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Hematotoxicological analysis of surface-modified and -unmodified chitosan nanoparticles

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Abstract: The increasing interest in using chitosan nanoparticles for controlled drug delivery is hampered by its blood incompatibility, especially for intravenous applications. This study investigated the effects of processing solvents (acetic acid/lactic acid), dispersing media (acidic medium/saline), and surface modifiers (polyethylene glycol, polyvinyl alcohol, and ethylenediaminetetraacetatic acid) on the hemocompatibility of chitosan. Blood compatibility of chitosan nanoparticles prepared by ionotropic gelation with altered surface chemistry was evaluated by assessing their hemolytic activity, platelet aggregation, coagulation, and cytokine induction. It was observed that nanoparticles prepared in lactic acid and dispersed in saline did not show hemolysis, platelet aggregation, or coagulation, whereas nanoparticles prepared in acetic acid showed strong hemolysis. Surface modifiers were not observed to significantly affect blood compatibility, with the exception of EDTA, which delayed blood clotting times. Thus, chitosan nanoparticles prepared in lactic acid and dispersed in saline may be an ideal nanocarrier for parenteral applications. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 2957–2966, 2013.

Key Words: chitosan nanoparticles, blood compatibility, surface modifiers, lactic acid, acetic acid

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INTRODUCTION

Chitosan [β (l-4)-2 amino-2-deoxy-D-glucose] is a natural copolymer of *N*-acetyl-D-glucosamine and glucosamine units obtained from the *N*-deacetylation of chitin, found in the exoskeleton of crustaceans and insects.[1] Chitosan with an average degree of deacetylation and a characteristic polycationic character is generally soluble at acidic pH conditions, with aqueous acetic acid as the most commonly used solvent. Owing to the excellent biological activity, biodegradability, biocompatibility, as well as the ability to be formulated into diverse morphologies such as films, fibers, micro-, or nanoparticles, chitosan finds immense applications in drug delivery, wound-healing dressings, artificial membranes, contact lenses or eye bandages, immobilization of enzymes and living cells, and so forth.[2–4] In applications of chitosan related to wound healing, several studies report the acceleration in wound-healing process owing to its influence on platelets.[5] This hemostatic effect of chitosan has been ascribed to its chemical structure, particularly the amino residue. Several authors report the use of chitosan microspheres and nanoparticles for various pharmaceutical applications.[6,7] Encapsulation of proteins, genes, vaccines, hydrophobic drugs, and so forth, in chitosan matrix has been carried out by adopting various methods of synthesis.[8,9] However, most of these reports have explored the use of chitosan carriers for the delivery of therapeutic molecules via the nasal or oral routes.[10,11] The high deacetylation of chitosan, presenting several glucosamine units imparts an increased cationic nature to it, which contributes to its mucoadhesive property and thereby applicability for oral drug delivery.[12,13] Nonetheless, the application of this natural polymeric material as an intravenous drug delivery nanocarrier is limited by its blood compatibility. From the early findings by Baier[14] and Amiji,[15] their conclusions imply the adverse effects of chitosan when used in parenteral applications. The introduction of chitosan into blood triggers a complex series of events including protein adsorption, platelet and leukocyte adhesion, and activation of coagulation pathway, leading to potential thrombogenicity.[16] The *in vivo* hemostatic effects of chitosan have been reported in detail by several researchers.[17–19]

To solve the problem of blood clotting of chitosan for use as an intravenous delivery vehicle, researchers have used various surface modifiers with reactive amino and

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hydroxyl groups. Hemocompatibility is found to improve by coupling chitosan with blood compatible molecules such as heparin, dextran sulfate, arginine, polyethylene glycol (PEG), methoxypoly(ethylene glycol) sulfonate, and ethylenediaminetetraacetatic acid (EDTA), thereby providing a steric barrier to undesirable protein adsorption.[20–24] In addition, derivatives of chitosan, that is, acylated, sulfonated, lauroyl sulfated, carboxypropionylated, hexanoylated, and oxidized chitosan, or chitosan modified with polymers such as polyethylene oxide, polyvinyl alcohol (PVA), polymethyl methacrylate, and so forth, were found to provide the necessary blood compatibility and enzymatic degradability to chitosan.[25–31] Most of these reports have discussed the influence of surface modifiers in improving the blood compatibility of chitosan films and membranes. Recently, Shelma and Sharma have investigated the effects of lauroyl sulfated chitosan in enhancing hemocompatibility of chitosan submicron particles.[30] Few studies, to date, have investigated the effect of surface modification in improving the blood compatibility of surface-modified chitosan nanoparticles.[22,32] Our study attempts to elucidate the effects of solvents, dispersing media and surface modifiers (PEG, PVA, and EDTA) on the surface charge and blood–cell interaction of chitosan nanoparticles by evaluating the percentage hemolysis, platelet aggregation, coagulation times, and cytokine induction. A comparative study between blood compatibility of chitosan nanoparticles with chitosan thin film and chitosan molecule is also done to understand the influence of dimensionality.

MATERIALS AND METHODS

Materials

Chitosan (low molecular weight, degree of deacetylation, $\geq$75%), sodium tripolyphosphate (TPP), PEG (MW = 200), and EDTA were purchased from Sigma Aldrich, USA, and PVA (MW = 2 kDa) from Acros Organics, USA. All other chemicals were of analytical grade and used without further purification.

