CHAPTER 3

RESULTS & DISCUSSION
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3.1. Synthesis, characterization, in vitro and in vivo evaluation of CS-PTH NPs, PEG-CS-PTH NPs and TCS-PTH NPs

3.1.1 Introduction

Osteoporosis is characterized by decreased density, fragility and porosity of bones resulting in frequent fractures. \textsuperscript{257} PTH 1-34 is approved by FDA for the treatment of osteoporosis \textsuperscript{236} since it induces anabolic responses in bone-forming cells and stimulates bone matrix production and suppresses osteoblast apoptosis in an intermittent pattern of administration. \textsuperscript{237,258} The peptide reaches peak serum concentrations about 30 min after subcutaneous injection of a 20 µg dose and declines to non-quantifiable concentrations within 3 h. The peptide is currently administered as a daily subcutaneous injection which results in a therapeutic intermittent pattern of serum concentration of PTH 1-34.\textsuperscript{66,75} In order to improve patient compliance, an alternate route of administration have to be made feasible over the daily painful injections. Oral route of administration can improve patient compliance and challenge for such an accomplishment is the ability of the peptide to survive the physiological conditions of the gastrointestinal tract and deliver therapeutic picogram levels of peptide in the systemic circulation. PTH 1-34 is entirely degraded by trypsin, chymotrypsin and pepsin within 5 minutes and rat small intestinal mucosa degrades approximately half of the peptide in 3 h.\textsuperscript{260} This precise characterization of the enzymatic barrier for oral PTH 1-34 administration justifies the need for development of PTH 1-34 delivery systems. The use of nanosystems as delivery agents con-
fers advantages such as simple and cost effective synthesis procedures, use of minimal peptide concentrations for encapsulation, protection of the peptide in the GI tract, efficient absorption of the nanoformulation across the microvillus, and systemic release of PTH 1-34. CS, a biopolymer finds immense applications in biomedical fields because of its biocompatibility and non toxicity. Certain biocompatible polyanionic substances such as sulfate, citrate, and tripolyphosphate can act as cross linkers in the preparation of biodegradable nano/microparticles of CS via ionic gelation. CS NPs exhibits strong electrostatic interaction with peptides and proteins, protecting them from enzymatic degradation and permitting its paracellular uptake making it suitable for oral delivery of PTH 1-34. CS-PTH NPs synthesized by ionic gelation may promote higher dissolution in the gastric pH and cause hemolysis during the in vivo experiments due to the positive charge of the exposed amino groups. Foreseeing such difficulties and to overcome this drawback basic CS-PTH NP system was modified in two ways, in one system the surface of the synthesized CS-PTH NPs was coated with PEG 200 and in the second CS was replaced with mucoadhesive thiolated CS (TCS) that resulted in a more hemocompatible and stable PEG-CS-PTH NPs and TCS-PTH NPs respectively. PEGylation of NP is reported to increase stability in gastric and biological fluids which can prolong its circulation and retard elimination. PEGylation can also facilitate the transport of bioactive macromolecules across the intestinal epithelium and improve drug bioavailability in vivo. The improved mucoadhesive properties of TCS is by the formation of covalent bonds between thiol groups of the polymer and cysteine rich sub domains of glycoprotein in the mucus layer. The longer residence time of TCS-PTH NPs at the absorption site contributes to increased absorption rate of the incorporated drug which supports non-invasive drug delivery. Based on all these properties, nanoformulations of PEGyated and thiolated CS will be efficient oral delivery vehicles for the osteoporosis therapeutic peptide PTH 1-34.
3.1.2. Research questions and Hypothesis

1. **Question:** What is the efficiency of entrapment of PTH 1-34 in Chitosan when TPP is added as a cross linker?
   **Hypothesis:** TPP interacts electrostatically with the Chitosan and PTH1-34 to initiate the nanoparticle formation in solution.

2. **Question:** What is the role of PEGylation on the *in vivo* hemocompatibility of the NPs?
   **Hypothesis:** Surface charge of the CS NPs will be reduced after coating PEG 200 that can enhance *in vivo* hemocompatibility.

3. **Question:** What is the role of Thiolation on the *in vivo* hemocompatibility of the NPs?
   **Hypothesis:** Thiolation reduces the free amine groups in the CS polymeric chain and reduces the surface charge of the CS NPs and confers *in vivo* hemocompatibility.

4. **Question:** What is the effect of nanoparticle entrapping on the bioactivity of PTH 1-34?
   **Hypothesis:** The NP systems release PTH 1-34 without affecting its bioactivity *in vitro* and *in vivo*.

5. **Question:** What will be the bioavailability of orally administered PTH 1-34 entrapped into CS, PEG-CS and TCS NPs when compared to the bare PTH 1-34?
   **Hypothesis:** Oral bioavailability of the entrapped PTH 1-34 will be larger than the bare PTH 1-34 because of the nature of the polymer used and will reach above 100pg/mL.

6. **Question:** What will be the influence of PEGylation and Thiolation on the *in vivo* release profile of PTH 1-34 when compared to CS-PTH 1-34 NPs?
   **Hypothesis:** CS-PTH 1-34 NPs with a higher positive charge and smaller size have a faster clearance hence shorten the *in vivo* release of PTH 1-34 whereas, modified PEGylated and Thiolated Chitosan NPs are in circulation for longer time and prolong the *in vivo* release of PTH 1-34.
3.1.3. Results and Discussion

3.1.3.1 Preparation and characterisation of CS NPs, CS-PTH NPs

The particle size data of CS NPs and CS-PTH NPs by DLS showed (Fig. 3.1.1(A)) a size range of 30-50 nm (average 40 nm) and 60-80 nm (average 70 nm) respectively. SEM (Figs. 3.1.1 (B), (C)) images of CS NPs and CS-PTH NPs show that the particle size is below 1 µm. AFM image (Fig. 3.1.2) shows that CS-PTH NPs have a size less than 500 nm without agglomeration. The AFM and SEM images clearly outline majority of CS-PTH NPs with spherical morphology and smooth surface. Stability of CS NPs and CS-PTH NPs were studied by zeta potential measurements and was around +60 and +40 mV respectively indicating the good stability of the NPs. Positive surface charge is due to the unreacted amine group of CS. A reduction in the charge after PTH 1-34 loading is observed due to the interaction of peptide and TPP with the amine group of CS. The cross-linking of CS would be dependent on the availability of the cationic sites and the negatively charged species. The mechanism of cross-linking of CS with TPP could be either by deprotonation or ionic interaction. Once the –NH₃⁺ group of the CS polymeric chains is sufficiently cross-linked by TPP and the reaction is kinetically no more favourable we observe the formation of a nanosuspension. The PTH 1-34 is a thirty-four amino acid peptide bearing both acidic and basic amino acids (pI-8.69). At the NP synthesis pH condition (which varies from of 5 to 3) these amino acids interact with –NH₃⁺ sites of CS as well as the δ⁻O=C developed due to the positive inductive effect of the –CH₃ groups of the 15% acetylated portions at carbon number 2 of the CS monomer. TPP dissolved in water generates (P₃O₁₀)⁵⁻ and OH⁻. When it is added as a cross linker more of the PTH 1-34 gets trapped into the CS NPs due to the ionic interaction. Hence PTH 1-34 loading is made feasible not only due to its interaction with CS but the cross linker as well.
Figure 3.1.1 (A) DLS showing the size distribution of (a) CS NPs and (b) CS-PTH NPs (B) SEM image of CS NPs  (C) SEM image of CS-PTH NP
Figure 3.1.2. (A) Atomic Force Microscopic image of CS-PTH NPs with majority of particles exhibiting spherical morphology (B) FT-IR spectra of CS (A), CS-NPs (B) PTH 1-34 (C) and CS-PTH NPs (D).
FT-IR spectrum of CS (Fig 3.1.2 A) peaks at 1646 cm\(^{-1}\) corresponding to amine stretching frequency and at 980 cm\(^{-1}\) depicting the saccharine in the polymer. Spectrum of CS NPs (Fig 3.1.2 B) shows a shift in peak from 1646 to 1639 cm\(^{-1}\) that indicates the interaction of the amine groups of CS with the \((P_3O_{10})^{5–}\) and \(OH^–\) of TPP. PTH 1-34 (Fig 3.1.2 C) has generated peaks at 1635 and 1480 cm\(^{-1}\) representing the amine stretching and C–N stretching vibration modes. The PTH 1-34 loading on CS NPs (Fig 3.1.2 D) was confirmed by a sharp peak at 980 and 3450 cm\(^{-1}\), which is indicative of the presence of the excess –NH\(_2\) and –COOH groups contributed by the amino acids from PTH 1-34. Moreover, the existence of 980 cm\(^{-1}\) peak in the observed FT-IR spectrum can be considered as a peak for the CS content in the peptide loaded nanoformulation. The 2857 and 2924 cm\(^{-1}\) peak is intense and it represents abundant –CH\(_2\) residues of the loaded PTH 1-34 moieties. The stretching frequencies for major functionalities have been shifted to lower frequency regions, as the TPP has interacted effectively with the protonated amine or amide residues in the nanoformulation. The stretching frequency is directly proportional to the wave number. Thus as the bond length is perturbed by the cross linker, the wave number decreases to lower frequency region.\(^{269,270}\)

