



## Chitosan nanoconstructs for improved oral delivery of low molecular weight heparin: In vitro and in vivo evaluation

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### ABSTRACT

The aim of present study was to investigate the potential of mucoadhesive polymer chitosan (CS) and *N*-trimethyl chitosan (TMC) based nanoparticulate systems for oral bioavailability enhancement of low molecular weight heparin (LMWH). The TMC was synthesized by methylation of chitosan followed by characterization using infrared spectroscopy and <sup>1</sup>H-NMR spectroscopy. The IR and NMR spectra of TMC confirmed the presence of trimethyl groups and estimated the degree of quaternization for TMC about 46%. TMC nanoparticles were then prepared by ionic gelation method. The developed CS-NPs and TMC-NPs were characterized for various parameters including morphology, particle size, zeta potential, entrapment efficiency, in vitro release behavior and storage stability at different temperature and simulated gastrointestinal tract conditions. The fluorescent microscopy study confirmed the higher particle uptake of TMC-NPs by gastrointestinal epithelium in comparison to the CS-NPs. The concentration of LMWH in the systemic circulation followed by oral administration of formulations was estimated using FXa chromogenic assay. A significant increase ( $p < 0.05$ ) in the oral bioavailability of LMWH was observed with TMC-NPs than both CS-NPs as well as plain LMWH solution. These findings suggested that TMC nanoparticles hold promise for oral delivery of LMWH and clinical applicability for the treatment of vascular disorders like deep vein thrombosis and pulmonary embolism, etc.

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### 1. Introduction

Low molecular weight heparin (LMWH) is the safest clinically used anticoagulant for the treatment of several vascular disorders such as deep vein thrombosis (DVT) and pulmonary embolism (PE) (Paliwal et al., 2011a). However, restricted choice of parental routes either intravenous or subcutaneous for its administration limits its wide clinical applicability. The non-invasive delivery of heparin is highly desired to overcome many problems associated with the use of parenteral formulation like patient inconvenience, hospitalization, risk of needle born infections etc. (Motlek and Youan, 2006). Oral drug delivery is the safest and most acceptable form of drug administration, which is highly admired by clinicians as well as patients. Although, many oral anticoagulants are regularly recommended in the medical practices by clinicians; however these anticoagulants are not much appreciated due to their high drug interactions and toxicological implications necessitating the development of safe and effective anticoagulants formulations based on some novel carrier concepts. Therefore, an oral

heparin formulation may be the best alternative of currently used parenteral heparin as well as other oral anticoagulants (Arbit et al., 2006; Paliwal et al., 2011b). Practically, oral delivery of LMWH is very limited due to its poor absorption across gastrointestinal tract because of high anionic charge density, giant molecular size, enzymatic degradation and first pass effect (Goldberg and Gomez-Orellana, 2003). Therefore, a specialized delivery vehicle is required for its safe oral delivery, which can protect it from harsh gastrointestinal tract (GIT) conditions vis a vis can increase its bioavailability. Several novel carrier systems have been reported for the oral delivery of LMWH (Paliwal et al., 2011a; Lanke et al., 2009; Chandy et al., 2002; Hoffart et al., 2006; Jiao et al., 2002; Kast et al., 2003).

Chitosan (CS), a natural cationic polymer, serves as an excellent biomaterial for delivery of large variety of macromolecules including protein/peptides, DNA, diagnostic agents and drug(s) potentially across the major routes of drug delivery (Calvo et al., 1997; Wadhwa et al., 2009; Illum, 1998). CS aids into paracellular transport of hydrophilic macromolecules mechanistically by widening of intercellular tight junctions of epithelium linings (Thanou et al., 2000). Being natural cationic polymer, it strongly interacts with anionic biomacromolecules and holds them tightly to deliver into systemic circulation without any toxicity to epithelium tissues. However, CS shows limited solubility at neutral or higher pH value. Therefore, CS derivatives have been developed

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and evaluated to overcome its solubility problems (i.e., <pH 6.5 only) and to improve its effectiveness as absorption enhancer at neutral pH values such as those found in major parts of the intestinal tract (Illum, 1998). The quaternization of CS into a derivative like N-trimethyl chitosan (TMC) tremendously resolved these problems and retained other desirable characteristics for oral absorption (Thanou et al., 2000). Further, TMC increases the permeation across intestinal epithelium barrier using similar mechanism to CS and hence may increase the absorption of drug as well as biomacromolecules without any toxicity.

