CHAPTER 2

MATERIALS & METHODS
CHAPTER 2

MATERIALS AND METHODS

CS-PTH NPs, TCS-PTH NPs and PEG-CS PTH NPs are the NP systems that have been synthesized. The materials used for the synthesis and the \textit{in vitro} and \textit{in vivo} evaluations of these NP systems have been included in section 2.1 and their methods of synthesis, \textit{in vitro} and \textit{in vivo} evaluation have been described under section 2.2

2.1 MATERIALS

Table 2.1.: \textit{List of Cell culture Medium}

<table>
<thead>
<tr>
<th>S.No</th>
<th>Cell culture Medium</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Minimum Essential Medium (MEM)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>2</td>
<td>Dulbecco’s modified eagle medium (DMEM)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3</td>
<td>Mc Coys 5A</td>
<td>GIBCO Bangalore</td>
</tr>
<tr>
<td>4</td>
<td>Osteoblast specific media and detach Kit</td>
<td>Promo cell</td>
</tr>
</tbody>
</table>
Table 2.2: List of Cell lines

<table>
<thead>
<tr>
<th>S.No</th>
<th>Cell Line</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mouse fibroblast cells -L929</td>
<td>NCCS, Pune</td>
</tr>
<tr>
<td>2</td>
<td>Rat osteosarcoma cell line UMR106</td>
<td>NCCS, Pune</td>
</tr>
<tr>
<td>3</td>
<td>Mouse embryo fibroblast cell line NIH3T3</td>
<td>NCCS, Pune</td>
</tr>
<tr>
<td>4</td>
<td>Human osteosarcoma cell Saos-2</td>
<td>NCCS, Pune</td>
</tr>
<tr>
<td>5</td>
<td>Primary human osteoblast HOB</td>
<td>PromoCell USA</td>
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</tbody>
</table>

Table 2.3: List of Biological assay Kits and reagents

<table>
<thead>
<tr>
<th>S.No</th>
<th>Assay Kit and Reagents</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactate dehydrogenase assay (LDH assay)</td>
<td>ALPCO diagnostics</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline phosphatase assay (ALP Assay) reagent</td>
<td>Kishgen Biosystems</td>
</tr>
<tr>
<td>3</td>
<td>Bicinchoninic acid assay (BCA) reagents</td>
<td>Sigma Aldrich.</td>
</tr>
<tr>
<td>4</td>
<td>Human recombinant PTH 1-34 ELISA Kit</td>
<td>ALPCO diagnostics</td>
</tr>
<tr>
<td>5</td>
<td>Human Osteocalcin ELISA Kit</td>
<td>Immunodiagnostic systems USA</td>
</tr>
<tr>
<td>6</td>
<td>Calcium calorimetric assay</td>
<td>Bio Vision</td>
</tr>
<tr>
<td>7</td>
<td>Cyclic AMP (cAMP) assay</td>
<td>Assay designs USA.</td>
</tr>
</tbody>
</table>
Table 2.4: List of Chemicals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemicals</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chitosan</td>
<td>Koyo chemical Co Ltd; Japan.</td>
</tr>
<tr>
<td>2</td>
<td>Human PTH 1-34 (4117.8 Da)</td>
<td>Genscript USA</td>
</tr>
<tr>
<td>3</td>
<td>Penta sodium tripolyphosphate(TPP)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>4</td>
<td>Poly (ethylene glycol) (PEG-200Mw).</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>5</td>
<td>Acetic acid</td>
<td>Merck</td>
</tr>
<tr>
<td>6</td>
<td>HCl</td>
<td>Merck</td>
</tr>
</tbody>
</table>

2.2 METHODS

2.2.1 Preparation CS-NPs

CS solution of 0.1% (w/v) was prepared in 1% acetic acid. TPP, 1.0% was added drop wise to 10 mL of CS with stirring. The resulting CS NP suspension was centrifuged at 10,000× g for 10 min. The pelleted particles were resuspended in deionized water with 10 sec sonication and lyophilized. 225

