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Preparation and Characterization of Chitosan Nanoparticles using Novel Emulsion Polymerization Method: Study of Degradation, Antibacterial Activity and In-Vitro Blood Compatibility

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ABSTRACT

Chitosan-TPP nanoparticles have been prepared using emulsion polymerization method devised by the authors, based on existing techniques. FTIR and XRD results showed characteristic peaks of components with maximum 44.6% crystallinity of chitosan-TPP nanoparticles. SEM and TEM studies determined the spherical shape of the particles with 20-90 nm size. Swelling ratio of both the particles in PBS and SBF was found to be in the range between 0.55-3.36 and 2.66-4.19. Percentage degradation of material in PBS in presence of enzyme papain has been calculated 46.6 and 80.0. In-vitro blood protein adsorption for the nanoparticles was found to be in the range between 0.052-0.351 mg/g. Percentage of haemolysis was found to be in between 3.31- 42.1 Antibacterial activity of material was also been checked against E. Coli and Bacillus Cereus while Percentage inhibition was found to in the range between of 11-35.

Keywords:— *chitosan, nanoparticles, antibacterial activity, enzymatic degradation, in-vitro blood compatibility.*

I. INTRODUCTION

Biopolymer, chitosan is the N-acetylated derivative of chitin. It is of commercial interest due to high percent of nitrogen (6.89%) compare to synthetically substituted cellulose (1.25%). Chitosan have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties etc. and it has been potentially using in textiles, membranes and medical aids [1]. Chitosan has also been used as material for enzyme immobilization [2]. Its desirable characteristic for immobilizing enzymes

include high affinity to proteins, availability of reactive functional groups for direct reaction with enzymes and for chemical modification, hydrophilicity, mechanical stability and rigidity. Chitosan provides ease of preparation in different geometrical configuration that in turns facilates the system with permeability and surface area suitable for a chosen biotransformation.

Attention has been paid to chitosan as a potential polysaccharide resource. It has been suggested that chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in tissue culture. [3, 4]

Chitosan has three type of reactive functional groups, an amino group as well as both primary and secondary hydroxyl group at the C-2, C-3 and C-6 positions. This special structure makes it exhibit chelation with various metal ions [5]. It has chemical modification of these groups and regeneration reaction gives to various novel bio-functional macromolecular products having original organization or new types of organization. Chitosan is reported to suppress viral infections in various biological systems. Cationic charges of amino groups in chitosan may have additional functions to activate the immune and defense systems in plants and animals [6]. It is a cationic polysaccharide and has gained increasing attention in pharmaceutical field due to its favorable biological properties [7, 8], mucoadhesive properties [9, 10]. Additionally, chitosan micro/nanoparticles can be easily prepared by ionic gelation method using tripolyphosphate (TPP) as precipitating agent [11]. The advantage of gelation method was attributed to mild condition without applying harmful organic solvent at room temperature in the procedure, and also could efficiently detain the bioactivity of macromolecules (protein, DNA etc) during encapsulation.

Systems using crosslinking of chitosan with tri polyphosphate (TPP) have been greatly used in controlled drug delivery studies. TPP is a non toxic salt, obtained from triple condensation of PO_4 groups. It acts by increasing pH and ionic strength of the solution forming gel and promoting ionic interaction between amino groups of chitosan and anionic groups of TPP [12]. In most of the chitosan-TPP systems chitosan is easily biodegradable with gel forming ability at low pH [13]. Moreover chitosan has antacid and antiulcer activities which prevent or weaken drug irritation in the stomach. Also, chitosan matrix formulations appear to float and gradually swell in an acid medium and TPP does not show any negative effect on these properties of chitosan. All these useful features make chitosan an ideal candidate for controlled drug release formulation [14].

Several chitosan dressing materials have been developed commercially for the healing of human and animal wounds as it stimulates the connective tissue formation *in-vitro* [15]. The growth of E. *Coli* was inhibited in the presence of more than 0.025% chitosan. It also inhibited growth of Fusarium Alternaria and Helnin – thosporium [16,17].

As chitosan has a wide range of applications it may be employed to solve numerous problems in environmental and biomedical engineering. Simultaneously nanoparticles are finding wide spread applications in all fields. The material of choice decides the multifunctional nature of the particles. [18,19].

The present work aimed to synthesized ionic crosslinked nanoparticles of chitosan and TPP using emulsion polymerization first time and study of their swelling kinetics, enzymatic degradation and antibacterial nature. The nanoparticles thus

prepared might be subjected as drug carrier species in near future.

II. EXPERIMENTAL

2.1 Materials

Chitosan, glacial acetic acid, sodium hydroxide was obtained from the E. Merck India, Sodium tri- polyphosphate (TPP) by Sigma Chemical Co. (USA). E. Coli (gram (-)^{ve}) MTCC 118 and Bacillus Cereus (gram (+) ^{ve}) F4810 from Science College, Department of Microbiology. All the chemicals were analytically grade so no further purification required.

