Journal of Drug Delivery Science and Technology 58 (2020) 101807

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Synthesis and characterization of pre-activated thiolated chitosan nanoparticles for oral delivery of octreotide

Sundus Maria[a], Hafiz Shoaib Sarwar[a,b], Muhammad Farhan Sohail[a,b], Muhammad Imran[a], Omer Salman Qureshi[c], Abida Raza[d], Nasir Mahmood Ahmad[e], Ali Iqbal[a], Gul Shahnaz[a,*]

[a] Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan
[b] Riphah Institute of Pharmaceutical Sciences (RIPS), Riphah International University, Lahore Campus, Lahore, Pakistan
[c] Department of Pharmacy, Faculty of Natural Sciences, Forman Christian College, Lahore, Pakistan
[d] NILOP Nanomedicine Research Labs, National Institute of Lasers and Optronics College, PIEAS, Islamabad, Pakistan
[e] School of Chemical and Materials Engineering, National University of Science and Technology, Islamabad, Pakistan

ARTICLE INFO

Keywords:
Octreotide
Preactivated
Thiolated chitosan
Oral delivery
Improved bioavailability

ABSTRACT

The study was designed to develop a nano-based delivery system for oral delivery of octreotide (OT), a peptide drug. The preactivated thiolated chitosan (PTCS) nanoparticles loaded with OT were successfully developed and characterized in vitro and *in vivo*. Chitosan (CS) was modified to thiolated chitosan (TCS) and preactivated thiolated chitosan (PTCS) by attaching thioglycolic acid and mercaptonicotinic acid to CS. The OT was encapsulated during NPs production, *via* ionic gelation technique. The PTCS-NPs had a mean size of 277 ± 19 nm, a zeta potential of +28.2 ± 3 mV with 0.2 PDI and a high payload of OT (89 ± 6%). The mucoadhesive properties of PTCS-NPs were 3- and 16-fold increased as compared to corresponding TCS and CS-NPs respectively. The sustained release of OT from PTCS-NPs over 24 h was observed with non-Fickian release kinetics. The improvement in relative oral bioavailability showed more than 4-fold improvement in elimination half-life, a ~3.4-fold enhancement in C_{max} and 7.2-fold enhanced $AUC_{(0-t)}$ as compared to OT solution. Thus the results suggested the success of PTCS-NPs a potential oral delivery system for macromolecules due to the improved mucosal residence time, sustained release and facilitated transport *via* intestinal tight junctions.

1. Introduction

Since the approval of insulin therapy in 1982, the pharmaceutical research and development of peptide and protein drugs have considerably increased, especially over the last two or three decades [1,2]. The availability of these drugs in the market has considerably increased and mainly includes protein hormones and their synthetic derivatives administered through invasive routes. The major disadvantages associated with the i.v. route include patient non-compliance due to pain at the injection site, high cost of sterile manufacturing and systemic side effects [3]. The oral drug delivery is superior to other routes of administration because of the ease of administration and patient compliance. However, the oral route is not suitable for delivering hydrophilic macromolecules such as proteins and peptides [4]. However, the oral delivery of peptides faces many challenges like extremely low oral bioavailability of 1–2%, enzymatic destruction in the gastrointestinal tract such as by pepsin and peptidases, large molecular weight and presence of both hydrophobic and hydrophilic groups which limit their cellular entry and exhibit poor permeability across the various mucosal membranes [5].

A number of approaches have been reported for successful oral delivery of peptide macromolecules in order to enhance their absorption from the small intestine and minimize their degradation by the proteolytic enzymes [2]. The addition of permeation enhancers, enzyme inhibitors, mucoadhesion, chemical modification and attachment of polyethylene glycol (PEG) enhances the protection against extreme conditions thus, improving the bioavailability of a peptide as reported with a 10% increased oral bioavailability of insulin through the pH-responsive gel [6,7]. A variety of formulation approaches are adopted in formulating oral peptide delivery systems as nano and microemulsions, liposomes, microspheres, and nanoparticles. The main advantages of nanoparticles (NPs) include versatility in materials, tunable properties, controlled release, and targeted drug delivery to enhance bioavailability, reducing the dosing frequency and associated side effects [8].

Octreotide (OT), the first somatostatin agonist introduced for

* Corresponding author.
E-mail address: gshahnaz@qau.edu.pk (G. Shahnaz).

https://doi.org/10.1016/j.jddst.2020.101807
Received 1 February 2020; Received in revised form 9 May 2020; Accepted 10 May 2020
Available online 16 May 2020
1773-2247/

clinical use, is an octapeptide synthetic analogue of somatostatin, the growth hormone inhibiting factor (GHIH). It inhibits the release of growth hormone, glucagon, thyroid-stimulating hormone, prolactin and numerous secondary hormones including, secretin, gastrin, motilin, cholecystokinin, gastric inhibitory polypeptide, vasoactive intestinal peptide, glucagon, and insulin [9]. OT is rapidly absorbed after subcutaneous application. Its half-life after subcutaneous administration is 2 h with C_{max} reaching in 30 min. Following IV injection, OT is eliminated from the body in two phases with half-life of 1 h and 40 min. OT is available as subcutaneous or i.v. injection under the brand name SANDOSTATIN®. As they have to be frequently administered owing to the short half-life (less than 2 h), they are not ideal for long-term administration in chronic diseases [10]. A lot of efforts have been put into reducing the frequency of dosing and the associated inconvenience by developing SANDOSTATIN® LAR® (long-acting release) comprising of polymeric microcapsules [11]. This formulation is only given once a month through intramuscular route and it is more suitable for the acromegalic patients that require daily dosing of OT. However, this formulation undergoes a lag time period of 14 days before it reaches the desired plasma levels and that requires daily administration of the formulation to cover this time lag [12].

Thiolated polymers are well known for their excellent mucoadhesion, permeation enhancing and *P-gp* inhibition properties that constitute a vast platform for the drug delivery applications especially for macromolecular drugs that encounter various biological barriers [13]. The thiol groups (-SH) present onto the backbone of thiolated polymers are responsible to impart the discrete properties. However, the conventional thiolated polymers exhibit premature oxidation of -SH groups leading to the formation of -S-S bonds thus, resulting in the decreased efficiency of these polymers. To overcome this drawback, pre-activated thiomers were introduced that protect the -SH groups from the premature oxidation and to maximize the effect of discrete properties of thiomers [14,15].

As described earlier, one of the challenges faced by the macromoleuclar drugs is the lack of their oral bioavailability due to high molecular weight, lack of intestinal permeability, enzymatic degradation and harsh pH variation within GIT. Therefore, macromolecular drugs inevitably constitute the invasive drug delivery system that requires high production cost and lack patient compliance. To maximize the therapeutic outcome and to minimize the cost associated with therapy, it is necessary to develop such a drug delivery system that can effectively deliver this macromolecular drug into the systemic circulation *via* the oral route. Therefore, the overall aim of this project is to enhance the oral bioavailability of OT by using preactivated TCS based NPs that can enhance the permeation of OT across the intestinal barrier.

2. Material and methods

2.1. Material

Chitosan (medium molecular weight with a degree of deacetylation 75-85%, viscosity 200-800 cps), sodium phosphate dibasic, thioglycolic acid (TGA), hydroxylamine, 1-Ethyle-3-(3-dimethylaminopropyl)carbodiimide) (EDAC), 3,3′-Dithiodipropionic acid, N-hydroxysuccinimide (NHS), sodium tripolyphosphate (TPP), sodium cyanoborohydride and sodium borohydride were purchased from Sigma-Aldrich, USA. N-hydroxysuccimide (NHS) from Scharlau, Germany and Ellman's reagent from Alfa Aesar, USA. OT acetate was obtained from Novartis Pharmaceuticals. All other solvents and chemicals used were of analytical-grade.

