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Vitamin B12 functionalized layer by layer calcium phosphate nanoparticles: A mucoadhesive and pH responsive carrier for improved oral delivery of insulin

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ABSTRACT

The present study investigates the potential of layer by layer coated calcium phosphate nanoparticles – for oral delivery of insulin where Vitamin B12 grafted chitosan and sodium alginate have been used as cationic and anionic polyelectrolyte respectively. The major emphasis has been given on the role Vitamin B12 conjugated chitosan as cationic polyelectrolyte (VitB12-Chi) in the delivery system. VitB12-Chi conjugate was prepared by carbodiimide reaction. The formulated VirB12-Chi-CPNPs were tested for *in vitro* and *in vivo* efficacy studies carried out in Caco-2 monolayers and diabetic rats. VitB12-Chi-CPNPs with particle size <250 nm and zeta potential + 32.56(±2.34) exhibited pH responsive insulin release at simulated gastric fluid and simulated intestinal fluid. Fluorescence microscopy and flow cytometry studies revealed higher uptake of VitB12-Chi-CPNPs in Caco-2 monolayer in comparison to Chi-CPNPs. Further reduction in TEER supported paracellular transport of insulin because of opening of tight epithelial junctions. *In vivo* intestinal uptake of FITC tagged Vit-B12-Chi-CPNPs from different intestinal segments supported paracellular and receptor mediated uptake of VitB12-Chi-CPNPs. Plasma insulin and blood glucose levels were measured in diabetic rats and showed about four fold increases in insulin bioavailability and sustained hypoglycemic effects up to 12 h of administration with VitB12-Chi-CPNPs in comparison to Chi-CPNPs. Results of the study revealed the potential of layer by layer nanoparticles for oral insulin delivery. The study also specifically highlighted the role of VitB12 as a pH sensitive and targeting ligand which significantly participated in enhancing insulin oral bioavailability.

Statement of significance

Oral delivery of insulin is always the most desirable approach for diabetic patients however it's also the most challenging in respect to formulation development due to harsh gastrointestinal conditions. Several groups have been working from decades for oral delivery of insulin. However the beauty of this prototype formulation is that it exhibits the pH responsive behavior in natural condition of gastrointestinal tract. It resists the release of insulin at gastric condition however stimulate the release at intestinal conditions. Apart from pH responsive behavior it utilizes multiple pathways to improve the overall bioavailability of insulin including paracellular transport and receptor mediated endocytosis.

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1. Introduction

Despite significant developments in the delivery of protein over the past several decades the subcutaneous injection of insulin remains to be only useful approach for the treatment of diabetic patients. Though recently Mankind Corporation has also got FDA approval for insulin inhaler (Afrezza™) but itself it is not considered a perfect substitute for injectable insulin. Subcutaneous injection of insulin has been used worldwide over many years but practically it also fails to mimic the glucose homeostasis as observed in normal subjects [1]. Thus effective alternative routes other than subcutaneous route which can mimic the natural physiology of insulin have always been an elusive goal for many investigators. Moreover, subcutaneous delivery is highly inconvenient and leads to poor patient compliance. Oral delivery of proteins is definitely the most preferred choice as it eliminates the pain caused by injection and thus most convenient method of drug administration [2]. Also orally delivered insulin mimics the biological action of natural insulin. Orally delivered insulin get absorbed from the intestinal mucosa and directly transport towards liver where it inhibits hepatic glucose production. Although oral delivery of insulin can be most effective route, subcutaneous route of insulin delivery is the only available option for diabetic patients due to lack of successful approach for its oral delivery [3]. The major hurdle in oral insulin delivery is its rapid degradation, inactivation and digestion by proteolytic enzymes which results in low bioavailability. In addition to this insulin is poorly permeable due to high molecular weight and lack of lipophilicity. Several delivery systems have been explored for the oral delivery of peptide based drug and have shown marked improvement in their absorption. But still successful oral delivery of peptide drugs is a big challenge. Several groups have worked on formulations like liposomes [4], microemulsions [5] and nanoparticles [6,7], for the oral delivery of insulin, but even till date no oral formulation is available in the market. The primary aim in oral delivery of insulin should be the development of biocompatible delivery systems which can improve the stability of insulin towards the enzymes and control the release to achieve its physiological concentration. Recent advances in oral drug delivery have aroused considerable research interest in development of layer by layer (LBL) coated nanoparticles (NPs). These advanced LBL coated NPs system have shown greater impact on oral bioavailability and stability issues related to protein delivery in GIT [8–10] and thus have further increased research attention on this matter. LBL nanoparticles are composed of oppositely charged polyelectrolyte (like chitosan, alginate, polyallylamine HCl, polyacrylic acid etc) deposited over a core. The present study also focuses on the development of LBL NPs. Layer-by-layer coating of NPs also provides better protection to insulin against gastric enzymes [11] as well as shows delayed release but for prolonged period of time.