2.2 Method

2.2.1 Deacetylation of chitin into chitosan

Chitin was refined twice by dissolving it in dilute HOAc (acetic acid) solution. The solution was filtered and chitosan was precipitated with aqueous sodium hydroxide. The precipitate was dried in vacuum at room temperature [20]. The degree of deacetylation was about 85% as determined by pH-metric titrimetry [21]. Brief process of deacetylation of chitin is shown in following reaction scheme given in Figure 1.

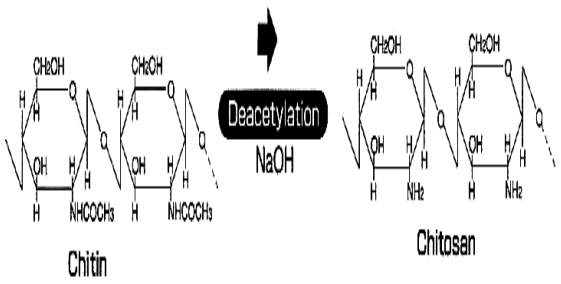

Figure 1. Reaction Scheme Showing the Deacetylation Process of Chitin.

2.2.2 Preparation of chitosan – TPP nanoparticles

The newly modified method is developed first time by H. Bundela and A.K. Bajpai using emulsion polymerization for the preparation of chitosan-TPP nanoparticles. Modification of the typical method [22-24] makes it easier, economic and time saving. In the method chitosan solution of different concentrations were prepared in 2% glacial acetic acid by addition of oil phase.

Solution of TPP was also prepared in distilled water and oil. Both chitosan and TPP solutions were gradually mixed together by mechanical agitation using mechanical *shaker (* Remi *Model No*. RS-24,. Remi Instrument Ltd., Mumbai) at 250 rpm Above mixture was left for shaking for about 3 hours. After a definite time period interval nanoparticles of chitosan-TPP obtained in the form of white sedimentation. The white viscous material washed with toluene and acetone simultaneously until white talcum powder like particles were not to be obtained. These fine chitosan-TPP particles were stored in air-tight polyethylene bags for further use. Figure 2 shows the crosslinking of chitosan moiety with TPP to yield chitosan- TPP particles.

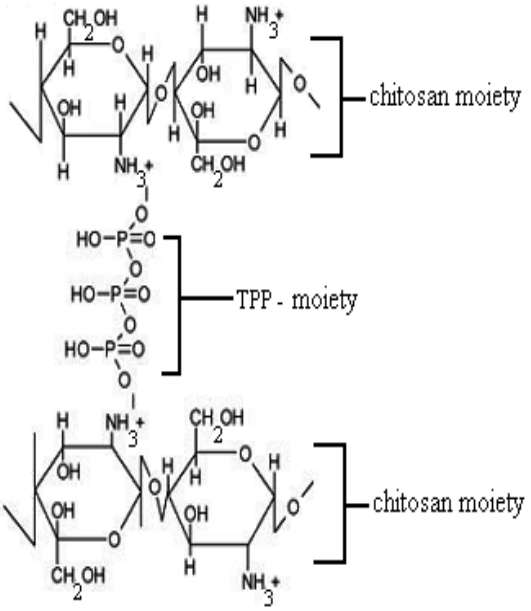

Figure 2: Figure showing the crosslinking of chitosan with TPP.

III. CHARACTERIZATION

3.1 Fourier tansform infrared spectroscopy (FTIR) - Studies

IR spectra of powdered specimens were recorded on a FTIR-8400S, Shimadzu spectrophotometer. Prior to analysis KBr pellets were prepared by mixing 1:10 of sample: KBr (wt/wt) by uniaxillary pressing of powders under vacuum. Spectra was obtained between 4400cm^{-1} - 450cm^{-1} at 2cm^{-1} resolution.

3.2 X-Rays Diffraction (XRD) – Studies

X-ray diffraction (XRD) analysis for phase identification was carried out by using Cu, Ka radiation (k = 1.5406 A°) in the XRD apparatus (Philips PW 1820) powder diffractometer. The diffraction data was collected at 2° to 85°, 2°θ values with a step size of 0.0 2° and within counting time of 2s step^{-1}.

3.4 Electron Microscopy studies

An insight morphology of the prepared nanoparticles was achieved by using SEM instrument (STEREO SCAN, 430, Lecica SEM, USA). Simultaneously TEM studies were performed on a TEM apparatus (MORGAGNI 268) to ensure the nanometer the size of chitosan-TPP particles.

