CHAPTER 1

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Osteoporosis is a condition in which, the bone mineral density is reduced, its micro architecture is deteriorated, and the amount and variety of proteins in it are altered. Different ways of treating the condition, such as with bisphosphonates decreases the risk of future fractures in those who have already sustained one due to osteoporosis. 1,2 Whereas, estrogen replacement therapy is only recommended in women with osteoporosis who also have menopausal symptoms. 3 On the other hand recombinant human parathyroid hormone (PTH 1-34) is administered subcutaneously on a daily basis in osteoporotic women. It increases bone mass and reduces vertebral and non vertebral fractures up to 65%. 4-6 But, painful daily injections confer poor patient compliance. Oral route is the most convenient route for the systemic delivery of pharmaceuticals and offers the advantages of self-administration with a high degree of patient acceptability. Oral administration of a tablet, capsule, suspension etc would be less expensive than injection if equivalent bioavailability could be achieved. 7-9 The gastrointestinal tract stretching from the oral cavity to the rectum offers a wide range of specialized epithelia that peptides can traverse. However, attempts to deliver peptides orally have not been widely successful. Bioavailability via this route is poor because peptides are susceptible to hydrolysis and modification at gastric pH levels, and they can be degraded by proteolytic enzymes in the gastrointestinal tract. 8-10 The main barrier in oral delivery is the rich mucus layer lining the GI tract which challenges the permeation of drugs or drug loaded vehicles. Hepatic first pass loss is yet another factor limiting bioavailability. 11-13
Oral delivery technology platform attempts to improve the bioavailability of peptide drugs by changing the physicochemical properties of the molecules. This can be achieved by adapting drug delivery systems that are mucoadhesives which can prolong release and the use of absorption enhancers such as salicylates, surfactants or fatty acids. Biodegradable polymeric nano and micro particles for matrix embedded controlled release of peptides is facilitated by hydrolysis or enzymatic digestion in the body. Uptake of the peptide or its loaded vehicles into the body can be paracellular, transcellular, receptor-mediated or via the M-cells of Peyer’s patches. The polymeric matrix also ensures the protection and retention of the bioactivity of the entrapped peptide.

The emphasis of this research work is on development of a biodegradable and biocompatible polymeric PEGylated and Thiolated CS NP system which can entrap and orally deliver PTH 1-34. Accomplishing this objective will help to overcome the challenges faced by the bare peptide in the GI tract and make a patient compliant oral formulation over the conventional injectable PTH 1-34.

1.1 REVIEW OF LITERATURE

1.1.1 Osteoporosis

The World Health Organization defines osteoporosis on the basis of the number of standard deviations below the peak adult bone mass. Bone mineral density (BMD) that is less than 2.5 standard deviations (SD) below the mean for young people may be considered osteoporosis.

1.1.1.1 Pathophysiology

Osteoporosis, (Fig.1.1) results due to a mismatch between bone resorption and bone formation. Bones are solid structures, but they are constantly being remodelled. Bone remodeling provides a mechanism for self-repair and adaptation to stress to the bone. This process has three primary functions i.e. to repair micro damage within the
skeleton, maintain skeletal strength, and supply calcium from the skeleton to maintain serum calcium. \textsuperscript{26,27}

Table 1.1. World Health Organization criteria for the diagnosis of osteoporosis based on bone mass or density category criteria

<table>
<thead>
<tr>
<th>Category</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>BMD 1 SD below average peak bone mass or higher</td>
</tr>
<tr>
<td>Osteopenia</td>
<td>BMD &lt;1 SD but &gt;2.5 SD below average peak bone mass</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>BMD &lt;2.5 SD below average peak bone mass</td>
</tr>
<tr>
<td>Severe osteoporosis</td>
<td>BMD &lt;2.5 SD below average peak bone mass with fragility fracture</td>
</tr>
</tbody>
</table>

Patients with osteoporosis may not know that they have the disease until their bones become so weak that a sudden strain, bump, or fall causes a hip fracture or a vertebra to collapse.\textsuperscript{27–30}
Bone remodeling consists of the removal of bone by the osteoclasts, followed by the synthesis and mineralization of new bone matrix by the osteoblasts within the cavity created. This is a coordinated process orchestrated by basic multicellular units, which include groups of osteoblasts, osteoclasts and osteocytes along with supporting vascular, nervous, and connective tissue. Osteoclast degrades bone by attaching to a bone surface and secreting acids and enzymes into the mineralized bone surface.\textsuperscript{31–34} Osteocytes are mature osteoblasts that become trapped within calcified bone. They play an important role in the osteogenic response to mechanical stimuli, and senses physical strains and initiate an appropriate modeling or remodeling response via the production of a cascade of chemical messengers.\textsuperscript{35–38} Under normal circumstances the sequence is always that of resorption followed by formation, and the amounts of bone resorbed and formed within individual remodeling units are closely balanced. With aging, less bone is formed by osteoblasts than is removed by osteoclasts.\textsuperscript{39,40} Estrogen deficiency increases osteoclast life span but decreases osteoblast life span, resulting in an increased rate of bone remodelling and a decreased rate of bone replacement by osteoblasts during menopause, which in turn increases the risk of osteoporosis in postmenopausal patients.\textsuperscript{41–44} The systemic calcium regulating hormones that influence bone remodelling include parathyroid hormone (PTH), calcitonin and vitamin D metabolites. PTH is a
hormone synthesized by the chief cells of the parathyroid gland and it has effects on both osteoclasts and osteoblasts (Fig.1.2).