2.2. Methods

2.2.1. Synthesis of thiolated chitosan (TCS)

TCS was successfully synthesized following the already reported method of our group [13]. Briefly, at first CS solution (1%, w/v) was prepared in a 1% acetic acid solution. To this, 50 mM EDAC was added followed by the addition of 500 mg of TGA while the pH was maintained at 5.0 using 1 M NaOH under continuous stirring for 4 h at room temperature. The reaction mixture was then dialyzed using cellulose membrane (Molecular weight (MW) Cut-off 12000) to get rid of unbound TGA. The dialysis was carried out once against 5 mM HCl, then two times using the exact same medium with added 1% NaCl and lastly twice against 1 mM HCl to adjust the final pH to 5.0. Afterward, the dialyzed solution was lyophilized and stored at 4 °C till further use.

2.2.2. Synthesis of preactivated thiolated chitosan

Preactivated TCS (PTCS) was prepared following the reported method by our group [15]. 2 g of mercaptonicotinic acid (MNA) was added in 50 mL of water followed by the addition of hydrogen peroxide (26 mM). The solution was agitated continuously for 1 h at pH 9.0. After the dimer was synthesized, it was dried by lyophilization and stored until further use. Then, 1 g of TCS was added in 100 mL water and a solution of a dimeric ligand in a concentration of 2.7 mM was added dropwise. The reaction mixture was allowed to stir for 2 h followed by the dialysis, as reported earlier.

2.2.3. Degree of conjugation

The degree of conjugation that refers to a number of thiol groups on the polymer backbone was calculated spectrophotometrically by Ellman reaction reported earlier by our group [14]. 0.5 mg of TCS was dissolved in 250 μL of distilled water and 250 μL of 0.5 M phosphate buffer (pH 8) and 500 μL of Ellman's reagent were added. The sample was incubated for 2 h at 37 °C while protected from light. The sample was analyzed spectrophotometrically using a microplate reader (PerkinElmer, USA) at 450 nm [16]. The number of thiol groups of PTCS was also calculated following the same method. The amount of conjugated MNA ligand was calculated by adding 1 mg of reduced glutathione (GSH) to the solution of 1 mg/mL of PTCS. And analyzed spectrophotometrically.

2.2.4. Fabrication of nanoparticles

The nanoformulations based on CS, TCS, and PTCS were prepared by ionic gelation method, as reported earlier with slight modifications to produce CS-NPs, TCS-NPs and PTCS-NPs respectively [14]. The polymeric solutions according to the concentrations as specified in Table 1 were prepared in 1% acetic acid solution separately for CS TCS and PTCS while TPP solution was prepared in the deionized water. To the 5 ml of polymeric solutions specified amount of OT was added with continuous stirring followed by dropwise addition of TPP solution until a bluish opalescent solution was obtained. The nanoparticulate suspension was then centrifuged at 13500 rpm for 20 min at 4 °C to obtain pellet of NPs. The pellet was redistributed in 3% mannitol solution, lyophilized and stored at 4 °C until further use.

10 different trials (Table 1) were prepared for each polymer (CS, TCS, and PTCS) to produce CS-NPs, TCS-NPs and PTCS-NPs (10 formulations each). One optimized formulation of each of the polymer was

Table 1
Concentrations of polymer solution, TPP solutions and drug for the development of nanoformulations CS-NPs, TCS-NPs and PTCS-NPs.

Formulations	polymer conc (%)	TPP conc (%)	Drug (μg)
1	0.1	0.1	100
2	0.15	0.1	100
3	0.2	0.2	200
4	0.25	0.2	200
5	0.3	0.3	300
6	0.35	0.3	300
7	0.4	0.4	400
8	0.5	0.5	500
9	0.2	0.1	200
10	0.3	0.1	300

chosen based upon particle size, zeta potential, polydispersity index (PDI) and entrapment efficacy for use in further experiments.

2.2.5. Particle size, polydispersity index, zeta potential, and morphology

The particle size, zeta potential and PDI (polydispersity index) of NPs were determined using zeta nanosizer ZSP (Malvern, UK). The lyophilized samples were re-distributed and diluted 10-fold with double deionized water prior to analysis. The surface morphology was analyzed through scanning electron microscopy (SEM) MIRA3 (TESCAN, Czech Republic) in order to confirm the size, shape, and morphology of the NPs. The NPs were attached *via* carbon tape to the grid followed by gold coating through gold sputter [17].

2.2.6. Entrapment efficiency

The entrapment efficiency (EE) of OT was determined by first separating the nanoparticles from the aqueous medium by centrifugation at 13500 rpm for 20 min at 4 °C using Eppendorf 5424R (Eppendorf, Germany) [18]. The amount of unbound OT in the supernatant was measured through RP-HPLC by following the method reported by wang et al.,. A gradient elution method was utilized with mobile phase A (0.1% v/v, TFA in water) and mobile phase B (0.1% v/v, TFA in acetonitrile). The gradient was 80:20 (A:B) to 65:35 (A:B) over 20 min with a flow rate of 1.0 mL/min. UV absorbance was measured at 220 nm. Octreotide solutions of known concentrations (0.01–0.15 mg/mL) in the same solvent system were used to generate calibration curve. The % entrapment efficiency was determined by the following equation.

$$EE(\%) = \frac{\text{Amount of drug added} - \text{Amount of drug in supernatant}}{\text{Amount of drug added}} \times 100$$

2.2.7. Evaluation of swelling behavior

The swelling behavior of CS-NPs, TCS-NPs and PTCS-NPs was investigated by using the gravimetric method [19]. For this purpose, the prepared nanoformulations (lyophilized) were compressed into a small tablet of 5 mm. The tablets were weighed and attached to the needle of a syringe and placed in a beaker containing phosphate buffer (pH 6.8) at 37 °C. At regular time intervals, the tablets were removed from the buffer medium and accurately weighed.

The amount of buffer absorbed was calculated as a function of time by using the equation:

$$\text{Swelling index}\,(\%) = \frac{Wf - Wo}{Wo} \times 100$$

Here Wo is the initial weight of the disc in dry state and W_f presents the wet weight of the swollen disc at time t.

2.2.8. In vitro drug release

In vitro release from all NPs and OT suspension was determined by the dialysis tube method at pH 2.0 for 2 h and at pH 6.8 for 48 h [20]. The known quantity of OT loaded nanoparticles was re-dispersed in 5 mL of buffer (pH 2 and pH 6.8 separately) and the dialysis membrane (Molecular weight (MW) Cut-off 12000) was immersed in a beaker containing dissolution medium maintained at 37 ± 0.5 °C. At specified time points, small volume media was withdrawn and replaced with an equal volume of freshly prepared media to maintain the sink conditions. The samples were analyzed spectrophotometrically at 280 nm for the amount of drug. The different kinetic models were applied to determine the mechanism of release from prepared nanoparticles [21].