3.1.3.2 Preparation and Characterization of PEG-CS-PTH NPs

SEM image of PEG-CS-PTH 1-34 NPs (200-250nm) is shown in figure 3.1.3 A. TEM image proves that such an aggregate (Fig. 3.1.3 B) is in turn formed of individual NP of approximately 20 nm in diameter (Fig. 3.1.3 C and D). Zeta potential measurement of +35 indicates the stability and surface charge of the 200-250nm sized PEG-CS-PTH NPs.

Figure 3.1.4 shows the FT-IR spectra of PEG, CS-PTH NPs and PEG-CS-PTH NPs. The PTH 1-34 loading on CS NPs was confirmed by a sharp peak at 980 and 3450 cm\(^{-1}\), which is indicative of the presence of the excess –NH\(_2\) and –COOH groups contributed by the amino acids from PTH 1-34. Moreover, the existence of 980 cm\(^{-1}\) peak in the observed FT-IR spectrum can be considered as a peak for the CS content in the peptide
loaded nanoformulation. The 2857 and 2924 cm\(^{-1}\) peak is intense and it represents abundant \(-\text{CH}_2\) residues of the loaded PTH 1-34 moieties. The stretching frequencies for major functionalities have been shifted to lower frequency regions, as the TPP has interacted effectively with the protonated amine or amide residues in the nanoformulation. The stretching frequency is directly proportional to the wave number. PEG coats the positively charged NP by electrostatic interaction. The \(-\text{NH}_3^+\) bending at 1570 cm\(^{-1}\) observed in the CS-PTH NPs spectra has narrowed which proves the interaction of \(-\text{NH}_3^+\) with the -\text{OH} groups of PEG. 2850-2960 cm\(^{-1}\) in the spectra of PEG marks the presence of alkanes in PEG, a similar less intense peak is observed at the same position in PEG-CS-PTH NPs but not in CS-PTH NPs spectra. This shows the contribution of alkanes by PEG in the PEG-CS-PTH NPs.\(^{271,272}\)

**Table 3: Zeta potential values of the synthesized nanoparticles**

<table>
<thead>
<tr>
<th>NANOPARTICLE</th>
<th>ZETA POTENTIAL</th>
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<tbody>
<tr>
<td>CS NPs</td>
<td>+60 mV</td>
</tr>
<tr>
<td>CS-PTH NPs</td>
<td>+40 mV</td>
</tr>
<tr>
<td>PEG-CS-PTH 1-34 NPs</td>
<td>+35 mV</td>
</tr>
<tr>
<td>TCS-PTH 1-34 NPs</td>
<td>+38 mV</td>
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</table>
Figure 3.1.3. (A) SEM of PEG-CS-PTH NPs showing particle size of 200-250 nm (B) TEM image of a 200-250nm PEG-CS-PTH NPs aggregate (C) and (D) TEM image showing that the cluster of PEG-CS-PTH 1-34 NPs are in turn formed of individual particles of approximately 20nm.
3.1.3.3 Preparation and Characterization of TCS-PTH 1-34 NPs

The DLS data of TCS-PTH 1-34 NPs (Fig. 3.1.5 (A)) shows a size range of 95 ± 10 nm and figure 3.1.5 (B) shows the AFM image depicting the surface morphology of the TCS-PTH 1-34 NPs. SEM (Fig. 3.1.5 (C)) image confirms the size, spherical morphology and smooth surface of the NPs. Zeta potential measurement was +38. These characters along with the presence of thiol group will ensure the muco adhesiveness and longer residence time of the peptide loaded nanoformulation in the gastrointestinal tract.
so that the PTH 1-34 reaches the blood stream effectively. The size of the NP and the presence of TCS would favour easy intestinal uptake by opening of the paracellular channel.

The acidic and basic amino acids of PTH 1-34, interact with –NH⁺₃ of the TCS. When TPP is added as a cross-linker more of the PTH 1-34 gets trapped into the TCS polymeric chain due to the ionic interaction. Once the –NH₃⁺ of the TCS polymeric chains are sufficiently cross-linked by TPP and the reaction is kinetically non feasible we observe the formation of a TCS-PTH 1-34 nanosuspension.¹⁴⁷

![Figure 3.1.5.](image)

**Figure 3.1.5.** (A) DLS of TCS-PTH 1-34 NPs, (B) AFM images of TCS-PTH 1-34 NPs showing spherical NPs. (C) SEM of TCS-PTH 1-34 NPs showing particle size of 90-100nm
FT-IR spectrum of PTH 1-34, TCS NPs and TCS-PTH 1-34 NPs is shown in figure 3.1.6. In the spectrum of TCS-PTH 1-34 NPs, the characteristic absorption peaks are at about 3410 cm\(^{-1}\) (\(-\text{O-H}\) 2924 cm\(^{-1}\) (\(-\text{C-H}\), 1623, 1088 cm\(^{-1}\) (\(-\text{C-N}\)), 895 cm\(^{-1}\) due to pyranosal ring could be easily observed. 1524 (amide II band), 1629 (amide I band) and 1250 cm\(^{-1}\) peak corresponds to thiol groups. The 2857 and 2924 cm\(^{-1}\) peak is intense and it represents abundant -CH\(_2\) residues of the loaded PTH 1-34 moieties. The stretching frequencies for major functionalities have been shifted to lower frequency regions, as the TPP has interacted effectively with the protonated amine or amide residues in the nanoformulation. The stretching frequency is directly proportional to the wave number.