Preparation of chitosan nanoparticles and thin film

Chitosan nanoparticles were prepared by the conventional ionotropic gelation technique using TPP as the crosslinking agent.[7] Briefly, 0.5 wt % of low-molecular-weight chitosan dissolved in 1% aqueous acetic acid (pH = 3.2 $\pm$ 0.2, ionic strength = 0.862 m*M*) or lactic acid (pH = 4.2 $\pm$ 0.2, ionic strength = 1.899 m*M*) solutions were ionically crosslinked with TPP (at an optimal weight ratio of 1:0.5) under magnetic stirring for 5 min at 800 rpm, yielding a colloidal suspension of chitosan nanoparticles in acidic medium. Probe sonication together with magnetic stirring yielded a uniform size distribution of the particles (step I). To prepare a series of surface-modified chitosan nanoparticles in acid media, 0.1 wt % of PEG, PVA, and EDTA was separately added to the colloidal suspension under magnetic stirring (step II). Subsequently, bare as well as surface-modified chitosan nanoparticles were prepared in saline medium as follows. Colloidal chitosan suspension prepared in step I was centrifuged at 20,000 rpm for 20 min (Hermle, Z36HK, High-Speed cooling centrifuge) and the pellet was repeatedly washed. The colloidal suspension was further surface modified by the addition of 0.1 wt % of PEG, PVA, and EDTA respectively, by an overnight incubation under stirring to ensure uniform dispersion and particle stability. The modified chitosan nanoparticles were further washed thrice by centrifugation and redispersed in 0.9 wt % saline (pH = 6 $\pm$ 0.2) under sonication, yielding surface-stabilized nanoparticles in saline.

Apart from nanoparticles, chitosan films were also prepared by casting 0.5 wt % chitosan solution in 1% lactic acid medium onto a glass slide and drying at 40°C for 1 h.[33] In addition, molecular chitosan solution (0.5 wt %) was prepared in 1% aqueous lactic acid. All the above samples prepared in acidic media, saline media, as well as the chitosan film and molecular chitosan were analyzed for their blood compatibility in comparison to an equivalent amount of chitosan powder.

Physicochemical characterizations

Particle size, morphology, and ζ-potential. Average size and size distribution of bare- and surface-modified chitosan nanoparticles prepared in acidic and saline media was measured by the dynamic laser light scattering (DLS) technique (Nicomp 380 ZLS, Particle Sizing Systems, CA) at a scattering angle of 90°. The intensity-weighted mean value was recorded as the average of three measurements. Laser Doppler anemometry using the same instrument was used to estimate ζ-potential or surface charge as well as stability of the colloidal suspensions.

Surface morphology of chitosan nanoparticles and film was observed using scanning electron microscopy (SEM) (JEOL, JSM 6490LA, Japan) and atomic force microscopy (AFM) (JEOL, SPM5200, Japan). The samples were sputter coated with platinum by the Auto Fine Platinum Coater (JEOL, JFC-1600, Japan) at 10 mA for 120 s before imaging in SEM. Samples for AFM measurements were drop casted on atomically flat mica sheet and imaged.

Fourier transform infrared spectrometric analysis. Surface modification of chitosan nanoparticles with various stabilizers was confirmed by assessing the samples using Fourier transform infrared (FTIR) spectroscopy by recording the absorbance of samples in the frequency range from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. The powder samples were mixed with KBr and pelletized before recording the IR spectra. The spectra for molecular chitosan, PEG, PVA, EDTA, and surface-modified nanoparticles were analyzed using Perkin Elmer Spectrum RX-1 FTIR spectrometer.

Thermogravimetric analysis. Thermogravimetric (TG) analysis was utilized to establish the presence of surface modifiers (PEG, PVA, and EDTA) on chitosan nanoparticles. Thermal analysis was performed by the simultaneous TG-DTA analysis using TG/DTA 6200 EXSTAR (Shimadzu, Japan). The mass of each samples analyzed was kept constant at 5 mg. The carrier gas used was nitrogen at a flow rate of 50 mL/min. The samples were heated from 25 to 520°C for recording the TG curve at a heating rate of 10°C/min.

Blood compatibility study

The blood compatibility studies of chitosan nanoparticles, molecular chitosan, as well as chitosan films were evaluated by studying the percentage of hemolysis, platelet aggregation, coagulation time, and cytokine activation as detailed below. The cell count of the whole blood used for the following assays was performed using hematology analyzer (Cell Dyn 3700, Abbott, USA). A detailed cell morphological analysis was performed using SEM (JEOL, JSM 6490LA, Japan).

Analysis of hemolytic properties. The extent of hemolytic property was studied by measuring the percentage of hemolysis using spectrometric techniques for the colorimetric determination of hemoglobin in whole blood.[34] Whole blood was obtained from healthy human volunteers (22–31 years old) after institutional ethical clearance and appropriate informed consent. Normal human blood was collected from three donors in vacutainers anticoagulated with Li heparin and pooled before the experiment. One milliliter of pooled blood was treated with 100 μL (0.5 mg/mL) of chitosan samples (nanoparticles prepared in acetic/lactic acid media and also dispersed in saline, chitosan molecular solution) and incubated at 37°C for 3 h under mild shaking conditions. Chitosan-treated blood was centrifuged at 4500 rpm (1900*g*) (Thermo Scientific, Sorvall, Legend Micro 17 Centrifuge) for 10 min at 22°C to yield plasma which was mixed with 0.01% sodium carbonate at a ratio of 1:10. Lactic acid and acetic acid were used as control samples, apart from 0.9% saline solution, which was used as the negative control (lysis, ~ 0%s), and 1% Triton X100 as the positive control (lysis, 100%). Chitosan powder and film were also tested as above by mixing 1 mL of pooled blood with the powder or by adding it onto the film. Optical absorbance was measured to quantify the hemoglobin released to estimate the extent of red cell lysis. The absorbance of blood samples (after exposure to various chitosan samples) were normalized with that of control blood. For evaluating the hemolytic properties of chitosan, the optical density was determined at the peak of the Soret band of hemoglobin (415 nm) and at 380 and 450 nm in a microplate reader (Powerwave XS-BioTek, USA) using UV transparent flat bottom 96-well plate (Corning NY14831). The % hemolysis was estimated from the formula for plasma hemoglobin calculated as follows[34]:

$$\text{Amount of plasma hemoglobin (mg/dL)} = \frac{2 \times A_{415} - (A_{380} + A_{450}) \times 1000 \times \text{dilution factor}}{(E \times 1.655)} \quad (1)$$

where A_{415}, A_{380}, and A_{450} are the absorbance values at 415, 380, and 450 nm, respectively. A_{415} gives the sorbent band based absorption of hemoglobin, whereas A_{380} and A_{450} are correction factors applied for uroporphyrin absorption measured in the same wavelength range. The molar absorptivity value of oxyhemoglobin at 415 nm, measured as E is 79.46 and 1.655, is the correction factor applied owing to the turbidity of plasma sample. The hemolytic property of nanoparticles was plotted as percentage hemolysis (calculated as per Eq. (2)) for various samples.

