Development of pH sensitive nanopectin/insulin based nanoemulsion for oral insulin drug delivery

Syed Muzammil Munawar[a], Dhandayuthabani Rajendiran[a], Sugantha Kumari Vinoji[b], Khaleel Basha Sabjan[*,c]

[a]*Department of Biochemistry, C. Abdul Hakeem College (Autonomous) Melvisharam 632 509, Ranipet District, TamilNadu, India.*

[b]*Department of Chemistry, Auxilium College, Vellore 632 006, India.*

[*,c]*Department of Chemistry and Biochemistry, C. Abdul Hakeem College (Autonomous) Melvisharam 632 509, Ranipet District, TamilNadu, India.*

Corresponding Author:-

Dr. Khaleel Basha Sabjan

Assistant Professor, Department of Chemistry and Biochemistry, C. Abdul Hakeem College (Autonomous) Melvisharam 632 509, Ranipet District, TamilNadu, India.

Mail id:- khaleelnano@gmail.com

Abstract

This work sought to create nanoemulsions uncoated and coated with nanopectin for oral insulin administration. Uncoated nanoemulsions were generated by sonication, homogenizing, water in oil in water (w/o/w) multiple emulsion, which was made of castor oil as emulsifier and span™20 as cosurfactant. The nanoemulsions were coated using the electrostatic self assembly process, resulting in a bulk nanoemulsion dispersion containing nanopectin. The coated nanoemulsions had a particle size of 200-400 nm and an insulin entrapment ratio of 99.3±0.5%. Transmission electron microscopy revealed that the synthesised nanopectin-insulin nanoemulsion consisted of spherical nanoparticles. Circular dichroism spectroscopy demonstrated insulin's structural stability under preparative stress. An *in-vitro* leakage analysis revealed that the nanoemulsions maintained their integrity in simulated gastric fluids, phosphate buffer saline, and simulated intestinal juices. Hypoglycemic effects were seen in both normal and diabetic rats. Furthermore, oral delivery of the coated nanoemulsions resulted in substantially longer hypoglycemic effects than subcutaneous (sc) insulin. The CCK-8 test demonstrated that the nanopectin-insulin nanoemulsion is safe for human colon adenocarcinoma (Caco-2) cells. A Caco-2 cell model was used to assess the apparent permeability (P_{app}) of insulin and nanopectin-insulin. Confocal imaging was employed in the colon to confirm enterocyte endocytosis. Finally, the nanoemulsion coated with nanopectin-insulin demonstrated promise as an oral delivery strategy for (protein) Insulin.

Key words: Nanoemulsion, Oral Insulin, Nanopectin, Caco-2 cells.

1. Introduction

The everlasting of their specific and powerful effects, an increasing number of peptides and proteins are being produced as medicinal medicines [1]. These are often supplied parenterally to increase bioavailability and not orally due to their rapid breakdown in the gastrointestinal tract (GIT) and poor permeability across the intestinal epithelium. Protecting against enzymatic degradation, improving permeability, and lengthening absorption time have all been used to improve oral bioavailability. Diabetes is one of the most prevalent diseases in modern Western society. Diabetics currently use insulin to control their blood sugar levels. It is administered straight into the bloodstream through injections. Subcutaneous insulin injections induce irritation, discomfort, allergic reactions, hyperinsuliemia, and lipodistrophy near the injection site. This painful procedure is unavoidable because stomach acid degrades protein-based compounds, including insulin, rendering oral insulin ingestion ineffective. According to recent predictions, roughly 285 million individuals globally, 6.6% of the 20-79 year age group would have diabetes by 2030, 438 million people, 7.8% of the adult population are predicted to have the disease. The highest gains will occur in regions dominated by developing economies. Insulin consists of two polypeptide chains and has a molecular weight of 5808 Da. The two chains are joined by two disulfide bonds. The pancreas' β-cells make and secrete it. Insulin's major function is to assist the transportation of glucose from the bloodstream to cells throughout the body. Insulin secretion is a tightly regulated procedure that is designed to keep healthy blood glucose levels in the bloodstream during both feeding and fasting times. Insulin has a polypeptide structure and is synthesised in pancreatic B cells. It serves several critical functions in the mammalian body. It can bind to receptors on the surface of cells. The receptors in cell membranes bind with each other via a disulfide link and are made up of four subunits. Two of these subunits are positioned in the cell surface membrane and are known as beta, while the other 2 are placed away from the cell surface and are known as alpha. Insulin binds to the first alpha subunits, causing autophosphorilation of beta subunits, which extend the cytoplasm *via* two subunit connections, and inducing the conversion of active protein kinase. In recent decades, lipid emulsions such as water in oil [w/o] microemulsions and multiple emulsions have been widely used in the enhancement of the oral bioavailability of peptides and proteins due to their protective ability; improving membrane fluidity and transiently opening the tight junctions induced by lipid constituents / surfactants. Following oral delivery, w/o microemulsions are rapidly converted into oil in water (o/w) emulsions in the gastric secretions. Phase inversion will then release the enclosed medicines, exposing them to gastrointestinal enzyme breakdown. Pectin can also diminish interfacial tension in oil-water emulsions. To avoid stomach degradation, many studies have delivered peptide/protein-loaded w/o microemulsions *via* duodenal injection [2]. Using pectin as an external layer of delivery vehicles in acidic environments like the stomach results in macromolecule aggregates. Controlled release formulations based on pectin can improve bioavailability of bioactive chemicals as prospective therapeutics for gastro-retentive systems and their application in the pharmaceutical industry has increased over the last decade [3].

Although water in oil in water (w/o/w) multiple emulsions are more stable in gastric fluids than w/o microemulsions, water-soluble medicines such as proteins and peptides are also rapidly released from multiple emulsions. Furthermore, the standard multiple emulsion droplet is quite big, measuring between 100 and 400 nm, limiting its uptake by intestinal epithelial or M cells. It may be concluded that current nanotechnologies employing w/o microemulsion and w/o/w multiple emulsions have inherent limitations when used to improve oral bioavailability of proteins and peptides. We believe that if proteins can be safely carried through the stomach, these emulsion-based technologies will be extremely effective for oral delivery. In this study, we reduced the size of w/o/w multiple emulsions to the nanoscale and coated them with polyelectrolyte polymers and polysaccharides.

Pectins are naturally degradable polymers that have been employed in the oral administration of peptides and proteins. Nanopectin and insulin can crosslink using the electrostatic self assembly approach to generate a nanoemulsion with protective properties as well as the ability to increase permeability and bioadhesion. In our investigation, insulin was used as the model protein medication. Improving insulin's oral bioavailability is a huge problem for pharmaceutical experts, yet success with oral insulin will be extremely beneficial to diabetes patients. Our study aims to reduce the size of numerous emulsion droplets through homogenization and sonication, and then coat the resulting nanoemulsion with nanopectin/insulin *via* electrostatic self assembly cross-linking to protect the nanoemulsion's stability in GIT. The primary goal was to determine how well this nanopectin/insulin-coated nanoemulsion technology improved insulin oral bioavailability. The encapsulation efficiency, size, and *in-vitro, in-vivo* stability in simulated gastric fluids, simulated intestine, and phosphate buffer saline were tested. The pharmacological bioavailability of nanopectin/insulin-coated nanoemulsion administered orally was assessed in both normal and diabetic rats. The purpose of this study was to develop a nanopectin-based nanoemulsion for oral insulin administration as a model drug. The main restriction of emulsion systems is thermodynamic instability, which can be overcome by modifying parameters like efficient emulsifiers. Emulsifiers influence the size distribution of droplets, the zeta potential, optical and physical properties, encapsulation efficiency, and stability. As a result, choosing the best emulsifier for producing optimized emulsions is crucial.