3.5 Swelling studies

The extent of swelling was determined by a conventional gravimetric procedure as reported in literature [25]. In a typical experiment, preweighed chitosan-TPP particles were allowed to swell in PBS (phosphate buffer saline) for a predetermined time period (up to equilibrium swelling), thereafter the particles were taken out from the solution and then gently pressed in-between two filter papers to remove excess of water and finally the particles were weighed using a sensitive balance (Denver instrument APX - 203). The swelling ratio was calculated by the following Equation (1).

$$\text{Swelling ratio} = \frac{\text{Weight of swollen particles}}{\text{Weight of dry particles}} = \frac{(Sw)}{(Sd)} \quad \ldots\ldots\ldots(1)$$

3.6 Swelling in Bio-Fluids

Simulated body fluid (SBF) is the most favored model solution simulating inorganic part of the blood plasma. Blood plasma is an aqueous saline solution that also contains variety of salts, sugars, amino acids and minerals along with blood cells. Plasma transports these materials needed by cells and materials that must be removed by cells. Inorganic part of blood plasma contains different ionic concentrations like sodium, calcium, magnesium, and chloride ions [26]. The ion concentrations of simulated body fluids are nearly equal to those of blood plasma. Simulated body fluids were prepared by dissolving various reagents like NaCl, K_2HPO_4, $MgSO_4$, $CaCl_2$, and D-glucose, KI into de-ionized water. Swelling experiments were performed as discussed earlier.

3.7 Degradation studies

3.7.1 Degradation in PBS

In a simple experiment preweighed amount of nanoparticles were immersed in 10 mL of PBS at 37°C for predetermined time period (till equilibrium swelling). After equilibrium swelling was achieved particles were taken out from PBS solution and then pressed between two filter papers gently to remove excess amount of water and weight of swollen particles was measured on a sensitive balance (Denver Instrument APX-203) at an appropriate time interval. The medium was changed daily to a fresh one and swollen particles were put into vial after weight measurements. The weight loss of the particles was recorded and percent

degradation was calculated using following formula given in Equation 2.

$$\text{Percent Degradation} = \frac{Wo - Wd}{Wo} \times 100 \quad \ldots\ldots(2)$$

where Wo = weight of swollen particle at equilibrium swelling

Wd = weight of swollen particles after degradation

3.7.2 Enzymatic Degradation

In a very simple experiment instead of PBS preweighed amount of particles were immersed in 10ml of deionized water containing enzyme, papain (from papya latex, crude, at concentration of 10mg/ mL.) at 37°C for a predetermined period (0-14 days). The degradation experiment was performed as described in section 3.7.1. Lisozyme and papain enzymes were found to be responsible for chitosan degradation [27]. The Percentage of enzymatic degradation has been calculated by the formula given in Equation 2.

3.8 In-vitro Blood compatibility

Nanoparticles are type of hydrogels which are three-dimensional crosslinked polymeric in structure, which can swell in an aqueous environment. Over the past 35 years, these materials have been extremely useful in biomedical and pharmaceutical applications mainly due to their biocompatibility, as well as their high water content and rubbery nature, which are similar to natural tissues [28]. A biomaterial is a substance used in medical devices for contact with the living body for the intended method of application and for intended time period. To acquire biocompatibility, the materials used in medical applications must meet certain regulatory requirements. The surface of biomaterials is believed to play an important role in determining biocompatibility. For materials that come into contact with blood, formation of clot is the most undesirable but frequently occurring event that restricts clinical acceptance of material to be used as biomaterial. Therefore, certain test procedures have been developed and they need to be employed to judge haemofriendly nature of materials before their *in-vivo* use.

3.8.1 Protein (BSA) Adsorption

Adsorption of BSA (Bovine serum albumin) onto the nanoparticles was performed by the batch contact process reported elsewhere [29]. For this experiment, protein (BSA) solutions were prepared in 0.5 M PBS at physiological pH 7.4. A fresh solution of BSA was always prepared prior to adsorption experiments. The definite weights of nanoparticles were equilibrated with PBS for 24 h. The adsorption was then carried out by gently shaking a BSA solution of known concentration containing preweighed and fully swollen composites. By taking fully swollen samples, the possibility of soaking of BSA solution within the particles becomes lesser. The shaking was performed so gently that no froth was produced, otherwise BSA would adsorb at air-water interface. After a definite time period, the samples were removed and adsorbed protein was assayed for the remaining concentration of BSA by recording absorbance at of protein solution at 272nm on a UV spectrophotometer (Systronics, model no. 2201, India).