2.2.9. Ex vivo permeation study

Ex vivo permeation study was carried out by using the everted sac method [13]. This study was performed on the intestine of rabbit obtained from local slaughter house, after carefully washing the intestine with Kreb's ringer solution. The intestine was then cut into small segments of 4–5 cm. One end of each sac was tied and filled with OT solution, and different NPs with a syringe needle and the other end was tied using suture. The filled intestinal segments were then immersed in the beakers containing 10 mL of the Kreb's ringer solution at 37 °C. At definite time intervals, samples were withdrawn and replaced with an equal volume of Kreb's solution. The collected samples were then collected and analyzed for quantification using HPLC method as described earlier. Apparent permeability (Papp) was calculated using the following formula:

$$\text{Apparent Permeability}\,(\mu g/cm^2) = \frac{\text{Concentration of OT} \times \text{Volume}}{\text{Mucosal Surface Area}}$$

The mucosal surface area was calculated by assuming intestine a cylinder and using the formula:

$$\text{Mucosal Surface Area}\,(cm^2) = \text{circumference}\,(\pi r^2) \times \text{length}$$

2.2.10. Ex vivo mucoadhesion study

Ex vivo mucoadhesion study was performed following the already reported method using a modified rotating cylinder [22]. The intestinal mucosa of rabbit was cut into small segments of 4–5 cm, washed thoroughly with saline and were mounted on glass slides using a cyanoacrylate adhesive. The tablets compressed of CS-NPs, TCS-NPs, PTCS-NPs were attached to these mucosal segments and the slides were carefully placed vertically in the beakers (250 mL capacity) containing phosphate buffer (pH 6.8). The beakers were placed in the water bath shaker and run for 3 h. The time of adhesion of the tablets was noted for 3 h.

2.2.11. Relative oral bioavailability studies

The animal investigations were conducted following the protocol approved by the Bioethical Committee of Quaid-i-Azam University Islamabad, Pakistan (Protocol No. DFBS/216-266/BEC-FBS-QAU-21). Healthy rabbits weighing 1500 ± 200 g were selected and kept in an animal house with free access to food and water a day prior to the experiment. The rabbits were divided into two groups (n = 5) and treated with PTCS-NPs and OT solution at the dose of 1 mg/kg in 100 μL of phosphate buffer. pH 7.4, using oral gavage needle. The blood samples were withdrawn from ear marginal vein of each rabbit at a predefined time interval (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h) using sterile syringe each time. The samples were transferred to 1.5 mL Eppendorf tube containing 100 μL anticoagulant (11% sodium citrate). The samples were centrifuged at 4000 rpm for 15 min to separate plasma. The plasma was stored at −20 °C until further used for analysis. The drug was extracted from plasma samples and was analyzed using the HPLC method developed and used for the measurement of encapsulation efficiency [23].

2.2.12. Stability studies

The stability of the formulations was analyzed for change in particle size and encapsulation efficiency over a period of 3 months while keeping them at varying stress conditions of −20, 4 and 37 °C [13]. The NPs were stored in lyophilized form and re-distributed into deionized water prior to evaluation.

2.2.13. Statistical analysis

For the measurement of significance of differences in the analysis of the results of variance (ANOVA) was performed followed by Tukey's post-hoc test and students' t-test were carried out where applicable. The results are expressed as mean ± SD of three independent experiments.

3. Results

3.1. Synthesis of TCS and preactivated TCS

Synthesis of TCS as described earlier was achieved by covalent attachment of the -NH_2 group of chitosan with the sulfhydryl group of TGA *via* amide bond formation as shown in Fig. 1. The covalent

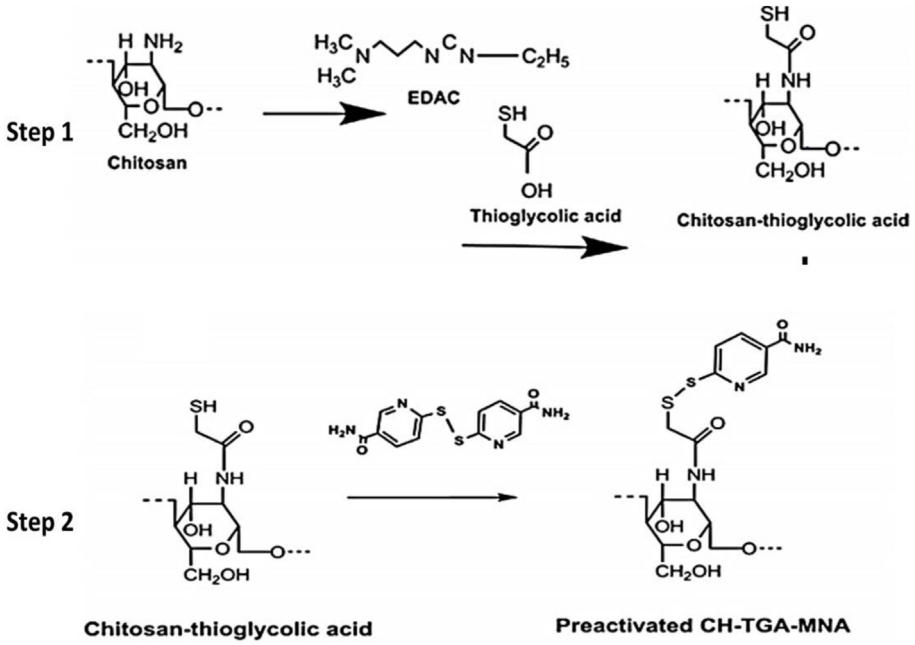

Fig. 1. Step 1: Synthetic pathway of the preparation of thiolated chitosan (TCS) and step 2 the modification of TCS to preactivated thiolated chitosan (PTCS).

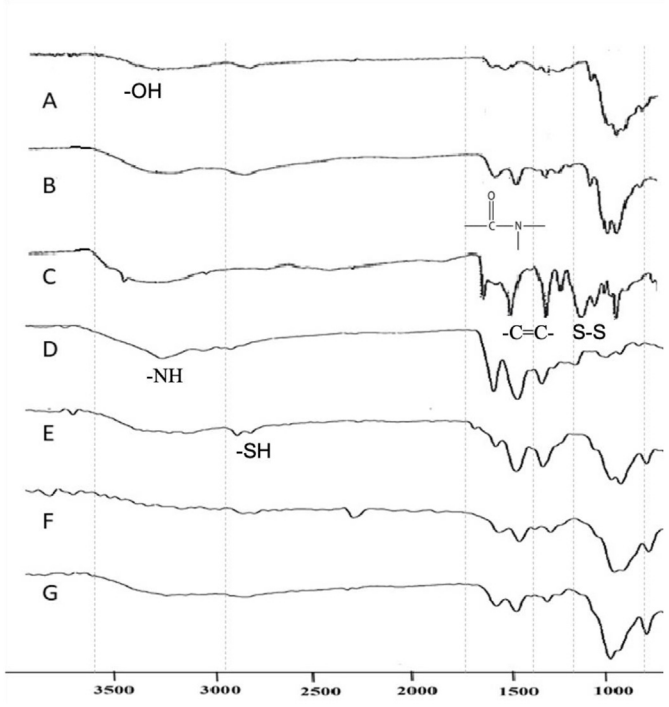

Fig. 2. FTIR spectra of (A) CS, (B) TCS, (C) PTCS, (D) OT, (E) CS-NP, (F) TCS-NPs and (G) PTCS-NPs.

conjugation of TGA *via* amide bond formation was confirmed by the FTIR analysis and is presented in Fig. 2. The peak at 1645 cm^{-1} (C=O, stretching amide) and deformation of the signal at 3225 cm^{-1} (-NH stretching amide) confirm the amide bond formation. The peak at 2496 cm^{-1} due to -SH stretching indicates the presence of thiol groups. The peaks at 808 cm^{-1} and 1245 cm^{-1} (S–S disulfide bond) in modified chitosan confirm the existence of mercaptans. The aromatic ring -C=C- is found at 1550 cm^{-1} and the thiol functional group of 6-MNA is found at 2515 cm^{-1}. The number of thiol groups immobilized on the polymer backbone was 334 ± 24 μmol per gram of TCS while the number of disulfide bonds formed was calculated to be 152 ± 15 μmol per gram of the TCS. These results confirmed the successful thiolation of CS. After freeze-drying, a white, cotton candy-like odorless TCS was obtained with improved water solubility. A similar synthesis shown in the second step of Fig. 1 was carried out for the preparation of PTCS by disulfide bond formation between the thiol groups of TCS and the aromatic ligand of the dimer (6,6′ DTNA). Hydrogen peroxide was added to 6-MNA resulting in the formation of the dimer, 6,6′-dithionicotinamide. The dimer was an off-white, gritty, odorless and water-soluble powder. Ellman's test revealed 40.05 ± 6 μmol per gram of the polymer which suggests successful conjugation.