![Bone Remodeling Cycle](http://genes.ccm.edu/releases/2005/feb05/img/bone.jpg)

**Figure 1.2:** The anabolic action of PTH 1-34 during bone remodeling

In addition, physiologically PTH is the most important regulator of extracellular calcium concentration and therefore some of its effects on bone are indirect.\(^45,46\) C cells of the thyroid gland produce calcitonin.\(^47-51\) It directly inhibits osteoclastic bone resorption and does not increase the deposition of calcium in the skeleton unless bone turnover is high. Growth hormone plays an important part in skeletal growth, increasing bone turnover, with a net increase in bone mass and in periosteal appositional growth. Sex hormones have marked effects on the skeleton and deficiencies are associated with low bone mass in both children and adults.\(^52,53\)

**1.1.1.2. Treatment and prevention of osteoporosis**

Risk of osteoporotic fracture may be reducing by various non pharmacologic and pharmacologic interventions. These interventions slow or stop bone loss, maintain bone strength, increase bone strength, or minimize or eliminate factors that may result
in fractures. Non pharmacologic measures are recommended for the population as a whole; pharmacologic interventions are usually reserved for patients at increased risk of fractures.\textsuperscript{54}

\textbf{(i) Non-pharmacologic interventions}

There are numerous non pharmacologic interventions that can be implemented to reduce the risk of bone loss and fracture.\textsuperscript{55,56} Non-pharmacologic interventions to reduce fracture risk are the following:

- Weight-bearing exercises and strengthening the muscles
- Prevention of falls
- Avoidance of excessive alcohol intake and tobacco
- Balanced diet and adequate calcium and vitamin D intake.

\textbf{(ii) Pharmacologic interventions}

The drugs which are used to treat osteoporosis increase BMD by inhibiting bone resorption while allowing bone formation to fill in pre-existing resorption cavities. These drugs prevent bone loss and, during a 3 to 5 year period, may increase BMD by up to 10\% (estrogen, bisphosphonates, calcitonin, and selective estrogen receptor modulators). However, there is a potential of optimizing osteoporosis therapy by using bone anabolic agents that directly stimulate bone formation (intermittent injections of parathyroid hormone, sodium fluoride, and strontium ranelate).\textsuperscript{57} Long-term hormone replacement therapy has been reported to be associated with increased risk of cancer in estrogen target tissues including the mammary gland and the uterus. The other available antiresorptive agents such as calcitonin and bisphosphonates, too, are not totally devoid of health hazards or are unacceptable due to their parenteral route of administration or high cost. Strontium ranelate reported to possess both antiresorptive and osteogenic properties, is poorly absorbed and increases incidence of venous thromboembolism. PTH 1-34 stimulates osteoblast function, increases gastrointestinal calcium ab-
sorption, increases renal tubular reabsorption of calcium, enhances bone turnover by initiating greater bone formation and poses only mild side effects which does not lead to the discontinuation of the drug.

1.1.2 Choice of therapeutic agent - Recombinant Human Parathyroid hormone

FDA has approved PTH 1-34 as an anabolic agent for osteoporosis. It is made of 34 amino acids and has a molecular weight of 4117 Dalton. Jin et al. studied the structure and the possible mechanism of PTH 1-34 specific receptor interaction. PTH 1-34, acts on cells via a common PTH/PTHRP receptor. They analysed and concluded that the extended helical conformation of PTH1-34 is the likely bioactive conformation (Fig.1.3). Friedl and Sheehan et al. have also proved that PTH 1-34 induces anabolic responses in bone-forming cells which stimulates bone matrix production and suppresses osteoblast apoptosis. Treatment for hypoparathyroidism using PTH 1-34 has been under recent investigation by Winer et al., where the intermittent pattern is not the criterion for treatment rather a continuous circulating level of PTH 1-34 is in demand.

1.1.2.1 Current treatment

The 20µg Teriparatide Forteo (Human recombinant PTH 1-34) is injected subcutaneously to achieve a peak peptide concentration of 100-150pg/mL (t½-1h), which then lowers to undetectable levels within 1-3h. The peptide is thus administered daily once, for a treatment span of 2 years. This intermittent dose and pattern of rise and fall has been tested and proved to enable PTH 1-34 to act as an anabolic hormone.
1.1.2 Drawback of the current treatment

- Poor patient compliance due to painful daily injections.
- Self administration of the injections in elderly people is difficult.

PTH 1-34 being vulnerable to enzymatic digestion in the GI tract restricts oral administration of the bare peptide.