3.8.2 Percent Haemolysis Test

Haemolysis experiments were performed on the surfaces of definite amount of prepared chitosan-TPP nanoparticles as described in literature [30]. In a typical experiment, preweighed amount of dry nanoparticles was equilibrated in normal saline water

(0.9% w/v NaCl) at 37°C for 24h and human ACD (acid citrate dextrose) blood (0.25mL) was added into the particles after 20min 2.0mL of saline water was added into specimens to stop haemolysis and all samples were incubated for 60 min at 37°C. Positive and negative controls were obtained by adding 0.25mL of human ACD blood and 0.9% NaCl to 2.0ml of double distilled water. Incubated samples were centrifuged for 45min, supernatant was taken and its absorbance at 545nm was recorded using a spectrophotometer. The percentage of haemolysis was calculated using the following relationship, given in Equation (3).

$$\text{Percent Haemolysis} = \frac{A_{\text{test-sample}} - A_{(-)\text{control}}}{A_{(+)\text{control}} - A_{(-)\text{control}}} \times 100 \quad \ldots\ldots(3)$$

where A = absorbance. The absorbance of positive and negative controls was found to be 1.73 and 0.048, respectively.

3.9 Anti bacterial Activity

3.9.1 Indicator bacteria and inoculum preparation

Strain of E. coli (gram (-) ^{ve}) MTCC 118 and Bacillus Cereus (gram (+) ^{ve}) F 4810 was obtained from collection of culture maintained in Microbiology Deapartment,, Govt. Science College Jabalpur. The cultures were maintained at 6°C on BHI (brain- heart in fusion) agar slants (Himedia, Mumbai, India) and subcultured every 15 day time intervals. Before use, the culture was successively propagated twice in BHI broth at 37°C. Cell suspension of culture, individually, were prepared from by 20h – hold BHI culture broth with appropriate dilution in 0.85 % saline, giving individual counts of 10^2 – 10^6 c.f.u./ml.

3.9.2 Bacterial growth inhibitory activity

Antibacterial activity of native chitosan and chitosan nanoparticles was studied against indicator bacterial strains, individually, in on nutrient broth. Chen et. al [31]

Bacterial activity was calculated using Equation (4) given below.

(C-T) / C × 100 …….. (4)

Where C= diameter of colony (Contol) T= diameter of colony (Tested bacteria with chitosan plaricle)

3.10 Result and Discussion

3.10.1 FTIR

FTIR spectra of TPP, chitosan and chitosan nanoparticles were analyzed. Figure 3(a) representing the FTIR- spectra of native TPP where measurements showed distinct ν3 (at around 1000-1100 cm^{-1}) and ν4 (at around 610 cm^{-1}) phosphate group [33, 34]. Figure 3(b) showing FTIR – spectra of native chitosan which reveals the characteristic band at 3449 cm^{-1} which is attributed to $-NH_2$ stretching vibration and the band for amide I at 1655 cm^{-1}. In crosslinked chitosan (Figure 3c) peak at 1655 cm^{-1} disappears and two new peaks at 1645-1554 cm^{-1} appears. The disappearance of peak could be attributed to the linkage between phosphoric and ammonium ions in chitosan-TPP particles.

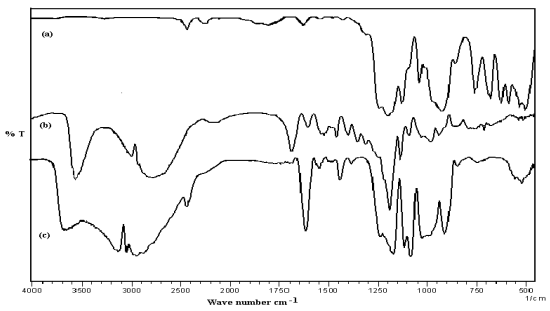

Figure 3. (a) FTIR – spectra of native TPP. (b) FTIR – spectra of native chitosan. (c) FTIR – spectra of chitosan-TPP nanoparticles.

The crosslinked chitosan also showed peak for P=O at 1155cm^{-1} [35, 36]. Peak at 1635cm^{-1} for – CONH and 1060cm^{-1} for C-O-C has been confirmed the presence of chitosan. Characteristic peaks are also present at 2934cm^{-1} and 2850cm^{-1} for asymmetric and symmetric stretching of methylene (-CH_2-) groups, for chitosan. On the other hand, the bands are around at 1544 and 1620cm^{-1} for N-H stretching can be observed for native and crosslinked chitosan with varying intensities. In nanoparticles peak appears at 1217cm^{-1} is probably due to stretching vibration of P=O [38].

3.10.2 XRD

XRD spectra of native chitosan (Figure 4 a) showed two prominent crystalline peaks at 10° (2°θ) and 20° (2°θ). In the case of crosslinked chitosan in Figure 4(b) there was a significant decrease in intensity of characteristic peaks of chitosan, which was in agreement with the study reported by Wan et al [39].