The present investigation was aimed to develop and characterize LMWH loaded CS nanoparticles (CS-NPs–LMWH) and TMC nanoparticles (TMC-NPs–LMWH) particularly designed for its oral bioavailability enhancement. The carrier systems were evaluated for particle shape, size, zeta potential, entrapment efficiency and in vitro drug release in the simulated GIT conditions. The storage stability studies were performed at different temperature and simulated pH conditions. The particle uptake study was performed using fluorescence microscopy. Plasma drug concentration profile followed by oral administration of the formulations was estimated using FXa chromogenic assay. The pharmacokinetic parameters were calculated using trapezoidal method.

## 2. Materials and methods

### 2.1. Materials

Chitosan (molecular weight range from 60 to 120 kDa and degree of deacetylation >80%), dialysis membrane (MWCO 10,000 Da), sodium tripolyphosphate (TPP) and Sephadex G-50 were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). LMWH (average molecular weight 4.35 kDa and anti-FXa activity 102 IU/mg) was provided as gift sample by Hebei Changshan Biochemical Pharmaceutical Ltd. (Shijiazhuang, China). The anti-FXa activity of LMWH was estimated by colorimetric assay using a Chromogenix Coatest LMW Heparin Kit® (Diapharma, USA). All other chemicals and reagents were purchased from Himedia, Bombay and were of analytical grade unless otherwise specified. The simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) were prepared according to the official methods of United State Pharmacopoeia (XXV).

### 2.2. Synthesis and characterization of N-trimethyl chitosan

TMC was synthesized following previously reported method of Sieval et al. (1998) with slight modification as per laboratory setup. Briefly, TMC was synthesized by methylation of chitosan using CH<sub>3</sub>I in a strong base (NaOH) and analyzed by infrared (IR) and 1H nuclear magnetic resonance (NMR) spectroscopy. The degree of quaternization (DQ) were calculated using the equations DQ (%) = [(CH<sub>3</sub>)<sub>3</sub>/(H) × 1/9] × 100 as reported earlier (Thanou et al., 2000). In the equations, (CH<sub>3</sub>)<sub>3</sub> represent integrals of the chemical shift of the hydrogen of the TMC group at 3.3 ppm of trimethyl amino group, whereas [H] is the integral of the H-1 peaks between 4.5 and 5.5 ppm, related to hydrogen atoms bound to C-1 of the CS molecule.

### 2.3. Development of LMWH loaded CS/TMC nanoparticles

The CS-NPs/TMC-NPs were prepared using ionotropic gelation method as reported earlier with slight modification (Wadhwa et al., 2010; Calvo et al., 1997). Briefly, TPP aqueous solution of varying concentration was added to CS/TMC solution (in 1%, v/v acetic acid) kept in a beaker. The solution was stirred for 30 min at 100 rpm with the help of magnetic stirrer (Remi instruments, India) at room

temperature. The CS/TMC to TPP ratio was varied in order to optimize the particle size and drug entrapment efficiency. The LMWH solution was added in to the TMC solution in order to get drug loaded CS-NPs/TMC-NPs. Free unentrapped LMWH was removed by ultracentrifugation (Hitachi, Japan) at 30,000 rpm for 30 min. Supernatant of dispersion was discarded and NPs were washed with distilled water at least 3 times for complete removal of free LMWH. The LMWH loaded NPs were then dispersed in to water, lyophilized and preserved till further use.

### 2.4. Characterization of nanoparticles

#### 2.4.1. Transmission electron microscopy (TEM)

The CS-NPs–LMWH and TMC-NPs–LMWH were examined for their morphological examination by transmission electron microscope (JEOL, Japan) using copper grid coated with carbon film. The 1% (w/v) solution of phosphotungstic acid was used as a negative stain.

#### 2.4.2. Size and zeta potential

The particle size and zeta potential of developed CS-NPs–LMWH and TMC-NPs–LMWH were measured by photon correlation spectroscopy (ZS90 zeta sizer, Malvern Instruments, UK). The particle-size distribution is reported as a polydispersity index (PDI).

#### 2.4.3. Entrapment efficiency

The entrapment efficiency of LMWH in CS-NPs and TMC-NPs was estimated by an indirect method. The amount of unentrapped LMWH in supernatant aqueous solution followed by centrifugation of dispersion was analyzed using Azure II colorimetric method reported earlier (Rawat et al., 2008). In brief, 500 µl aqueous samples were reacted with 4.5 ml of Azure II solution (0.001%, w/v) and were determined by spectrophotometrically with UV-spectrophotometer (Cintra 10, Japan) at 530 nm.