2.2.2 Preparation of CS-PTH NPs

10 mL CS solution of 0.1% (w/v) was prepared in 1% acetic acid into which 0.05 mg PTH 1-34 (5×10^{-2} mg/mL or 10^{-5} M) was added and incubated in cold for 3 h under constant stirring. To this mixture TPP, 1.0% was added drop wise until a nanosuspension was obtained. The suspension obtained thereafter was centrifuged at 10,000× g for 10 min. The pelleted particles were resuspended in deionized water. 110,226
2.2.3. Preparation of PEG-CS-PTH NPs

10 mL CS solution of 0.1 % (w/v) was prepared in 0.3 % acetic acid into which 0.5 mg PTH 1-34 (5x10^{-2} mg/mL or 10^{-5} M) was added and incubated at 4º C for 4 h under constant stirring. To this mixture 1.0 % TPP was added drop wise to obtain a nanosuspension. PEG 200 was added to the polymer in a 1:2 w/w ratio and stirred for another hour. The suspension obtained thereafter was centrifuged at 15,000 x g for 20 minutes. The pelleted particles were resuspended in deionized water.

2.2.4 Preparation of TCS-NPs

TCS-NPs were obtained as a result of ionic cross-linking reaction of TCS solution with TPP. TCS solution was dissolved in water and cross linked with TPP solution for a weight ratio of TCS to TPP as 3:1. The resulting turbid suspension was kept for half an hour stirring and TCS NPs were separated from the stable suspension through centrifugation at 13000rpm for 30 minutes at 15°C (Hermle Labortechnik). The pellet was redispersed in saline and was used for further characterization and studies.

2.2.5 Preparation of TCS-PTH NPs

10 mL TCS solution of 0.05 % (w/v) was prepared in water into which 0.5 mg PTH 1-34 (5x10^{-2} mg/mL or 10^{-5} M) (Genscript USA) was added and incubated in cold for 4 h under constant stirring. To this mixture 1.0 % sodium tripolyphosphate (TPP) from Sigma Aldrich was added drop wise to obtain a nanosuspension. The suspension obtained was centrifuged at 20,000 rpm at 4ºC for 45 min. The pelleted particles were resuspended in deionized water.

2.2.6. Characterization

The mean size and zeta potential of CS NPs, CS-PTH NPs, PEG-CS-PTH NPs, TCS NPs and TCS-PTH NPs were determined by photon correlation spectroscopy using a Zeta Plus particle analyzer (Brookhaven Instrument Corp., Holtsville, NY, USA).
Size distribution of the synthesized NPs before and after peptide loading was analyzed by dynamic light scattering (DLS) using DLS-ZP/Particle Sizer Nicomp™380 ZLS. The surface morphology was evaluated using Scanning electron microscope (SEM); JEOLJSM-6490LA. Fourier Transform Infrared Spectroscopy (FT-IR) spectrum was used to identify the potential interaction between the different components and their functionalities in the NP system using Perkin Elmer Spectrum RXI Fourier Transform Infrared spectrophotometer applying the KBr method. Atomic force microscope (AFM) using JEOL JSPM-5200 and transmission electron microscope (TEM); JEOL JEM 2100 have been used for confirming and detailing the size and characters of the PEG-CS-PTH NPs synthesized in this work.

2.2.7. Entrapment Efficiency and percentage loading of peptide

After the peptide-loaded nanoformulation was prepared the unentrapped peptide was separated from the NPs by ultra-centrifugation at 20,000 rpm at 4 °C for 45 min. To determine the entrapment efficiency of PTH 1-34 in the CS based NP system the supernatant was collected and quantified using PTH 1-34 ELISA kit. The entrapment efficiency percentage was calculated based on the ratio of the amount of drug present in the NPs to the amount of drug used in the loading process, which followed the equation given below.

\[
\text{Entrapment Efficiency \%} = \left(\frac{\text{Total amount of PTH 1-34} - \text{Free PTH 1-34}}{\text{Total amount of PTH 1-34}}\right) \times 100
\]

