifying by excess poly-anion.

The results showed that chitosan concentration may be up to 4mg/ml and TPP concentration should less than 1.5 mg/ml for the formation of nanoparticles and the size ranges from 168–682 nm. The CS concentration was in favor of Calvo *et al.* [3]; Koukaras *et al.* [13]. According to Calvo *et al.* [3], the final concentration of CS can be up to 4 mg/ml, while max TPP concentration is only 0.75mg/ml. They noted that the minimum size (260 nm) being obtained for the lowest CS and TPP concentrations. Koukaras *et al.* [13] find the optimum CS/TPP w/w ratio 4:1, which gave nanoparticles with sizes of 340 nm, while for other CS/TPP ratios, the size of the nanoparticles tended to increase. This behavior is in agreement with those results obtained by Zhang *et al.* [14], who found an optimum ratio of 5:1. The same behavior was reported by Fan *et al.* [15] in their recent work on low-molecular-weight chitosan. According to Aydın and Pulat [16], minimum criteria for nanoparticles formation is that chitosan concentration should be <2.5 mg/ml and it should not exceed TPP concentration. They obtained nanoparticles in the range of 15–2393 nm. In 2013, Vimal *et al.* [17] also used ionic gelation method and obtained smaller CS/TPP nanoparticle 30-60nm. In 2006, Lam *et al.* [18] prepared the CS/TPP nanoparticles with the size of 50–70 nm. Slightly bigger sized CS/TPP nanoparticles have been prepared [19,20]. The ratio of CS and TPP was 5:1 Gan Q and Wang [18]; Mohammadpour *et al.* [21]. They obtained a 260 nm size particle. In 2009 Csaba *et al.* [22] used ionic gelation method and obtained smaller CS/TPP nanoparticles (93 nm) using low molecular weight chitosan. Above studies suggest that nanoparticles can be of different size and can be formed by different ratio of CS/TPP but these studies didn't reveal the functional aspect of nanoparticles of different sizes, it is hard to say the effect of size and ratio on nanoparticles efficacy. We had selected CS/TPP ratio 5:1 for further study. In effect, a 5:1 chitosan to TPP ratio is high enough to observe a colloidal solution but not too high as to drag the zeta potential of the particles too low.

Characterization of prepared CSNPs by U.V. spectrophotometer showed a peak at 226 nm. This may be due to the presence of amido group in chitosan. In 2014, Krishnaveni and priya [23] obtained a peak at 310 nm for chitosan nanoparticle. In 2005 Liu *et al.* [24] obtained a peak of chitosan at 201 nm.

SEM analysis revealed that size of CSNPs ranges from 80 to 100 nm. Morphologically the CSNPs nanoparticles prepared in the present work were found to be spherical in shape as observed by Yang [11]; Gan Q and Wang [19]. It is noteworthy that hydrodynamic diameter of particles measured by DLS was higher than size estimated by microscopy particularly because of high swelling capacity of CSNPs. In DLS we get the hydrodynamic radius of the particle whereas by SEM we get an estimation of the projected area diameter. In DLS when a dispersed particle passes through a liquid medium, a thin electric dipole layer of the solvent adheres to its surface. This layer influences the movement of the particle in the medium. Therefore, hydrodynamic diameter gives us information of the inorganic core along with coating material and the solvent layer attached to the particle as it moves under the influence of Brownian motion. At the core, DLS provides excellent ensemble statistics for an average size (by intensity), average poly-dispersity index (PDI), and a moderately peak-resolved distribution by mathematical inversion. While estimating the size by SEM, this

hydration layer was not present hence; we get information only about the inorganic core. The difference occurs as DLS measures the dispersion in water and even the dust particles in the sample may change the readings. Therefore we get greater size of nanoparticles in DLS analysis.

Zeta sizer also measures zeta potential. Zeta potential is the surface charge which greatly influences particle stability in suspension through the electronic repulsion between particles. It can also determine nanoparticle interaction *in vivo* condition with the cell membrane of bacteria, which is usually negatively charged. The result showed the zeta potential of CSNPs was 50.3 mV. The higher zeta potential indicates that CSNPs was fairly stable. It seems likely that the long amino groups hinder anion adsorption and keep high the value of the electrical double layer thickness, and thus prevent aggregation.

FTIR characterization reveals the intermolecular interaction of CSNPs. IR Spectroscopy is an extremely effective method for determining the presence or absence of a wide variety of functional groups in a molecule. According to the results of FTIR analysis in CS spectrum, the peak of OH group was seen at 3424–3269 cm^{-1} becomes wider i.e. 3424–3069 cm^{-1} indicated, the H bonding is enhanced. The band1651 cm^{-1} (C=O stretching in amide group, amide I vibration), and 1592 cm^{-1} (N-H bending in amide group, amide II vibration), respectively in pure CS, shifts to 1628 cm^{-1} and 1526 cm^{-1} for CSNP due to the interaction between phosphoric groups of TPP and amino groups of CS in nanoparticles. The 1592 cm^{-1} peak of the (NH_2) bending vibration is sharper than the peak at 1651 cm^{-1}, which shows the high degree of deacetylation of the chitosan. The peak of PO_4^{-2} group of TPP 1138–888 cm^{-1} was also seen in chitosan nanoparticles. Thus it is postulated that polyphosphoric groups of sodium polyphosphate interact with the ammonium groups of chitosan, which serves to enhance both the -inter and intra-molecular interaction in chitosan nanoparticles [25]. Similar results were observed by Lam *et al.* [18] and Mohammadpour *et al.* [20]. Lam *et al.* [21] observed the peaks at 1650 cm^{-1} and 1636 cm^{-1} for the amino group in CS and CS/TPP, respectively and Mohammadpour *et al.* [20] found that the 1595 cm^{-1} peak of N H bending vibration shifts to 1540 cm^{-1} in CS/TPP nanoparticles after addition of TPP.

CONCLUSIONS

Chitosan is highly effective in nanoparticles production or nanoparticles formation. The chitosan concentration should be less than or equal to 4 mg/ml for selected TPP concentrations. The minimum size (168 nm) was obtained for the lowest CS and TPP concentration (0.5 mg/ml) and maximum size (682 nm) having CS (4 mg/ml) and TPP (1 mg/ml). The prepared CSNPs were also incorporated with silver ion to enhance their properties. The prepared CSNPs were characterized by various systems. UV-Vis spectroscopy showed absorption peak of CSNPs at 226 nm. The morphology of CSNPs was observed by SEM and the results revealed that CSNPs have homogenous morphology and spherical in shape. The size of CSNPs (selected concentration) was 216 nm. The zeta potential for CSNPs was 50.3 by DLS analysis i.e. formed nanoparticles was fairly stable. The CSNPs spectrum obtained from FTIR showed that the peak of OH group of chitosan becomes wider and the peak of PO_4^{-2} group of TPP was also seen in chitosan nanoparticles.

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