#### 2.4.4. In vitro release study

The in vitro release profile of LMWH from both CS-NPs–LMWH and TMC-NPs–LMWH was determined in different GIT simulated conditions (SGF pH 1.2 and SIF pH 7.4) using previously reported method (Paliwal et al., 2011b). Briefly, LMWH loaded NPs dispersion was suspended in 10 ml of respective medium in a flask incubated in a water bath at 37 °C under magnetic stirring at 100 rpm. The samples were withdrawn after regular time intervals followed by replacement with equal volume of fresh medium. The withdrawn samples were assayed for LMWH content using Azure II assay.

#### 2.4.5. Stability study

The LMWH loaded CS-NPs and TMC-NPs formulations were studied for stability studies at various temperatures (4 ± 1 °C, 25 ± 1 °C) and simulated GIT pH conditions (SGF pH 1.2, SIF pH 7.4). These formulations were examined and estimated at regular time intervals for any change in particle size and drug content.

#### 2.4.6. In vivo study

The in vivo studies were carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India). All the study protocols were approved by the local institutional animal ethics committee (Approval No. Animal Eths. Comm./10/87/13).

##### 2.4.6.1. Particle uptake study.

Fluorescence microscopy was performed on Sprague-Dawley albino rats for the confirmation of particle uptake of CS-NPs–LMWH and TMC-NPs–LMWH by the intestinal epithelium. The 6-carboxy fluorescein (6-CF) was used as

a fluorescent marker and was loaded into the NPs replacing LMWH. After 1 h of oral administration of 6-CF loaded NPs formulation, the animals were anesthetized and sacrificed. The small intestine was removed followed by microtomy and viewed under a fluorescence microscope (Nikon Eclipse, E200).

**2.4.6.2. Plasma profile and pharmacokinetics study.** The plasma drug concentration after oral administration of the formulations was estimated on albino rats of either sex weighing in the range of 100–125 g. Animals were divided into the five groups and were fasted over night with free access to water prior to the formulation administration. Animals were anesthetized for a very short time with diethyl ether and formulation was administrated orally with help of canula to each animal in a group. Group I, II and III received equivalent oral dose (50 mg/Kg body weight) of CS-NPs–LMWH, TMC-NPs–LMWH and plain LMWH formulation by oral route respectively. Group IV received LMWH by intravenous route with dose of 1 mg/Kg. Group V served as control and received phosphate buffer solution orally. The blood samples were collected from retro-orbital plexus at different time points and were analyzed for LMWH concentration by anti-FXa chromogenic assay. The different pharmacokinetic parameters were estimated from the time versus plasma concentration profile. The overall bioavailability (*F*) of LMWH was calculated by comparing its pharmacokinetic data obtained from oral administration with the results obtained from intravenous injection.

## 2.5. Statistical analysis

The results were expressed as mean  $\pm$  standard deviation. Student's *t*-test was conducted on data for statistical analysis. Comparison between different groups was made by a one-way ANOVA test. The level of significance was chosen as less than 0.05 (i.e.,  $p < 0.05$ ).