1.1.3 Oral peptide delivery

The first chemical synthesis of a therapeutic peptide was that of oxytocin in 1953 and recombinant human insulin in 1982. Over the years several therapeutic peptides have been commercially synthesized and marketed for treatment in humans. Few of the subcutaneously administered FDA approved peptides are enfuvirtide for treatment of
HIV infection; oxytocin for inducing uterine contractions at labor; exenatide for glucoregulatory effects\textsuperscript{71,72} and PTH 1-34 for treatment of osteoporosis.\textsuperscript{58,73–75}

The current subcutaneous injections of PTH 1-34 holds poor patient compliance and has urged the need for more compliant oral delivery vehicles for the peptide without compromising its biological functions. Researchers are advancing in formulating oral therapeutics of PTH 1-34. The use of absorption enhancers such as 8-(N-2-hydroxy-5-chlorobenzoyl)-amino-caprylic acid (5-CNAC) and PLGA microparticles for oral administration has been documented whereas the use of polymeric nanoformulation have not been reported.\textsuperscript{76,77} Oral drug delivery is the most common method of drug administration with high levels of patient acceptance.\textsuperscript{78–80} Over the last few decades, various natural polymers have been applied for oral insulin delivery, using advanced nanotechnology.\textsuperscript{81–84} Mukhopadhyay \textit{et al.}, and Sarmento \textit{et al.}, have investigated the use of polymers from natural as well as synthetic sources as oral insulin delivery vehicles. The biopolymer chitosan (CS) has been widely studied in oral insulin delivery due to its favorable properties such as biocompatibility, biodegradability, non-immunogenicity and non-toxicity.\textsuperscript{85,86} Sung \textit{et al.}, and Wong \textit{et al.}, worked on the oral delivery of peptides and proteins and the challenges faced by these biomolecules in the GI tract. Chemical barriers such as the acidic gastric pH and the presence of proteolytic enzymes in the stomach and intestine limit the effective absorption of external proteins and peptides within the GI tract. Absorption can even be physically hindered by the absorption barrier consisting of a layer of columnar epithelial cells joined at the apical surface by a tight junction complex. The presence of negative charges in the junction complex leads to segregation of the apical layer from the basolateral compartment of the epithelial cells, making the intestinal environment selective for particles based on size and charge. Nanoparticles (NPs) are able to overcome these barriers and deliver proteins and peptides. CS and modified CS NPs are highly promising agents for oral peptide and protein delivery.\textsuperscript{87,88} The presence of poly ethylene glycol (PEG), whether alone or grafted to CS, improved the stability of the nanocapsules in the gastrointestinal fluids. Using the Caco-2 model cell line it was observed that the PEGylation of CS re-
duced the cytotoxicity of the nanocapsules. In addition, these nanocapsules did not cause a significant change in the transepithelial resistance of the monolayer. Prego et al., experimentally proved in vivo, the capacity of CS-PEG nanocapsules to enhance and prolong the intestinal absorption of salmon calcitonin. Despite the preference of oral delivery, administration of therapeutic proteins have been extremely difficult. Increasing the bioavailability of oral protein drugs to the therapeutically acceptable level is still a challenging goal. Poor membrane permeability, high molecular weight, and enzymatic degradation of protein drugs have remained an unsolved issues. Among diverse strategies, nanotechnology has provided a glimpse of hope in oral delivery of protein drugs. NPs have advantages, such as small size, high surface area, and modification using functional groups for high capacity or selectivity. NPs with peptidic ligands are especially worthy of notice because they can be used for specific targeting in the GI tract.

1.1.3.1 Recent advances in oral delivery of PTH 1-34

Leone Bay et al., investigated the oral absorption of PTH in rats and monkeys facilitated by the novel delivery agent, N-[8-(2-hydroxy-4-methoxy)bensoyl]amino caprylic acid (4-MOAC). Monkeys were administered an aqueous solution containing 4-MOAC and PTH and mean peak serum PTH concentrations of about 3000 pg/mL were obtained. The relative bioavailability of oral PTH was 2.1% relative to subcutaneous administration. The biological activity of the orally-delivered PTH was further evaluated in a rat model of osteoporosis which showed that the bone formed following oral PTH/4-MOAC administration was comparable to that formed following PTH injections. In the study conducted by Hammerle et al., they demonstrated the potential therapeutically relevant PTH 1-34 systemic exposure levels, after oral administration of PTH1-34 formulated with the absorption enhancer 5-CNAC. High doses of 2.5 and 5 mg of oral PTH-34 was required to achieved exposure levels closest to that achieved by 20 μg PTH 1-34 administered subcutaneously. The loss of peptide and the cost effectiveness of such a formulation is therefore questioned.
1.1.4 Nanotechnology for the oral delivery of peptides

Polymeric NPs, which could be nanospheres or nanocapsules, consist of the drug dispersed in an amorphous form within a biodegradable polymer matrix. The biodegradability of the matrix helps to avoid its accumulation on repeated dosing.

NPs are efficient as sustained or controlled drug release systems, its advantages are mentioned below \(^{90,92,93}\):

1. Maintenance of optimum therapeutic drug concentration in the blood with minimum fluctuation.
2. Predictable and reproducible release rates for extended duration.
3. Enhancement of activity duration for short half-life drugs
4. Elimination of side effects, frequent dosing and wastage of drug
5. Optimize the therapy and better patient compliance.
6. Economy: In comparisons with immediate dosage products the cost of treatment over an extended time period may less and also a decrease in nursing time and hospitalization.