2. Materials and Methods

2.1 Materials

All analytical-grade chemicals were used in our research study. Pectin low molecular weight, Monobasic sodium phosphate, span™20, Dibasic sodium phosphate, Glycerol, were purchased from Sigma Aldrich. Castor oil was supplied by Sigma-Aldrich Mumbai. Insulin, Biphasic isophane, (40 I.U/ml, 10 ml), [(Human Mixtard, Monocomponent Biosynthetic *r*-DNA insulin obtained from Torrent Pharmaceuticals Ltd), (Novo Nordisk India Pvt Ltd)]. Caco-2 (human epithelial colorectal adenocarcinoma cell) was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). For all the experiments, milli-Q deionized water was used. All other chemicals used were of Good and Analytical grade.

2.2 Preparation of w/o/w multiple emulsion

The electrostatic self assembly method was used with minor changes to manufacture monodispersed nanopectin by [4]. To create the nanoemulsion, adequate amounts of sodium chloride (100 mM) were mixed with pectin (15%) in milli-Q deionized water and sonicated for 15-45 minutes until lumpid, turbid, and clear opalescent. After that, the mixture was magnetically swirled for 10 minutes at 50°C. Glycerol (15µl) was then added to the aforementioned mixture drop by drop and centrifuged at 10,000 rpm for 40 minutes at 20ºC. The pellet was resuspended in (100 µl) of 100 mM NaCl and rinsed twice with milli-Q deionized water. The acquired nanopectin was further investigated.

2.3 Preparation of Nanopectin/Insulin-coated nanoemulsion

To create the w/o/w emulsion, a two-step technique was used [5]. In summary, the w/o/w primary emulsion was created by combining 800 µL of insulin solution, 150 mg insulin dispersed in 10 mL of phosphate buffer solution (PBS, 0.2 M) at pH 7.4 (USP 34), and 8 g of the oily phase composed of castor oil as surfactant and span-20 as cosurfactant, and stirring for 15 minutes at 800 rpm. A modified emulsification / electrostatic self assembly approach was used to manufacture insulin-loaded nanoemulsions. The primary emulsion was then blended in various concentration ratios (1:1, 1:3, 3:3) with previously made nanopectin (Scheme 1). After 15 minutes of continuous spinning at 600 rpm, the nanoemulsion was formed. The optimum generated nanoemulsion ratio of 3:3 was chosen for further investigation. The adjusted 3:3 ratio of w/o/w multiple emulsion was extruded into nanoscale using high pressure homogenization (AH 100, ATS Engineering Inc, Brampton, ON, Canada). The electrostatic self assembly approach was then used to create the nanopecin-insulin coated nanoemulsion.

Pectin Nanopectin Castor oil Insulin Stirring Sonication 3:3 Nanoemulsion (formed) (emulsifier/span20)

Scheme 1. Preparation of nanoemulsion

3. Determination of entrapment ratio

The entrapment ratio (ER) was calculated indirectly after destroying the coated nanoemulsion by measuring the leakage ratio. In short, 1 mL of nanoemulsion was dissolved in 4 mL of methanol. The ultimate pH was 3.0 after adding 100 µL of HCl (pH 1.0) and vigorously mixing. Milli Q water was added to produce 10 mL, then properly mixed. After centrifugation at 16000 g for 10 minutes, the clear supernatant was used to test insulin using HPLC.

Permeation tests were carried out in identical amounts of isotonic calcium chloride 204 mEq/L, pH 7.4 solutions to investigate the effect of calcium binding on intestinal permeability. At ½ hour, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, and 8 hours, an aliquot of 1 mL of medium was removed from the receptor tube and replaced with an equal volume of fresh medium. The insulin content of the samples was tested using a previously described HPLC method. The amount of insulin encapsulated in the nanoemulsion was estimated as the difference between the total amount used to make the formulation and the amount of free insulin remaining in the outer aqueous phase after centrifugation.

$$\text{ER } (\%) = \frac{The\ total\ amount\ of\ Insulin - Amount\ of\ free\ Insulin\ in\ the\ Supernatant}{The\ total\ amount\ of\ Insulin\ added} \times 100$$

4. Determination of insulin

Insulin levels were determined using a reversed-phase HPLC ultraviolet (UV) technique [31]. The HPLC system (1100 series, Agilent Technologies, Santa Clara, CA, USA) consisted of a quaternary pump, a degasser, an auto-sampler, a column heater, and a tunable UV detector. A C18 column Zorbax, 5 m, 4.6 mm 150 mm, Agilent, and a C18 pre-column 2 mm 20 minutes, Alltech, were utilized for detection at 25°C. The mobile phase consisted of acetonitrile and 0.57% phosphoric acid solution adjusted to pH 2.25 with triethylamine in a volume ratio of 26:74. The detection wavelength was λmax 220 nm and the flow rate was set to 1.0 mL/min.

5. Characterization of coated and uncoated nanoemulsion

5.1 Size and zeta potential

The average particle size and size distribution of the nanoemulsions were assessed using dynamic light scattering (DLS) on a NICOMP 380 DLS device (Santa Barbara, CA, USA). Before measurement, the nanoemulsion was 500 times diluted in deionized water. At 25°C, the zeta potential was determined using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were collected using foldable capillary cells equipped with gold electrodes. Three measurements were obtained, and the software automatically determined the number of runs for each. The data were provided as mean standard deviation (SD).

5.2 Physical stability of nanoemulsion

The stability of the produced nanoemulsions was assessed using the centrifugal acceleration method [6]. A 5 mL Eppendorf tube was filled with 4 mL of nanoemulsion, which was then spun at 3000 g for 10 minutes in a desktop centrifuge (Anke TGL-16G, Shanghai). A 0.8 mL sample of subnatant was softly and steadily pipetted from the tube's bottom. The samples were then vortexed for 20 seconds before being transferred to a 50 mL volumetric flask and diluted with milli-Q water to achieve the necessary final volume. At λmax 500 nm, the absorbance of diluted nanoemulsions was determined spectrophotometrically. The centrifugal stability constant (K_e) was determined using the method [6].

$$ke = \frac{A_0 - A}{A_0} \times 100\%$$

where A_0 and A are the absorbance of the diluted nanoemulsion before and after centrifugation, respectively.

5.3 Transmission electron microscopy (TEM)

Transmission electron microscopy was used to study the morphologies of uncoated and nanopectin-insulin nanoemulsions (coated). A drop of nanoemulsion was put on 300-mesh carbon-coated copper grids, and any surplus water was wiped away with blotting paper. Before drying at room temperature, the stain was treated with a drop of 1% phosphotungstic acid for 60 seconds. The samples were evaluated at an acceleration voltage of 120 kV. The photos were acquired with a final magnification of 12,000 times.

5.4 Conformational stability of insulin

The conformational stability of insulin was assessed using circular dichroism (CD) spectroscopy (Jasco J-810, Jasco Corp, Tokyo, Japan). The coated nanoemulsion of 1 mL was destroyed by adding 4 mL of methanol and a pH 1.0 hydrochloric acid solution to 10 mL. After 10 minutes of centrifugation at 16,000 g, insulin was isolated from the supernatant. After that, the insulin was separated with a Supelco ENVI-18 solid-phase extraction column (Sigma-Aldrich) [7]. To summarize, the ENVI-18 column was activated with methanol before being balanced with a solvent. The 1 mL supernatant mixture was then plated. 3 mL of 40% aqueous methanol was used to remove impurities, followed by 30 mL of 60% aqueous methanol to elute the insulin. To perform the circular dichroism test, the insulin concentration was raised to 30 µg/mL using a hydrochloric acid solution. At 20°C, spectra were collected with a 0.5 nm step size, a maximum wavelength range of 200-300 nm, a band width of 3 nm, a scanning speed of 500 nm/minute, and a reaction time of 0.25 seconds. The Jasco w32 secondary structure estimation software version 1.0 was used to calculate secondary structure content.