The NP pellet is lyophilised and the weight of the pellet determines the yield of NPs from the formulation. Once the yield was determined, the percentage of PTH 1-34 in the CS based NP systems were calculated as follows.
Chapter 2  Materials & Methods

(Eq.2)

\[
\% \text{ Drug Loading} = \frac{\text{Total amount of PTH 1-34-Free PTH 1-34}}{\text{Yield of PTH 1-34 loaded NPs}} \times 100
\]

2.2.8. In vitro Peptide Release Studies

In vitro PTH 1-34 release from the three PTH 1-34 loaded CS based NP systems was determined at pH 7.5, 6.8 and 3.4. The NPs suspension was pelletized and redispersed in 10 mL of 0.9% saline with a final peptide concentration of 20µg/mL. From this 1mL nanoformulation was added to 20 mL of PBS at pH 7.5 corresponding to the pH and volume of rat blood. Another 1mL was added to 3.4mL simulated rat gastric fluid pH 3.4 and simulated rat intestinal fluid pH 6.8 (enzyme free) corresponding to that of overnight starved rats. Beakers were incubated at 37 °C under gentle shaking. At definite time intervals, 1mL of the release solution was collected and replaced with the fresh solutions. The sample was centrifuged and the supernatant was analysed using PTH 1-34 ELISA kit.

(Eq.3)

\[
\text{Peptide release \%} = \frac{\text{Amount of PTH 1-34 released at time \text{“}t\text{”}}} {\text{Amount of PTH 1-34 loaded in the nanoformulation}} \times 100
\]

Thereafter a cumulative graph of the percentage of peptide released was plotted against each time period.

2.2.8.1. Kinetic modelling of in-vitro drug release in phosphate buffer

The drug release kinetics can be explained using various kinetic models such as zero order, first order, Higuchi, Korsmeyer Peppas model etc.\textsuperscript{230}
Table 2.5: Depicts the different kinetic models of drug release

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Order</td>
<td>Cumulative percentage of drug release vs time</td>
</tr>
<tr>
<td>First Order</td>
<td>Log cumulative percentage of drug remaining vs time</td>
</tr>
<tr>
<td>Higuchi model</td>
<td>Cumulative percentage of drug release vs square root of time</td>
</tr>
<tr>
<td>Korsmeyer Peppas model</td>
<td>Log cumulative percentage of drug released vs log time</td>
</tr>
</tbody>
</table>

The experimental drug release data was fitted to these models and the corresponding linear regression coefficients (R) were determined, considering the model giving an R value closer to unity as the best fit.\textsuperscript{231} Drug release mechanism from the polymeric matrix was explained by fitting the data to Korsmeyer-Peppas model. To study the release kinetics, data obtained from the in vitro drug release studies were plotted in various kinetic models (Table 2.5). The plots were drawn using Microsoft excel and the regression equations were obtained for each plot. The linearity of the plots was obtained from the value of regression coefficient R.
• **Zero order kinetics**

A zero order release can be predicted by using the equation

\[ Q_t = Q_0 - K_0 t \]  
(Eq.4)

where \( Q_0 \) : initial amount of drug present in solution  
\( Q_t \) : Amount of drug release at time \( t \)  
\( K_0 \) : zero order release rate constant

A graph of cumulative percentage of drug release v/s time would yield a straight line with a slope equal to \( K_0 \).

• **First Order kinetics**

The first order describes the release from a system where the release rate is concentration dependent it can be described by the following equation:

\[ \ln Q = \ln Q_0 - K_1 t \]  
(Eq.5)

where \( K_1 \) : First order release rate constant

• **Higuchi model kinetics**

The drug release can be predicted by the following equation:

\[ Q = K t^{\frac{1}{2}} \]  
(Eq.6)

Where \( K \) : Higuchi dissolution constant  
\( t \) : Time in hours

The model predicts that the drug release from the dosage form is directly proportional to the square root of time.
• **Korsmeyer Peppas model**

To evaluate the mechanism of drug release, the in-vitro release data was plotted in Korsmeyer equation as log cumulative percentage of drug release vs log time and the exponent ‘n’ was calculated through the slope of the straight line.

\[
\frac{M_t}{M_\alpha} = K_t^n
\]  
(Eq.7)

where \( \frac{M_t}{M_\alpha} \): Fractional solute release  
\( t \): Release time  
\( K_t \): Kinetic constant  
n : An exponent which indicates the mechanism of drug release.