1.1.4.1 Diffusion controlled release systems

In these types of systems, the rate of drug release is controlled by diffusion of the polymer in aqueous medium which diffuse the dissolved drug from a gel layer of polymeric net-work.\(^ {94}\) The length of diffusion layer was maintained by using swellable or non-swellable or insoluble matrix material such as polyvinyl chloride, beeswax, sodium alginate, CS etc. The drug release from matrix system occurs by hydration or swelling of the polymeric membrane forms thick hydrodynamic gel layer followed by diffusion in a constant manner.\(^ {95}\) Dissolution rate has been first described by Noyes and Witney in 1897. Factors that affect dissolution are temperature, surface active substances, crystal shape, pH and particle size. According to the Nernst Brunner equation dissolution rate is proportional to the surface area of the active drug substance in contact with the dissolution medium.
\[ \frac{dw}{dt} = \frac{(D/h)X S(C_s - C_t)}{h} \]  

\( \frac{dw}{dt} \): Dissolution rate (mg/s)  
\( D \): Diffusion coefficient (cm/s)  
\( h \): Effective diffusion layer thickness (cm)  
\( S \): Effective surface area of the solid drug (cm\(^2\))  
\( C_s \): Saturated concentration (mg/mL)  
\( C_t \): Concentration of solute at time \( t \) (mg/mL)  
\( C_s - C_t \): Concentration gradient (mg/mL)

It can be deduced from the formula that the changing particle size of the active drug substance, it is possible to change the specific surface area and the dissolution rate of the active drug substance in body fluids. \(^96,97\)

### 1.1.4.2 Formulation principles of polymeric NP

Polymeric NPs can be prepared by various methods. \(^98,99,100\) NPs have been mostly prepared by three methods:

1. Dispersion of preformed polymers  
2. Polymerization of monomers  
3. Ionic gelation and coacervation of hydrophilic polymers

NPs constituted of synthetic polymers are usually prepared by dispersion of preformed polymers. Formulation of NPs with natural polymers is performed by ionic gelation (CS) or by coacervation. These are mild methods presenting the advantage to produce organic solvent free formulations.

The main criteria in choosing polymer for drug delivery are:

1. Size of the NP required  
2. Inherent properties of the drug which is its aqueous solubility and stability of the drug  
3. Surface characterization such as charge and permeability.
4. Degree of biodegradability biocompatibility and toxicity
5. Desired drug release profile
6. Antigenicity of the final product

1.1.5 Choice of polymer

The nature of polymers constituting the formulation significantly influences the NP size and their release profile. Although all natural polymers generally provide a relatively quick drug release synthetic polymers enable extended drug release over periods from days to several weeks. Polymeric materials such as poly (lactic acid), poly (glycolic acid), polycaprolactone, polysaccharides (particularly CS), poly (acrylic acid) family, proteins or polypeptides (such as gelatin), have been applied for the preparation of nanoparticles. Among them, polysaccharides are the most popular polymeric materials to prepare NPs for drug delivery. In nature, polysaccharides have various resources from algal origin (e.g., alginate), plant origin (e.g., pectin, guar gum), microbial origin (e.g., dextran, xanthan gum), and animal origin (CS, chondroitin). Polysaccharides have a large number of reactive groups, a wide range of molecular weight (MW), varying chemical composition, which contribute to their diversity in structure and in property. Polysaccharides can be divided into polyelectrolytes and nonpolyelectrolytes, the former can be further divided into positively charged polysaccharides (CS) and negatively charged polysaccharides (alginate, heparin, hyaluronic acid, pectin, etc.).

1.1.5.1 Chitosan

The European Pharmacopoeia has included CS since 2002 as a novel excipient in drug delivery systems due to its highlight features of easy enzymatic biodegradability, non toxicity and biocompatibility. CS is insoluble at alkaline and neutral pH whereas at acidic pH the amino groups get protonated that promotes easy solubility.
Janes and Alonso, Jintapattanakit et al., and Tiyaboonchai et al., have demonstrated that CS enhances the paracellular route of absorption, which is important for the transport of hydrophilic compounds such as therapeutic peptides across the membrane.\textsuperscript{84,110,111} The mechanisms underlying this permeation enhancing effect was experimentally proved by Bernkop-Schnürer et al., and others based on the positive charge of CS, which interact with the cell membrane resulting in the structural reorganization of tight junction-associated proteins.\textsuperscript{108,112,113} In the presence of the mucus layer, however, this permeation enhancing effect is comparatively lower, as CS cannot reach the epithelium due to size limited diffusion and/or competitive charge interactions with mucins.\textsuperscript{81,108,114–116} The permeation enhancing effect of CS can be strongly improved by the immobilization of thiol groups.\textsuperscript{114,117–122}
1.1.5.1.1 Methods of Preparing CS NPs

Different methods have been used to prepare CS particulate systems. Selection of any of the methods depends upon factors such as particle size requirement, thermal and chemical stability of the active agent, reproducibility of the release kinetic profiles, stability of the final product and residual toxicity associated with the final product. However, selection of any of these methods depends upon the nature of the active molecule as well as the type of the delivery device.