5.5 *In-vitro* drug release

The rate and extent of insulin release as a model drug were examined in both simulated intestinal fluid (SIF, pH 6.8, duodenum pH) and phosphate-buffered saline (PBS, pH 7.4, colon pH) to determine the feasibility of the proposed delivery method for oral peptide and protein delivery. To investigate the *in-vitro* release of insulin from polysaccharide-like nanopectin, 30 mg of lyophilized nanoemulsion was mixed with 500 mL of SIF solution and shaken at 50 rpm. The temperature was maintained at 37°C±5°C. To ensure the sink condition, the releasing medium was designed to be somewhat large. At predetermined intervals, 1 mL aliquots were taken and replaced with preheated blank medium. For 20 minutes, the samples were centrifuged at 14,000 rpm. Instead of adding blank medium after aliquot withdrawal, the supernatant was tested for insulin concentration, and the sediment was dispersed in 1 mL blank media before being reintroduced into the release medium. Because the insulin release from the nanoemulsion's polymeric mesh did not complete within the first few hours, the unreleased insulin in the withdrawn nanoemulsion was also released. The insulin concentration in the supernatant was determined using the previously published HPLC method. To further understand the process of peptide release from the nanoparticulate drug delivery system, *in-vitro* insulin release data were fitted to the Ritger-Peppas model.

$$\frac{M_t}{M_\infty} = Kt^n$$

where M_t and M_∞ are the cumulative insulin release at time (*t*) and infinite time, respectively, *K* is a constant related to the structural and geometric properties of the device, and '*n*' is an exponent reflecting the diffusion mechanism. The number of calculated values for 'n' was used to classify the release mechanism. As a result, if n = 0.45, the release mechanism is Fickian / case I diffusion 0.45, < n < 0.89, non-Fickian / anomalous transport and n = 0.89, diffusion and zero-order (case II) transport.

5.6 *In-vitro* cytotoxicity of nanoemulsions

Caco-2 cells develop under high glucose circumstances. Dulbecco's modified Eagle medium contains 10% foetal bovine serum, 1% v/v non-essential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin. Caco-2 cells were cultured for 21 days to stimulate cell monolayer formation. Cells were cultivated at 37°C in 5% CO_2 in a culture medium that was changed every two days for the first 15 days and every day for the final week [8]. The MTT assay was used for 24 hours to examine the cytotoxicity of a nanopectin-insulin nanoemulsion on Caco-2 cells. Caco-2 cells ($1x10^5$ cells/well) were seeded in 6-well plates for 24 hours with sterilized glass cover slips for confocal microscopy analysis. The cells were treated with insulin-FITC and insulin-FITC loaded nanopectin-insulin nanoemulsion at staggered time intervals, for a total insulin-FITC concentration of 10 µg/mL. Cell monolayers were completely cleansed with PBS pH 7.4 before being examined under a confocal microscope (CLSM, Carl Zeiss Microscopy GmbH, Germany). The endocytic inhibitors chlorpromazine HCl (5 µg/mL), nystatin (50 µg/mL), amiloride (13.3 µg/mL), and colchicine (10 µM) were administered to seeded Caco-2 cells separately for 1 hour at 37°C. Caco-2 cells were grown with heparinases (5U) to study adsorptive absorption. Pretreatment of cells with a 1 mM folic acid solution inhibited receptor-mediated endocytosis. The cells were then seeded with a nanopectin-insulin nanoemulsion containing 10 µg/mL insulin for 4 hours at 37°C. Before being lysed with 0.1% Triton X-100, the cells were washed 3 times with ice-cold PBS. In the absence of inhibitors, the manufacturer's suggested ELISA kit was utilized as a control to measure intracellular insulin content absorption.

5.7 Transepithelial electrical resistance (TEER) and insulin transport studies

Caco-2 cell lines were cultured in transmembrane inserts with 0.4 µm pore size (Millipore) and 5×10^5 cell density/well. TEER studies were conducted using a previously reported approach. The TEER of the cell monolayer was measured at specific time intervals at 37°C using either plain nanopectin nanoemulsion or nanopectin-insulin loaded nanoemulsion at a concentration of 10 mg/mL/well. According to some reports [9], aspart insulin, a new and short-acting analogue of human insulin, was also loaded in either the plain nanopectin or nanopectin-insulin loaded nanoemulsion with the same concentration. Aspart insulin has a higher potential to pass through the lumen epithelium due to its monomeric nature and linear conformation in aqueous medium. We tested both human insulin and aspart insulin (Alpha Diagnostic International) in identical conditions and compared the findings to see whether our nanoparticulate technique outperformed aspart insulin. As a control, a simple solution containing both human and aspart insulin was administered to the cell layer's donor chamber. For insulin transport investigations, the donor chamber medium was replaced with new media containing insulin-laden plain nanopectin or nanopectin-insulin loaded nanoemulsion (10 mg/well).

Aliquots from the receiver chamber were collected at predefined time intervals over a 4-hour period, and the insulin content was measured using a customised ELISA kit that was unique to and sensitive to both conventional human insulin and aspart insulin. The average results for all instances assessed in triplicate were presented. The apparent permeability co-efficient (P_{app}) of insulin was calculated using the following equation. In this equation, dQ/dt indicates the rate of permeability, A is the filter membrane's surface area, and C0 is the starting insulin concentration in the apical chamber.

$$Papp = \frac{\frac{dQ}{dt}}{A \times C_0}$$

5.8 Pharmacokinetics study

The animal procedure was approved by the institution's ethical committee. Male albino wistar rats weighing 180g±10%, aged 2-3 months, were housed in room temperature plastic cages with a 12-hour light/dark cycle. They were fed rat chow and had unlimited access to water. The CSLM approach was utilized to duplicate the intestinal absorption and localization of an insulin-FITC solution and a nanopectin-insulin nanoemulsion with 10 μg/mL insulin [10]. Male albino rats were given a single intraperitoneal injection of 60 mg/kg streptozotocin to cause diabetes. The diabetic rats in the study had blood glucose levels greater than 250 mg/dL. Thirty diabetic rats (n=6) were divided into five main groups. The glucose levels in rat blood were determined with a colorimetric glucose assay kit (Cell Biolabs, Inc., USA). At different time intervals, the percentage drop from the initial value was calculated. The total drop in blood glucose level (TD%) was calculated using the equation below [11].

$$\text{TD } (\%) = \frac{AUEC_c - AUEC_s}{AUEC_c}$$

where $AUEC_c$ is the area beneath the curve after oral administration of PBS to diabetic rats / control diabetic and $AUEC_s$ is the area beneath the curve after oral administration of nanopectin-insulin nanoemulsion and insulin SC injection, respectively. In groups 1 and 3, plasma insulin levels were carefully determined using an ELISA, and the pharmacokinetic parameters C_{max}, T_{max}, AUC, mean residence time (MRT), and relative bioavailability (F%) were taken into account.

6. Statistical Data Analysis

The results were provided as mean standard deviation. For group comparison, a one-way analysis of variance (ANOVA) was employed (SPSS 16.0, IBM Corp, Amonk, NY). The difference was considered statistically significant when the P value was less than 0.05.