$$\% \text{ Hemolysis} = \frac{\text{Plasma Hb value of sample}}{\text{Total Hb value of blood}} \quad (2)$$

Platelet aggregation assay. Platelet aggregation upon interaction with chitosan was studied by means of electrical impedance method to calculate the distribution of the platelets. NCL Method, ITA-2 Version-1.0, was adopted for the study. Briefly, human blood obtained from three healthy volunteers was collected in vacutainers anticoagulated with Li heparin. The whole blood was centrifuged at 2500 rpm (1000*g*) (Eppendorf, Centrifuge 5702 R) for 12 min at 22°C to obtain platelet-rich plasma (PRP). Chitosan samples as used earlier in hemolysis assay were added to PRP in 1:10 (0.05 mg/mL) volume ratio and incubated at 37°C for 3 h. Untreated PRP was used as a negative control and collagen (1 mg/mL) treated PRP was used as a positive control. The platelet count was measured using a hematology analyzer (Cell Dyn 3700, Abbott, USA). Chitosan powder and film were also tested by mixing 1 mL of PRP with the powder or by adding it onto the film. One milliliter of PRP mixed with 0.9% saline was used as the negative control.

Coagulation assay. The NCL Method, ITA-12 Version-1.0, was followed for coagulation assay. Human blood obtained from three healthy volunteers was collected as detailed earlier. The whole blood was centrifuged at 4500 rpm (3000*g*) (Eppendorf, Centrifuge 5702 R) for 12 min at 22°C to obtain the plasma. Chitosan samples were added to the plasma in 1:10 (0.05 mg/mL) volume ratio and incubated at 37°C for 3 h, centrifuged at 1000 rpm for 10 min after which the reagents, that is, neoplastine for determining prothrombin time (PT) and activator (phospholipids) and calcium chloride for activated partial thromboplastin time (APTT) studies were added to the plasma samples. Clotting time was optically measured using a coagulation analyzer (AMAX-Destiny Plus, Amelung, USA). One milliliter of plasma mixed with 0.9% saline was used as the negative control.

Cytokine activation. The cytokine activation of chitosan samples was determined by cytometric bead assay adopting the NCL method ITA-10 for the study. For this, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density gradient centrifugation and the isolated PBMCs were seeded into 96-well plates at a density of 1×10^5 cells/well, followed by treatment with particles and incubated for 24 h. PBS and 1 μg/mL lipopolysacharide (Sigma, St. Louis, USA) were served as negative control and positive control, respectively. Further for incubation, PBMCs were centrifuged and the supernatant (100 μL) was analyzed by flow cytometry according to the supplier's instructions for the quantification of different cytokines.

Blood cell morphological analysis using SEM. The morphology of blood cells after interaction with chitosan

TABLE I. Particle Size of Chitosan Nanoparticles of Varying Surface Chemistries Prepared in 1% Acetic Acid/Lactic Acid Media and Redispersed in Saline

	Particle Size (nm)			
Samples	NP in AA	NP from AA in Saline	NP in LA	NP from LA in Saline
Bare chitosan (C Bare NP)	180 ± 25	160 ± 20	150 ± 25	140 ± 10
PEG–chitosan (C-PEG NP)	120 ± 15	130 ± 10	110 ± 12	105 ± 15
PVA–chitosan (C-PVANP)	110 ± 20	138 ± 22	110 ± 20	102 ± 22
EDTA–chitosan (C-EDTA NP)	124 ± 16	125 ± 25	124 ± 16	118 ± 25

Abbreviations: AA, acetic Acid; LA, lactic Acid.

nanoparticles, chitosan powder, and chitosan film was studied using SEM. One milliliter of whole blood collected for hemolysis assay was used for these studies.

Chitosan film was rinsed thrice with deionized water and prewarmed at 37°C and 0.1 mL of whole blood was dropped onto the chitosan film.[35] After 1-h incubation at 37°C, the film was washed with PBS to remove any nonadherent cells. The adhered cells were fixed with 2% formaldehyde for 30 s. After thorough washing with PBS, the erythrocyte-attached films were dried at 37°C and sputter coated with platinum by the Auto Fine Platinum Coater (JEOL, JFC-1600, Japan) at 10 mA for 120 s before imaging in SEM.

In brief, 100 μL (0.5 mg/mL) of chitosan nanoparticles dispersed in saline was mixed with 1 mL of whole blood and incubated at 37°C for 1 h. This was dropped onto a cover slip coated with platelet-free plasma and dried. The cells were fixed with 2% paraformaldehyde, dried at 37°C, and sputter coated prior to SEM studies. As controls, the whole blood was also similarly fixed and sputter coated for SEM imaging.

Statistical analysis

Statistical analyses of the values are expressed as the mean ± standard deviation of three independent experiments. The data were analyzed using Student's *t*-test and ANOVA. In all cases, the degree of significance is indicated when appropriate ($^*p < 0.05$).

RESULTS AND DISCUSSION

Particle size, ζ-potential, and morphology

Chitosan nanoparticles prepared in this study using acetic or lactic acid as the solvent, and with various surface modifiers, under identical conditions by ionotropic gelation technique, were compared to that of the saline-dispersed nanoparticles for their size distribution and ζ-potential. The results are summarized in Tables I and II.