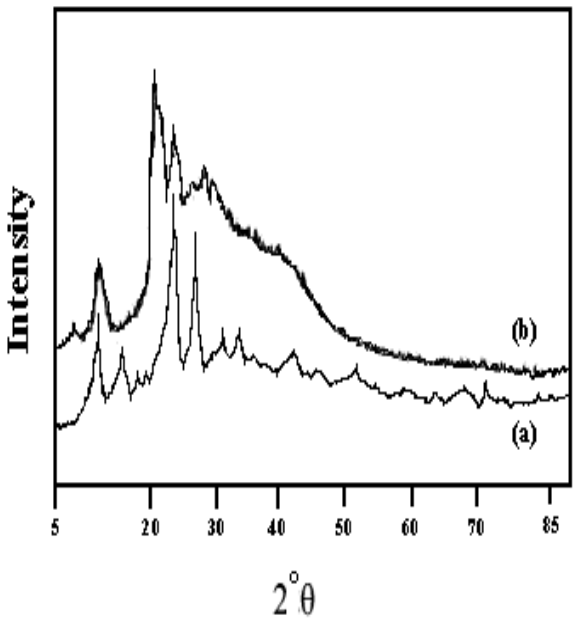

Figure 4. (a) X-ray Diffraction Spectra of Native Chitosa (b) X-ray Diffraction Spectra of Chitosan-TPP Nanoparticles

The distinct difference in the diffraction patterns of chitosan and crosslinked chitosan could be attributed to modification in the arrangement of the molecules in crystal lattice. In chitosan crosslinked with TPP at 10° (2°θ) and 20° (2°θ) are suppressed might be due to amophization.

Physical and mechanical properties of the polymers are profoundly dependent on degree of crystallinity. All the X-ray diffraction methods reported in literature for calculating the crystallinity in polymer are based on following assumptions:

- The scattering capability of crystallite is equal to in amorphous with same mass.
- The intensity of the X-rays scattered from a specimen is approximately equal to sum of the crystalline form and amorphous in specimen.

Percent crystallinity has been calculated for both chitosan and chitosan-TPP nanoparticles using expression given in literature [40]. The numerical formula to calculate % crystallinity (%X) has been given in the following Equation (5),

Percentage Crystallinity = (Ic / Ia + K) × 100 ..... (5)

where Ic and Ia are the integrated intensities of crystalline and amorphous peaks, K is a constant taken as unity [41]. Areas of the peaks were determined by the "cut and weight metho". The relation between integrated intensities and area of crystalline and amorphous peaks has been evaluated from the literature [42]. It has been found that the Percentage crystallinity of the chitosan and chitosan-TPP nanoparticles was calculated about 61.18% and 44.6% The net decrease in the crystalline nature of chitosan nanoparticles as compared to the native chitosan is may be due to formation of more amorphous regions within the polymer chains after crosslinking.

3.10.3 Electron Microscopy Studies

The result of scanning electron microscopy has been shown in Figure 5(a) that clearly reveals the spherical geometry of the nanoparticles. Figure 5(b) showed the particle size in the range between 20-90 nm when they were subjected to transmission electron microscopy measurements.

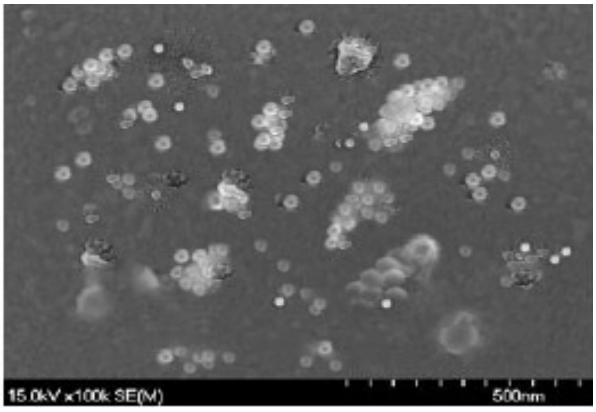

Figure 5. (a) SEM Image of Chitosan-TPP Nanoparticles

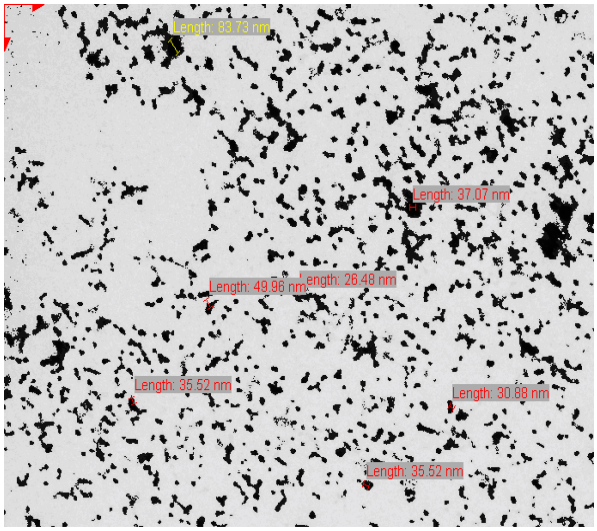

Figure 5. (b) TEM Image of Chitosan-TPP Nanoparticles Showing Size of Particles.