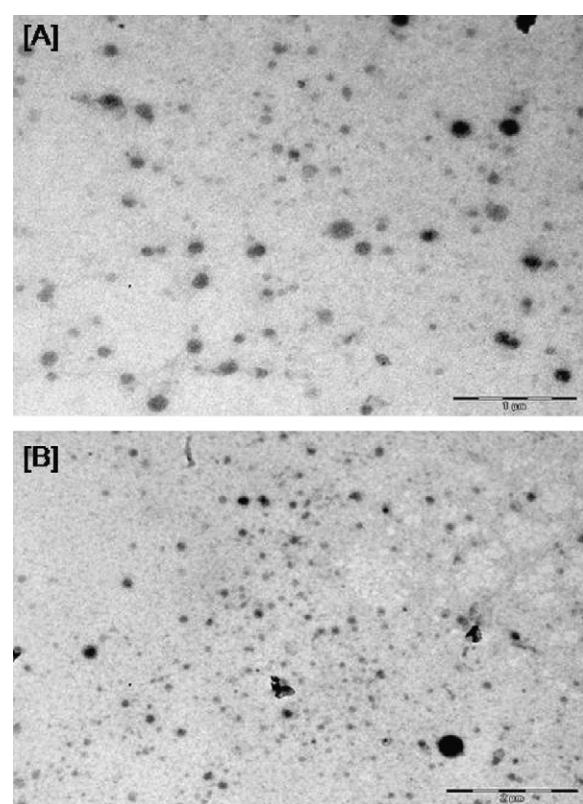
## 3. Results

### 3.1. Synthesis and characterization of *N*-trimethyl chitosan

TMC was synthesized by methylation of chitosan with methyl iodide in basic media. The IR spectrum of TMC showed presence of band at  $1408\text{ cm}^{-1}$  confirming asymmetric angular deformation of C–H bonds. A characteristic band of N–H bond of amino group, which was observed in the spectrum of chitosan was found to be very weak or almost absent in the TMC spectra because of N-methylation. Additionally, a newer peak was also observed at  $1628\text{ cm}^{-1}$  assigned to the quaternary ammonium salt. The  $^1\text{H}$  NMR spectra of TMC showed dimethylated amine peak at 2.5 ppm and trimethylated amine (i.e., quaternized amine) peak at 3.7 ppm. These results were in accordance with the results of the previous reports (Sieval et al., 1998). The degree of quaternization of synthesized TMC was calculated using  $^1\text{H}$  NMR data and was found to be 46%.

### 3.2. Preparation and characterization of CS/TMC nanoparticles

The CS and TMC nanoparticles were prepared by gelation of polycationic molecules of CS/TMC with polyanionic TPP molecules. The TEM study revealed that both CS-NPs–LMWH and TMC-NPs–LMWH were uniform in morphological appearance with homogenous shading around them (Fig. 1). The polymer to TPP ratio was optimized on the basis of particle size, PDI and entrapment efficiency. Optimum ratio (polymer to TPP; 3:1) produced CS-NPs–LMWH and TMC-NPs–LMWH of size 148.8 and 183.4 nm, respectively with minimum PDI than other ratios (Table 1). The highest entrapment efficiency of LMWH was also achieved at



**Fig. 1.** Transmission electron microphotograph of LMWH loaded nanoparticles (A) CS-NPs (bar 1  $\mu\text{m}$ ); (B) TMC-NPs (bar 2  $\mu\text{m}$ ). Particles are uniformly distributed and are homogenously shaded by stain. Each time minimum three observations were made.

this ratio, i.e., 72.6 and 71.9% in the case of CS-NPs–LMWH and TMC-NPs–LMWH, respectively. Zeta potential describes stability of dispersion as well as interactions of nanoparticles with epithelium linings in the form of mucoadhesion. Optimized ratio of polymer to TPP (3:1) produced nanoparticles possessing a high positive zeta potential, i.e., 24.3 and 21.1 mV in the case of CS-NPs–LMWH and TMC-NPs–LMWH, respectively.

### 3.3. In vitro drug release

Fig. 2(A and B) represents cumulative release of LMWH at different time point in vitro using simulated GIT mediums from CS-NPs–LMWH and TMC-NPs–LMWH formulations respectively. In SGF, CS-NPs–LMWH formulation showed 7.2% and 26.8% release of LMWH in 2 h and 24 h, respectively. Whereas in SIF, CS-NPs–LMWH showed 9.1% and 10.1% release of LMWH in 6 h and 24 h, respectively. At the same time, in SGF, TMC-NPs–LMWH showed 4.4% and 16.4% release of LMWH within 2 h and 24 h, respectively. In SIF, 8.4% and 12.2% release of LMWH was shown by TMC-NPs–LMWH in 6 h and 24 h respectively.

### 3.4. Stability study

The developed nanoparticulate systems were tested for the stability parameters at different storage conditions in terms of change in particle size and entrapment efficiency (Table 2). The average particle size of both CS-NPs–LMWH and TMC-NPs–LMWH formulations were increased from 148.8 to 210.4 nm and 183.4 to 248.3 nm respectively after 60 days storage at room temperature. The entrapment efficiency of CS-NPs–LMWH and TMC-NPs–LMWH stored at room temperature was decreased from 72.6% to 63.4% and 71.9% to 64.2% respectively after 60 days. Both the CS-NPs–LMWH and

**Table 1**Optimization parameters of LMWH loaded CS/TMC nanoparticles. Data represents mean  $\pm$  S.D. ( $n=4$ ).

Formulation	Polymer:TPP ratio	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
CS-NPs	2:1	386.4 $\pm$ 12.1	0.242 $\pm$ 0.082	18.2 $\pm$ 3.4	50.1 $\pm$ 2.8
	3:1	148.8 $\pm$ 9.2	0.103 $\pm$ 0.009	24.3 $\pm$ 5.1	72.6 $\pm$ 3.5
	4:1	289.2 $\pm$ 8.5	0.313 $\pm$ 0.104	27.2 $\pm$ 4.0	54.7 $\pm$ 4.2
TMC-NPs	2:1	406.2 $\pm$ 14.4	0.294 $\pm$ 0.096	16.7 $\pm$ 4.8	48.4 $\pm$ 3.1
	3:1	183.4 $\pm$ 10.1	0.111 $\pm$ 0.017	21.1 $\pm$ 6.5	71.9 $\pm$ 4.8
	4:1	367.4 $\pm$ 10.9	0.352 $\pm$ 0.126	23.8 $\pm$ 5.3	56.1 $\pm$ 3.2