When \( n \) less than 0.5 the drug diffuses through the polymer matrix by a fickian (Case 1) diffusion mechanism. For \( n \) less than 0.5 less than 1 an anomalous (non-fickian) mechanism occurs; \( n=1 \) indicates a zero order (Case 2) and \( n \) greater than 1 indicates non-fickian super class 2 release mechanism.

**2.3 Cell Culture**

The preliminary biocompatibility tests of CS NPs and CS-PTH NPs were conducted on normal fibroblast cell lines and human and rat osteosarcoma cells. Further biological assays of the three systems were carried out using primary human osteoblast cells (HOB) which have transmembrane cell surface receptors for PTH 1-34.

L929, NIH3T3 and UMR 106 were maintained in DMEM and Saos-2 in McCoy’s 5A, supplemented with 10% fetal bovine serum (FBS). The cells were incubated in 5% \( \text{CO}_2 \) incubator at 37 °C. After attaining confluency, the cells were detached from the flask with Trypsin-EDTA. The cell suspension was centrifuged at 3000 rpm for 3 min and then resuspended in its respective growth medium for further studies.

HOB cells were maintained in osteoblast growth media. The cells were incubated in 5% \( \text{CO}_2 \) incubator at 37 °C. After attaining confluency, the cells were detached from
Chapter 2

Materials & Methods

the flask using osteoblast specific detach kit. The cell suspension was centrifuged at 3000 rpm for 3 minutes and then resuspended in media for further assays.

2.4 Biocompatibility assays

To test the biocompatibility of the synthesised NPs using the MTT and LDH assays; the normal fibroblasts, osteosarcoma and the primary osteoblasts were seeded at a density of 10,000 cells/well into a 96 well plate. After attaining 90% confluency, the cells were incubated for 24 hours with a serial dilution of the NP suspensions that were freshly synthesized in a sterile environment.

Table 2.6: Concentrations of NPs tested

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tested Concentrations (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS NPs</td>
<td>0.1, 0.01, 0.001</td>
</tr>
<tr>
<td>CS-PTH NPs</td>
<td>0.02, 0.002, 0.0002</td>
</tr>
<tr>
<td>PEG-CS NPs</td>
<td>0.02, 0.002, 0.0002</td>
</tr>
<tr>
<td>TCS-PTH NPs</td>
<td>0.03, 0.003, 0.0003</td>
</tr>
<tr>
<td>TCS NPs</td>
<td>0.5, 0.05, 0.005</td>
</tr>
</tbody>
</table>

Media taken as the positive and triton –X 100 as negative controls for MTT assay and vice versa for LDH assays. The biocompatibility tests conducted are detailed below.

2.4.1 MTT Assay

MTT assay was performed to evaluate the viability of cell due to the effect of the prepared NPs and it is a colorimetric test based on the selective ability of viable cells to reduce the tetrazolium component of MTT in to purple coloured formazan crystals. After
the exposure of cells to different concentrations of the three different nanoformulations
the cells were incubated with MTT solution for 4h followed by 1h incubation with solu-
ibilisation buffer. Then the absorbance of the solution was measured at a wavelength of
570 nm using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS). Tripli-
cate samples were analyzed for each experiment. Cell viability was expressed as the per-
centage of the negative control calculated as

\[ \text{Viability (\%)} = \frac{N_t}{N_c} \times 100 \]  
(Eq.8)

\( N_t \) is the absorbance of cells treated with sample and \( N_c \) is the absorbance of the untreat-
ed cells.