Currently chitosan (Chi) and its derivatives are much investigated polyelectrolytes in LBL drug delivery systems [12]. Chitosan is a cationic polysaccharide and a copolymer of β (1 → 4) linked glucosamine and N-acetyl glucosamine. In the present study chitosan has been selected as positively charged polyelectrolyte because of several reasons. First, it is non-toxic, biocompatible and biodegradable. Second, it has reactive amino groups by which delivery system can be subjected to chemical modification to obtain a derivative with the desired characteristics. Third, chitosan itself is mucoadhesive and can adhere the NPs on epithelial surfaces of GIT and show pH responsive behavior which helps in transiently opening tight junctions between contiguous epithelial cells [13,14]. Furthermore to improve the insulin bioavailability Vitamin B12 (VitB12) conjugation has been carried out in present study as it seems to further improve the absorption of NPs by receptor mediated endocytosis in epithelial cells [15,16]. These VitB12 conjugated nanoparticles use body's natural VitB12 transport system i.e. VitB12-IF-IFR (intrinsic factor receptor) which are present in ileocytes of intestine for systemic uptake of VitB12 [17]. Further reason of selecting VitB12 is due to its pKa (∼1.8) which leads change in zeta potential profiles of particles as function of pH. Calcium phosphate nanoparticles (CPNPs) were selected as a core because calcium phosphate has been found to be highly compatible with insulin. These ceramic nanoparticles are the most biocompatible, biodegradable and cost effective systems compared to any other novel drug delivery system. They can be synthesized very easily and also show pH sensitive behavior with complete dissolution at low pH (<5). LBL coated nanoparticles when comes in contact of acidic pH of stomach the core get dissolved leaving behind the flexible insulin loaded Vit-B12 conjugated polyelectrolyte capsule. These LBL systems also bear specific advantage over delivery systems including liposomes or emulsion as they provide a robust and tunable compartment for active molecule encapsulation [18].

Previously, Chalasani et al. in 2007 reported the development of VitB12 conjugated dextran nanoparticle and observed higher levels of insulin in plasma as compared to standard insulin solution [19]. Their objective was to investigate the effect of VitB12 as a targeting ligand that can bind to intrinsic factor and enhance the absorption mediated by intrinsic factor receptors. However in the present investigation we are reporting the pH sensitive behavior of VitB12 conjugated chitosan which is due to the pKa of VitB12. The delivery system has been hypothesized to show pH responsive behavior and expected to retard the release of insulin at gastric pH but stimulate the release at intestinal pH due layer rearrangement in LBL coatings. Also another advantage is that conjugation of VitB12 to chitosan significantly increase its solubility at neutral or slight alkaline pH. Moreover in the present formulation we are first time reporting the use of VitB12 in multilayered nanoparticles and the delivery systems is expected to enhance absorption by multiple pathways apart from the IF receptor mediated endocytosis. In short the aim of the present study was to develop VitB12 functionalized insulin loaded layer by layer calcium phosphate nanoparticles and to thoroughly investigate their potential for the oral delivery of insulin.

2. Materials and method

2.1. Materials

Chitosan with a degree of N-deacetylation of 75–85% (65–90 kDa), alginate (sodium salt, 15–20 cP, 1% in H_2O), Vitamin B12, FITC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), succinic acid, succinic anhydride, trifluoroacetic acid (TFA), fluorescein isothiocyanate (FITC), Human Insulin, Tween 80, Pluronic F68, Bradford reagent and Bovine serum albumin were purchased from Sigma Aldrich (St. Louis, USA). Calcium nitrate, ammonium per sulfate and dialysis bag (cut off mol. wt. 12,000 Dalton) were purchased from Himedia. Analytical grade sodium chloride, sodium phosphate dibasic anhydrous, sodium sulfate anhydrous, calcium chloride dihydrate, sodium bicarbonate, methylene chloride and glacial acetic acid, were purchased from SD fine Chem India.

2.2. Synthesis of VitB12 grafted chitosan and characterization

Conjugation of Vitamin B12 with chitosan (VitB12-Chi) was carried out using method reported previously by Jain et al. with slight modification. VitB12 (1 Mol) was dissolved in 5 ml of dry DMSO by stirring at room temperature (RT) followed by addition of (3 Mol) succinic anhydride with 1 Mol of DMAP and reaction was stirred for 24 h at RT in anhydrous conditions. Formed product (succinylated Vit-B12) was separated by precipitation using acetone and

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