![Figure 3.1.6. FTIR of CS, TCS, TCS–PTH NPs, PTH 1-34 and TCS NPs](image-url)
3.1.3.4 Entrapment Efficiency and Percentage Loading

The entrapment efficiency of CS-PTH NPs with 0.1 w/v CS and 0.05mg/mL PTH 1-34 was 40 % and the yield of NPs was 9 mg based on which the loading efficiency was calculated as 0.8181 %. The entrapment efficiency of PEG-CS-PTH NPs with 0.1 w/v CS and 0.05mg/mL PTH 1-34 was 40 % and the yield of NPs was 14.2 mg based on which the loading efficiency was calculated as 1.408 %. The entrapment efficiency of TCS-PTH 1-34 NPs with 0.5 w/v TCS and 0.05mg/mL PTH 1-34 was 60% and the yield of NPs was 5.2mg based on which the loading efficiency was calculated as 5.76% .

Trials were conducted with different concentrations of polymer to enhance the entrapment and loading efficiency but the NPs generated were above 300nm and showed a concentration dependent size increase. This was an undesirable result for the application of these systems for the oral delivery of PTH 1-34 where size of NP influences the intestinal uptake.274

Based on the entrapment and loading efficiencies, the volume and dose of nanosuspension to be orally administered in rats was calculated. 20µg subcutaneous administration of teriparatide is the conventional osteoporosis therapy, to obtain this concentration the CS-PTH NPs and PEG-CS-PTH NPs were redispersed in 10 mL water or saline. PTH 1-34 concentration of 20µg was calculated to be present in 1mL of these nanosuspensions respectively. Hence 1 mL of the CS-PTH NP and PEG-CS-PTH NP suspensions containing 20µg was decided for oral administration in rats. The TCS-PTH NP suspension was reconstituted in order to obtain a 20 µg PTH 1-34 containing 1mL nanosuspension from the initial concentration of 30 µg
3.1.3.5 *In vitro* peptide release and release kinetics of CS-PTH NPs, PEG-CS-PTH NPs and TCS-PTH NPs

The *in vitro* release profile of the formulation in pH 3.4, 6.8 and 7.5 that corresponds to the blood, intestinal and gastric pH of the fasting state rat physiological pH is a means to evaluate the stability and release pattern of the nanoformulations. The possible loss of PTH 1-34 in the gastric pH can also be measured from the *in vitro* release data. But reproducing the same in the *in vivo* environment is not possible as other influential factors such as gastric residence time, mucus from the goblet cells and the morphology of the stomach determines the fate of the NPs and the release of the peptide.\textsuperscript{275,276}

**Figure 3.1.7** shows the cumulative *in vitro* release profile of PTH 1-34 at pH 3.4, pH 6.8 (volume 3.4ml), and pH 7.5 (volume 20ml) from CS-PTH NPs. The cumulated release at the end of 2 h at pH 3.4 was 3000 pg/mL. A cumulated release of 3000 pg/mL is observed only at the end of 24h at pH 6.8. The cumulative *in vitro* release of 6000-7000 pg/mL of PTH 1-34 at pH 7.5 (volume 20ml) from CS-PTH NPs which was observed by 48h.

**Table 3.1** shows that the kinetics of drug release at different pH from CS-PTH NPs had a higher correlation to the Kormeyer Peppas kinetic model. Its equation predicts that the fractional release of the drug is exponentially related to the release time and describes the release of drug from spherical NP. The plot of the log(drug released) vs log (time ) yields slope $n$(diffusion exponent). The value of $n$ for pH 3.4,6.8 and 7.5 is observed as 0.5<$n$<1.0 that indicates anomalous non Fickian diffusion mechanism for the release of the drug from the NPs.\textsuperscript{77,230}
Figure 3.1.7. Cumulative in vitro release of PTH 1-34 from the CS-PTH NPs at pH 3.4, pH 6.8, and pH 7.5 and which is the gastric, intestinal and blood pH of rats (12h fasting)
Table 3.1: Kinetic modeling of CS-PTH NPs

<table>
<thead>
<tr>
<th>pH</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Korsmeyer Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Q_t = Q_0 - K_0 t$</td>
<td>$\ln Q = \ln Q_0 - K_1 t$</td>
<td>$Q = K_t^{\frac{1}{n}}$</td>
<td>$M_t / M_\infty = K_t^n$</td>
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<tr>
<td></td>
<td>$R^2$</td>
<td>$R^2$</td>
<td>$R^2$</td>
<td>$R^2$</td>
</tr>
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<td>3.4</td>
<td>0.8889</td>
<td>0.889</td>
<td>0.9534</td>
<td><strong>0.9579</strong></td>
</tr>
<tr>
<td>6.8</td>
<td>0.879</td>
<td>0.9138</td>
<td>0.9867</td>
<td><strong>0.9871</strong></td>
</tr>
<tr>
<td>7.5</td>
<td>0.6481</td>
<td>0.6918</td>
<td>0.8796</td>
<td><strong>0.9563</strong></td>
</tr>
</tbody>
</table>

Figure 3.1.8 shows the cumulative in vitro release profile of PTH 1-34 at pH 3.4, pH 6.8 (volume 3.4ml), and pH 7.5 (volume 20ml) from PEG-CS-PTH NPs. The cumulated release at the end of 2h at pH 3.4 was 2500pg/mL. A cumulated release of 3000pg/mL is observed only at the end of 24h at pH 6.8. The cumulative in vitro release of 5000-6000 pg/mL of PTH 1-34 at pH 7.5 (volume 20ml) from PEG-CS-PTH NPs which released by 48h.

Table 3.2 shows that the kinetics of drug release at different pH from PEG-CS-PTH NPs had a higher correlation to the Korsmeyer Peppas kinetic model. Its equation predicts that the fractional release of the drug is exponentially related to the release time and describes the release of drug from spherical NP. The plot of the log(drug released) vs log (time) yields slope $n$ (diffusion exponent). The value of $n$ for pH 3.4, 6.8 and 7.5 is
observed as $0.5 < n < 1.0$ that indicates anomalous non Fickian diffusion mechanism for the release of the drug from the NPs. 277,278

Figure 3.1.8. Cumulative in vitro release of PTH 1-34 from the PEG-CS-PTH NPs at pH 3.4, pH 6.8, and pH 7.5 and which is the gastric, intestinal and blood pH of rats (12h fasting).
Figure 3.1.9, shows the in vitro release profile of PTH 1-34 at pH 7.5, 6.8 and 3.4 from TCS-PTH 1-34 NPs. The cumulated release at the end of 2h at pH 3.4 was 2500pg/mL. A cumulated release of 500 pg/mL is observed only at the end of 24h at pH 6.8. The cumulative in vitro release of 2500 pg/mL of PTH 1-34 at pH 7.5 (volume 20ml) from PEG-CS-PTH NPs was observed at the end of 48h.