**Table 2**Effect of storage temperature on particle size and entrapment efficiency of LMWH loaded chitosan based nanoparticulate systems after 60 days. Data represents mean  $\pm$  S.D. ( $n=4$ ).

Formulation	Size (nm)			Entrapment efficiency (%)		
	0 day Room temp.	After 60 days 4 $\pm$ 1 °C	0 day 25 $\pm$ 1 °C	After 60 days Room temp.	4 $\pm$ 1 °C	25 $\pm$ 1 °C
CS-NPs	148.8 $\pm$ 9.2	154.2 $\pm$ 4.3	210.4 $\pm$ 6.1	72.6 $\pm$ 3.5	68.4 $\pm$ 3.2	63.4 $\pm$ 4.2
TMC-NPs	183.4 $\pm$ 10.1	196.4 $\pm$ 3.4	248.3 $\pm$ 8.2	71.9 $\pm$ 4.8	70.2 $\pm$ 2.8	64.2 $\pm$ 3.2

**Table 3**Effect of pH environments resembling simulated GIT conditions on particle size and entrapment efficiency of LMWH loaded CS-NPs and TMC-NPs. Data represents mean  $\pm$  S.D. ( $n=4$ ).

Formulations	Size (nm)			Entrapment efficiency (%)		
	0 h Initially	2 h SGF (pH 1.2)	6 h SIF (pH 7.4)	0 h Initially	2 h SGF (pH 1.2)	6 h SIF (pH 7.4)
CS-NPs	148.8 $\pm$ 9.2	326.2 $\pm$ 10.1	242.5 $\pm$ 8.6	72.6 $\pm$ 3.5	43.2 $\pm$ 4.1	52.3 $\pm$ 4.4
TMC-NPs	183.4 $\pm$ 10.1	247.4 $\pm$ 11.1	204.3 $\pm$ 7.5	71.9 $\pm$ 4.8	62.8 $\pm$ 2.9	67.8 $\pm$ 5.6

TMC-NPs–LMWH showed comparatively better stability at refrigerated conditions than room temperature in terms of increase in particle size and decrease in entrapment efficiency. The CS-NPs–LMWH and TMC-NPs–LMWH, when kept in SGF (pH 1.2) medium for 2 h, showed a sudden increase in the average particle size (i.e., from 148.8 to 326.2 nm and 183.4 to 247.4 nm, respectively) and decrease in the entrapment efficiency (i.e., from 72.6 to 43.2 and 71.9 to 62.8%, respectively) (Table 3). Whereas, when kept in SIF (pH 7.4) medium for 6 h, showed a comparatively less increase in the average particle size (i.e., from 148.8 to 242.5 nm and 183.4 to 204.3 nm in case of CS-NPs–LMWH and TMC-NPs–LMWH, respectively) and also less change in the entrapment efficiency (i.e., from 72.6% to 52.3% and 71.9% to 67.8%, respectively) in comparison to the SGF condition.

### 3.5. Particle uptake study

The CS/TMC nanoparticles both were remained attached to the epithelium linings after 1 h of oral administration of developed formulations as shown in Fig. 3. Fig. 3(A) demonstrates control group treated with 6-CF solution, where almost negligible fluorescence in the tissue was observed. The CS-NPs loaded with 6-CF were significantly associated the intestinal tissues as shown in Fig. 3(B). The TMC-NPs showed highest association of nanoparticles with the gut tissues Fig. 3C.