7. Results and Discussions

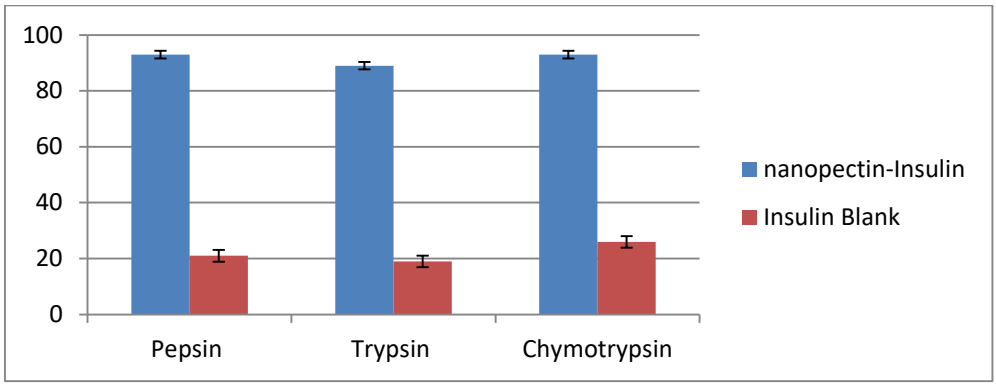
7.1 Insulin Encapsulation efficiency

The insulin encapsulation efficiency was 48.3%±0.5%. This high level is due to the pH influence of nanoemulsion components. Insulin has an isoelectric point of roughly pH 5.3, while pectin has pKa values ranging from 3.5 to 4.5. As a result, at the final pH of 4.5, substantial electrostatic self assembly attractions may occur, resulting in a high EE of the oppositely charged protein.

Furthermore, calcium ions interact with nanopectin residues and can form ionic bridges with insulin's negatively charged carboxylic acids, increasing the bond between nanopectin and insulin. The use of emulsifiers such as castor oil and cosurfactants such as span20 can also improve insulin encapsulation efficiency due to emulsifier molecules' steric stabilisation.

7.2 Size and Zeta potential

The synthesis of nanopectin/insulin-coated nanoemulsion in a 3:3 ratio was straightforward and reliable, thanks to the electrostatic self assembly approach. Pectin's surface net charge remained positive at pH >4.5 (ζ-potential = +20.88±1.67 mV). The ζ-potential, represents the electrical potential at the hydrodynamic shear plane relative to the solvent. Our analysis focused on ζ-potential to determine the charge state of polyelectrolytes at a specific working pH. Abodinar *et al.*, investigated the conformation and chain flexibility of polyelectrolytes in dilute solution, as well as their ionic strength dependence, employing -potential measurements as a function of ionic strength. The ζ-potential parameter is not a substitute for potentiometric or conductimetric studies due to its sensitivity to polyelectrolyte structural and conformational characteristics. The pH-dependence (ζ-potential) of pectin matches to the known pKa range of 3.5-4.5. Pectin's anionic property decreases below pH 4.5 due to carboxylic group dissociation in its structure. Any polymeric nanoparticulate delivery system designed for oral peptide delivery, such as Insulin, must meet basic criteria such as adequate Insulin encapsulation, high drug encapsulation efficiency, and drug loading efficiency. As a result, even after an appropriate and realistic amount of polymeric nanoemulsion is administered orally, low insulin EE prevents the nanoparticulate delivery system from carrying out the necessary therapeutic effects. Submicron carriers have previously been employed to demonstrate insulin absorption across intestinal membranes [12-14]. The impact of nanoemulsion size is clear, raising the likelihood that larger particles will stay in the Peyer's patches, which are densely packed with M-cells, resulting in slower activity. Smaller nanoemulsions, if suitably charged, can pass through the lymphatic system and into the systemic circulation. Nanoemulsions should be less than 1 μm, specifically less than 200 nm, for efficient uptake by M-cells. As a result, the first step in our research was to reduce the w/o/w emulsion droplets to nanoscale dimensions. Calcium is necessary for strong connections and the integrity of intestinal membranes. To summarize, the nanoemulsion's potential to enhance mucosal transport would be beneficial. Tight junctions can also be opened to compensate for cell shrinkage caused by the increased water absorption capabilities of nanopectin. The ability of the nanopectin-insulin nanoemulsion to withstand gastrointestinal enzyme breakdown is essential in determining oral insulin bioavailability. There was no significant variation in particle size / zeta potential in the presence of pepsin, trypsin, or chymotrypsin (Graph 1), and the nanopectin-insulin nanoemulsion retained more than 75-80% of the entrapped / encapsulated insulin in the three different biological enzymes. Under the same conditions, however, more than 78-80% of the naked insulin was destroyed. The ability of the nanopectin-insulin nanoemulsion to endure harsh gastrointestinal conditions could be attributed to nanopectin carboxylic / amino groups that bond with Ca^{2++}, a recognized cofactor for enzyme function. Finally, the lack of significant changes ($p > 0.05$) in EE%, particle size, and zeta potential at different time intervals (Table 1) and after 6-month storage at 5°C indicated the insulin nanoemulsion's physical durability.



Graph 1. Effect of entrapment efficiency

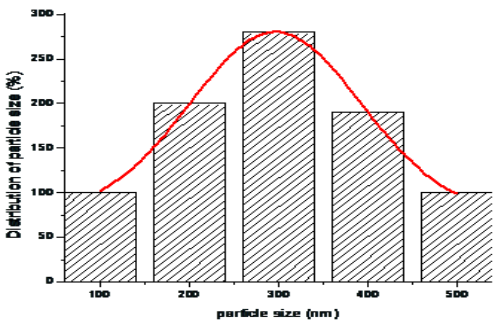
The carboxylate groups in pectin were expected to engage electrostatically with oppositely charged groups in Insulin to form a stable nanoemulsion through electrostatic self-assembly under regulated conditions. The nanoemulsion's ζ-potential is proportional to the surface charge density values due to the electrostatic self-assembly of nanopectin and Insulin. The particle size measured was 200-400 nm (Fig. 1a,1b), with an average Zeta potential (ζ) of +20.88±1.67 mV (Table 1). The encapsulation by nanopectin-insulin nanoemulsion resulted in a significant decrease in [15]. At gut pH, nanopectin's carboxyl groups may have carried a negative charge, improving its ability to chelate cations such as calcium as well as dissociation of the pectin structure's carboxylic groups, a critical co-factor for proteolytic enzymes that inhibits their activity. Ca^{2++} binding empathy has also been demonstrated to disrupt cellular actin and adherents, activate protein kinases, and thereby open cellular tight junctions. Transmission electron micrographs show that nanoemulsions have a smooth structure with no fractures or pores (Fig. 1b). Finally, the lack of significant changes ($p > 0.05$) in EE%, particle size, and zeta potential at various time intervals, as well as after 6-month storage at 5°C, demonstrated the insulin nanoemulsion's physical stability.

7.3 Physical stability

Nanoemulsion bioavailability is significantly influenced by particle size and dispersion, as well as physical stability. In our work, we looked at how homogenization, sonication, protein concentration, w/o/w ratio, and pH affected particle size, polydispersity index (PI), and physical stability of nanoemulsions.

7.4 Morphology of the coated nanoemulsion

The TEM image revealed a nanoemulsion size of 200-400 nm, consistent with dynamic light scattering measurements of 258.0 nm (Polydispersion Index [PI] = 0.290) for the uncoated nanoemulsion and 200-400 nm (PI = 0.211±0.014) for the coated nanoemulsion. The droplet shape of the uncoated nanoemulsion was quasicircular with a smooth profile (Fig 1a, 1b). However, the nanoemulsion's coating had a rough and uneven outline. This occurrence is consistent with reports from others [16]. Cross-linking between nanopectin and protein may have altered surface tension, resulting in surface shrinkage and reduced particle size in nanoemulsions.



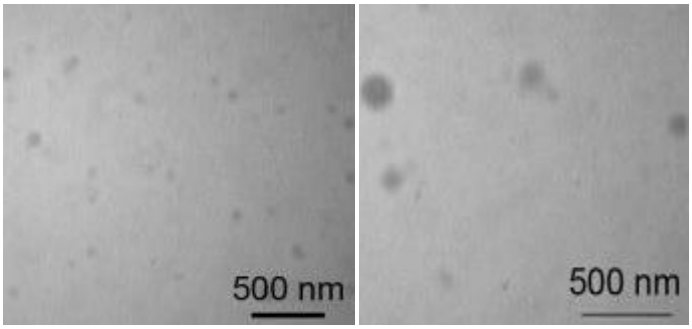


Figure 1a. Histogram of nanoemulsion Figure 1b.TEM of uncoated, coated nanoemulsion

Table 1. Physico-chemical Characterization of nanopectin-insulin nanoemulsion

Parameter	Particle size (nm)	PDI	Zeta potential	Calcium binding efficiency*
Result ± SD	200-400	0.211 ± 0.014	+20.88±1.67 mV	26.01 ± 2.05

Results are mean of three replicates ± standard deviation. *Data were presented as % to the initial Ca^{2++}.