Table 3.3 shows that the kinetics of drug release at different pH from PEG-CS-PTH NPs had a higher correlation to the Kormeyer Peppas kinetic model. Its equation predicts that the fractional release of the drug is exponentially related to the release time and describes the release of drug from spherical NP. The plot of the log(drug released) vs log (time )yields slope n(diffusion exponent). The value of n for pH 3.4,6.8 and 7.5 is observed as 0.5<n<1.0 that indicates anomalous non Fickian diffusion mechanism for the release of the drug from the NPs.279
Figure 3.1.9. Cumulative in vitro release of PTH 1-34 from the TCS-PTH NPs at pH 3.4, pH 6.8, and pH 7.5 and which is the gastric, intestinal and blood pH of rats (12h fasting).
Table 3.3 Kinetic modeling of TCS-PTH NPs

<table>
<thead>
<tr>
<th>pH</th>
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<th>Higuchi</th>
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<td>$Q_t = Q_0 - K_0 t$</td>
<td>$\ln Q = \ln Q_0 - K_1 t$</td>
<td>$Q = K_t^{\frac{1}{2}}$</td>
<td>$M_t/M_\infty = K_t^n$</td>
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<td>3.4</td>
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<td>6.8</td>
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</tr>
<tr>
<td>7.5</td>
<td>0.4719</td>
<td>0.525</td>
<td>0.7224</td>
<td>0.856</td>
</tr>
</tbody>
</table>

Over all the mechanism of in vitro release in 20mL PBS (pH 7.5) is due to surface desorption of peptide, followed by swelling, relaxation or erosion making the cross linker interaction feeble enabling further peptide release.\(^{280,281}\) 50% amino acids in PTH 1-34 such as aspartic acid, glutamate, histidine, lysine, asparagine, glutamine etc has a tendency of forming intermolecular hydrogen bonding and gelling/cross linking in aqueous solution.\(^{282,283}\) When there is a change in the pH of the released medium from 7.5 to 3.4 or 6.8 this gelling/cross linking phenomenon is more pronounced and the release of the PTH 1-34 from the CS based NPs is retarded. The released peptide may even become unavailable for detection due to gelation and sedimentation at the NPs centrifugation force during the experimental procedure for analysing the supernatant for the presence of released PTH 1-34.

The in vitro release profile of the formulation at pH 3.4, 6.8, and 7.5 indicates its stability and cumulative release pattern. The mechanism of in vitro release is assumed to
be the imbibition of water and swelling making the cross linker interaction feeble, resulting in the release of peptide. The PTH 1-34 release at pH 3.4 was conducted only up to 2h as gastric clearance of empty stomach falls between a minimum of 30 minutes to a maximum of 2h. The release at pH 6.8 was extended to 24h as the tendency of intestine is to retain substances this long. All the three formulations show that the acidic pH has a greater influence on the rate of release of the peptide from the nanoformulation due to its dissolution.

3.1.3.6 Biological Assays

The main advantage to using primary human cells is their clinical applicability and the reduced need for accounting for interspecies differences, as is the case when other animal cell sources or cell lines are used. Other cell lines used for preliminary evaluation of NPs include SaOs-2, cells which have a mature osteoblast phenotype. Cytokine and growth factor expression of SaOs-2 cells have been shown to be similar to primary normal human osteoblast cells. In 1994 the FDA presented guidelines for preclinical and clinical evaluation of agents used in the treatment or prevention of postmenopausal osteoporosis, with the recommendation of using rat as an animal model for this evaluation. Rat osteoblast cells serve as a model in vivo and in vitro research. The UMR-106 cell line is a clonal derivative of a transplantable rat osteosarcoma that had been induced by injection of radiophosphorous.

3.1.3.7 Biocompatibility and Blood compatibility assays

The MTT and LDH results (Fig. 3.1.10,3.1.11,3.1.12) proves that CS NPs,CS-PTH NPs,PEG-CS-PTH NPs and TCS-PTH NPs at different concentrations tested on cells for a period of 24 h were found non-toxic and the cell viability had been unaffected by the nanoformulation. The mitochondrial activity of the HOB cells used to test the PEG-CS PTH NPs and TCS-PTH NPs was efficient to produce formazan salts even after exposure to the samples and there were no significant cell death. The intactness of the
cells and absence of plasma membrane damage was confirmed as the levels of LDH in the medium was negligible compared to the positive control.

Figure 3.1.10. (A) MTT assay and (B) LDH assay CS NPs concentrations of ((a) 0.005 mg/mL (b) 0.0005 mg/mL (c) 0.00005 mg/mL and CS NPs concentrations corresponding to the PTH 1-34 loaded concentrations (d) 0.1 mg/mL (e) 0.01 mg/mL (f) 0.001 mg/mL along with negative control (g) Media (h) triton –X 100.
Figure 3.1.1. (A) MTT assay; (B) LDH assay (a) 0.05 mg/mL, (b) 0.005 mg/mL, (c) 0.0005 mg/mL PTH 1-34 loaded PEGylated CS NPs and CS NP concentrations corresponding to the PTH 1-34 loaded concentrations, (d) 0.1 mg/mL, (e) 0.01 mg/mL, (f) 0.001 mg/mL along with (g) media and (h) triton –X 100
Figure 3.1.12. (A) MTT assay (B) LDH assay (a) 0.03 mg/mL, (b) 0.003 mg/mL, (c) 0.0003 mg/mL PTH 1-34 loaded TCS NPs and TCS NPs concentrations corresponding to the PTH 1-34 loaded concentrations, (d) 0.5 mg/mL, (e) 0.05 mg/mL, (f) 0.005 mg/mL along with (g) media and (h) triton –X 100.
Hemocompatibility is a significant index for biomaterials because the materials might be exposed in blood environment and may damage the erythrocytes in certain degree or causes the formation of thrombus.\textsuperscript{232} Hemolysis assay was carried out to evaluate the blood compatibility of relevant concentrations of the CS NPs, CS-PTH NPs, PEG-CS-PTH NPs and TCS-PTH NPs. Damage of the erythrocytes due to the sample was insignificant as plasma haemoglobin content detected in the sample treated blood was way below the detection limits of the spectrophotometer (BioTek Power Wave XS) and that of the positive control was 88%. This indicated that percentage haemolysis of the sample was far below 5%, the critical safe haemolytic percentage for biomaterials according to ISO/TR 7406.\textsuperscript{290} The APTT and PT results (Fig. 3.1.13 (A), (B) and (C)) showed that the CS-PTH NPs have not affected the intrinsic and extrinsic clotting pathways.
Figure 3.1.13: (A) Activated partial thromboplastin time APTT, (B) Photograph showing the negligible hemolytic effect of the PTH 1-34 loaded CS NPs of concentrations (a) 0.005 mg/mL (b) 0.0005 mg/mL (c) 0.00005 mg/mL and CS NPs concentrations corresponding to the PTH 1-34 loaded concentrations (d) 0.1 mg/mL (e) 0.01 mg/mL (f) 0.001 mg/mL along with negative control (g) Saline and positive control (H) triton –X 100 and (C) Prothrombin time test
The APTT and PT results for PEG-CS-PTH NPs were 33.8 and 18.5 seconds respectively and TCS-PTH 1-34 NPs shows a value of 34.9 and 15.3 seconds respectively, all of which are around the normal range (Fig 3.1.14, 3.1.15).

Figure 3.1.14. Blood compatibility results of the PEG-CS-PTH NPs and CS NPs (A) APTT, (B) PT and (C) Haemolysis assay
Figure 3.1.15. Haemolysis assay (a) 0.03 mg/mL, (b) 0.003 mg/mL, (c) 0.0003 mg/mL PTH 1-34 loaded TCS-PTH 1-34 NPs and TCS NP concentrations corresponding to the PTH 1-34 loaded concentrations, (d) 0.5 mg/mL, (e) 0.05 mg/mL, (f) 0.005 mg/mL along with negative control, (g) Saline and positive control and (h) triton –X 100 (B) APTT and (C) PT.
3.1.3.8 Biochemical markers

The bioactivity of the released PTH 1-34 from the nanoformulations is determined by evaluating the biochemical markers that are synthesized, released or assimilated by HOB cells on exposure to the PTH 1-34 loaded nanoformulations.