(i) Emulsion cross-linking

This method exploits the reactive functional amine group of CS to cross-link with the available reactive groups of the cross-linking agent. In this method, water-in-oil (w/o) emulsion is prepared by emulsifying the CS aqueous solution in the oil phase. A suitable surfactant is used to stabilize the aqueous droplets. Thereafter stable emulsion is cross-linked by using an appropriate cross-linking agent to harden the droplets. Microspheres are filtered and washed repeatedly with alcohol and then dried. This method is helpful in controlling the size of the particles by controlling the size of aqueous droplets. However, the particle size of final product is dependent on the extent of cross-linking agent used while hardening along with the speed of stirring.\(^{123-125}\)

(ii) Coacervation/precipitation

The physicochemical property of CS is utilized in this method since it is insoluble in alkaline pH medium, but precipitates/coacervates when it comes in contact with alkaline solution. CS solution is blown into an alkali solution like sodium hydroxide, methanol or ethanediamine using a compressed air nozzle to form coacervate droplets. Separation and purification of particles are performed by filtration/centrifugation followed by successive washing with hot and cold water. Variation in compressed air pressure or spray-nozzle diameter can be done to control the size of the particles. Mao et al., has used this technique to prepare CS-DNA NPs.\(^{126,127}\)
(iii) Spray-drying

Spray-drying is a popular method to produce powders, granules or agglomerates from the mixture of drug and excipient solutions as well as suspensions. The method is based on drying of atomized droplets in a stream of hot air. Briefly, CS is dissolved in aqueous acetic acid solution; drug is then dissolved or dispersed in the solution followed by the addition of a suitable cross-linking agent. This solution or dispersion is then atomized in a stream of hot air which leads to the formation of small droplets, from which solvent evaporates instantaneously leading to the formation of free flowing particles. 128, 129

(iv) Emulsion-droplet coalescence method

This emulsion-droplet coalescence method, developed by Tokumitsu and associates, combines the principles of both emulsion cross-linking and precipitation. 130 Instead of cross-linking the stable droplets, precipitation is induced by allowing coalescence of CS droplets with sodium hydroxide droplets. Two separate emulsions are prepared, one containing aqueous solution of CS along with drug is produced in liquid paraffin oil, and another containing CS aqueous solution of sodium hydroxide is produced in the same manner. The emulsions are mixed under high-speed stirring; droplets of each emulsion collide at random and coalesce, forming small sized particles that precipitate. The particle size increased with the decrease in degree of deacetylation of CS which in turn decreased the drug content. Completely deacetylated CS has been observed to produce particle size of 452 nm with 45% drug loading. 131

(v) Ionic gelation

The reversible physical cross-linking by electrostatic interaction, instead of chemical cross-linking, decreases the potential toxicity impact of reagents and other undesirable effects. For example, the electrostatic interaction of polyanion, tripolyphosphate (TPP) with the cationic CS. 132–134 For ionic gelation, CS is dissolved in aqueous acidic
solution which quaternizes the CS amino groups making it soluble; this solution is then added drop wise under constant stirring to polyanionic TPP solution. The complexation between oppositely charged species causes the CS to undergo ionic gelation and precipitate as spherical particles. Various formulations of CS NPs produced by the ionic gelation of TPP and CS were studied by Xu and Du. 132

(iv) *Reverse micellar method*

Reverse micelles are thermodynamically stable liquid mixtures of water, oil and surfactant. The NPs prepared by conventional emulsion polymerization methods are usually large (>200 nm), with a broad size range. Ultrafine polymeric NPs with narrow size distribution could be achieved by using reverse micellar medium. A rapid dynamic equilibrium maintains the size, polydispersity and thermodynamic stability of these droplets. To prepare reverse micelles, the surfactant is dissolved in an organic solvent followed by the addition of CS and drug under constant vortexing into which a cross-linking agent is added with constant stirring. 135–137

From all these NP preparation methods, ionic gelation has been adapted in this work. Ionic gelation, enables biopolymers to encapsulate large number of micro and macro therapeutic molecules in their hydrogel meshwork structure. The utilization of expensive and toxic organic solvents in the encapsulation process has been drastically reduced due to evolution of ionic gelation. Hence it provides an eco friendly pharmaceutical product development process in the preparation of NPs. 138,139

1.1.5.2 Modification of CS

(i)*Thiolated CS*

Mucoadhesive thiolated CS (TCS) is obtained by immobilizing thiol groups on the primary amino group at the 2-position of the glucosamine subunits of CS (Fig.1.5). Sulfhydryl bearing agents covalently attach to this primary amino group via the for-
mation of amide bonds. The carboxylic acid group of thioglycolic acid reacts with the primary amino group of CS for the formation of this amide bond.\textsuperscript{119,140} The improved mucoadhesive properties of TCS is explained by the formation of covalent bonds between thiol groups of the polymer and cysteine rich sub domains of glycoprotein in the mucus layer.\textsuperscript{117,141} The longer residence time of the mucoadhesive TCS NPs at the absorption site is believed to contribute to increased absorption rate of the incorporated drug which supports non-invasive drug delivery.\textsuperscript{142–144} TCS NPs have a high surface area to volume ratio which is a driving force for faster diffusion of drug loaded NPs from its site of administration.\textsuperscript{148} It was proved by Banerjee et al., that the renal clearance of NPs of size less than 100-150nm is feeble and can outwit the macrophages in the body to be in circulation for long.\textsuperscript{149} Hence TCS NPs are suitable for nasal and oral delivery of peptides and proteins.