### 3.6. Plasma concentration profile and pharmacokinetics

The plasma concentration of LMWH followed by oral absorption was estimated using anti-FXa assay (Fig. 4). The plain LMWH solution showed maximum anti-FXa activity ( $C_{max}$ ) of 0.14 IU/ml after 30 min ( $t_{max}$ ) of oral administration with the  $AUC_{0-6h}$  value 27.5  $\mu$ g/ml/min (Table 4). In comparison to this, CS-NPs–LMWH formulation showed a shift in  $t_{max}$  value from 30 to 180 min with significantly enhanced  $C_{max}$  value, i.e.,  $0.20 \pm 0.03$  IU/ml and

$AUC_{0-6h}$  42.2  $\mu$ g/ml/min. However, among all, TMC-NPs–LMWH showed improved pharmacokinetic parameters, i.e.,  $C_{max}$ ,  $t_{max}$  and  $AUC_{0-6h}$  were 0.36 IU/ml, 180 min and 67.8  $\mu$ g/ml/min respectively. The bioactivity of LMWH followed by absorption in the form of blood clotting time was also estimated at different time points after oral administration of formulations (Fig. 5). The CS-NPs showed significant increase in the clotting time (22 s) in comparison to plain LMWH (14 s). However, TMC-NPs showed higher clotting time profile (27 s) than CS-NPs.

## 4. Discussion

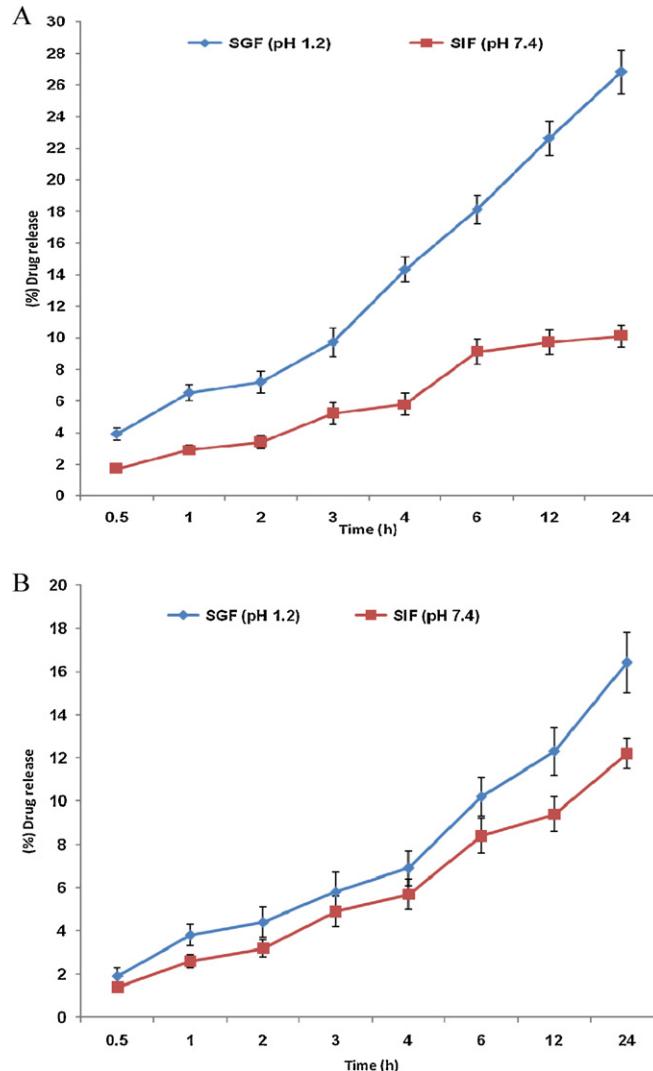
Oral delivery of biomacromolecules like LMWH is one of the most desirable tasks among formulation scientists. The designing of a suitable carrier system for the oral delivery of therapeutic molecules is controlled by the factors such as charge density, hydrophilicity, large size and interactions of molecules with GIT pH conditions and enzymatic flora (Goldberg and Gomez-Orellana, 2003; Paliwal et al., 2011a,b). In the present investigation, chitosan based carrier systems were developed and evaluated for the oral bioavailability enhancement of LMWH and hence to develop an effective formulation approach for the non-invasive, non-hospitalization based treatment of vascular disorders like DVT and PE. Chitosan and its derivative, i.e., TMC, possess cationic charge and thus favor charge based interactions resulting in to the increased mucoadhesiveness to the developed system.

TMC was synthesized using two-step synthesis protocol in which chitosan was methylated using methyl iodide. The synthesis of TMC was confirmed by characteristics peaks of N-CH<sub>3</sub> group at 1628 cm<sup>-1</sup> in IR spectrum. The degree of quaternization was estimated using <sup>1</sup>H NMR spectroscopy. The CS and TMC nanoparticles were prepared using ionic gelation technique of cationic polymer with anionic tri-polyphosphate counter ion. The polymer to TPP ratio was optimized on the basis of parameters such as particle size, PDI and entrapment efficiency and was found to be

**Table 4**

Pharmacokinetic parameters of LMWH loaded CS/TMC nanoparticles after oral administration in albino rats. Data represents mean  $\pm$  S.D. ( $n=6$ ).