As summarized in Table I, the solvent medium (acid medium/saline) did not have considerable effects on the particle size. DLS technique for measuring particle size, in general, yields only an estimate of the hydrodynamic diameter and hence the variations in size measured in our experiments are only marginal.

However, as summarized in Table II, it is clear that the solvent medium used affected the surface charge of the particles significantly. In the case of bare chitosan nanoparticles prepared in acetic acid, the surface charge was highly positive (+47 mV), indicating the protonation of NH_2 functional groups of glucosamine units to NH_3^+ ions in acidic media. For particles dispersed in saline media, the positive charge on chitosan was reduced to +20 mV. Yet, another important observation was the reduction in particle size as well as the ζ-potential when the surface of chitosan was modified with stabilizers such as PEG, PVA, or EDTA. A reduction in ζ-potential in case of surface-modified particles can be attributed to the neutralizing effect of the stabilizers on the NH_3^+ ions of glucosamine units in chitosan. The ζ-potential values of these surface-modified samples were significantly reduced further when dispersed in saline. This can be well correlated with Eqs. (3) and (4) for electrophoretic mobility and ζ-potential as well as the concept of Helmholtz plane and Gouy layer.

$$\mu = \frac{2\varepsilon_r\varepsilon_0\varsigma}{3\pi\eta} \tag{3}$$

$$\varsigma = \frac{Q}{4\pi\varepsilon_r a(1+\kappa a)} \text{ and } \kappa = \sqrt{\frac{e^2\Sigma n_i z_i^2}{\varepsilon_r\varepsilon_0 kT}} \tag{4}$$

where μ is electrophoretic mobility, ζ is ζ-potential, Q is charge on the particle, a is radius of the particle, ε_r is relative dielectric constant of the medium, n_i and z_i are bulk concentration and valence of the "*i*"th ion in the system.

TABLE II. ζ-Potential of Chitosan Nanoparticles of Varying Surface Chemistries Prepared in 1% Acetic Acid/Lactic Acid Media and Redispersed in Saline

ζ-Potential (mV)				
Samples	NP in AA	NP from AA in Saline	NP in LA	NP from LA in Saline
Bare chitosan (C Bare NP)	47.74 ± 1.0	20.7 ± 0.8	38.94 ± 1.8	26.5 ± 0.7
PEG–chitosan (C-PEG NP)	39.46 ± 0.4	1.91 ± 1.5	25.99 ± 0.2	2.18 ± 1.5
PVA–chitosan (C-PVANP)	36.18 ± 0.7	−2.3 ± 0.5	28.56 ± 0.5	−1.98 ± 0.3
EDTA–chitosan (C-EDTA NP)	30.74 ± 0.5	−4.92 ± 1.6	24.23 ± 1.2	−5.43 ± 1.1

Abbreviations: AA, acetic Acid; LA, lactic Acid.

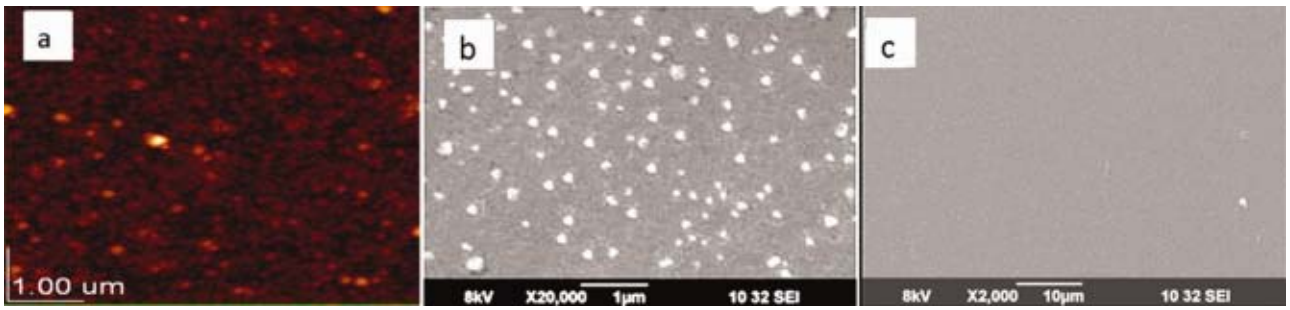

FIGURE 1. AFM and SEM images of (a and b) chitosan nanoparticles, (c) chitosan film. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The value of *Q*, that is, the charge on the surface of particles varies depending on the counter ions in the solvent. The counter ions in acidic medium protonates the NH_2 groups of glucosamine units in chitosan, resulting in its highly positive surface charge. Upon redispersing the acid prepared particles in 0.9% saline, deprotonation occurs. We suppose that the Cl^- counter ions in saline migrate to the Gouy layer and interacts with the NH_3^+ groups, resulting in the deprotonation and thereby a significant drop in ζ-potential toward negative values. This reduction in ζ-potential from a highly positive to a negative value would better aid the blood compatibility of surface-modified chitosan nanoparticles, as will be discussed in the ensuing sections.

It is well established that size as well as surface charge of nanoparticles critically alters their biodistribution and fate when injected into the blood stream.[36] The preparatory conditions also significantly influence the physicochemical characteristics of the nanoparticles formed. The role of surface modifiers in altering the size distribution as well as the surface charge of nanoparticles is also vital to its clearance from circulation.[37,38] Chitosan nanoparticles, which have emerged as an efficient natural polymeric nanomaterial for drug, gene, and protein delivery applications, are reported to have positive surface charge.[39,40] However, our studies clearly indicate that appropriate surface modification of chitosan nanoparticles can help to alter their surface charge, which in turn can modify their blood-contacting properties.