Krauland et al., developed a microparticulate delivery system based on a TCS conjugate for the nasal application of insulin.\textsuperscript{145} Gioconda et al., synthesized CS-6-mercaptanoticnic acid, a TCS nanoparticulate formulation with strong mucoadhesive properties and tested its potential for oral insulin delivery. He observed that the area under the curve (AUC) of insulin after oral administration in rats of TCS NPs was 4-fold improved compared to unmodified CS NPs.\textsuperscript{146} The ability of TCS NPs to inhibit P-glycoprotein was proved by Palmberger et al., in the oral delivery of efflux pump substrate acyclovir.\textsuperscript{147}

Based on all these experimental proofs, PTH 1-34 can be successfully entrapped and administered orally using TCS NPs. The charge difference between the PTH 1-34 and the TCS at the working pH being opposite will favors strong electrostatic interaction to efficiently entrap the peptide and form , TCS-PTH NPs.\textsuperscript{150}
(ii) **PEGylated CS**

CS-PTH NPs due to the exposed amine groups may favor higher dissolution in the gastric pH, and could also result in hemolysis if the CS concentration is increased while administering a dose, due to which using this system for *in vivo* oral delivery is limited. To overcome this drawback, modifying the surface with PEG was the appropriate solution ([Fig.1.6](#)). Hu *et al.* and Malhotra *et al.* have reported that PEGylation of NP increases stability in gastric and biological fluids which can prolong the particles circulation and retard elimination.\(^{151,152}\) PEGylation can also facilitate the transport of bioactive macromolecules across the intestinal epithelium and improve drug bioavailability *in vivo*.\(^{153,154}\) The absorption-molecular weight profiles examined using different-sized PEGs were different between the small and large intestines; the large-intestinal absorption of PEGs with molecular weights larger than 300 was poor, while PEGs with molecular weights up to 600 were relatively well absorbed in the small in-
testine.\textsuperscript{89,155,156} So to ensure maximum absorption low molecular weight PEG 200 is ideal.

![Figure 1.6: Structure of PEGylated CS](image)

1.1.6 Influence of the physiological and morphological feature of the GI tract on the absorption of drugs.

The primary functions of gastrointestinal tract (GI) is secretion, digestion and absorption.\textsuperscript{157} The GI barrier allows, to passing rapidly into systemic circulation of most nutrients and vitamins by passive diffusion; but controls entry of higher molecular weight medicaments and toxins. The oral administration of these materials, passes into GI and gets distributed into different organs or tissues, followed by absorption and eliminated from the body. After oral ingestion the materials are stored in the stomach and mixed with GI-fluid, converted into liquid mass and then passed gradually into the upper small intestine. Thus, the GI represents a primary barrier for oral absorption of
drugs. The GI consists of three major anatomical regions: the stomach, the small intestine which includes the duodenum, jejunum, ileum, and the large intestine.  

![Diagram of digestive system](image)

**Figure 1.7: Diagrammatic representation of digestive system of rat and human**

The drug absorption at the site of action depends on particular properties of GI-fluid with respect to pH, enzymes, electrolytes, fluidity and surface features. The stomach’s acidic pH 1-3 is due to secretion of hydrochloric acid, which favors absorption of acidic drugs. The small intestine, with its enormous absorptive area of between 200 and 600 m is invariably the principal site of drug absorption. In contrast, the perfusion rate of blood to the small intestine is 6 to 10 times that of stomach and the pH range of 5 to 7.5 is most favorable for sustained or controlled release dosage forms. The small intestine provides certain significant properties for drug absorption such as high permeability, slow peristaltic movement and long transit time. Large intestine
having small absorptive area usually plays very little role in the absorption of drugs. Some of the other factors such as drug solubility and dissolution rate, particle size, effective surface area, lipophilicity of the drug, pKa of the drug, polymorphic nature of the drug also influence the drug absorption from oral route.

A rat’s digestive system differs from that of a human digestive system in two ways: it does not have a gallbladder and it has an enlarged cecum or large intestine (Fig. 1.7). \(^{164,165}\) The fed fast states of the stomach and intestine, regulates the pH and gastric emptying. An understanding of which is very crucial for the oral dosing of nanoformulation which falls under categories of postprandial, pre-prandial fed state and fasting states of the experimental animals.

**1.1.6.1 Fate of NP in the gastrointestinal tract**

The fate of orally delivered NP is determined by various physiological conditions that they have to undergo. The interaction between nanocarriers and the contents of the stomach or the intestine can lead to the degradation of these particles. Consequently the nanocarriers need to be resistant and stable in the GI tract. The stomach is characterized by an acidic pH ranging between 1.5 and 5 for the fasted and fed state respectively and the presence of pepsin. In the small intestine the pH is in the range of between 5.5 to 7 and several digestive enzymes such as trypsin, chymotrypsin, carboxypeptidase, amylase, lactase and lipase as well as bile salts are present. Furthermore the stability of nanocarriers can be affected by the residence time, the volume of fluid available in the GI tract and its motility.\(^{166,167}\)
1.1.6.2 Transport across the intestinal epithelium

After entering the small intestine from the stomach different mechanisms are employed to transport nanocarriers across the intestinal barrier.\textsuperscript{168}

1.1.6.2.1. Paracellular transport

Paracellular transport is the passive diffusion through intercellular spaces.\textsuperscript{168–170} Tight junctions between two cells form an almost impermeable barrier. These tight junctions are composed of a group of cytosolic proteins, principally including occludins, claudins, actin and zona occludens-1. Between these protein complexes paracellular spaces are defined. Consequently the paracellular transport between the epithelial cells is limited by intercellular space whose pore diameter has been estimated to be between 3 and 10Å. In order to allow drug passage, tight junctions need to be opened.\textsuperscript{171,172}