2.4.2 LDH Assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme, which is pre-

sent in all cells. When the plasma membrane is damaged, LDH is rapidly released into
the culture supernatant. The LDH cytotoxicity detection kit provides a simple and precise
calorimetric method to measure the LDH activity. To test the biocompatibility of the syn-
thesised NPs After the incubation period the 96 well plate as a whole was centrifuged
(SORVALL LEGEND X 1R centrifuge) and the cell free culture supernatant was collect-
ed and incubated for 30 min at room temperature with the reaction mixture from the kit.
The absorbance of the solution was measured at a wavelength of 490 nm using a Beck-
mann Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were ana-
lyzed for each experiment. Toxicity of the samples was expressed as the percentage of
the positive control calculated as

\[ \text{Toxicity (\%)} = \frac{N_t}{N_c} \times 100 \]  
(Eq.9)

\( N_t \) is the absorbance of cells treated with sample and \( N_c \) is the absorbance of the cells
 treated with 1% Triton-X 100.
Chapter 2

Materials & Methods

2.5 Blood Compatibility Studies

Blood compatibility of CS NPs, CS PTH NPs, PEG-CS-PTH NPs and TCS NPs with PTH 1-34 ranging from 30-10 µg/mL was evaluated via the hemolysis assay and the blood clotting effects were evaluated by the Prothrombin time (PT) and activated partial thromboplastin time (APTT), Triton-X 100 (1%) and saline were the positive and negative controls respectively. Each concentration was evaluated in triplicate.

2.5.1 Hemolysis assay

Fresh human blood was used in this study. 1.5 mL acid citrate dextrose (ACD) was added to 10 mL fresh blood. To 1 mL of this blood sample, 100 µL of samples were added and incubated for 2 h with shaking in an incubator chamber at 37 °C. The samples were spin down at 4500 rpm for 10 min to obtain the plasma (Plasma would be red in colour if haemolysis happened). The plasma were collected (100 µL plasma+ 1 mL Na₂CO₃ (0.01%)). The OD values were read at 450, 380 and 415 nm (BioTek Power Wave XS). The plasma haemoglobin can be found out according to the following protocol as explained in the literatures.

\[
Plasma\ Hb = \frac{[2A_{415}-(A_{380}+A_{450})\times1000\times\text{Dilution factor}]}{E \times 1.655} \quad (\text{Eq.10})
\]

Here A_{415} corresponds to the absorption band of hemoglobin, A_{380} and A_{450} are the correction factors applied for uroporphyrin absorption falling in the same wavelength range. ‘E’ corresponds to the molar absorptivity value of Hb (79.46), 1.655 corresponds to the correction factor accounting for plasma sample turbidity. The plasma and total Hb values can be used for calculating the percentage hemolysis.²³²,²³³

\[
\text{Hemolysis (\%)} = \frac{\text{Plasma hemoglobin content in test}}{\text{Total hemoglobin content}} \times 100 \quad (\text{Eq.11})
\]
2.5.2 Prothrombin time (PT) and activated partial thromboplastin time (aPTT)

The coagulating effect of drug loaded NPs on plasma was determined by plasma coagulation assay, which is performed by two tests, i.e., prothrombin time (PT) and activated partial thromboplastin time (aPTT). Fresh blood was collected in ACD containing tubes. It was then centrifuged at 4000 rpm at 25°C for 15 minutes (Ependorf centrifuge 5810 R) to obtain the platelet-poor plasma (PPP). The experimental protocol involves the incubation of 0.9ml of PPP with 0.1ml of different concentrations of NPs samples (0.2 to 2 mg/ml) for 20-30 minutes at 37°C. 100µl of prothrombin reagent (Diagnostica stago, France) was added to 50 µl of treated plasma and the time taken for the plasma to coagulate, i.e., PT was measured. In case of activated partial thromboplastin time (aPTT) measurement, 50 µl of aPTT activator (Diagnostica stago, France) was added to 50 µl of plasma and incubated for 180 seconds before the addition of 50 µl of 0.025 M CaCl₂. After CaCl₂ treatment, the time taken by plasma to coagulate was measured as aPTT. The experiments were carried out using the saline treated PPP as the negative controls and it was done in triplicates to confirm the data. The experiment was done in triplicates and saline treated platelet poor plasma served as negative control.²³⁴