The size and surface morphology of bare chitosan nanoparticles as well as the morphology of chitosan film analyzed using SEM is shown in Figure 1. AFM and SEM display clear spherical morphology of the nanoparticles having an approximate size of $<$200 nm as shown in Figure 1(a,b). Surface-modified chitosan nanoparticles also possessed similar morphology as well as size (data not shown). Chitosan films prepared by casting on glass slides displayed a featureless morphology as shown in Figure 1(c).

FTIR spectral analysis

FTIR spectroscopy was carried out to verify the surface modification of chitosan with stabilizers such as PEG, PVA, and EDTA. Figure 2 shows the FTIR spectra of bare chitosan nanoparticles and the stabilizers PEG, PVA, and EDTA and a comparison with the modified chitosan nanoparticles. The presence of the broad band between 3000 and 3500 cm^{-1} corresponding to —OH groups present in PEG, PVA, and EDTA is apparent in all the surface-modified chitosan samples (region I). Similarly, the characteristic bending vibration of NH_2 group in chitosan nanoparticles (C-Bare NP) is evident in the FTIR spectra at 1580–1650 cm^{-1} (region II).[41] This characteristic vibration band is also observed in the spectra of all the surface-modified nanoparticles. The N—H bending frequency of the —NH_2 group in chitosan appears slightly shifted to higher wavenumbers and significantly reduced in intensity for PEG, PVA, and EDTA surface coating, respectively. This could be owing to the interactions occurring between the amino group and the hydroxyl group of the stabilizers (PEG and PVA). For EDTA–chitosan (C NP EDTA), the stretching vibrations of carboxylic group of EDTA at 1700–1725 cm^{-1} appears merged with the bending vibrations of NH_2 groups in chitosan, which is owing to the chelation of carboxylic groups of EDTA with protonated amine group of chitosan.[42] Thus, surface-modified chitosan nanoparticles exhibited the characteristic bands of both chitosan as well as the surface modifiers in their FTIR spectra.

TG analysis

TG analysis was also done as a measure to confirm surface modification on chitosan nanoparticles with PEG, PVA, and

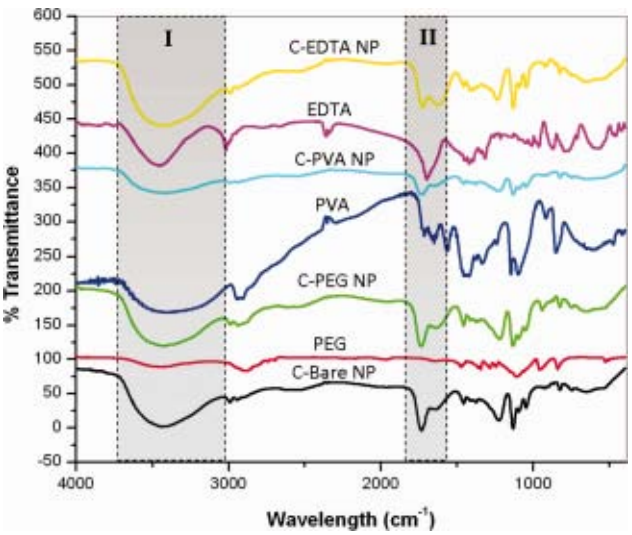

FIGURE 2. FTIR spectra of chitosan- and surface-modified chitosan nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

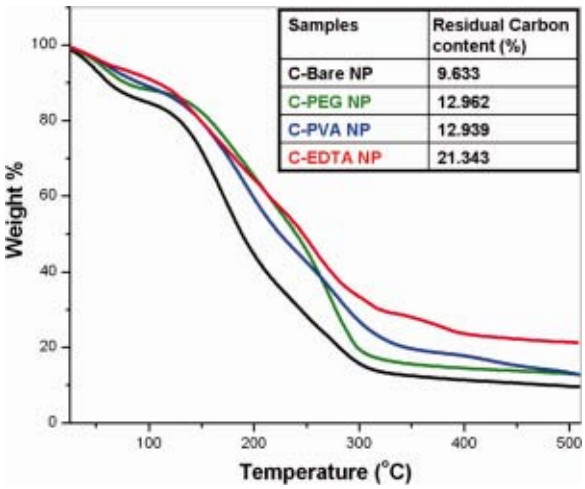

FIGURE 3. TG analysis of chitosan- and surface-modified chitosan nanoparticles to understand the residual carbon content. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

EDTA by looking at the residual carbon content. The weight of all the samples for analysis was kept same (5 mg). From the analysis, the residual carbon content of bare chitosan was found to be 9.633% and that of PEG-, PVA-, and EDTA-coated chitosan were 12.962, 12.939, and 21.344%, respectively (Fig. 3). The increase in carbon content in the coated samples when compared to bare chitosan is rendered by the surface coating. The higher carbon content in the EDTA coating may be owing to the better interaction of the anionic EDTA with the cationic chitosan nanoparticles. The results from residual carbon content analysis by TGA prove that by physical adsorption of PEG/PVA/EDTA, a coating is formed on the chitosan nanoparticles.

Blood compatibility study

Under normal conditions, blood is in contact with the endothelium that has inherent anticoagulant and antithrombotic properties. Any foreign material which lacks such properties would promote thrombosis.[43] Surface charge of biomaterials (nanoparticles) decides the fate of its interaction with blood, resulting in either clot formation or excellent blood compatibility.[44] In this study, we have evaluated how the surface charge of chitosan nanoparticles, the medium of preparation, as well as dimensionality modulate its hemolytic, coagulation, and platelet aggregation properties.

Analysis of hemolytic properties. The percentage hemolysis of chitosan samples (0.05 mg/mL) is graphically shown in Figure 4. Nanoparticles prepared in acetic acid medium showed high % hemolysis compared to those prepared in lactic acid medium, whereas the saline-dispersed nanoparticles were found to be hemocompatible. Chitosan film as well as the molecular solution treated with whole blood showed complete hemostasis, whereas chitosan in its powder form showed no hemolysis.