PTH receptor is a trans membrane receptor located on the cell surface of osteoblast cells. PTH 1-34 activates cAMP dependant protein kinase and calcium dependent protein kinase.\textsuperscript{60,291} PTH receptor can be internalised and can influence gene transcription processes. Osteocalcin is produced by osteoblasts, it is often used as a marker for the bone formation process. During treatment with anabolic bone formation drugs such as teriparatide, it has been observed that higher serum-osteocalcin levels are relatively well correlated with increases in bone mineral density. Osteocalcin is used as a preliminary biomarker on the effectiveness of a given drug on bone formation. study the effectiveness of a glycoprotein called lactoferrin on bone formation used osteocalcin as a measure of osteoblast activity.\textsuperscript{238} Imai et al studied the exchangeability, location, and amount of the total calcium in bone cells in relation to their osteoblastic activity.\textsuperscript{292} The cells closest to the forming bone had the highest alkaline phosphatase activity and the highest Ca\textsuperscript{2+} content.\textsuperscript{64} ALP release, regulation of calcium synthesis, release of osteocalcin and hormonal trigger of second messengers have been reported for \textit{in vitro} and \textit{in vivo} studies on the effect of PTH 1-34, PTH 1-84 and PTH related peptides.\textsuperscript{260,293,294} Thus an evaluation of cAMP, osteocalcin, ALP and calcium will prove the bioactivity of the released PTH 1-34 from the nanoformulations(\textbf{Fig.3.1.16}).
Figure 3.1.16: Diagram depicting the molecular mechanism of the anabolic effect of PTH 1-34 on human primary osteoblast cell.

3.1.3.8.1 In vitro bone specific alkaline phosphatase activity

The ALP produced by the HOB cells treated with test and controls for 3 day was 400ng/mL. There was an increase and variation in ALP released by the 7th day. Cells grown as controls and those treated with CS NPs had released 1200ng/mL ALP which is the basic quantity this population of primary human osteoblast cells can produce in 7 days. Whereas CS-PTH NPs, PEG-CS-PTH NPs and TCS-PTH 1-34 NPs have released sufficient peptide to stimulate an increased production of 1800ng/mL, 1600ng/mL and 1500ng/mL ALP respectively in 7 days (Fig. 3.1.17, 3.1.18, 3.1.19). Concentration of ALP produced by the HOB treated with bare PTH 1-34 alone was 2400ng/mL which shows the peptides ability to stimulate ALP production in the HOB cells.
Figure 3.1.17. 3 and 7 day bone specific alkaline phosphatase detected by the ALP assay. 
# indicates the $p < 0.05$ when compared to 3 day CS-PTH NPs treated HOB cells and
*indicates $p$ value $< 0.05$ when compared to 7 day CS-PTH NPs treated HOB cells.

Figure 3.1.18. 3 and 7 day bone specific alkaline phosphatase detected by the ALP assay. 
# indicates the $p < 0.05$ when compared to 3 day PEG-CS-PTH NPs treated HOB cells and
*indicates $p$ value $< 0.05$ when compared to 7 day PEG-CS-PTH NPs treated HOB cells.
Figure 3.1.19. 3 and 7 day bone specific alkaline phosphatase detected by the ALP assay. # indicates the p < 0.05 when compared to 3 day TCS-PTH 1-34 NPs treated HOB cells and * indicates p value < 0.05 when compared to 7 day TCS-PTH 1-34 NPs treated HOB cells.

CS in tissue engineering scaffolds are well known to be osteoconductive and activates ALP production, but here, in the NP form these effects of CS are less pronounced. Thus it is primarily the PTH 1-34 released from the NPs in slow and sustained manner that has stimulated the ALP production and release from the HOB cells. This is proof that the PTH 1-34 released from the NPs are bioactive and stimulate their receptors for signaling ALP synthesis and release. The PTH 1-34 cell surface receptor stimulation and the downstream second messenger signaling is confirmed by quantifying the cAMP produced by the HOB cells.

3.1.3.8.2 Cyclic adenosine monophosphate (cAMP) assay

cAMP, a second messenger is produced by HOB cells in response to PTH 1-34. CAMP concentrations of 1.5 - 3 pgmol/mL is produced in response to PTH 1-34 and the PTH released from the CS based nanoformulations respectively as shown in figure 3.1.20,3.1.21,3.1.22. This heightened level of cAMP in peptide and peptide loaded NP treated HOB cells compared to ≤ 0.5pgmol/mL in the CS NPs, TCS NPs and cells in me-
dia alone indicates the hormonal stimulation of the transmembrane PTH 1-34 receptors on the HOB and the extent of signalling happening within the cells. This pathway is the most crucial in deciding the anabolic effect of PTH 1-34 on HOB cells.

Figure 3.1.20  cAMP assay *indicates p value < 0.05 when compared to 7 day CS-PTH NPs treated HOB cells.

Figure 3.1.21. cAMP assay *indicates p value < 0.05 when compared to 7 day PEG-CS-PTH NPs treated HOB cells.
Figure 3.1.2. cAMP assay * indicates p value < 0.05 when compared to 7 day TCS-PTH 1-34 NPs treated HOB cells.

Stimulation of cAMP production by the TCS-PTH NPs is observed to be higher than the CS-PTH NPs and PEG - CS-PTH NPs treated cells. This could be due to the influence of thiol group in acting as redox switches in regulating signaling pathways. Reversible oxidative thiol modifications have been found to modulate the function of proteins involved in many different pathways such as gene transcription, translation to metabolism and signal transduction. Hence TCS-PTH NPs stimulate cAMP production due to influence of the released PTH 1-34 as well as the thiol groups in the TCS.\textsuperscript{299,300} The bioactivity of the released PTH 1-34 can be further proved by determining the influx of calcium ions by the primary osteoblast cells.

3.1.3.8.3 Calcium Assay

The calcium calorimetric assay shows the influx of calcium due to the effect of PTH 1-34. The cells treated with CS-PTH NPs, PEG-CS-PTH NPs and TCS-PTH NPs had 27, 28.09 and 31.5 µg/mL intracellular calcium respectively whereas control cells had less than 15 µg/mL intracellular calcium (Fig 3.1.23 and 3.1.24). The calcium calorimetric assay shows the influx of calcium due to the effect of PTH 1-34. PTH stimulates
both the influx and efflux of calcium depending on the dose administered. These high levels of intracellular calcium in HOB cells treated with the TCS-PTH 1-34 NPs and PEG-CS-PTH NPs also marks the beginning of mineralization.\textsuperscript{292,301}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_1_23.png}
\caption{Calcium assay *indicates $p$ value $< 0.05$ when compared to 7 day CS-PTH NPs treated HOB cells.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_1_24.png}
\caption{Calcium assay*indicates $p$ value $< 0.05$ when compared to 7 day PEG-CS-PTH NPs treated HOB cells.}
\end{figure}
Figure 3.1.25. Calcium assay * indicates p value < 0.05 when compared to 7 day TCS-PTH 1-34 NPs treated HOB cells

3.1.3.8.4 Osteocalcin

The level of osteocalcin produced by CS-PTH NPs, PEG-CS-PTH NPs and TCS-PTH NPs treated HOB cells is 2.5, 3 and 2.8 ng/mL respectively. The control groups show a value between 2 and 2.5 ng/mL. Osteocalcin is used as a preliminary biomarker on the effectiveness of a given drug on bone formation. The transcription and maturation of osteocalcin is dependent on the availability of vitamins D and K. In vitro degradation of the synthesized osteocalcin could be the reason for such an observation.238,239
Figure 3.1.26. Osteocalcin assay *indicates p value < 0.05 when compared to 7 day CS-PTH NPs treated HOB cells.