2.6 Biological assays

50,000 cells per well of 6 well plates was seeded with HOB and on attaining 70% confluence were treated in triplicates with 20 µg/mL PTH 1-34 containing CS-PTH NPs, PEG-CS-PTH NPs, and TCS- PTH NPs and bare CS- NPs and TCS- NPs for a period of 7 days. Cells untreated were kept as controls. Thereafter the following biological assays were performed.

2.6.1 In vitro Alkaline Phosphatase Activity

Bone alkaline phosphatase (ALP) is known to be an important biochemical marker of bone formation. ALP synthesized and released from an osteoblast cell is indicative of the anabolic effect of the PTH 1-34 on the osteoblast.
Chapter 2

Materials & Methods

The media was removed and the cells were treated with 1% Triton-X 100 and incubated for 2 h. Thereafter sonicated for 15 min and centrifuged to obtain the supernatant, which is the transferred to a 96 well plate to be treated with ALP substrate paranitrophenol-phosphate and glycine buffer. After incubating for 30 min in dark stop solution is added (5 M NaOH) and the absorbance was taken at 405 and 490 nm (BioTek Power Wave XS) and concentrations are determined from a standard calibration curve.\textsuperscript{235,236}

2.6.2 Calcium calorimetric assay

$\text{Ca}^{2+}$ sequestration and release into and out of the cytoplasm, functions as a signal for many cellular processes. Depending on the level of PTH 1-34 in the extracellular medium, $\text{Ca}^{2+}$ may be effluxed or influxed by the HOB. The cell lysate was analyzed using the calcium calorimetric assay kit for quantifying the intracellular calcium. Depending on the level of PTH 1-34 in the extracellular medium, $\text{Ca}^{2+}$ may be effluxed or influxed by the HOB. The media used for the experiment on HOB cells was collected and analysed for calcium using the calcium calorimetric assay kit (Biovision).\textsuperscript{236,237}

2.6.3 Human Osteocalcin assay

The detection of extracellular levels of osteocalcin produced and released by HOB cells is valuable for the study of bone metabolism. The osteocalcin ELISA is a solid phase Enzyme Linked Immuno Sorbet Assay (Immunodiagnostic systems USA) performed on microplates. The media used for the experiments is collected and analysed for osteocalcin using the specific ELISA kit. The detection of extracellular levels of osteocalcin produced and released by HOB is valuable for the study of osteoblast metabolism.\textsuperscript{238,239}
2.6.4 Cyclic adenosine monophosphate second messenger detection assay
(cAMP assay)

Adenosine 3’, 5’-cyclic monophosphate (cAMP) is an important “second messenger” involved in many physiological processes. cAMP activity assay kit (PromoKine) utilizes a recombinant protein G coated 96-well plate to anchor cAMP to the plate. The media from the cell culture wells was removed and the cells were lysed with 0.1M HCl, centrifuged and the supernatant was lyophilised. These samples were assayed following the instructions in the kit and the absorbance was read in a spectrophotometer at 405nm (BioTek Power Wave XS) and the cAMP concentrations are determined from a standard calibration curve. The results were compared to the controls.240,241

2.7 In vivo evaluation following oral administration

In vivo experiments were carried out following the approved protocols for the pharmacokinetic evaluation of oral uptake of PTH 1-34 from CS-PTH NPs. PEG-CS-PTH NPs and TCS PTH NPs. The use of female Sprague dawley rats 242 for the in vivo experiments was approved by the institutional ethical committee for animal studies (IAEC/2011/1/3).