It is very evident from the graph that 1% acetic acid caused $\sim$ 30% hemolysis, which can be attributed to the ability of acetic acid to swell erythrocytes and cause lysis.[45] Further, nanoparticles prepared in lactic acid exhibited low hemolytic property. This can be owing to the inherent biocompatibility of lactic acid, which is a common metabolite in glucose metabolism and also a major component of lactate Ringer's solution. Surface modification of nanoparticles has also resulted in a uniform improvement in the hemocompatibility of chitosan as shown in Figure 3. As expected, the hemolytic activity was observed to be the lowest for nanoparticles dispersed in saline.

The hemolysis assay could not be performed on chitosan film samples owing to the formation of a thick clot upon addition of blood onto the film, implying its hemostatic nature. This observation is consonant with the hemostatic effects of chitosan membranes and films, already reported in the literature.[19,18,46] On the contrary, chitosan molecular solution was hemolytic, whereas chitosan powder did not cause any hemolysis. This change in behavior of the different forms of chitosan can be explained on the basis of the protonation of its amine group and thereby its surface charge. Chitosan molecular solution in 1% acid possesses more or less complete protonated amines, which in turn render the molecule highly positively charged, thereby influencing its interaction with red blood cells. In contrast, chitosan powder sample contains free nonprotonated amine groups which results in their altered hemolytic behavior. Thus, our results on the hemolytic activity of different forms of chitosan clearly illustrate the effect of surface charge on hemolysis.

Platelet aggregation assay. An early indicator of thrombogenic activity of any blood-contacting material is its platelet adhesion property.[47] Figure 5 shows the platelet aggregation results carried out on various forms of chitosan. As chitosan nanoparticles prepared in acetic acid showed a high % hemolytic activity, all further tests were carried out by using particles prepared only in lactic acid. Nanoparticles in 1% lactic acid medium showed high platelet aggregation compared to that dispersed in saline. This can be attributed to the high positive surface charge on nanoparticles prepared

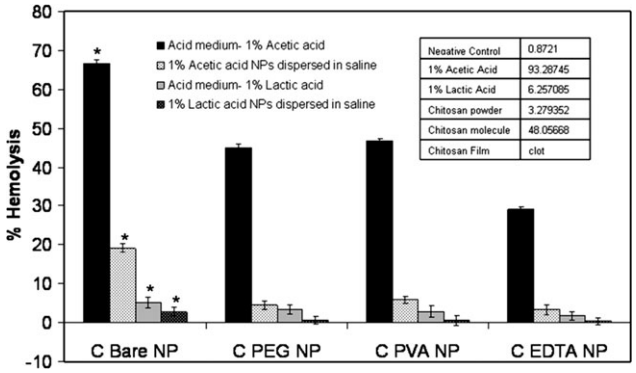

FIGURE 4. Percentage hemolysis: Nanoparticles in 1% acetic acid and lactic acid are represented as (■) and (▩) and nanoparticles prepared in 1% acetic acid and lactic acid redispersed in saline are represented as (□) and (▩), respectively. The degree of significance compared to C-Bare NP is indicated when appropriate ($*p < 0.05$).

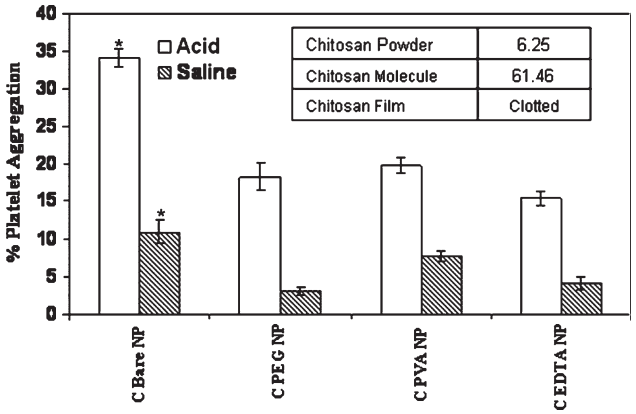

FIGURE 5. Percentage platelet aggregation of chitosan samples. When appropriate, the degree of significance is indicated compared to C-Bare NP (*$p < 0.5$).

in acid medium (Table II), which increases its affinity toward platelet adhesion proteins. Positively charged surfaces of biomaterials in contact with blood have been reported to induce the formation of primary platelet clots, whereas surfaces with negatively charged groups generally possess excellent blood compatibility.[48] Among all saline-dispersed nanoparticles, PEG-coated chitosan exhibited the least platelet aggregation. A plausible reason for this can be the steric hindrance provided by the PEG chain, resulting in particles with a reduced surface charge of 1.9 ± 1.5 mV. Such an appreciable improvement in platelet compatibility of chitosan upon surface modification is in concord with the previous reports on chitosan surface modification.[28,49,50] However, studies by Xue et al.[51] have shown that chitosan nanoparticles having a comparatively high surface charge of 14.1 ± 0.6 mV also exhibit platelet compatibility. Hence, surface charge density of nanoparticles is a vital factor that dictates its platelet compatibility.

It has been previously reported that platelets readily aggregate onto positively charged membranes than negative or neutral surfaces.[52] In this study, such an effect is explained with regard to various surface-modified nanoparticles and its comparison with the film surface, molecular solution, and powder form, which helps to comprehend the effect clearly. In contrast to the thick clot formed by chitosan film and chitosan molecular solution which showed 61.5% aggregation, chitosan powder samples showed platelet aggregation very similar to that of saline-dispersed surface-modified nanoparticles. This undeniably supports the reports on effect of surface charge on platelet aggregation.[47]