Volkeimer introduced the concept of persorption of particles, and suggested a passage of particles by the mechanical lesion of the tight junction. This results in enhanced paracellular permeability.\textsuperscript{173–175} Two hypotheses can be proposed to explain the paracellular drug transport. Nanocarriers infiltrate the mucus layer, then break and release their components which open up the tight junction, allowing the released drug to permeate through the paracellular route. Alternatively, the intact nanocarriers adhere to the tight junctions and open up the paracellular channels and the encapsulated drugs are transported between the paracellular cells. According to the second hypothesis the drug remains encapsulated during the mucus layer transport to reach the tight junction which is an advantage.\textsuperscript{176,177}

1.1.6.2.2 Transcellular transport

After crossing the mucus, nanocarriers have to reach the surface of the enterocytes where they could develop adhesive interaction and be translocated by intestinal cells. To cross the intestinal barrier, nanocarriers can be transported according to
different passive and active transcellular pathways.\textsuperscript{178,179} The later is divided in three main steps

- Uptake process at the apical side of the cell
- Transport through the cell
- Release at the basolateral side of the cell

These different transport stages, across the epithelial cells happen through enterocytes or M-cells. Passive transcellular transport includes diffusion across the cells. This transport by diffusion is only feasible for small molecules, due to the limited size of the pores in the cell membrane, that can be opened or closed by the conformational change of the membrane protein.\textsuperscript{180–183} The rate of absorption is governed by the Fick’s law which depends along other parameters on the physiochemical properties of the molecule and on the concentration gradient across the cells. Passive transport has been used to transport small lipophilic molecules across the phospholipidic bilayer and the membrane bound regions of the cell membrane. Theoretically supramolecular structures such as nanocarriers cannot diffuse through the cells by passive diffusion due to their size. Transport by energy dependent active transcellular process should also be considered. Indeed large objects such as macro molecules and particles are internalized by an active mechanism in which portion of the membrane extends and envelopes the object drawing it into the cell to form a vacuole (cytosis). Active transport which is illustrated by different energy dependent mechanisms is mostly used to transport drugs encapsulated in nanocarriers.\textsuperscript{184–186}
Figure 1.8: Diagrammatic representation of (A) intestinal section and the microvilli and (B) different modes of NP uptake across the epithelial cells lining the microvilli of the intestine

1.1.6.2.3 Transport via M cells

M cells have been identified in the intestinal epithelium. These are specialized cells of the mucosa-associated lymphoid tissues that consist of lymphoid follicles arranged to form distinct structures such as Peyer’s patches. Cells are characterized by their ability to transport antigens from the lumen of the small intestine to cells of the immune system. Indeed the invagination of cells at the basolateral side contains lymphocytes and some macrophages which are then distributed throughout the whole body. M cells can endocytose particles by different mechanisms (fluid phase adsorptive phagocytosis) and then exocytose particles across the basolateral membrane into the lymphoid tissue. These cells have sparse microvilli, glycocalyx and an absence of mucus which relate to the hydrophilic property of the intestinal barrier, and facilitate the adherence of both microorganisms and inert particle to their surfaces.

Particles with size up to 1 micrometer can also be taken up by M cells compared with normal epithelial cells which have reduced level of membrane hydrolase activity. This can influence the uptake of protein containing or protein decorated NPs. These
cells also enhance transcytosis which makes them interesting for oral drug delivery applications.\textsuperscript{189–193}

1.1.7 Bioavailability assessment methods

Bioavailability is the measurement of the rate and extent of drug that is systemically available. Hence, pharmacokinetic parameters that give information on the amount of drug reaching the systemic circulation (extent) and the time taken to reach the systemic circulation (rate) are used as measures for assessing bioavailability.

**Direct measures of Bioavailability based on plasma drug concentrations:**

Drug concentrations in the blood and plasma are the most direct methods of determining the systemic availability of a drug. The pharmacokinetic parameters that describe the rate and extent of absorption and systemic exposure based on plasma drug concentration data are summarized below.

(a) The area under the plasma drug concentration and time curve (AUC\textsubscript{0-t},) (units = pg.h/ml): AUC is the measure of the extent of drug bioavailability. This gives a measure of the total systemic exposure. AUC can be obtained by a numerical integration method such as the trapezoidal rule.

(b) The peak plasma drug concentration (C\textsubscript{max}) (units = n=pg/ml): The C\textsubscript{max} is also a measure of the extent of bioavailability or peak exposure and indicates concentration required for a therapeutic or toxic response. It relates to peak exposure of the drug. C\textsubscript{max} is obtained directly from the plasma concentration time profile.

(c) The time to peak plasma drug concentration (T\textsubscript{max}) (units=hours, minutes, etc.): The T\textsubscript{max} is a measure of the rate of drug absorption and is the time required to reach the maximum drug concentration after drug administration. The T\textsubscript{max} is obtained directly from the plasma concentration time profile.\textsuperscript{194}
1.1.8 Properties of nanocarriers affecting their bio distribution

1.1.8.1 Size

The biodistribution of a nanocarrier depends on its physical and chemical properties. Particle size and size distribution are the most important characteristics of NPs related to their biodistribution. Nano formulations increase the dissolution rate of the drug compound and improve bioavailability and reduce variability due to food effects. Thus nanocrystal dispersion (particles of 100-300nm size) improves dissolution rate and oral bio-availability in comparison with micro-sized suspension. Size is also an important parameter governing the entry of the nano carriers in the cell and their fate in the sub-cellular domain. Generally NP have relatively high cell uptake when compared with micro-particles. Particles with a diameter below 50 nm showed a higher degree of uptake by mammalian cells than large particles. NPs have been shown to greatly improve cellular uptake over microspheres which were only taken up by phagocytic M-cells in the Peyer’s patches. 100 nm particles experienced a 2.5 fold greater uptake rate by Caco-2 cells than microparticles.