Table 2.7: Details of the animals used for the experiments

<table>
<thead>
<tr>
<th>Species</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
</tr>
<tr>
<td>Weight</td>
<td>200–250 g</td>
</tr>
<tr>
<td>Age</td>
<td>9-12 weeks (Adult stage)</td>
</tr>
<tr>
<td>Number of groups</td>
<td>5 (n=3)</td>
</tr>
</tbody>
</table>
Chapter 2  

Materials & Methods

2.7.1 Dose

The dose of PTH 1-34 and the PTH 1-34 loaded NPs was calculated based on its current clinical administrations and preclinical animal studies. The range of PTH 1-34 used in the earlier experiments range from 20-200 µg/animal/day which in certain experiments have gone upto 600 µg/animal/day and the current clinical administration of PTH 1-34 (Teriparatide Forteo) is 20µg/day. Based on these data the single dose of peptide used in the following in vivo experiment was fixed as 20ug/animal.

20µg/mL PTH 1-34 loaded NPs was orally administered through oral gavage (1mL) in each set of animal; n=3 (animals were subjected to an overnight fast). Blood was drawn (~150 µl) by cannulation at different time intervals from retro orbital sinus. The serum separated was immediately analyzed using Human PTH 1-34 ELISA kit. The results were compared with control PTH 1-34 and CS-PTH 1-34 NPs administered orally. The cross reactivity of endogenous rat PTH 1-34 was also measured.

2.7.2 Pharmacokinetic analyses

The following pharmacokinetic parameters were calculated for PTH 1-34 using Pharmacokinetics version 2.

- **AUC<sub>0-t</sub>**: The area under the plasma concentration versus time curve, from time zero to the last measurable concentration, as calculated by the linear trapezoidal method.
- **AUC<sub>0-12</sub>**: The area under the plasma concentration versus time curve, from time zero to 12 h
- **AUC<sub>0-∞</sub>**: The area under the plasma concentration versus time curve, from time zero to infinity. AUC<sub>0-∞</sub> is calculated as the sum of AUC<sub>0-t</sub> plus the ratio of the last measurable plasma concentration to the elimination rate constant.
- **C<sub>max</sub>**: Maximum measured plasma concentration over the time span specified.
- **T<sub>max</sub>**: Time of the maximum measured plasma concentration. If the maximum value occurs at more than 1 time point, T<sub>max</sub> is defined as the first time point with this value.
• **K<sub>e1</sub>:** Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve. The parameter will be calculated by linear least-square regression analysis using the maximum number of points in the terminal log-linear phase (e.g. three or more non-zero plasma concentrations).

• **T<sub>1/2</sub>:** The apparent first-order terminal elimination half-life will be calculated as 0.693/K<sub>e1</sub>.

### 2.8 In vivo Near Infrared Imaging

*In vivo* imaging using near infrared imaging system of rats orally administered with CS-PTH NPs, PEG-CS-PTH NPs and TCS PTH 1-34 NPs conjugated to indocyanin green (ICG) helped to track the path and residence time of formulation in the gastrointestinal tract.\(^ {248-250}\)

#### 2.8.1 Preparation of ICG conjugated PTH 1-34 loaded CS, PEG-CS and TCS NPs

The procedure followed for conjugation of the NIR dye indocyanin green is as explained in 2.2.2, 2.2.3 and 2.2.5 where ICG (0.2mg/mL) is added along with PTH 1-34 prior to incubation on ice. The prepared ICG conjugated peptide loaded nanoformulations were independently orally administrerd in Sprague dawley rats and imaged from the 5<sup>th</sup> minute of administration to the 48<sup>th</sup> hour using a Near Infrared Imaging system (Kodak *in vivo* multispectral FX Pro).\(^ {251-253}\)

### 2.9 Statistical analysis

All the above experiments were conducted three times independently with triplicates and the results were expressed as mean ± standard deviation. The standard deviation values are indicated as error bars in the corresponding graphs. Student’s T-test was performed to find the statistical significance of the values. A probability of p< 0.05 was considered to be statistically significant.\(^ {254-256}\)