7.5 Conformational stability of insulin

Particle size and dispersion, as well as physical stability, are widely recognized as having a significant impact on nanoemulsion bioavailability. In this study, we investigated how the oil-to-water ratio and pH affected particle size, polydispersity index (PDI), calcium binding efficacy, and physical stability of nanoemulsions. High temperature, mechanical manipulation, and exposure to organic solvents can all disrupt protein/peptide structure, which is critical for optimum therapeutic efficacy. As a result, when altering proteins or peptides, conformational stability must be considered. Circular dichroism spectroscopy is a powerful method for analyzing protein and peptide secondary structures, including the α-helix and β-fold, which can indicate insulin effectiveness. Circular dichroism is a form of dichroism. Figure 2 illustrates spectroscopic spectra for insulin secondary structures. The spectra of insulin mixed with nanoemulsion and coated nanoemulsion were identical to those of insulin solution, with a peak valley around 209 nm and a shoulder around 225 nm. Insulin secondary structures were consistent among samples, with 21%-22% α-helix and 27%-29% β-folds. This secondary structural analysis demonstrates that insulin's conformational shape remained consistent throughout the manufacturing of nanopectin-insulin coated nanoemulsions [17].

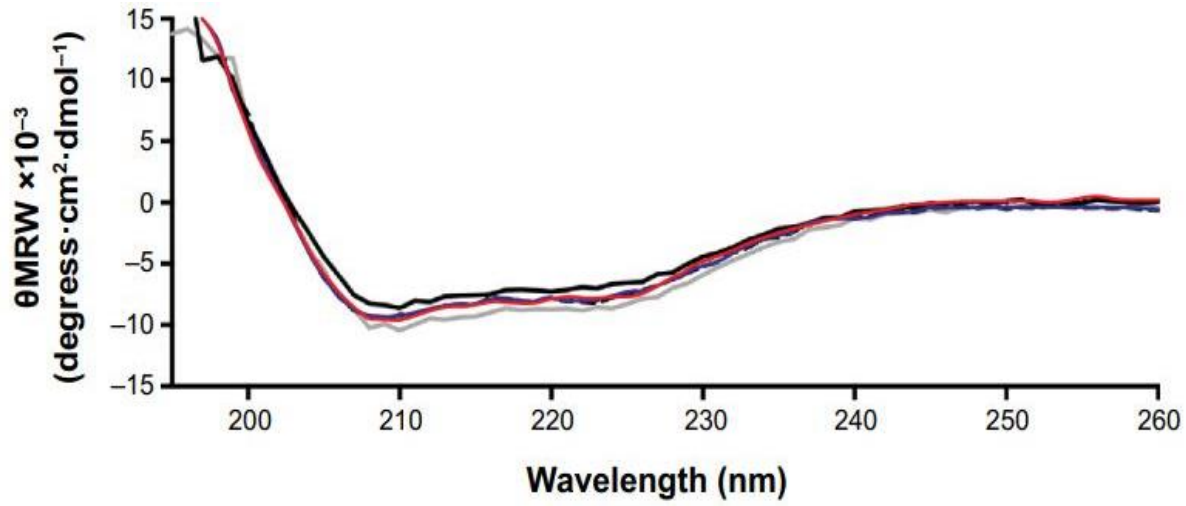
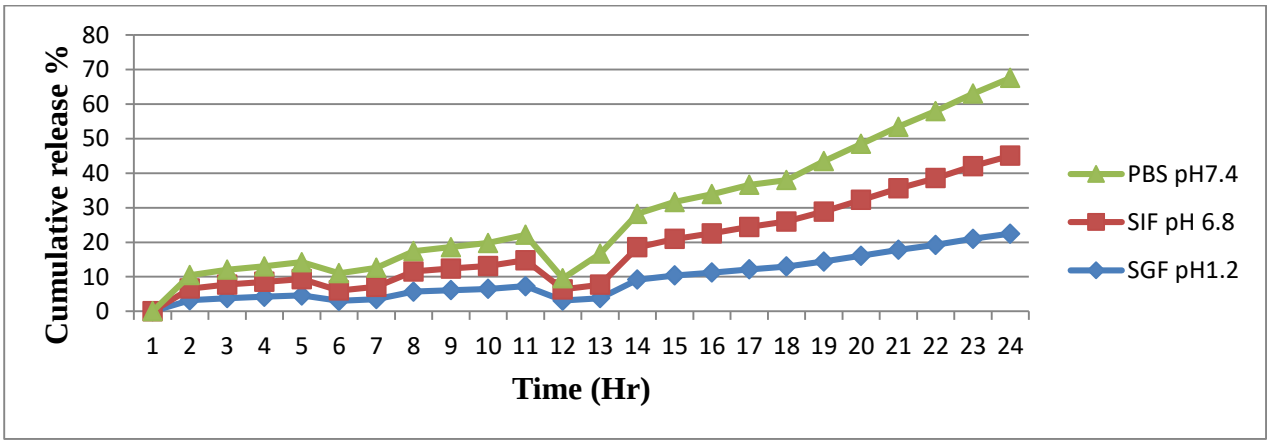


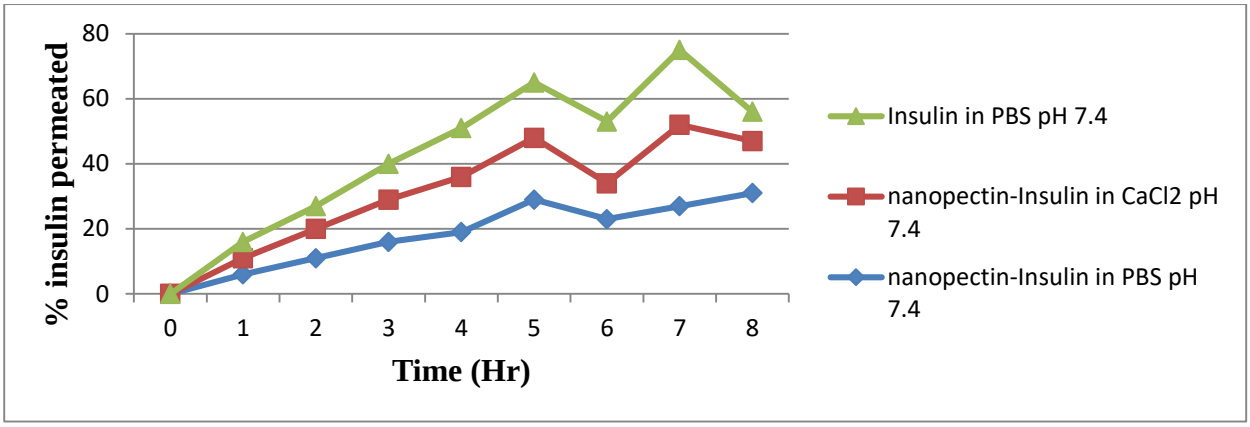
Figure 2 CD spectra of nanoemulsion

7.6 *In-vitro* release study

Graph 2 depicts the *in-vitro* release profile of model peptide insulin from an optimized nanoemulsion delivery device loaded with the embedded peptide during a post-loading operation. The release profile was investigated at SIF pH 6.8, SGF pH 1.2, and phosphate-buffered saline pH 7.4. Both media showed a burst release of insulin during the first 30 minutes, then declined but remained steady over the next 24 hours [18]. During the burst release phase, approximately 33%±6% of total insulin is released at pH 7.4, 20%±2.1% at pH 6.8, and 22%±2.3% at pH 1.2.