3.2. Fabrication and characterization of nanoparticles

The nanoparticles were fabricated successfully *via* the ionic gelation method. Out of 10 nanoformulations of each of CS-NPs, TCS-NPs and PTCS-NPs, the formulations with suitable particle size, zeta potential, PDI and entrapment efficacy were selected for further studies. The results of various features of selected nanoformulations are shown in Table 2. The size of the CS-NPs, TCS-NPs and PTCS-NPs was found to be 180 nm, 431 nm and 277 nm with PDI of 0.2, 0.196 and 0.2 respectively. The PDI of all formulations was found to be less than 0.5 which indicates a homogenous dispersion. All formulations showed a positive zeta potential that can be attributed to the unreacted -NH_2 groups of chitosan. The % entrapment efficiency (EE) was in the range of 85%–91%. The SEM analysis was performed to confirm the shape and size of the nanoformulations as shown in Fig. 3. The particle size of CS-NPs, TCS-NPs and PTCS-NPs were found to be 180 ± 11, 431 ± 17 and 277 ± 19 nm respectively. The particle size increased due to attachment of thiol groups to the polymeric backbone.

Table 2
Physicochemical characterization including particle size, % entrapment efficiency, polydispersity index and zeta potential of optimized octreotide loaded unmodified, thiolated and preactivated thiolated nanoparticles.

Formulations	Particle size (nm)	Encapsulation Efficiency %	PDI	Zeta Potential (mv)
CS-NPs	180 ± 11	85 ± 5.2	0.200 ± 0.03	+25.0 ± 3.6
TCS-NPs	431 ± 17	91 ± 6.4	0.196 ± 0.01	+33.3 ± 4.2
PTCS-NPs	277 ± 19	89 ± 6.0	0.200 ± 0.02	+28.2 ± 3.5

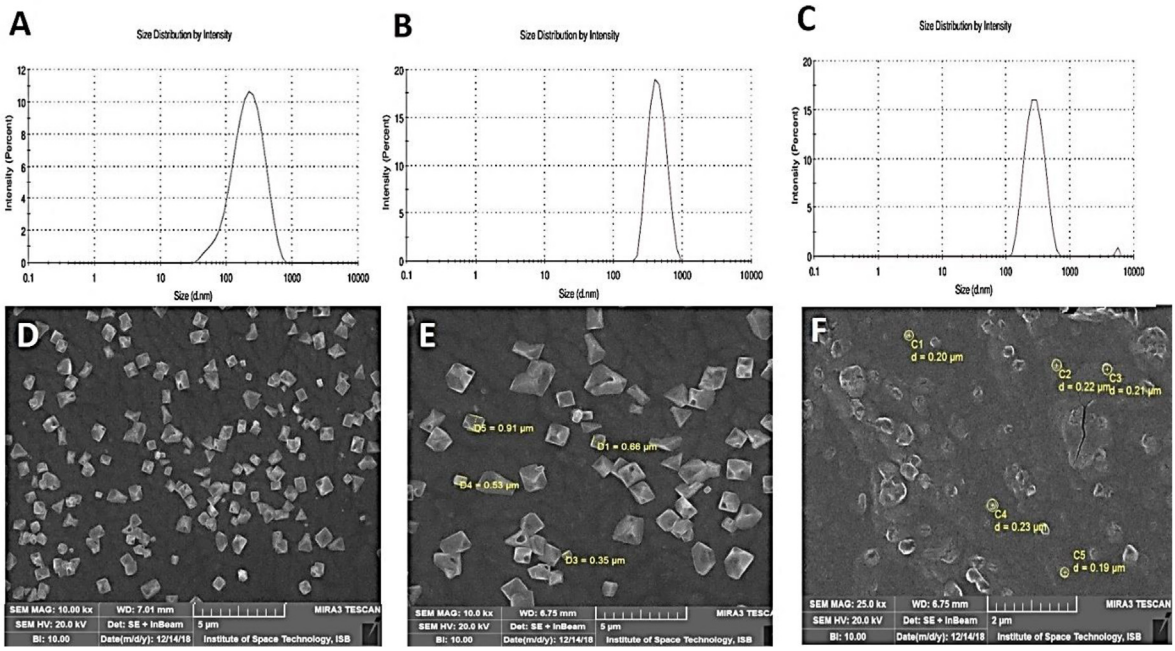

Fig. 3. Particle size analysis using zeta sizer (a) CS-NPs nanoparticles, (b) TCS-NPs, (c) PTCS-NPs. Scanning electron microscopy (SEM) of (d) CS-NPs, (e) TCS-NPs and (f) PTCS-NPs.

3.3. Ex vivo mucoadhesion study

Ex vivo mucoadhesion study was performed for 3 h to evaluate the increase in the mucoadhesiveness of PTCS-NPs, TCS-NPs as compared to that of CS-NPs. The compressed tablets of the respective formulations were allowed to be attached with the intestinal mucosa and the time during which they remained attached was calculated and is presented in Fig. 4 a. PTCS-NPs based tablets were able to remain attached to the intestinal mucosae for 148 ± 9.53 min as compared to 56 ± 5.3 min for TCS-NPs and 9.5 ± 2.1 for CS-NPs. These results indicated that pre-activation resulted in significantly ($p < 0.05$) improved mucoadhesion accounting up to 15.5-fold for PTCS-NPs and 5.8-fold for TC-NPs compared to CS-NPs. Even the mucoadhesion was significantly ($p < 0.005$) improved for PTCS-NPs when compared to TCS-NPs indicating a 2.6-fold higher mucoadhesion. These results support the concept pre-activation to protect the -SH groups ultimately leading to the improved mucoadhesion.

3.4. Swelling behavior

The study was carried out for 2 h to demonstrate the influence of thiolation and preactivation on the swelling properties of the polymer (Fig. 4b). The buffer uptake behavior showed a rapid swelling and disintegration of CS-NPs tablets and eroded within 180 min by

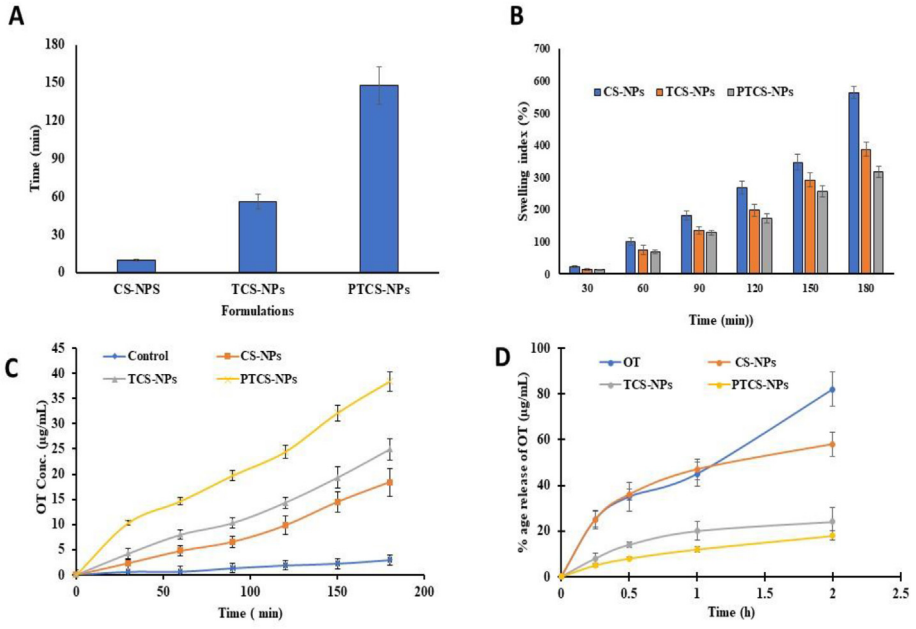

Fig. 4. Characterization of polymeric nanoparticles: (A) mucoadhesion studies (B) swelling studies and (C) permeation enhancement and (D) drug release studies at pH 20 of CS-NPs, TCS-NPs and PTCS-NPs. The results are shown as mean ± S.D. of three experiments.