Coagulation assay. Blood coagulation results from a series of proteolytic reactions involving the stepwise activation of coagulation factors present in the plasma.[14] The effect of chitosan samples on the intrinsic and extrinsic pathways of coagulation cascade was studied by measuring the APTT and PT, respectively, upon treatment with platelet poor plasma. Figure 6 shows the effects of chitosan samples on the above coagulation times. We observed that chitosan samples in acid medium did not induce any clot formation up to 60 s for PT as well as APTT studies. This could be because of the strong affinity of nanoparticles for clotting factors in plasma owing to which the coagulation cascade was not initiated. This corroborates with the observations of Song et al.[32] that acidic pH prolongs blood coagulation times. In contrast, saline-dispersed nanoparticle-treated plasma showed clotting times that were comparable to the instrument control coagulation times (14.6 s for PT and 32.2 s for APTT). This is an indication that bare- as well as surface-modified chitosan samples are nonthrombogenic, and do not interact with the coagulation factor proteins and cause no interference with the plasma coagulation pathway, only when dispersed in saline. However, a delay was observed in clotting for EDTA surface-modified nanoparticles in comparison with PEG and PVA, which can be attributed to the inherent anticoagulant property of EDTA. Similar effects of blood protein adsorption changes owing to differences in surface chemistry have also been observed by

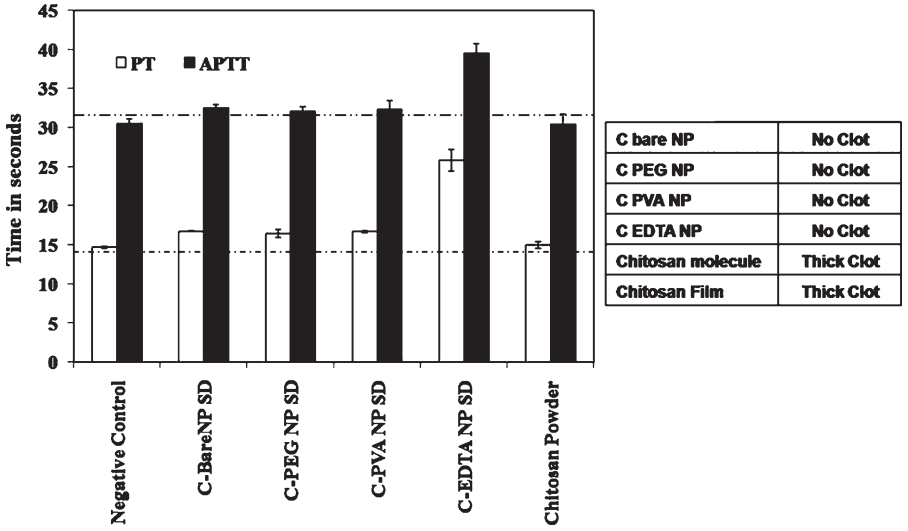

FIGURE 6. Coagulation assay results: PT and APTT; dotted line represents instrument control values (PT 14 s and APTT 32.2 s).

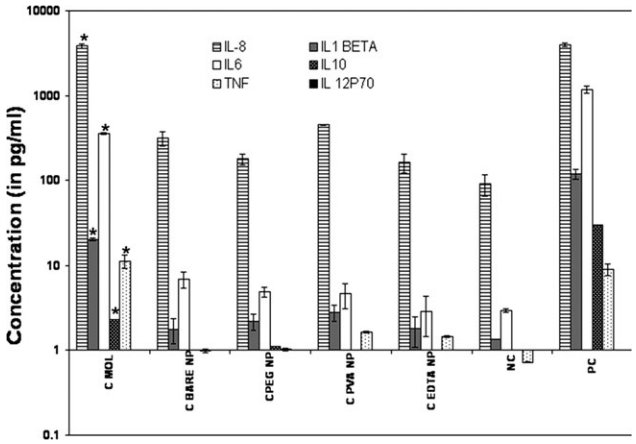

FIGURE 7. Cytokine induction results for chitosan samples. The degree of significance compared to chitosan molecule is indicated when appropriate (*$p < 0.05$).

Benesch and Tengvall,[16] wherein it was shown that large amounts of fibrinogen and other plasma proteins bind to chitosan and not to acetylated chitosan, proving the influence of surface chemistry on protein adsorption.

Blood coagulation studies carried out similarly on chitosan film as well as the molecular solution showed visual clot formation even before the addition of the neoplastine and activators, implying their hemostatic effects. In contrast, for chitosan powder, coagulation times nearly equivalent to the instrument control values were measured. These results are in strong support of the platelet aggregation noticed for chitosan samples, which implies the influence of surface charge on blood coagulation and hemostasis. It should also be noted that the appreciable blood compatibility of chitosan nanoparticles dispersed in saline were observed for concentrations of 0.5 mg/mL and concentrations above 0.5 mg/mL are likely to show variations from these results.

Cytokine activation. Intravenous delivery of drugs encapsulated nanocarriers can elicit both inflammatory and immune responses through activation of the immune system.[53,54] Ideally, materials selected for use as nanocarriers for drugs, genes, and so forth, should not induce any such immune response. In this study, this was studied by measuring the expression levels of the major proinflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10, and TNF-α from chitosan-treated PBMCs using flow cytometry. These cytokines are soluble proteins that principally bind to transmembrane receptors and elicit cell proliferation, differentiation, apoptosis, or release other soluble proteins that are involved in