Figure 3.1.27. Osteocalcin assay *indicates p value < 0.05 when compared to 7 day PEG-CS-PTH NPs treated HOB cells.
Chapter 3

Results & Discussion

Figure 3.1.28. Osteocalcin assay * indicates p value < 0.05 when compared to 7 day TCS-PTH 1-34 NPs treated HOB cells

3.1.3.9 In vivo evaluation following oral administration

In figure 3.1.29, 3.1.30 and 3.1.31., the results of human PTH 1-34 ELISA tests on the blood samples collected from the Sprague dawley rats at various time points after the oral administration (20 µg PTH 1-34 loaded NPs) is shown. Figure 3.1.30 and 3.1.31 shows that the PEG-CS-PTH NPs efficiently delivers a bioavailable concentration of 100-120pg/mL PTH 1-34 and TCS-PTH NPs delivered 150-160pg/mL PTH 1-34 upto 12h in the 48h period of study. The point of interest is the detection of PTH 1-34 in the rat blood within 5 minutes of oral administration of the nanoformulation and a maintained steady serum concentration which falls by the 12th hour.

CS-PTH NPs is observed to be efficient in delivering 100-160pg/mL PTH 1-34 from the 5th minute to 30 minutes and thereafter the level falls to negligible levels by 1h. Hemolysis was observed in the initial half an hour of the blood samples collected after oral administration of CS-PTH NPs (Fig.3.1.29). 1mL of CS-PTH NPs orally administered in rats had a CS concentration of 1mg/mL which was observed to initiate haemolysis for the first half an hour following which the condition cleared. The high positive charge on the CS-PTH NPs interacts with the negatively charged sialic acid residues of
RBC and causes the clumping and haemolysis. The animals were kept under observation and no further ill effects were observed. PEG-CS-PTH NPs and TCS-PTH NPs showed no such hemolytic effects after oral administration.

Bare PTH 1-34 (20µg) administered orally and the endogenous PTH 1-34 in naive rat that has a cross reactivity with the human PTH 1-34 ELISA kit shows a concentration less than 30pg/mL throughout the study period which proves that there is no intestinal uptake of bare PTH 1-34 from the digestive tract of rats.

**Figure 3.1.29.** The pharmacokinetic profile of PTH 1-34 after oral administration of the CS-PTH NPs and PTH 1-34 in Female Sprague dawley rats (overnight fast); (Inset: Hemolysis observed in the initial 30 min of blood draw).
Figure 3.1.30. The pharmacokinetic profile of PTH 1-34 after oral administration of the PEG-CS-PTH NPs, CS-PTH NPs, PTH 1-34 in Female Sprague dawley rats (overnight fast)
Figure 3.1.3. The pharmacokinetic profile of PTH 1-34 after oral administration of the TCS-PTH NPs, CS-PTH NPs, PTH 1-34 in Female Sprague dawley rats (overnight fast)

The justification for the peptide uptake is that the water molecules form a close, hydrogen-bonded layer with the soluble PTH 1-34 backbone as well as its hydrophilic side chain amino acid groups. Transcellular PTH 1-34 permeation would involve breakdown of these hydrogen bonds. Overcoming the energy requirement for this process might be an important determinant of transcellular peptide diffusion which would involve the desolvation of the PTH 1-34, whereas the alternate paracellular mode doesn’t require the solute to leave the aqueous environment and, hence depends predominantly on size-sieving of the molecules. Based on perfusion studies with hydrophilic solutes inulin, glucose, and creatinine; paracellular pores are estimated to have a pore radius of 50 Å with a cross sectional area per unit path length of 4.3 cm per cm length of intestine, this fact
supports the easy paracellular uptake of the NPs. Another interesting observation is the higher permeation of hydrophilic peptides with net positive, rather than negative, charge. Partition coefficient (P) of the drug molecule between n-octanol and water is also an important factor determining the mode of peptide and protein absorption through the intestine. Log P values less than zero indicates a hydrophilic molecule, which is more likely to follow the paracellular pathway which uses aqueous channels for molecular diffusion. The 4117 Da low molecular weight, linear PTH 1-34 with a P value of -3.10 and a net positive charge has a high probability to follow the paracellular pathway of absorption from the intestinal lumen hence making its presence in rat blood merely after 5 minutes of oral administration.

The prolonged circulation of PTH 1-34 might be due to its absorption into the lacteals in the microvillus. The higher porosity of lymphatic capillaries over blood vessel endothelium directs even macromolecules to lymphatic circulation. The interest in lymphatic absorption is linked to the fact that it outflanks the hepatic first-pass metabolism that any blood-absorbed component must undergo, in this way elevating bioavailability.

The nanoformulation is expected to have survived the gastric environment of the overnight fasted rat stomach (fasting pH of 3.4) due to PEGylation of the nanoformulation as well as the speedier gastric emptying. PEG also has the property to enhance paracellular transport. The surface charge of PEG-CS-PTH NP is +35 hence will bind to cell membranes. The positive charges on the CS, interacts with tight junction protein followed by redistribution of filamentous actin, and superficial destabilization of the plasma membrane and decrease the trans-epithelial electrical resistance of cell monolayers to increase paracellular permeability. This work proves that PEG-CS-PTH NPs has successfully protected and delivered 100-120pg/mL PTH 1-34 orally in rats. These findings affirmatively imply the potential of this system to release biologically functional levels of peptide in the in vivo rat models used here. The anabolic action of such a sustained low dose of PTH 1-34 in osteoporotic rat models or other relevant higher animal models will be insightful.
to conclude the therapeutic efficiency of this orally administered PEG-CS-PTH nanofor-
mulation.

The *in vivo* results prove the efficiency of TCS NPs to deliver and improve the
half life of the osteoporosis drug PTH 1-34 orally by crossing the barriers of the gastroin-
testinal tract meanwhile protecting the precious peptide within. The blood level of PTH
1-34 obtained by the oral administration of TCS-PTH 1-34 NPs was observed even at 48
h which is a significant result over subcutaneously administered PTH 1-34 that has a
clearance time of 3h. It shows that the TCS-PTH 1-34 NPs efficiently delivered a maxi-
mum concentration of 146pg/mL PTH 1-34 which falls by the 12th h for a study con-
ducted for 48h whereas the control PTH 1-34 30µg/mL administered orally has failed to
reach the systemic circulation. The circulating endogenous levels of rat PTH 1- 34 was
assayed using the human PTH 1-34 ELISA the samples showed cross reactivity with the
ELISA plate and this proves that the PTH 1-34 administered orally fails to reach the sys-
temic circulation and TCS-PTH 1-34 NPs efficiently delivered the peptide orally.