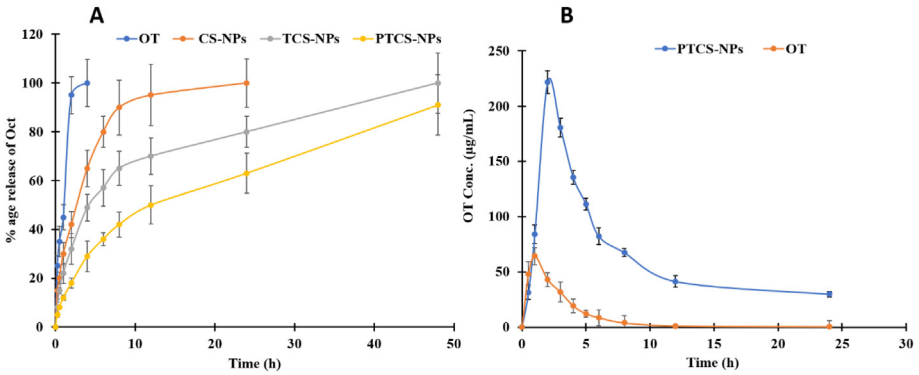

Fig. 5. *In vitro* release studies of octreotide from OT, CS-NPs, TCS-NPs and PTCS-NPs nanoparticles showing sustained release from PTCS-NPs over 48 h, (B) oral bioavailability studies of OT from PTCS-NPs and OT solution showing plasma level of OT following oral administration to rabbits at the dose of 1 mg of OT/Kg. The results are shown as mean ± S.D. of three results.

absorbing the maximum amount of water as reflected by the swelling index. Whereas the disc made of TCS-NPs and PTCS-NPs displayed a controlled swelling over a period of 180 min and absorbed less water as compared to the CS-NPs. with no sign of erosion. The decreased water uptake by TCS-NPs and PTCS-NPs might be due to the presence of thiol groups that developed into the disulfide bonds resulting in disulfide crosslinked matrix, thus, providing the controlled swelling behavior as compared to CS-NPs.led. The water-absorbing capacity of PTCS-NPs was further decreased compared to TCS-NPs due to the addition of lipophilic aromatic ligand, displaying a constant and slow uptake of water.

3.5. In vitro drug release studies

OT release from suspension and nanoparticle formulations was evaluated for 2 h at gastric medium and results are shown in Fig. 4D. The data indicated a fast release of octreotide in case of CS-NPs showing up to 50% of OT release while the TCS-NPs and PTCS-NPs exhibited much slower drug release, less than 20% of total OT. The drug release studies for 48 h at pH 6.8 using dialysis method maintaining the sink conditions are presented in Fig. 5a. The data indicated that PTCS-NPs showed a significantly ($p < 0.05$) slower release in comparison to TCS-NPs and CS-NPs. Within 6 h, more than 80% of the drug was released out of CS-NPs in comparison to 70% for the TCS-NPs and 50% for the PTCS-NPs. Various release kinetic models were applied to the OT release data from in vitro studies and are shown in Table 3. The data indicated the OT release from CS-NPs followed the first-order release as evident from the R^2 Values. Whereas, TCS-NPs and PTCS-NPs followed the Higuchi model with Fickian drug release as the value of $n < 0.45$. While CS-NPs followed non-Fickian drug release ($n > 0.45$).

3.6. Ex vivo permeation study

Ex vivo permeation study was conducted to evaluate the permeation of the OT solution, CS-NPs, TCS and PTCS-NPs and improvement in the permeation is presented in the form of Papp in Table 4. The amount of drug permeated with respect to time is shown in Fig. 4. The results indicated significant ($p < 0.05$) improvement in the permeation of OT in the case of PTCS-NPs across the intestinal mucosa compared to the OT solution. PTCS-NPs, TCS-NPs and CS-NPs indicated a 4.1-fold, 2.1-fold and 1.9-folds enhanced permeation compared to the OT after 3 h.

Table 4
Permeation studies of OT, OT loaded unmodified chitosan nanoparticles, thiolated chitosan and pre-activated thiolated chitosan nanoparticles.

Formulations	Apparent Permeability Coefficient Papp × 10^6 (cm/s)	Enhancement ratio
OT	10.25	
CS-NPs	19.47	1.9
TCS-NPs	25.65	2.5
PTCS-NPs	42.05	4.1

3.7. Relative oral bioavailability studies

The relative oral bioavailability studies were carried out in rabbits and the plasma OT level-time curve is shown Fig. 5b. The significant (0.05) differences were observed in the pharmacokinetic parameters presented in Table 5. PTCS-NPs exhibited C_{max} of 221.65 µg/mL with T_{max} of 2 h as compared to native OT solution that exhibited a C_{max} of 64.30 µg/mL with T_{max} of 1 h. The elimination half-life of PTCS-NPs was found to be 14.91 h against 3.72 h for OT indicating 4-fold improvement. The $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf,}$ and $AUMC_{0\text{-}inf}$ in the case of PTCS-NPs were found to be 1562.83, 2124.50 and 36794.5 µg/mL respectively, While, for the OT the values for the respective parameters were found to be 215.65, 218.77 and 847.31.

3.8. Stability studies

The TCS-NPs and PTCS-NPs formulations were found to be comparatively more stable than the CS-NPs formulation, however, they did face aggregation of particles during long term storage. Table 6 represents the 3 months' stability data of OT loaded NPs, kept under different storage temperatures i.e. −20, 4 and 25 °C. After 3 months, the PTCS-NPs were found to be stable in terms of particle size, PDI and encapsulation efficiency. There were no significant changes except for a slight increase in particle size. However, after 2 months statistically significant increase ($p \leq 0.05$) in the average size and PDI was observed for CSNPs.

Table 3
Octreotide kinetic models to determine the mechanism of drug release from nanoparticles at pH 6.8.

	Zero order		First order		Korsmeyer-Peppas		Higuchi	
Formulation	R^2	Ko	R^2	K_1	R_2	*N	R_2	KH
OT	0.678	30.90	0.953	0.877	0.936	0.502	0.936	53.701
CS-NPs	0.198	6.095	0.987	0.286	0.940	0.360	0.874	26.157
TCS-NPs	0.152	2.771	0.935	0.137	0.966	0.345	0.876	17.310
PTCS-NPs	0.651	2.292	0.946	0.060	0.993	0.437	0.992	13.453

Table 5
Results of *in vivo* relative oral bioavailability and important pharmacokinetic parameters obtained after oral administration of PTCS-NPs to rabbit through oral gavage.