3.10.4 Swelling Studies

The swelling results of chitosan-TPP nanoparticles in PBS are shown Figure 6. Chitosan with pka of 6.3 is polycationic when dissolved in acid and presents $-NH_3^+$ sites. Sodium tripolyphosphate ($Na_5P_3O_{10}$) dissolved in water dissociates to give both hydroxyl and phosphoric ions. The crosslinking of the chitosan would be dependent on availability of the cationic sites and negatively charged species.

Figure 6(a) shows the effect of chitosan concentration on the swelling ratio of nanoparticles in PBS. In the present study as amount of chitosan was varied from 0.5g to 2.0g with definite amount of TPP (3.0g) extent of swelling increases from by 0.5-1.5g. It may be due to fact that up to this concentration porous hydrogel network is formed within the chitosan-TPP nanoparticles that permit solvent molecules to enter into the nanoparticles network easily. Since chitosan-TPP particles have been prepared either by deprotonation or by ionic interaction.

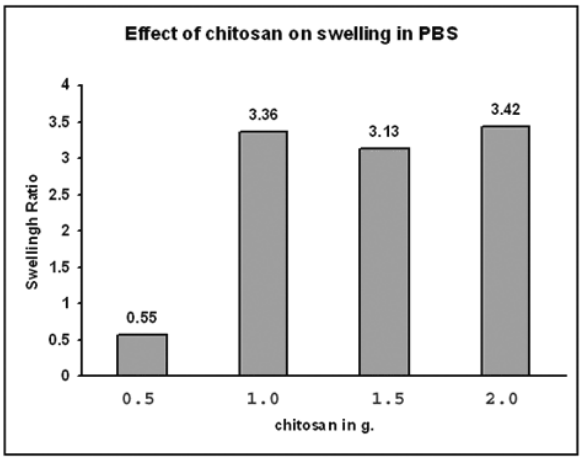

Figure 6 (a). Graph showing the effect of chitosan concentration on swelling ratio of chitosan-TPP nanoparticles in PBS.

Mutual interaction between the groups of polymer chain and solvent would affect the swelling. Till 1.5g of chitosan concentration polymer chains of nanoparticles are quite flexible for entry of solvent molecules and system shows increase in swelling ratio. At 2.0g of chitosan concentration, swelling ratio decreases. Although the chitosan is a hydrophilic polymer but its greater amount (2.0g) results in more dense polymer hydrogel system and does not allow passage

of solvent molecules within the polymer network of nanoparticles feasible.

An important fact has also been observed that for higher concentrations of chitosan, formation of nanoparticles become difficult with definite amount of TPP and water. At higher concentrations of chitosan a gel like deposition was obtained which is strictly due to increase in the viscosity of the reaction mixture. Enhanced viscosity of the reaction system results decrease in the agitation speed required to the formation of nanoparticles.

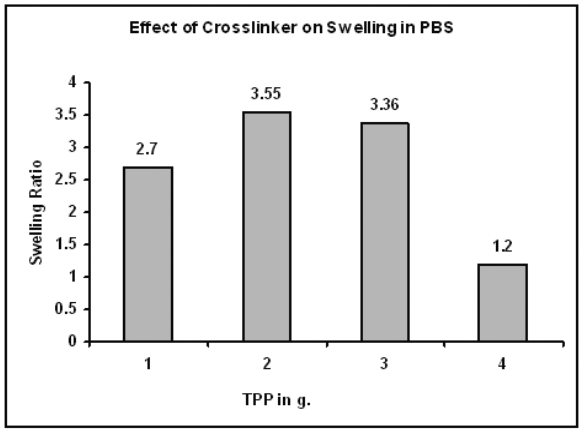

Figure 6(b): Graph Showing the Effect of TPP Concentration on Swelling Ratio of Chitosan-TPP Nanoparticles in PBS.

In Figure 6(b) the effect of crosslinker concentration is shown on the swelling ratio of chitosan-TPP particles in PBS, as the amount of crosslinker increases in feed mixture the swelling ratio decreases. It has been seen that for the low concentrations of TPP (1.0g and 2.0g). The yield of nanoparticles was very low with poor quality of product material while for 4 g. of TPP concentration the swelling ratio was found to be very less due to the greater amount of crosslinking which may results in the formation of more compact polymer network and restricts the feasible transport of solvent molecules. For 3.0g of TPP concentration in the feed mixture fair level of swelling ratio was achieved with good quality and yield of nanoparticles.

Beside all above discussion it is worthy to mention here that in the swelling phenomenon of chitosan-TPP nanoparticles, many volume and phase transitions were induced independently by pH and ionic strength changes. However the swelling results that have been reported in the present study, had helped the authors to decide control set of nanoparticles with fine quality, good yield and desirable swelling properties so that they can be used as good drug delivery vehicles in future.