To elaborate, once the formulation is adhered to mucus that lines the gastrointes-
tinal tract, the significant presence of PTH 1-34 in blood could be attributed to the para-
cellular uptake of the free peptide or the peptide loaded nanoformulation as such or the
in situ released peptide. The justification is that the water molecules form a close, hydro-
gen-bonded layer with the soluble PTH 1-34 backbone as well as its hydrophilic side
chain amino acid groups. Transcellular PTH 1-34 permeation would involve breakdown
of these hydrogen bonds. Overcoming the energy requirement for this process might be
an important determinant of transcellular peptide diffusion which would involve the
desolvation of the PTH 1-34, whereas the alternate paracellular mode doesn’t require the
solute to leave the aqueous environment and, hence depends predominantly on size-
sieving of the molecules.\textsuperscript{310}

The surface charge of TCS-PTH 1-34 NP is +38 helps to bind to cell membranes.
The positive charges on the nanoformulation, interacts with occludin and ZO-1 that are
tight junction protein followed by redistribution of filamentous actin, and superficial de-
stabilization of the plasma membrane and decrease the trans-epithelial electrical re-
Chapter 3

Results & Discussion

Resistance of cell monolayers to increase paracellular permeability. PTH 1-34 administered orally as a control in rats was not significantly taken up by the rats gastrointestinal tract.

3.1.3.9.1 In vivo pharmacokinetics

Estimation and calculation of pharmacokinetic parameters was performed using the PK solver version 2 software for one compartmental extravascular model. Prior to evaluating the pharmacokinetic data an important observation made is stated below. The human PTH 1-34 ELISA used in these experiments showed a cross reactivity to endogenous rat PTH. The cross-reactants were diluted in the 0 pg/mL Standard and measured using the High Sensitivity Human PTH 1-34 ELISA Kit. The results are expressed as % cross-reactivity relative to the human PTH 1-34 standards contained in the kit.

Table 3.4 Shows the cross reactivity of the Rat PTH 1-34 to Human PTH 1-34 ELISA Kit.

<table>
<thead>
<tr>
<th>CROSS-REACTANT</th>
<th>CROSS-REACTIVITY (weight%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PTH(1-34)</td>
<td>100</td>
</tr>
<tr>
<td>Rat PTH (1-34)</td>
<td>43</td>
</tr>
<tr>
<td>Rat PTH (1-84)</td>
<td>6.8</td>
</tr>
</tbody>
</table>

The human PTH 1-34 detected in the Human PTH 1-34 ELISA after bare PTH 1-34 oral administration in rats was in fact the endogenous rat PTH 1-34 and PTH 1-84. Hence the data interpretation stands on the fact that orally administered PTH 1-34 completely perishes in the GI tract of rat and none is absorbed into the blood plasma. The human PTH 1-34 loaded in the NPs on the other hand was protected and delivered efficiently into the systemic circulation.

The maximum plasma concentration ($C_{max}$) of PTH 1-34 bare and the three different nanoformulation and the corresponding peak time ($T_{max}$) were determined by the in-
inspection of the individual drug plasma concentration-time profiles. $T_{\text{max}}$ or the peak time gives an indication of the rate of absorption. Its value decreases as the rate of absorption increases. $C_{\text{max}}$ the peak plasma concentration that gives an indication whether the drug is sufficiently absorbed systemically to provide a therapeutic response. $C_{\text{max}}$ increases as the dose and the absorption increases. It is observed here that maximum concentration of PTH 1-34 in the plasma delivered orally from the CS based NPs is in the therapeutic range for osteoporosis. The area under the curve to the last measurable concentration (AUC0-t) was calculated by the linear trapezoidal rule. AUC gives a measure of the extend of absorption or the amount of drug that reaches the systemic circulation which extrapolates to infinity the ability of the PTH 1-34 loaded nanoformulation to release PTH 1-34. The degradation of CS and its derivatives *in vivo* over a period of time will diminish the potential of the NPs to deliver PTH for prolonged period of time.

**Table 3.5.** *In vivo* pharmacokinetic parameters of CS-PTH NPs obtained from plasma after oral administration in female Sprague dawley rats.

<table>
<thead>
<tr>
<th>S.NO</th>
<th>PARAMETERS</th>
<th>PTH 1-34</th>
<th>CS-PTH NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$T_{\text{max}}$(h)</td>
<td>0.629 ± 0.002</td>
<td>0.022 ± 0.013</td>
</tr>
<tr>
<td>2</td>
<td>$C_{\text{max}}$( pg/mL)</td>
<td>35.48 ± 3.3</td>
<td>180.35 ± 3.36</td>
</tr>
<tr>
<td>3</td>
<td>AUC 0-48h (pg/mL.h)</td>
<td>906.704 ± 82.2</td>
<td>245.81 ± 6.34</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters derived for the CS-PTH NPs and the respective bare PTH 1-34 solutions subsequent to oral administration are summarized in **Table 3.5**. The plasma concentration vs. time profiles of PTH 1-34 and CS-PTH NPs administered orally as in **Fig. 3.1.29** clearly exhibit remarkable differences between the bare and
nanoformulations. The plasma PTH 1-34 concentration profile of the bare PTH 1-34 and the CS-PTH 1-34 NPs showed a peak plasma concentration; C\text{max} of 35.48 ± 3.3 and 180.35 ± 3.36 pg/mL. The CS-PTH 1-34 NPs has delivered this concentration of peptide within 5 minutes after oral administration as evident from the T\text{max} value of 0.022 h. The AUC\text{0-48} values of PTH 1-34 and CS-PTH NPs was found to be 906.704 ± 82.2 and 245.81 ± 6.34 pg/mL.h respectively. Based on the cross reactivity of the human specific PTH 1-34 ELISA as shown in table 3.4 the bare oral PTH 1-34 administered falsely shows its presence for an extended period of time. The plasma concentration-time profile of PTH 1-34 from CS-PTH 1-34 NPs declined with time and was completely cleared in 3h. The CS-PTH NPs absorbed into the systemic circulation owing to its positive surface charge triggered sudden hemolysis in rats (fig 3.1.30 inset) and the body’s natural mechanism of clearance of lysed erythrocytes would have cleared the NPs from circulation.\textsuperscript{311}

Table 3.6. In vivo pharmacokinetic parameters of PEG-CS-PTH NPs obtained from plasma after oral administration in female Sprague dawley rats.

<table>
<thead>
<tr>
<th>S.NO</th>
<th>PARAMETERS</th>
<th>PTH 1-34</th>
<th>PEG-CS-PTH NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T\text{max}(h)</td>
<td>0.629 ± 0.002</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>C\text{max}( pg/mL)</td>
<td>35.48 ± 3.3</td>
<td>125.33 ± 5.64</td>
</tr>
<tr>
<td>3</td>
<td>AUC 0-48h (pg/mL.h)</td>
<td>906.704 ± 82.2</td>
<td>6007.92 ± 254.32</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters derived for the PEG-CS-PTH NPs and the respective bare PTH 1-34 solutions subsequent to oral administration are summarized in Table 3.6. The plasma concentration vs. time profiles of PTH 1-34 and PEG-CS-PTH NPs administered orally as in Fig. 3.1.30 clearly exhibit remarkable differences between
the bare and nanoformulations. The plasma PTH 1-34 concentration profile of the bare PTH 1-34 showed a peak plasma concentration; \( C_{\text{max}} \) of 35.48 ± 3.3 and PEG-CS-PTH 1-34 NPs \( C_{\text{max}} \) of 125.33 ± 5.64 pg/mL was obtained at a \( T_{\text{max}} \) of 1.04 ±0.05 h. The AUC\(_{0-48}\) values of PTH 1-34 and PEG-CS-PTH NPs was found to be 906.704 ± 82.2 and 6007.92 ± 254.32 pg/mL.h respectively. Based on the cross reactivity of the human specific PTH 1-34 ELISA as shown in \textbf{table 3.4} the bare oral PTH 1-34 administered falsely shows its presence for an extended period of time. The plasma concentration-time profile of PTH 1-34 from PEG-CS-PTH 1-34 NPs showed a prolonged release pattern as evident from the AUC\(_{0-48}\) which shows that PEGylation of the CS NP systems enhances its circulation and evades the macrophage based clearance,\(^{312}\) due which a prolonged release of PTH 1-34 was observed from the NPs system.