1.1.8.2 Surface properties

In order to increase the percentage of nanocarrier absorption by the intestinal cells, nanocarriers have to remain intact during a GI transit. Hydrophilic polymers such as PEG form a sterically stabilizing crown on the surface of a nanocarriers that protects the particles against degradation. Positively charged NPs interacts with the cell membrane and penetrates the lipid bilayer of cells with ease. CS and its PEGylated and thiolated modifications can generate NPs of desired size and surface properties for oral delivery of PTH 1-34.
1.2. Thesis scope

NPs composed of naturally occurring biodegradable polymers have emerged as potential carriers of various therapeutic agents for controlled drug delivery through the oral route.\textsuperscript{205–212} CS, a cationic polysaccharide, is one of such biodegradable polymers, which has been extensively exploited for the preparation of NPs for oral controlled delivery.\textsuperscript{87} However, the high solubility of CS at low pH impedes the delivery of proteins to the intestine.\textsuperscript{85,213,214} To overcome this problem, CS derivatives have been developed in the long run, which not only protect the proteins from degradation in the GI but also provide a sustained release delivery platform. NPs should remain intact during their transit through the stomach for the effective delivery of proteins and peptides and should be able to protect their degradation by proteolytic enzymes.\textsuperscript{110,215,216} Although CS is one of the most commonly exploited polymer for the oral delivery of insulin, the focus has recently shifted from CS to CS derivatized biomaterial for the oral delivery of proteins and peptides.\textsuperscript{87,119,217,218} The use of CS derivatives for oral NP preparation vastly improves properties, such as better drug retention capability, improved permeation, enhanced mucoadhesion and sustained release of therapeutic agents.\textsuperscript{83,219,220} CS has free amino and hydroxyl groups which enable substitution or modification with different chemical entities to form CS derivatives with desired properties for oral drug delivery.\textsuperscript{221} CS derivatized polymers are primarily the TCS, PEG-CS, quaternized CS derivatives, CS cyclodextrin complexes and CS combined with other peptides.\textsuperscript{117–119,151,222}

PTH 1-34 is used for the treatment of osteoporosis, osteoarthritis and is in trials for the treatment of hypoparathyroidism.\textsuperscript{58,60,223,224} The current subcutaneous injections of PTH 1-34 holds poor patent compliance and has urged the need for more compliant oral delivery vehicles for the peptide without compromising its biological functions.

Researchers are advancing in formulating oral therapeutics of PTH 1-34. The use of absorption enhancers such as 8-(N-2-hydroxy-5-chlorobenzoyl)-amino-caprylic acid (5-CNAC) and PLGA microparticles for oral administration has been documented whereas the use of polymeric nanoformulation have not been reported.\textsuperscript{76,77}
Therefore this PhD thesis mainly focuses on the challenges in entrapping small peptide PTH 1-34 into the CS, TCS and PEGylated CS polymeric NPs. *In vitro* and *in vivo* comparison between the modified and unmodified CS NP system has been depicted as proof of concept. A detailed account of the synthesis and characterization of the nanoformulation, *in vitro* toxicity profile and a record of the bioactivity of the released peptide has been experimentally proven in Human Primary osteoblast(HOB) and osteosarcoma cell lines. All the formulations were orally administered independently in female Sprague dawley rats and the bioavailability and pharmacokinetic profile of the peptide was determined using the human specific PTH 1-34 ELISA kit and PK deduction software. The GI transit of the nanoformulations were parallely tracked.

![Figure 1.9: Schematic representation of the research strategy](image_url)
1.3 Objectives of the work

The major objective of this research work is to develop a biodegradable biocompatible polymer based NP systems for the entrapment and oral delivery of PTH 1-34, to overcome the challenges faced by the bare peptide in the GI tract and to reduce the dose of administration of the fairly expensive peptide which in turn will make a patient compliant formulation over the conventional injectable PTH 1-34.

Figure 1.10: A representation of the overall objectives of the thesis
1.3.1 The specific objectives of this study

1. To achieve the same blood level (120-150pg/mL) by oral route as that of the subcutaneous injection at a dosage of 20ug/day.

2. To develop a biodegradable, biocompatible polymer based NP systems for the entrapment and oral delivery of PTH 1-34

3. To overcome the challenges faced by the bare peptide in the GI tract

4. To reduce the dose of administration of the fairly expensive peptide

5. Make a patient compliant formulation over the conventional injectable PTH 1-34

6. Obtain short term release from the nanoformulation to maximise anabolic response

1.4 Research Questions and Hypotheses

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.
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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.

1.5 Thesis outline

- In Chapter 1 the first part is the introduction of the thesis and the second part is the comprehensive review of relevant literature for this thesis.
- In Chapter 2 the materials and experimental methods used in the research study of this thesis is described.
- In Chapter 3 the results and discussion of the research studies conducted are described with specific introductions and research question.
- In Chapter 4, the work as described in this thesis is summarized and the future perspectives are discussed.
- In Chapter 5, the bibliography of the citations in the thesis are listed