Parameter	Unit	OT	PTCS
$t_{1/2}$	h	3.72	14.91
T_{max}	h	1	2
C_{max}	μg/mL	64.30	221.65
AUC $_{0\text{-}t}$	μg/mL*h	215.65	1562.83
AUC $_{0\text{-}inf}$	μg/mL*h	218.77	2124.50
AUMC $_{0\text{-}inf}$	μg/mL*h^2	847.31	36794.35
MRT $_{0\text{-}inf}$	h	3.87	17.32

4. Discussion

Upon oral administration, the gastrointestinal epithelial layer acts as a physical barrier due to its lipophilic nature resulting in bioavailability as low as 1–2%. The biochemical barrier comprises enzymatic degradation by peptidases [24]. OT, a peptide drug that has multiple clinical indications, is the synthetic analogue of the naturally occurring growth hormone somatostatin. Somatostatin is rapidly degraded in plasma, OT has a half-life of 2 h. The commercially available Sandostatin ® is given *via* the IV or SC route and because of the short half-life, it requires multiple administrations a day [25].

The use of NPs is becoming common to diminish all the mentioned problems. The NPs based on thiomers is a new method that prolongs the residence time at the gastro-intestinal (GI) mucosal layer and ensuring optimal bioavailability [26]. These thiomer NPs provide strong mucoadhesion by forming S–S bonding to the cysteine parts in GI mucosal layer. Mucus layer with a thickness of 15–600 μm, is a complex aqueous gel composed of glycoproteins that provide barrier properties to the underlying membranes [27,28]. However, the thiolated polymers have free thiol groups that are comparatively unstable in solutions and gels as they are subjected to thiol oxidation at pH $>$ 5 unless sealed under inert conditions. This premature oxidation of the thiol groups before even reaching the mucosal layer can affect the interaction between thiomer and mucus, thereby, reducing the efficacy of thiomers. In order to resolve this issue, the concept of pre-activation was introduced. It was shown that coupling of 2-mercaptonicotinic acid through disulfide bond formation can prevent thiol oxidation, which leads to even further improved mucoadhesion. Pre-activation improves the stability of thiol groups towards oxidation as well as enhances the mucoadhesive and cohesive properties of thiomers over a broader pH range [29]. A wide range of polymers is available to synthesize mucoadhesive drug delivery systems. Among these, Chitosan has been studied extensively as a drug delivery mucoadhesive polymer due to its properties such as high biocompatibility, low toxicity, and biodegradability. Chitosan is, therefore, an ideal drug delivery system for hydrophilic drugs. To improve the mucoadhesive and permeation properties along with prevention from inter and intrachain disulfide linkage of the TCS, pre-activated TCS was synthesized by coupling the 2-mercaptonicotinic acid dimer that provides free thiol groups *via* disulfide exchange mechanism [30].

The results from FTIR analysis Fig. 2 confirmed the development of amide bind formation that indicates the successful conjugation of the carboxyl group of the MNA with amino group of CS. Furthermore, the appearance of a peak at 2450 cm^{-1} indicated the thiol groups. The presence of thiol groups was further quantified by the Ellman's reagent. The number of thiol groups was less than disulfide bonds indicates the successful pre-activation of thiol groups while in the case of conventional TCS the free thiol groups are greater than the disulfide bonds.

OT loaded nanoformulations based on CS, TCS and PTCS were produced by ionic gelation 'method to produce CS-NPs, TCS-NPs and PTCS-NPs respectively. Ionic gelation method was selected due to mild conditions of the process that is pre-requisite for the incorporation of the macromolecular drugs like OT to avoid denaturation. Thus, harsh parameters like organic solvents, high temperature, sonication, and extreme pH were avoided. The nanoparticles were optimized for entrapment efficiency, particle size, and polydispersity index, Entrapment efficiency varied from 50 to 90% with varying concentrations of polymer, TPP, and drug. It increased with the increase in the concentration of TPP and polymer. The reason for this was due to the formation of the intact matrix of nanospheres by higher concentrations of polymer and cross-linker that entrap more drugs by dispersion in the matrix. Also, OT is a hydrophilic drug that is entrapped in a matrix formed by CS. Due to the hydrophilic nature of the polymer and cross-linker, more drug diffuses and attaches to the polymeric network. Thus, higher concentrations of polymer and cross-linker entrap more drugs and hinder its backward diffusion from the matrix surface. The particle size significantly increased with an increase in polymer concentration because of the increase in consistency at higher concentrations [31]. The, concentration of TPP also had an impact, low concentrations of 0.1% and 0.2% yielded small-sized nanoparticles and vice versa. The values of PDI were in the range of 0.1–0.4. All the formulations were noted to have positive zeta potential due to free amino groups of chitosan. The zeta potential is very critical in controlling the stability of nanosuspensions that is why a zeta potential within a suitable range is required. The zeta potential of all the nanoformulations was above +20 mV indicating sufficient electro repulsive forces between similar charges leading to the stable nanosuspension.

The ultimate purpose of designing the drug delivery systems is to tailor the drug release profile according to the designed objectives. Therefore, it is of extreme importance to validate the release of drugs form the developed systems to ensure that the loaded drug will be available at the target site. The *invitro* release profile from OT suspension, CS-NPs, TCS-NPs, PTCS-NPs, and NPs was examined by the dialysis membrane method for 2 h at gastric pH followed by 24 h at intestinal pH as shown in Figs. 4d and 5a respectively. The properties of dialysis membrane effects the drug release kinetics especially the MW

Table 6
3 months stability data of Octreotide loaded, unmodified chitosan nanoparticles, thiolated chitosan and preactivated thiolated chitosan nanoparticles based on changes in particle size, PDI and encapsulation efficiency performed at different storage conditions i.e., −20, 4 and 37 °C.

Formulation	Temp °C	Particle size (nm)			Polydispersity Index (PDI)			Encapsulation Efficiency (%)		
		1 month	2 month	3 month	1 month	2 month	3 month	1 month	2 month	3 month
CS-NPs	−20 °C	270.5 ± 0.3*	288.8 ± 0.6*	345.4 ± 0.6*	0.56 ± 0.05*	0.57 ± 0.07*	0.59 ± 0.08*	74.49 ± 3.8	69.87 ± 6.5	60.43 ± 5.8
TCS-NPs		281.5 ± 0.3*	293.8 ± 0.6*	300.4 ± 0.9*	0.12 ± 0.05*	0.25 ± 0.07*	0.38 ± 0.08*	71.49 ± 3.8	69.87 ± 6.5	66.43 ± 4.6
PTCS-NPs		200.5 ± 0.2*	250.6 ± 0.4*	290.8 ± 0.4*	0.28 ± 0.04*	0.31 ± 0.02*	0.32 ± 0.08*	95.47 ± 6.7	89.45 ± 8.4	67.39 ± 7.3
CS-NPs	4 °C	317.5 ± 0.3*	345.8 ± 0.6*	460.4 ± 0.8*	0.51 ± 0.05*	0.53 ± 0.07*	0.55 ± 0.08*	75.49 ± 3.8	70.87 ± 6.5	65.73 ± 6.6
TCS-NPs		284.5 ± 0.3*	296.8 ± 0.6*	320.4 ± 0.4*	0.31 ± 0.05*	0.34 ± 0.07*	0.36 ± 0.08*	88.49 ± 3.8	87.87 ± 6.5	82.73 ± 6.6
PTCS-NPs		250.7 ± 0.3*	266.2 ± 0.4*	296.6 ± 0.6*	0.33 ± 0.04*	0.39 ± 0.07*	0.40 ± 0.07*	92.47 ± 6.7	90.76 ± 5.9	89.94 ± 8.2
CS-NPs	25 °C	297.5 ± 0.3*	347.8 ± 0.6*	469.4 ± 0.3*	0.42 ± 0.09*	0.46 ± 0.04*	0.48 ± 0.08*	79.93 ± 3.8	75.74 ± 8.4	69.67 ± 6.7
TCS-NPs		350.7 ± 0.6*	365.9 ± 0.5*	412.8 ± 0.4	0.32 ± 0.04*	0.35 ± 0.03*	0.39 ± 0.03*	86.47 ± 6.7	82.54 ± 5.3	76.52 ± 6.8
PTCS-NPs		253.7 ± 0.6*	312.9 ± 0.5*	396.8 ± 0.4	0.29 ± 0.04*	0.38 ± 0.03*	0.39 ± 0.03*	90.47 ± 6.7	85.54 ± 5.3	80.52 ± 5.2