\textbf{Table 3.7.} \textit{In vivo pharmacokinetic parameters of TCS-PTH NPs obtained from plasma after oral administration in female Sprague dawley rats.}

<table>
<thead>
<tr>
<th>S.NO</th>
<th>PARAMETERS</th>
<th>PTH 1-34</th>
<th>TCS-PTH NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( T_{\text{max}} ) (h)</td>
<td>0.629 ± 0.002</td>
<td>0.58 ± 0.0018</td>
</tr>
<tr>
<td>2</td>
<td>( C_{\text{max}} ) (pg/mL)</td>
<td>35.48 ± 3.3</td>
<td>146.12 ± 4.23</td>
</tr>
<tr>
<td>3</td>
<td>AUC 0-48h (pg/mL.h)</td>
<td>906.704 ± 82.2</td>
<td>7009.01 ± 302.7</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters derived for the TCS- PTH NPs and the respective bare PTH 1-34 solutions subsequent to oral administration are summarized in \textbf{Table 3.7}. The plasma concentration vs. time profiles of PTH 1-34 and TCS-PTH NPs administered orally as in \textbf{Fig. 3.1.31} clearly exhibit remarkable differences between the bare and nanoformulations. The plasma PTH 1-34 concentration profile of the bare PTH 1-34
showed a peak plasma concentration; C\text{max} of 35.48 ± 3.3 and TCS-PTH 1-34 NPs C\text{max} of 146.12 ± 4.23 pg/mL was obtained at a T\text{max} of 0.58 ± 0.00018 h. The AUC\text{0-48} values of PTH 1-34 and TCS-PTH NPs was found to be 906.704 ± 82.2 and 7009.01 ± 302.7 pg/mL.h respectively. Based on the cross reactivity of the human specific PTH 1-34 ELISA as shown in table 3.4 the bare oral PTH 1-34 administered falsely shows its presence for an extended period of time. The plasma concentration-time profile of PTH 1-34 from TCS-PTH 1-34 NPs showed a prolonged release pattern as evident from the AUC\text{0-48}. This proves that like PEGylation, a thiolated modification of CS can also prevent hemolysis post administration. This will prolong the circulation of NPs and enable the prolonged release of entrapped peptide.

3.1.3.9.2 In vivo Near infrared imaging

The bioavailability of orally administered drugs is determined by several factors. The nature of the drug administered orally, its vulnerability to degradation in the GI tract and the optimum conditions for efficient intestinal absorption are the factors that decide its oral bioavailability. For drugs that are prone to gastric degradation, quick gastric emptying is necessary for the drug to reach the intestine to ensure maximum drug absorption. This can be achieved when the stomach is empty as after a 12h overnight fast in our experiments and the gastric retention is poor especially when the dosage is a nanosuspension. The drug and its carrier polymer is therefore protected and reaches the site of absorption without much change. A 12h fast, prior to orally administering ICG conjugated PTH 1-34 loaded nanosuspensions in Female Sprague dawley rats, stands relevant to a physicians choice of suggesting a pill in the morning on an empty stomach in humans. The NIR image obtained after the oral administration of ICG-CS-PTH NPs is compared with that of the ICG-PEG-CS-PTH NPs and PEG-TCS PTH NPs. These NIR images are proof of concept that the gastric retention is poor and gastric emptying is quick when the stomach is devoid of food.
The animals were anesthetized only after the nanosuspension was swallowed in order to prevent asphyxiation and was immediately imaged to track the gastrointestinal transit of the administered ICG conjugated nanosuspension. The NIR image (Fig 3.1.32) first panel shows the gastric entry of ICG-CS-PTH NPs within minutes after oral administration and maximum intensity was observed at the 3rd h. These images align well with the plasma PTH 1-34 concentration obtained after the oral administration of CS-PTH NPs that peaks at 0.022h and declines by 3h (Fig.3.1.29). The NIR image of the rats, orally administered with ICG-PEG-CS-PTH NPs revealed its entry into the absorptive site, the intestine within 30 minutes due to speedy gastric emptying of the nanoformulation. The mucoadhesive property of the nanoformulation is quite evident as the IR emission of the ICG-PEG-CS-PTH NPs is observed even after 24 hours in the gastrointestinal tract. The NIR images in the second panel of Figure 3.1.33 support the fact that the TCS-PTH NPs has exhibited high mucoadhesion up to 24h. The speedy gastric emptying into the intestine is also evident in the images that prevent the deterioration of peptide loaded nanoformulation in the stomach. Once the formulation is adhered to mucus that lines the gastrointestinal tract, the significant presence of PTH 1-34 in blood could be attributed to the paracellular uptake of the peptide loaded nanoformulation as such or the in situ released peptide.

**ICG-CS-PTH 1-34 NPs**

**ICG-PEG-CS-PTH 1-34 NPs**

**Figure 3.1.32.** In vivo NIR image of rats orally administered with ICG-PEG-CS-PTH NPs is compared to the in vivo NIR image of rats orally administered with ICG-CS-PTH.
The areas marked A & B are the junction of stomach and duodenum and the small intestine respectively.

**Figure 3.1.33.** In vivo NIR image of rats orally administered with ICG-TCS-PTH NPs is compared to the in vivo NIR image of rats orally administered with ICG-CS-PTH NPs

**To conclude and analyze the hypothesis based on the obtained results:-**

1. TPP interacts electrostatically with the peptide and polymer and resulted in the formation of PTH 1-34 loaded CS, PEG-CS and TCS NPs.

2. Surface charge of the CS - PTH NPs was reduced after coating PEG 200 which enhanced its *in vivo* hemocompatibility.

3. Substituting CS with TCS reduced the surface charge of the CS-PTH NP system and enhanced its *in vivo* hemocompatibility.

4. *In vitro* biological assays in HOB cells treated with these nanoformulations proved the bioactivity of the released PTH 1-34.
Chapter 3  Results & Discussion

5. The entrapment and loading efficiency, in vivo drug released in circulation was higher for TCS PTH1-34 NP system compared to CS and PEG -CS PTH 1-34 NP systems.

6. CS-PTH NPs delivered 100-160pg/mL PTH 1-34 and cleared from blood in 3h but it caused hemolysis in rats.

7. CS-PTH 1-34 NPs with a higher positive charge and smaller size had a faster clearance hence shortened the in vivo release of PTH 1-34.

8. PEG-CS-PTH NPs delivered 100-120pg/mL PTH 1-34 up to 12 hours.

9. TCS PTH 1-34 NPs efficiently delivered 150-160pg/mL PTH 1-34 and the release was observed for a period of 12h.

10. Modified PEGylated and Thiolated Chitosan NPs were in circulation for longer time and prolonged the in vivo release of PTH 1-34 but no haemolysis was observed.