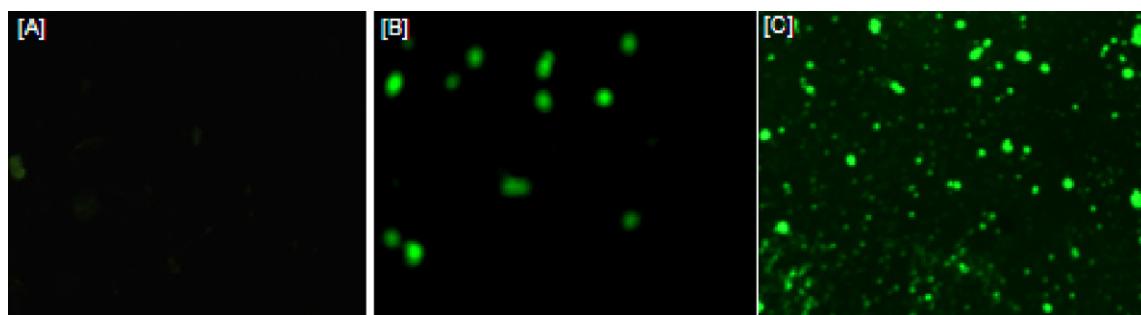
Formulation	Dose (mg/kg)	$C_{\max}$ (IU/ml)	$t_{\max}$ (min)	$AUC_{0-6\text{ h}}$ ( $\mu\text{g}/\text{ml}/\text{min}$ )	$F$ (%)
LMWH (i.v.)	1	0.64 $\pm$ 0.04	–	344.7 $\pm$ 16.2	–
LMWH (oral)	50	0.14 $\pm$ 0.02	30	27.5 $\pm$ 2.1	0.16
CS-NPs–LMWH (oral)	50	0.20 $\pm$ 0.03	180	42.2 $\pm$ 3.4	0.24
TMC-NPs–LMWH (oral)	50	0.36 $\pm$ 0.05	180	67.8 $\pm$ 4.2	0.39



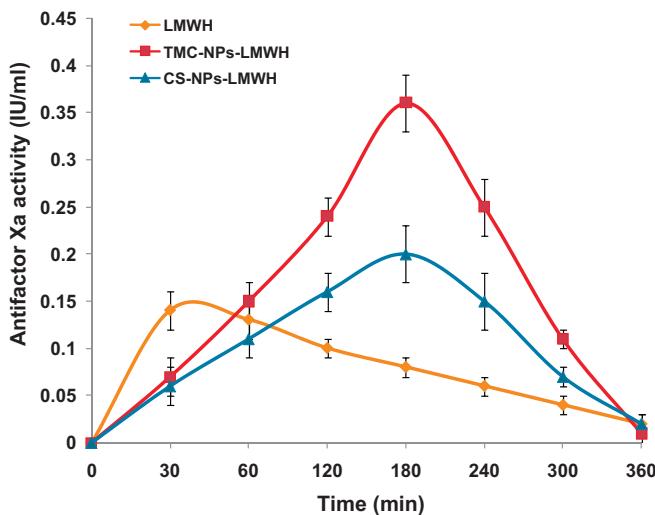
**Fig. 2.** In vitro release profile of LMWH loaded nanoparticulate systems in different simulated pH environments (A) CS-NPs; (B) TMC-NPs. Data represents mean  $\pm$  S.D. ( $n=3$ ).

optimum at 3:1 molar ratio in case of both the CS-NPs–LMWH and TMC-NPs–LMWH (Table 1). The TEM images confirmed about the morphological uniformity with almost homogenous shading of developed nanoparticles (Fig. 1). The optimized LMWH loaded NPs showed particle size range below 200 nm in both the cases with more than 72% entrapment efficiency confirming that at this molar ratio a tight cross linking between polymer and TPP is generated (Table 1). The optimized formulation possessed sufficiently higher positive zeta potential for long-term interactions of nanoparticles with epithelium linings and their colloidal stability as well. The drug release patterns of nanoparticles were determined in both the GIT simulated conditions, i.e., SGF (pH 1.2) and SIF (pH 7.4). The CS-NPs–LMWH showed higher release rate than TMC-NPs–LMWH in SGF condition (Fig. 2A). This may be attributed to higher solubility of CS at low pH than TMC. Whereas, in case of SIF, the drug release rate of TMC-NPs–LMWH was slightly higher than that of CS-NPs–LMWH (Fig. 2B). After 1 h of oral administration, a significant particle uptake was confirmed by fluorescent microscopic studies suggesting higher accumulation of 6-CF loaded TMC-NPs than CS-NPs (Fig. 3). These results demonstrated utility of the developed nanoparticles for oral delivery of bioactives.