Graph 2. Nanopectin-Insulin release profile from PBS pH 7.4, SIF pH6.8, and SGF pH1.2

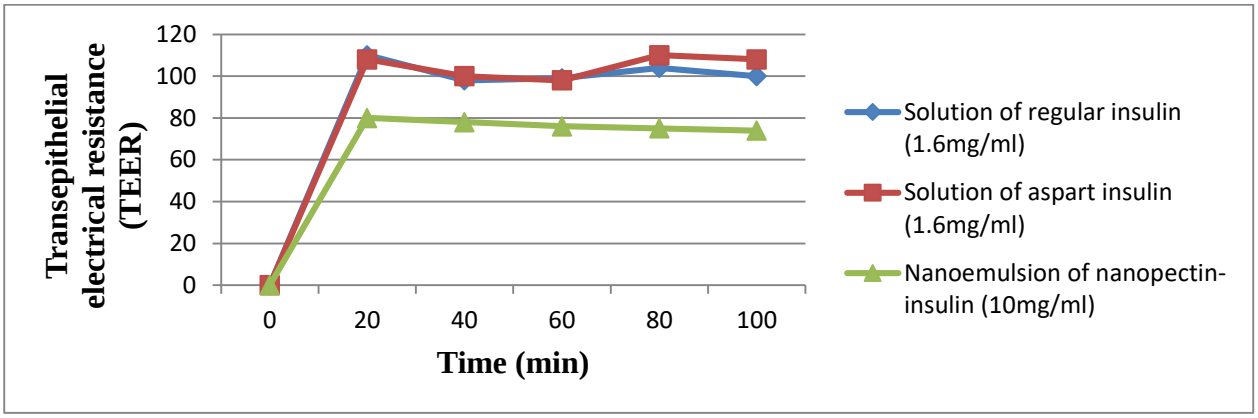


Graph 3. Insulin permeability in the rat gut and the influence of several stomach enzymes

During optimization testing, we determined that nanoemulsions made with certain polysaccharide and emulsifier-cosurfactant concentrations had the highest loading efficiency, release behaviour, and total release extent. Graph 2 shows that insulin release from the optimised nanoemulsion was nearly complete after 24 hours, with 86% of the implanted insulin released in phosphate-buffered saline pH 7.4 at this time. However, burst and total insulin release were lower in SIF pH 6.8, SIF pH 1.2, and phosphate-buffered medium, with 20%±2.1% burst release and 78%±6.5% total release in SIF pH 6.8 and 1.2, respectively. At pH 6.8, several of nanopectin's primary carboxy groups may still be protonated, retaining cationic charges, attracting negatively charged insulin and delaying release. At pH 7.4, this formulation demonstrated extremely high burst and total release. Graph 3 demonstrates the effect of different stomach enzymes on *ex-vivo* insulin permeability in the rat gut. Due to its amphiphilic character, the polysaccharide in this formulation is water soluble at all pH levels [18]. Aside from normal solubility, the carbohydrate's cationic charge may have been lost at this pH, preventing it from binding and retaining negatively charged insulin. This rapid peptide release from the nanoemulsion's polymeric mesh is advantageous for peptides with short half-lives, such as insulin, since it permits the delivery system to maintain therapeutic advantages. Other studies discovered that the mechanism of drug release from erodible, hydrophilic polymer matrices is a complex process involving a variety of factors such as water penetration into the polymeric matrix, solubilization / erosion of the polymeric formulation, polymer swelling, and drug dissolution from the enlarged matrix. Drug release from polymeric formulations with swelling properties is normally non-Fickian / anomalous, but, for large molecules like peptides and proteins, the story may be different, especially when ionic charges interact between the carrier polymer and the enclosed moiety. To explore the release mechanism of insulin as an ionic charge peptide from the synthesised and produced nanoemulsion, the parameter "n" for the Ritger-Peppas equation was calculated. The optimised formulation yielded a correlation coefficient of 0.8213 ($R \geq 0.99$), indicating that the release data fits the empirical equation. The "n" release exponent ranged from 0.86 to 0.89, indicating non-Fickian / anomalous transport ($0.45<n<0.89$).

7.7 Transepithelial electrical resistance (TEER) and insulin transport studies

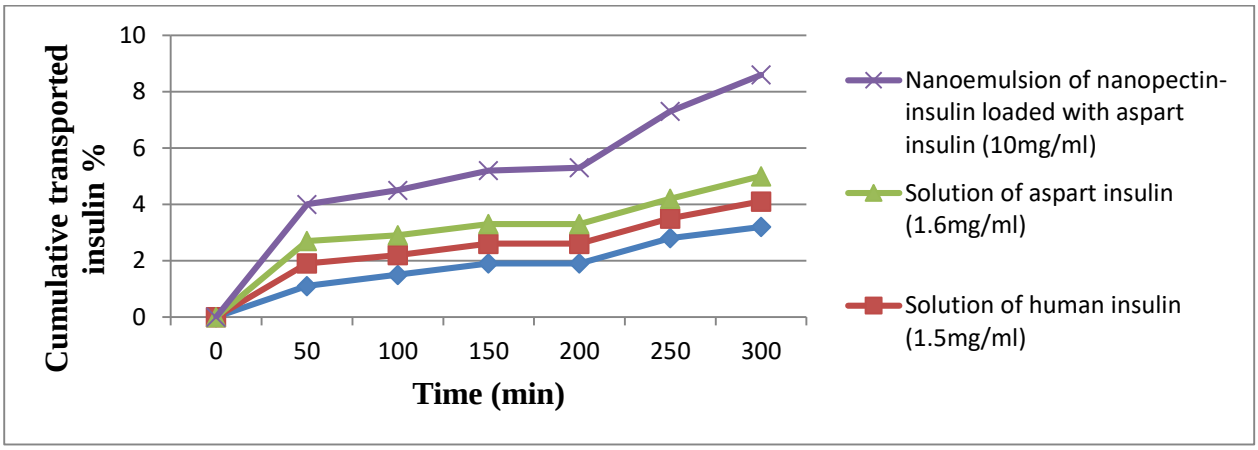
In-vitro Caco-2 cell lines were not affected by either uncoated nanopectin or coated/encapsulated nanopectin-insulin, according to MTT cytotoxicity assays. The particles had nearly 100% cell viability as compared to the control media. Graph 4 shows that after 2 hours of exposure to Caco-2 cells with either uncoated nanopectin or coated/encapsulated nanopectin-insulin at a dose of 10 mg/mL/well, there was a significant reduction in TEER of ~86 and 41%, respectively, compared to the initial values of coated/encapsulated nanopectin-insulin or uncoated nanopectin. TEER values were significantly reduced in the presence of both native coated and uncoated nanoemulsions, regardless of whether the embedded peptide was regular human insulin or aspart insulin, but there was no significant difference in the presence of a simple solution of regular human insulin or a simple solution of aspart insulin. The presence of nanpectin nanoemulsion reduces TEER, a well-known scientific fact validated by several researches. Our findings, however, revealed a considerable and dramatic difference in TEER decrease between uncoated and coated nanopectin nanoemulsions. According to Graph 4, the TEER reduction in the presence of coated encapsulated nanopectin-insulin was more than double that of the uncoated nanopectin nanoemulsion.