cut-off value cannot be overlooked. The basic premise of the utilizing dialysis membrane is that the drug that is released from the nanoformulations will diffuse rapidly from the one compartment, through membrane, to the other where it is pooled and analyzed. Thus, the membranes with sufficiently high molecular weight cut-off as compared to the released drug is often selected to allow the free and rapid diffusion of the drug. Furthermore, the solubility of the drug also effects the transport across the membrane. Generally, the drug with high solubility diffuse rapidly whereas poorly water-soluble drugs experience a lag-time in drug release followed by exponential increase due to limited dissolution inside the membrane. The drug, OT, being reported, possess high solubility and MW of 1079 g/mol while the dialysis membrane was having MW cut-off 14 KD, so that it should not be a limiting factor in drug transport. At gastric pH the CS-NPs indicated a faster drug release as compared to TCS-NPs and PTCS-NPs. the CS-NPs indicated a cumulative drug release of 50% compared to less than 20% of both TCS-NPs and PTCS-NPs. In case of drug release behavior at intestinal pH the nanoformulations exhibited prolonged release behavior. The drug release from NPs was in order of CS-NPs > TCS-NPs > PTCS-NPs. The CSNPs displayed high cumulative drug release due to lack of modification with thiol moieties which attributed to the faster diffusion of drug from the nanoparticles. Upon modification with thiol groups, the release rate was slower and prolonged due to the formation of disulfide linkages within the polymer matrix thus providing a more crosslinked matrix with limited swelling and creation of pores for the release of drug. Within 6 h, more than 80% of the drug was released out of CS-NPs in comparison to 70% for the TCS-NPs and 50% for the PTCS-NPs. Thus, the prepared NPs indicated suitable drug release properties that can be utilized to deliver OT. The evaluation of drug release kinetic modeling indicated that PTCS-NPs, TCS-NPs and CS-NPs followed Fickian drug release while OT followed non-Fickian drug release as value of $n > 0.45$.

Swelling, mucoadhesion and permeation enhancement are the discrete properties of thiomers with high pharmaceutical value especially in case of delivering the macromolecular drugs. Swelling and mucoadhesion are well correlated with each other. Swelling of the polymer chains in necessary for the mucoadhesion as the thiol groups will be able to adhere to the mucus in the aqueous medium and ionization of the cationic polymer that shows interaction with the negatively charged mucus. However, the swelling must be within a specific range as too much swelling will result in the bursting of the proper shape of the nanocarriers and loss of integrity. In this regard the TCS-NPs and PTCS-NPs showed controlled swelling due to development of disulfide bonds between the polymeric matrix. Mucoadhesion is one of the approaches adopted to overcome the physiological barriers of oral drug delivery that is the presence of mucus provides an additional protective layer against the enterocyte and hinders the absorption of macromolecular drugs. The mucoadhesive nanocarriers remain adhere to the mucus for a longer period of time thus easily penetrate the mucus to be taken up by the enterocytes or to be permeated. The thiol moieties attached to the polymer are critical for the improvement of the permeation and mucoadhesion effect (Vetter A et al., 2010). Mucoadhesion has three stages: wetting, swelling and entanglement of polymer with mucus layer. When attached to the mucus layer the polymer takes water from the surrounding layer, swell, gets adsorbed and strengthens the mucoadhesion [13]. Excess of water absorption also causes over the swelling and as a result detaches the polymer from the mucus layer. Thus, for strong mucoadhesion, moderate water absorption property is necessary [32]. The mucoadhesive property of PTCS-NPs was 3-and 16-fold increased as compared to corresponding TCS-NPs and CS-NPs. The presence of thiol groups on TCS-NPs results in the formation of disulfide bond with the cysteine groups of the mucin. These disulfide bonds help in strengthening the mucoadhesive force between nanoparticles and mucous surface. There was, a gradual decrease in mucoadhesive property over 3 h. The, decrease was however, significant for the CS-NPs as compared to TCS-NPs and PTCS-NPs.

The presence of tight junction in the intestinal cells limits the oral absorption of high molecular weight drugs including the macromolecular drug OT. Therefore, development of specific functional group modified polymer-based nanocarriers that can interact with the tight junction is a suitable strategy to enhance the permeation across the intestine. One of the remarkable properties of the thiomers is to enhance the permeation across the intestinal cells that make them ideal candidates for the oral delivery of macromolecules. The results of the permeation enhancing study indicated that PTCS-NPs exhibited an increased permeation by 1.9-, 2.5- and 4.1-folds compared to TCS-NPs, CS-NPs and OT solutions respectively. The significantly enhanced permeation across the intestinal mucosa in case of thiolated NPs is due to the ability of thiomers to interact with protein tyrosin phosphatase (PTP). It was reported that inhibition of PTP is due to the ability of thiomers to develop disulfide bonds (S–S) between the surface thiol groups of thiolated polymer and cysteine-rich subunits of PTP [15]. Consequently, a higher degree of tyrosine phosphorylation of membrane proteins leads to the opening of tight junctions resulting in increased permeation of NPs across the intestinal cells. The results of the permeation study showed enhanced permeation for the PTCS-NPs nanoparticles that might be due to the paracellular transport across the intestinal cells. Over 50% of the drug permeated the intestinal mucosa. The results confirmed enhanced permeation and mucoadhesion for PTCS-NPs.

The purpose of utilizing the TCS, especially of PTCS is to achieve the mucoadhesion, enhanced permeation and controlled release of OT as it has a very short elimination half-life and eliminated very quickly. The incorporation of the OT into the NPs enables them to achieve a longer half-life due to enhanced permeation and retention effect. The oral bioavailability studies were carried out to validate the concept of enhanced oral bioavailability of OT in case of PTCS-NPs compared to that free OT solution. The results indicated a significantly enhanced bioavailability of OT with favorable pharmacokinetic parameters. PTCS-NPs had more than 4-fold improvement in elimination half-life, and a ~3.4-fold enhancement in maximum plasma concentration compared to OT solution. The relative oral bioavailability of OT loaded PTCS-NPs calculated on the basis of $AUC_{(0\text{-}t)}$ and found to be 7.2-fold enhanced as compared to OT solution. The enhanced bioavailability of OT in case of PTCS-NPs is likely due to improved residence time, controlled swelling, sustained release and paracellular transport across the intestinal cells as thiolated polymer are well known for opening the tight junctions of the intestinal cells thus contributing to the enhanced permeation via paracellular transport. All these properties are being mediated by the -SH groups of thiolated polymers with the added advantage of pre-activation that protected the -SH groups from premature oxidation.

The results of stability showed better stability of PTCS-NPs as compared to other NPs except slight changes in size due to aggregates formation in suspension form. The temperature did not significantly affect the stability of the NPs. Thus, based on the above results and above discussion it is obvious that PTCS-NPs based nanocarriers are suitable drug delivery approaches to achieve the oral delivery of macromolecular drugs including OT.