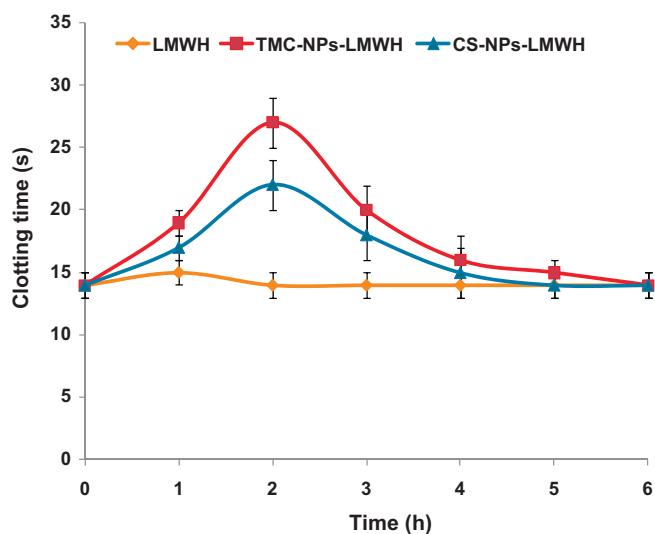
The long-term storage stability and effect of pH on the stability of CS-NPs–LMWH and TMC-NPs–LMWH were also determined. The particles were found to be more stable at refrigerated conditions than room temperature condition. The temperature induced particle aggregation may be the cause of increased particle size and decreased entrapment efficiency. Additionally, low pH environment was found to be comparatively unfavorable for both the formulations, i.e., CS-NPs–LMWH and TMC-NPs–LMWH when kept for about 2 h in the terms of increased particle size and decreased entrapment efficiency. However, even after 6 h in SIF a comparatively less change in both the parameters was observed. This suggested that even after 6 h of oral administration, average particle size range was less than 250 nm and entrapment efficiency above 50% in case of both the NPs. The in vivo evaluation of formulations for their bioavailability enhancing potential was carried out on albino rats. The plasma drug concentration of LMWH was estimated in the terms of anti-FXa activity. Both the chitosan based systems significantly enhanced the bioavailability of LMWH (Fig. 4). A significantly higher ( $p<0.05$ ) clotting time of blood than plain LMWH after oral administration of CS-NPs–LMWH and TMC-NPs–LMWH was observed (Fig. 5). The CS-NPs–LMWH



**Fig. 3.** Fluorescent photographs of microtome section of intestinal epithelium after 1 h of oral administration of LMWH loaded 6-CF labeled nanoparticles (A) control (B) CS-NPs; (C) TMC-NPs.



**Fig. 4.** Anti-FXa activity vs time profiles of different LMWH loaded CS-NPs and TMC-NPs formulations after oral administration in equivalent dose of 50 mg/Kg. Data represents mean  $\pm$  S.D ( $n=6$ ).



**Fig. 5.** Clotting time profile of LMWH loaded CS-NPs and TMC-NPs formulations after oral administration in equivalent dose of 50 mg/Kg. Data represents mean  $\pm$  S.D ( $n=6-8$ ).

and TMC-NPs-LMWH enhanced 1.5 and 2.4 times bioavailability in comparison to plain LMWH solution, respectively. However, TMC-NPs-LMWH showed better absorption of LMWH than CS-NPs-LMWH in term of increased anti-FXa activity as well as clotting time profile. This may be attributed due to better association of TMC-NPs-LMWH with gastrointestinal tract linings and hence increased permeation of LMWH via paracellular mechanism of drug absorption.

## 5. Conclusion

From the present study, it was concluded that chitosan based nanocarriers could be successfully used for developing oral heparin

formulation. The methyl derivative of chitosan, i.e., TMC successfully enhances the bioavailability of LMWH. A significant uptake of LMWH loaded nanoparticles by GIT proposed them suitable carrier for its oral administration. The higher stability of TMC-NPs-LMWH than CS-NPs-LMWH in the SGF and SIF mediums along with its capability to be solubilized at all the pH range along GIT make them more suitable biomaterial nanosconstructs than CS-NPs for oral heparin therapeutics. However, further evaluation of these nanocarriers in the treatment of DVT animal model will be assessed for their clinical potential in future studies.

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