Graph 4. Outcome of diverse formulations on TEER

The concentration of insulin in transwell receptor chamber samples was measured using an ELISA kit designed for conventional human insulin and aspart insulin. Calculating the cumulative transferred insulin in the receiver chamber and comparing it to the initial concentration of insulin in the donor chamber, insulin translocation across the cell monolayer was found to be ~0% for simple solutions of ordinary or aspart insulin, A nanopectin nanoemulsion loaded with ordinary human insulin yields around 6%, while aspart insulin yields 6.5%. A coated nanopectin-insulin nanoemulsion loaded with regular human insulin yields approximately 19%, while aspart insulin yields around 17.8%. This is the highest degree of insulin translocated from the donor side of Caco-2 cells seen in cell culture research thus far. The aggregate transit of multiple insulins is shown in Graph 5. Our findings, as well as release tests, show that the nanoparticulate nanoemulsion system and its specific features, rather than the kind of insulin, influence how much insulin is given across the cell monolayer (Graph 5). Our findings contradict those of another study, which hypothesized that aspart insulin is easier to administer orally because it is monomeric. Another noteworthy discovery in this work is that when insulin translocation is stopped by withdrawing insulin-containing

samples from the receiver chamber and incubating them in fresh micro tubs for 24 hours, the concentration of assayable insulin increases over time.

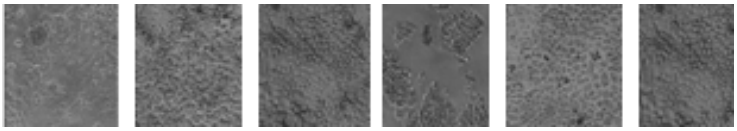


Graph 5. Collective transported formulation of insulin

The influence of nanoemulsion size is obvious, raising the possibility that larger particles may linger in the Peyer's patches, which are densely packed with M-cells, resulting in slower activity. Smaller nanoemulsions can cross the lymphatic pathway and enter the systemic circulation if properly charged. The findings show that this delivery technique was designed with great care, taking use of the extraordinary tight-junction opening and unique cell penetration potentials of cell penetrating peptides. As previously stated, this prefabricated nanoparticulate nanoemulsion system may be simply loaded with a large number of peptides, proteins, nucleotides, and other big and hydrophilic molecules in a simple and modest post-loading process because of nanoparticulate properties such as muco-adhesion and tight-junction opening properties of nanopectin, as well as direct and energy-independent [19]. The embedded peptide will be translocated once the nanopectin-insulin coated nanoemulsion is exposed to biological barriers. The impact of nanoemulsion size is clear, raising the likelihood that larger particles will stay in the Peyer's patches, which are densely packed with M-cells, resulting in slower activity. Smaller nanoemulsions can cross the lymphatic route and enter the systemic circulation if charged correctly. The data reveal that this delivery approach was created with great care, taking use of the amazing tight-junction opening and unique cell penetration potentials of cell penetrating peptides. This nanoemulsion nanoparticulate system looks to have great promise for noninvasive delivery of peptides, proteins, vaccines, and nucleotides, as well as medication delivery to the blood-brain barrier and diagnostic and contrast media distribution from blood-brain barrier compartments.

7.8 *In-vitro* cytotoxicity assessment

Cell viability was greater than 91% at concentrations comparable to 10 µg/mL insulin (Fig. 3). Cell viability decreased significantly ($p<0.05$) with increasing insulin nanoemulsion concentrations, possibly due to increased nanopectin content. In general, the resulting nanoemulsion's moderate positive charge value adds to its safety [20]. As a result, a nanopectin-insulin nanoemulsion containing insulin equivalent to 10 µg/mL was employed in subsequent studies.



(a) (b) (c) (d) (e) (f)

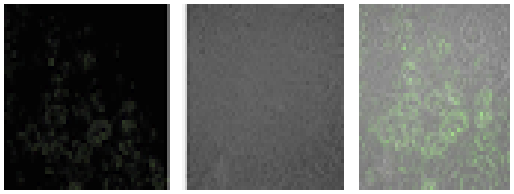
Figure 3. Images of Light microscope as Caco-2 exposure to (a) insulin-FITC for 5 hr, nanopectin-insulin for 1,2,3,4 and 5 hours

7.9 *In-vitro* Caco-2 cellular uptake

After 5 hours of incubation with insulin-FITC, Caco-2 cells produced no fluorescence (Fig. 3a-3f). Insulin's large molecular weight inhibits cellular internalisation. In contrast, CLSM micrographs of a nanopectin-insulin nanoemulsion exhibited a large number of green fluorescent spherical particles, indicating insulin cellular internalisation. Furthermore, the intensity of the fluorescent dye rises with incubation time.

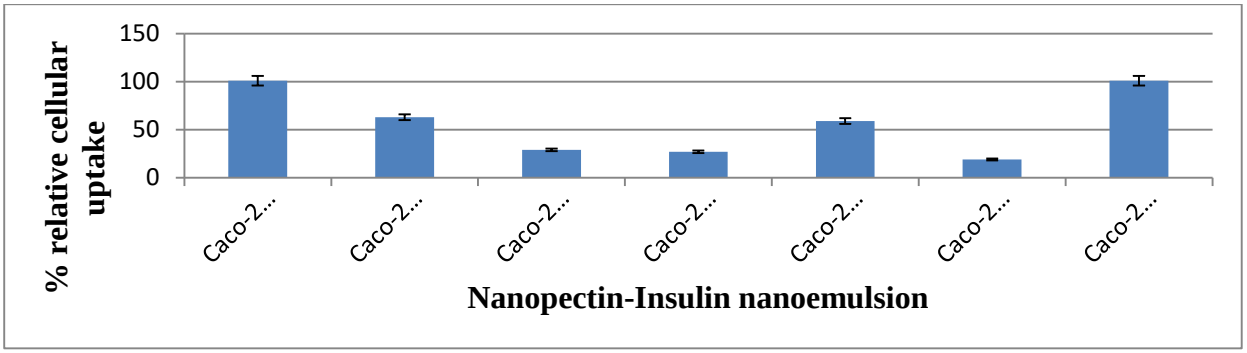
7.10 Tracking the insulin-Nanopectin nanoemulsion cellular uptake pathway

The results of this experiment are displayed in Figure 4. Nanopectin dramatically reduced insulin absorption in Caco-2 cells ($p<0.05$), indicating that the nanopectin-insulin nanoemulsion can enhance insulin internalisation. Caco-2 cells were pre-treated with various chemical agents to determine the mechanism of nanoparticle uptake, with chlorpromazine, nystatin, amiloride, and colchicine showing clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and microtubular-mediated endocytosis, respectively. Except for chlorpromazine, all inhibitors dramatically reduced cellular internalisation of insulin nanoparticles ($p>0.05$) (Graph 6), demonstrating that clathrin-mediated endocytosis is not a transporter mechanism for insulin-Nanopectin nanoemulsions. Nystatin, on the other hand, is a cholesterol-binding drug that inhibits sag formation and thus caveolae-mediated endocytosis [20]. Prior study has shown that caveolae-mediated endocytosis involves separating caveolae from the plasma membrane in order to transfer nanocargo to caveosomes while avoiding the degradative lysosomal pathway. Amiloride also inhibits macropinocytosis by preventing Na^+/H^+ exchange in the plasma membrane, which is a necessary step in the process. Colchicine inhibits microtubule polymerization by binding irreversibly to tubulin, hence decreasing microtubule-mediated endocytosis. Microtubules, in general, create a cytoplasmic network that participates in endocytic vesicle trafficking because of the presence of heparan sulphate proteoglycans, the cell membrane is negatively charged. Heparinases are enzymes that hydrolyze different proteoglycans found on cell membranes, thereby mitigating their detrimental effects. Pre-treatment of Caco-2 cells with enzymes resulted in an 80% reduction in insulin cellular internalisation due to decreased electrostatic contact between the less negative cell membrane and the cationic nanoparticles. Finally, by targeting Vitamin B_{12} receptors, the particles are conveyed through non-destructive caveolae-mediated endocytosis.