The results of swelling of chitosan-TPP nanoparticles in simulated biological fluids have been shown in Table 1. The swelling of the particles in biofluids was found to be comparable with the values that have been found in PBS.

3.10.5 Degradation Studies

Degradation is an important factor for the material to be biocompatible. The percent degradation results of control set of chitosan-TPP nanoparticles (1g chitosan, 3g TPP) have been shown in the Figure 7 (with white hollow columns).

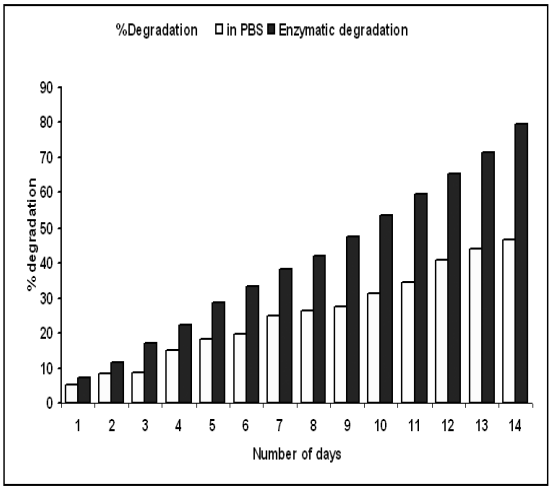

Figure 7. Graph showing results of % degradation of chitosan-TPP (1.0 g chitosan, 3.0 g TPP) in PBS and in presence of enzyme papain.

The results clearly indicate, in the absence of any enzyme (only in PBS medium) particles show maximum 46.6% degradation till 14th day. The results are quite satisfactory and reveal self degradation tendency of chitosan-TPP nanoparticles. It is obvious that the material which will come in contact with body environment will also be in contact with different enzymes present inside the animal body. As, enzymes are specific for their substrates they selectively affect the substrates that come in contact with them. The results of enzymatic degradation for the control set of chitosan – TPP particles have been shown in Figure 7 (with black solid columns). The results show the maximum degradation of the particles in the presence of enzyme papain is about 80% that is almost double of the degradation results that found in PBS environment only [43].

Table 1 : Swelling of Chitosan-TPP Nanoparticles of Composition (a) in Bio-Fluids.

S. No.	Bio-fluids	Swelling Ratio (Sr)		
		First day	Second day	Third day
1	Saline water	2.58	3.20	3.22
2	Synthetic Urine	3.97	4.20	4.19
3	15% KI	2.93	3.50	3.53
4	5% Urea	2.15	2.85	2.96
5	D-glucose	2.64	2.60	2.66
6	PBS	3.11	3.35	3.36

Table 1 Showing the Swelling of Chitosan-TPP Nanoparticles of Composition (a) in Bio-Fluids. (a) chitosan = 1g, TPP = 3 g.

3.10.6 In-vitro blood compatibility

Results for the BSA (bovine serum albumin) adsorption and percent haemolysis have been shown in the Table 2. It has been observed that the optimum protein adsorption was obtained for the nanoparticle system containing 1g chitosan and 3g TPP with optimum haemolysis. It may be possible that for greater amount of chitosan (2.0g) and crosslinker (4.0g TPP) the swelling capacity of the system is lesser and so the blood compatibility. Since the swelling properties of the system directly affect the blood compatible behavior of the material. It has been reported that the systems having good water content able to achieve reversible protein adsorption onto the material surface and inturns reduce the chances of blood coagulation [44, 45]. The control set of chitosan- TPP nanoparticles (chitosan 1.0g, TPP 3.0g) showed fair level of blood compatibility. Also the protein adsorption and percent haemolysis for 1g chitosan and 1g TPP containing system is 0.092mg/g and 3.3% respectively that may be attributed to the lesser amount of crosslinking with greater swelling.

Table 2: Results of *in-vitro* Blood Compatibility of Different Compositions of Chitosan-TPP Nanoparticles

S. No.	chito-san (g)	TPP (g)	BSA Adsorp-tion mg/g	Percent Hae-molysis
1	0.5	3.0	0.241	15.2
2	1.0	3.0	0.052	17.5
3	1.5	3.0	0.171	18.7
4	2.0	3.0	0.135	28.9
5	1.0	1.0	0.092	3.31
6	1.0	2.0	0.172	32.7
7	1.0	4.0	0.351	42.1

Table 2 showing the *in-vitro* blood compatibility results of different compositions of chitosan-TPP nanoparticles. It is well known fact that greater amount of crosslinking enhances compactness of the system to reduce water absorption capacity simultaneously it also

reduces smoothness of the polymer system. Increased roughness of the polymer surface causes destructive interactions among the material surface and blood components and thus results reduced blood compatibility. It is also evident from the results that the system having lesser amount of polymer and crosslinker content showing fair level of blood compatibility though the yield and quality of the material has been reported to be found excellent in case of control set only.