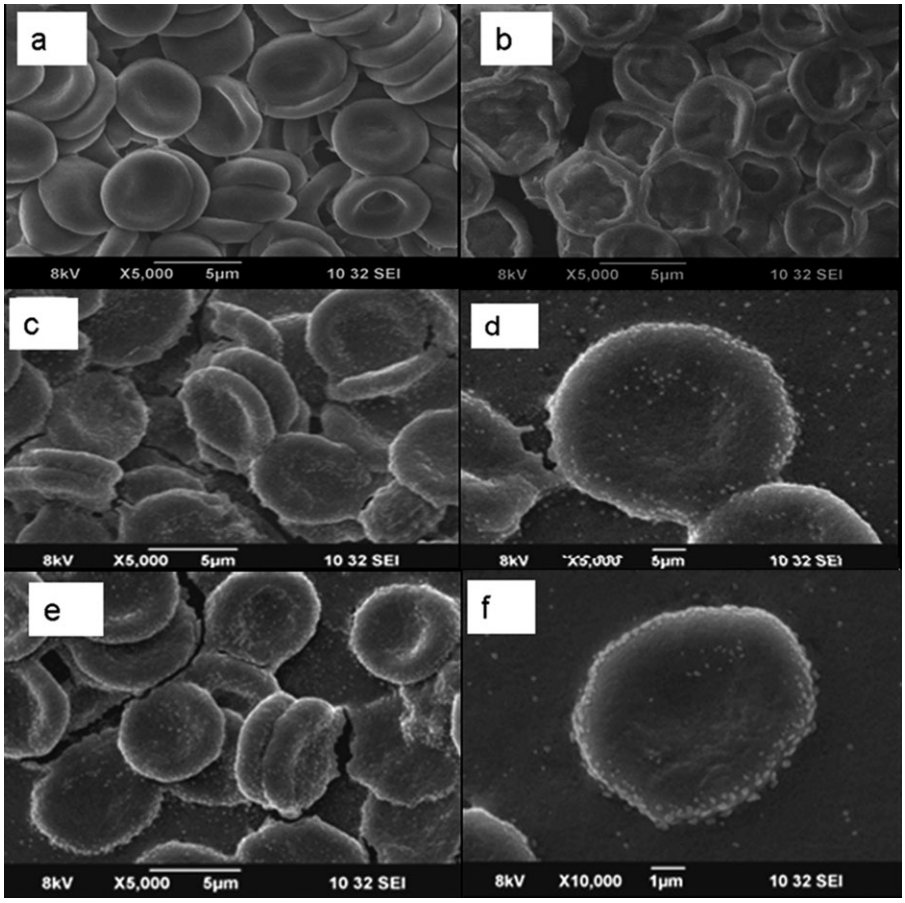

FIGURE 8. Representative SEM images of (a) control erythrocytes at high magnification (b) erythrocytes on chitosan film at high magnification; (c) erythrocytes treated with bare chitosan nanoparticles at low magnification and (d) at high magnification; (e) erythrocytes treated with PEG coated chitosan nanoparticles at low magnification and (f) at high magnification.

various signal transduction pathways responsible for inducing inflammation.[55] Figure 7 shows the results of cytokine analysis carried out on the chitosan samples for a concentration of 5 mg/mL and 24 h of incubation. Concentration of IL-8 which plays an important role in acute inflammation was nearly 42-fold higher in molecular chitosan-treated PBMCs (represented as C MOL in Fig. 7), whereas it was comparatively less (1.5- to 5-fold) for PBMCs treated with saline-dispersed chitosan nanoparticles. The activation of proinflammatory cytokines such as IL-1β, IL-6, IL-10, and TNF also followed a similar trend as that of IL-8 induction, for all the chitosan samples. These observations again have a bearing on their surface chemistry and thereby the surface charge, which in turn modulates their interaction with immune cells. Thus, saline-dispersed chitosan samples did not elicit any significant increase in the secretion level of these cytokines compared to that of untreated cells, implying that chitosan nanoparticles may not cause serious inflammatory response when appropriately prepared. Our results provide supporting evidence for the fact that cationic particles induce inflammation to a great extent than anionic and neutral ones.[53] Also, among the various surface-modified chitosan samples, cytokine activation was observed to be less in PEG- and EDTA-modified chitosan than PVA–chitosan nanoparticles. It has been reported that an intraperitoneal administration of chitosan induces significant inflammatory responses through indirect chemotactic activity.[56] Lin et al.[56] have observed that chitosan–carboxymethyl dextran nanoparticles with varying degree of deacetylation induced varying levels of serum cytokines when studied *in vitro* on tumor and normal cell lines. Thus, surface chemistry can significantly alter the inflammatory response of nanoparticles.

Blood cell morphological analysis. Our observations on the hemolysis activity of chitosan were elucidated by SEM. Figure 8 shows the representative SEM images obtained for the interaction of blood cells (erythrocytes) with bare- and PEG-coated chitosan nanoparticles (dispersed in saline) as well as chitosan film. To the best of our knowledge, our study appears to be the first to explain and correlate the blood compatibility of chitosan nanoparticles in terms of cellular morphology and molecular level interaction.

A significant difference in blood-contacting properties of two-dimensional chitosan film and chitosan nanoparticles was observed throughout the study. Bare- as well as surface-modified chitosan nanoparticles prepared in lactic acid medium and redispersed in saline were compatible with erythrocytes, whereas those on chitosan film had a significant change in morphology as shown in Figure 8. This morphological variation validates our explanation regarding the influence of dimensionality as well as dispersing media on blood-contacting properties, specifically the hemolytic activity. Hence, from our results, it is apparent that both chemical and physical modifications of chitosan decide its safety for blood-contacting applications and thereby its suitability for intravenous administration.

CONCLUSIONS

In summary, this study highlights the effect of solvents, surface charge, and dimensionality on the blood compatibility of chitosan. A significant observation from the blood compatibility study was that bare- as well as surface-modified chitosan nanoparticles dispersed in saline exhibited appreciable blood compatibility in contrast to chitosan film which was hemostatic. This well explains the effect of dimensionality on blood compatibility of chitosan. Another distinct attribute that significantly altered the blood-contacting properties of chitosan was its surface charge, which varied with the choice of surface modifiers and dispersing medium. The blood compatibility results of chitosan powder which lack protonated amines justify this explanation. Hence, for chitosan to be used as an intravenous drug delivery vehicle, it is very critical that its molecular solutions are prepared in a biocompatible solvent such as lactic acid and the nanoparticles be appropriately surface modified with PEG, EDTA, or PVA and dispersed in biological fluids. However, for *in vivo* applications, covalent conjugation of the aforementioned stabilizers would provide better stability in circulation.

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