5. Conclusion

The results of the study support the utilization of preactivated thiolated chitosan for the improvement in the oral bioavailability of OT, where preactivation of -SH groups of the thiolated polymers provides increased mucoadhesion and permeation enhancement compared to the thiolated chitosan. The thiolated nanoformulations exhibited high drug loading efficiency than that of non-thiolated nanoformulation (CS) while mucoadhesion and permeation enhancement of PTCS-NPs was even higher as compared to that of TCS due to the preactivation of -SH groups. PTCS-NPs provided 7.2-fold increased oral bioavailability as compared to the OT thus, validated the concept of the increased bioavailability of OT by utilizing preactivated thiolated chitosan. Based on

the findings, OT loaded PTCS-NPs seems to be a promising platform for the oral delivery of macromolecular drugs.

CRediT authorship contribution statement

Sundus Maria: Data curation, Formal analysis, Writing - original draft. **Hafiz Shoaib Sarwar:** Formal analysis, Writing - original draft. **Muhammad Farhan Sohail:** Conceptualization, Supervision, Writing - review & editing, Writing - original draft. **Muhammad Imran:** Project administration, Software, Formal analysis. **Omer Salman Qureshi:** Formal analysis. **Abida Raza:** Formal analysis. **Nasir Mahmood Ahmad:** Funding acquisition, Project administration, Supervision. **Ali Iqbal:** Formal analysis, Validation. **Gul Shahnaz:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration Conflict of interests

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2020.101807.

References

[1] D.J. McClements, Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and microparticle systems: a review, Adv. Colloid Interface Sci. 253 (2018) 1–22.
[2] C.Y. Wong, H. Al-Salami, C.R. Dass, Microparticles, microcapsules and microspheres: a review of recent developments and prospects for oral delivery of insulin, Int. J. Pharm. 537 (1–2) (2018) 223–244.
[3] A.C. Anselmo, Y. Gokarn, S. Mitragotri, Non-invasive delivery strategies for biologics, Nat. Rev. Drug Discov. 18 (2018) 19–40.
[4] R.M. Pinto, et al., Oral administration of lipid-based delivery systems to combat infectious diseases, Nanoparticles in Life Sciences and Biomedicine, Jenny Stanford Publishing, 2018, pp. 75–110.
[5] P. Tyagi, S. Pechenov, J.A. Subramony, Oral peptide delivery: translational challenges due to physiological effects, J. Contr. Release 287 (2018) 167–176.
[6] S. Maher, et al., Application of permeation enhancers in oral delivery of macromolecules: an update, Pharmaceutics 11 (1) (2019) 41.
[7] S. Hassan, et al., Evolution and clinical translation of drug delivery nanomaterials, Nano Today 15 (2017) 91–106.
[8] N. Erdoğar, S. Akkın, E. Bilensoy, Nanocapsules for drug delivery: an updated review of the last decade, Recent Pat. Drug Deliv. Formulation 12 (4) (2018) 252–266.
[9] H.-Y. Zhang, et al., Tumor targeted delivery of octreotide-periplogenin conjugate: synthesis, in vitro and in vivo evaluation, Int. J. Pharm. 502 (1–2) (2016) 98–106.
[10] P. Marbach, et al., From somatostatin to Sandostatin®: pharmacodynamics and pharmacokinetics, Digestion 54 (Suppl. 1) (1993) 9–13.
[11] N. Ayati, et al., Sandostatin LAR therapy differentially alters 68Ga-DOTATATE uptake in normal tissues compared to primary tumors and metastatic lesions, J. Nucl. Med. 117 (2017) 192203.
[12] J. Varshosaz, et al., PEGylated trimethylchitosan emulsomes conjugated to octreotide for targeted delivery of sorafenib to hepatocellular carcinoma cells of HepG2, J. Liposome Res. (2019) 1–16.
[13] M.F. Sohail, et al., Folate grafted thiolated chitosan enveloped nanoliposomes with enhanced oral bioavailability and anticancer activity of docetaxel, J. Mater. Chem. B 4 (37) (2016) 6240–6248.
[14] M. Sajjad, et al., Folate-functionalized thiomeric nanoparticles for enhanced docetaxel cytotoxicity and improved oral bioavailability, AAPS PharmSciTech 20 (2) (2019) 81.
[15] J. Iqbal, et al., Preactivated thiomers as mucoadhesive polymers for drug delivery, Biomaterials 33 (5) (2012) 1528–1535.
[16] H.S. Sarwar, et al., Mannosylated thiolated polyethylenimine nanoparticles for the enhanced efficacy of antimonial drug against Leishmaniasis, Nanomedicine 13 (1) (2018) 25–41.
[17] M.F. Sohail, et al., Polymeric nanocapsules embedded with ultra-small silver nanoclusters for synergistic pharmacology and improved oral delivery of Docetaxel, Sci. Rep. 8 (1) (2018) 13304.
[18] A.H. Ghassemi, et al., Controlled release of octreotide and assessment of peptide acylation from poly (D, L-lactide-co-hydroxymethyl glycolide) compared to PLGA microspheres, Pharmaceut. Res. 29 (1) (2012) 110–120.
[19] G. Shahnaz, et al., Synthesis, characterization, mucoadhesion and biocompatibility of thiolated carboxymethyl dextran–cysteine conjugate, J. Contr. Release 144 (1) (2010) 32–38.
[20] F. Novelli, et al., Self-assembly and drug release study of linear l, d-oligopeptide-poly (ethylene glycol) conjugates, New biotechnology 37 (2017) 99–107.
[21] M.F. Sohail, et al., Development and in vitro evaluation of flurbiprofen microcapsules prepared by modified solvent evaporation technique, Trop. J. Pharmaceut. Res. 13 (7) (2014) 1031–1038.
[22] F. Laffleur, et al., Evaluation of functional characteristics of preactivated thiolated chitosan as potential therapeutic agent for dry mouth syndrome, Acta Biomater. 21 (2015) 123–131.
[23] A.M. Sindi, In situ gel loaded with curcumin-based fluvastatin nanostructured carrier effective against squamous tongue carcinoma, Int. J. Res. Pharm. Sci. 10 (3) (2019) 2225–2233.
[24] A. Muheem, et al., A review on the strategies for oral delivery of proteins and peptides and their clinical perspectives, Saudi Pharmaceut. J. 24 (4) (2016) 413–428.
[25] D.J. Brayden, M.-J. Alonso, Oral delivery of peptides: opportunities and issues for translation, Adv. Drug Deliv. Rev. 106 (2016) 193–195.
[26] A.A. Date, J. Hanes, L.M. Ensign, Nanoparticles for oral delivery: design, evaluation and state-of-the-art, J. Contr. Release 240 (2016) 504–526.
[27] N. Ijssennagger, R. van der Meer, S.W. van Mil, Sulfide as a mucus barrier-breaker in inflammatory bowel disease? Trends Mol. Med. 22 (3) (2016) 190–199.
[28] Z. Ahmad, et al., Fabrication and characterization of thiolated chitosan microneedle patch for transdermal delivery of tacrolimus, AAPS PharmSciTech 21 (2) (2020) 1–12.
[29] T.A. Ahmed, B.M. Aljaeid, Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery, Drug Des. Dev. Ther. 10 (2016) 483.
[30] S. Ahmed, S. Ikram, Chitosan & its derivatives: a review in recent innovations, Int. J. Pharmaceut. Sci. Res. 6 (1) (2015) 14.
[31] H. Ghafar, et al., Development and characterization of bioadhesive film embedded with lignocaine and calcium fluoride nanoparticles, AAPS PharmSciTech 21 (2) (2020) 1–12.
[32] D.A. López, Characterization of gelatin/chitosan scaffold blended with aloe vera and snail mucus for biomedical purpose, Int. J. Biol. Macromol. 92 (2016) 645–653.