3.10.7 Antibacterial activity assessment

Since chitosan is only soluble in acidic media, the precipitation of chitosan solution in acetic acid occurred upon addition to bacterial suspension, while chitosan nanoparticles could be well distributed in bacterial suspension after a slight shock for a nice dispersion. Bacteria can adhere to the surface of chitosan and chitosan nanoparticles significantly in short time thus chitosan and chitosan nanoparticles exhibit anti-bacterial activity. According to the literature, [46, 47] chitosan possess antimicrobial activity against a number of gram-negative and gram-positive bacteria.

Table 3: Growth Inhibitory Effect of Native Chitosan and Chitosan-TPP Nanoparticles

Sr.	Compound	Concen-tration in mg.	% Inhibition of indicator bacterium	
			E.Coli	B. Cer-eus
1	Native chitosan	0.05	09	12
2		0.07	11	19
3		0.09	16	17
4	Chitosan-TPP nanoparti-cles	0.05	11	17
5		0.06	19	10
6		0.07	25	32
7		0.08	16	22
8		0.09	35	19

Table 3 Showing Growth Inhibitory Effect of Native Chitosan and Chitosan-TPP Nanoparticles (Sontrol Set = 1 g Chitosan, 3 g TPP) Towards E.Coli (10^4 CFU . mL^{-1}), B. Cereus (10^6 CFU . mL^{-1})

Growth inhibitory effect of native chitosan and chitosan-TPP nanoparticles (control set = 1 g chitosan, 3 g TPP) towards E.Coli (10^4 CFU . mL^{-1}), B. cereus (10^6 CFU . mL^{-1})

In Table 3 anti-bacterial activity of chitosan nanoparticles was compared with chitosan. Table shows the MIC and MBC of chitosan and chitosan nanoparticles against strain of E.coli (gram (-) ^{ve}) MTCC 118 and Bacillus Cereus (gram (+) ^{ve}) F4810. According to the data, the antibacterial activity of chitosan nanoparticles is significantly higher than that of chitosan.

The study resolved the initial purpose of authors to check antibacterial activity of the prepared material towards gram positive & negative bacterial strains. The results of antibacterial activity found to be quite interesting with maximum 32% and 35% of zone inhibition for the E.Coli and B. Cereus bacterial strains respectively.

3.11 Conclusion

Nanosized chitosan-TPP particles have been prepared by the emulsion polymerization method. The FTIR- studies reveals the presence of characteristic packs of native materials such as chitosan and TPP. The disappearance of –NH- str. peak of chitosan was found in chitosan-TPP particles clearly shows the crosslinking of chitosan with TPP. XRD studies show the characteristic peaks of chitosan phase at $10^{o}\theta$ and $20^{o}\theta$ for native chitosan and chitosan-TPP nanoparticles. The suppression of XRD peaks in nanoparticles reveals the decrease in crystallinity of chitosan moiety in the nanoparticles. The percentage crystallinity

of native chitosan and chitosan-TPP nanoparticles was calculated as 61.18% and 44.6% respectively. SEM and TEM studies show spherical shape of nanoparticles with size in between 20-90nm.

Swelling of the nanoparticles was checked in PBS and also in SBF. The results found to be quite satisfactory. The nanoparticles with composition 1g chitosan and 3g TPP shows optimum swelling while greater amount of chitosan (2.0g and more) results in viscous reaction mixture that restricts the formation of nanoparticles. Higher amount of crosslinker (4.0g TPP) showed poor swelling ratio that is not desirable for the material to be subjected as drug delivery system.

Degradation is an obvious and essential phenomenon for the biomaterials. The percentage degradation of the material was checked in PBS and in the presence of enzyme papain. It was found that the chitosan–TPP particles shows self degradation about 46.6% in 14 days in PBS. The results of percentage degradation in presence of enzyme were calculated about 80% in 14 days. The results of *in-vitro* blood compatibility showed protein adsorption about 0.052 mg/g and percent haemolysis was about 17.5% for the control set. In-*vitro* blood protein adsorption and percent haemolysis for the chitosan-TPP nanoparticles having composition 1g chitosan and 1g TPP were found to be 0.092 mg/g and 3.31% respectively. The results of blood compatibility tests *in-vitro* are quite satisfactory. The material was subjected to check its resistance against the bacterial *strains* of E.Coli (gram (-) ^{ve}) MTCC 118 and Bacillus Cereus (gram (+) ^{ve}) F4810 and it was found that the chitosan- TPP nanoparticles are more effective against the described bacterial strains as compare to the native chitosan.

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