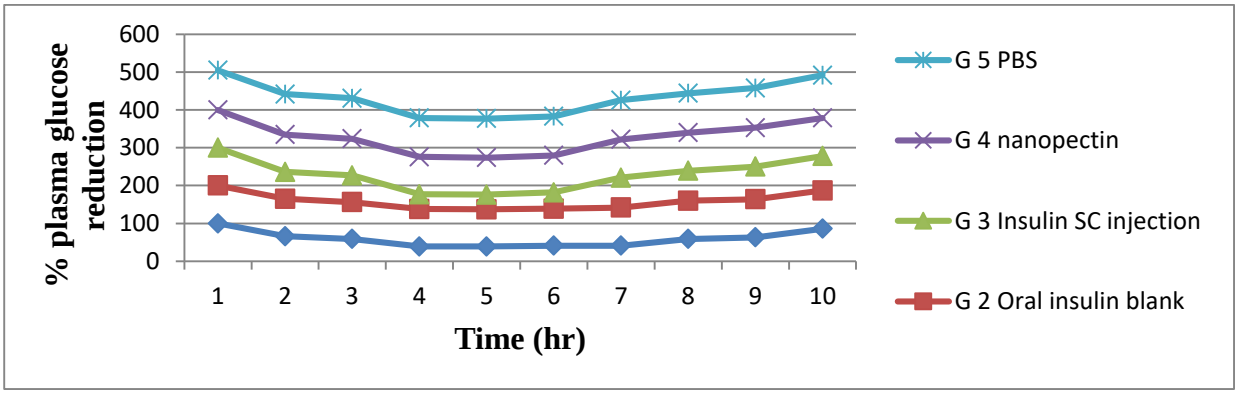


(a) (b) (c)

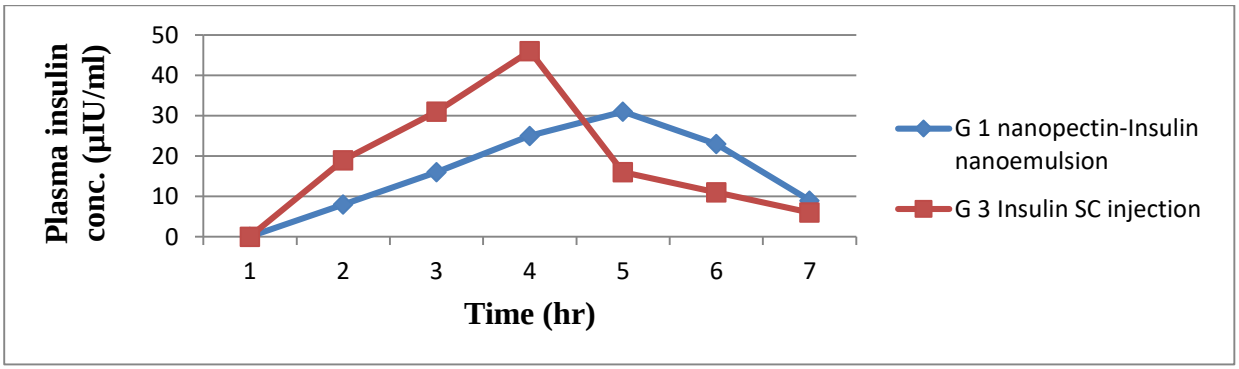
Figure 4. Confocal (CS) of rat intestinal mucosa and orally (a) insulin-FITC (b), and (c) nanopectin-insulin-FITC



Graph 6. The effect of various endocytosis inhibitors on Caco-2 cellular insulin uptake after incubation with a nanopectin-insulin nanoemulsion



Graph 7. (%) drop in plasma glucose after treatment



Graph 8. Plasma insulin concentrations after oral nanopectin-insulin and SC injection

Table 2. Pharmacodynamics of oral nanopectin-insulin, SC insulin delivery

Groups	Initial plasma glucose conc. mg/dL)±SE	Pharmacodynamics parameters ± SE			
		Cmin (mg/dL)	Tmin (hr)	AUEC0-∞ (mg h/dL)	Total decrease in p.glu (%)
Group 1 received oral nanopectin-Insulin*	259.83±5.32	118.66±8.38	6hr	1690.11±3.47	32.41±2.74
Group 3 received insulin SC injection**	255.83±4.98	91.33±4.24	4hr	1814.59±3.24	27.45±2.55

Table 3. Pharmacokinetics - after oral nanopectin-insulin treatment, SC insulin

Groups	Pharmacodynamics parameters ± SE				
	Cmax (μIU/mL)	Tmax (h)	AUC0-∞ (μIU h/mL)	MRT (h)	Relative bioavailability
Group 1 oral nanopectin-Insulin*	31.04±1.11	6hr	502.67±15.74	13.28±1.02	17.04%±1.34
Group 3 received insulin SC injection**	43.91±2.06	4hr	294.61±19.67	8.10±0.32	100

Results are the mean of six replicate±SE. *Insulin dose 50 IU/Kg. **Insulin dose 5 IU/Kg.

7.11 Intestinal uptake

The rat intestinal mucosa showed no histological alterations following oral administration of the nanopectin-insulin nanoemulsion, showing histocompatibility. There was no fluorescence in any part of the colon after administering insulin-FITC solution orally (Fig. 4a,b,c). This could be owing to the fact that insulin degrades rapidly in the gastrointestinal tract and cannot pass intestinal walls. In contrast, fluorescence was observed throughout the digestive tract. A nanopectin-insulin nanoemulsion, as previously demonstrated, may protect insulin against enzymatic breakdown in the gastrointestinal system. Surprisingly, the nanoemulsion was found deep within the submucosa and muscle regions, as well as on the mucosal surface. The electrostatic interaction between the positively charged nanoemulsion and the negatively charged cell membrane allowed the nanoemulsion to stay on the mucosa. Furthermore, a nanopectin-insulin nanoemulsion was found in the Peyer's patches. Previous research discovered that nanoemulsion internalisation in Peyer's patches was predominantly achieved by adsorptive endocytosis. The ultimate properties of nanopectin, such as mucoadhesion and penetration enhancement *via* opening cellular tight junctions, allow the nanoemulsion to penetrate the intestinal wall [21]. These findings demonstrated that the paracellular route included both transcellular and receptor-mediated endocytosis. These findings are consistent with those of the cellular absorption mechanism.

7.12 *In-vivo* pharmacodynamics and pharmacokinetic studies

The plasma% glucose reduction after oral administration of insulin solution and plain nanopectin-insulin nanoemulsion was comparable to that after oral PBS, indicating that they had no hypoglycemic effect (Graphs 7 and 8). Oral administration of insulin solution, as previously stated, is susceptible to stomach enzymatic harshness and decreased intestinal absorption. In contrast, insulin-Nanopectin nanoemulsion and insulin subcutaneous injection significantly reduced plasma glucose levels compared to the control group ($p<0.05$). Subcutaneous insulin injection resulted in a considerable drop in plasma glucose levels until 4 hours of hypoglycemia shock, after which they quickly returned to baseline at 12 hours. After 6 hours (T_{min}) of oral administration of insulin-Nanopectin nanoemulsion, plasma glucose levels significantly decreased from 259.83±5.32 to 118.66±8.38 mg/dL (C_{min}) ($p <0.05$), and returned to baseline after 24 hours. These data provide support for the nanoemulsion's delayed and long-lasting hypoglycemic effect. The $AUEC_{-\infty}$ after oral treatment with nanopectin-insulin nanoemulsion was 1690.11±3.47 mg/dL, yielding in a total drop in plasma glucose level of 32.41±2.74%, compared to 27.45±2.55% with subcutaneous injection (Table 2). Subcutaneous insulin injection elevated blood insulin concentrations to a maximum (C_{max}) of 43.90±2.06 µIU/mL after 4 hours (T_{max}), indicating maximum hypoglycemic activity (Table 3). Following that, serum insulin levels plummeted dramatically. In contrast, nanopectin-insulin nanoemulsion absorption was slower, with a T_{max} of 6 hours, although blood insulin levels were higher for 24 hours (Graph 8). Rats fed nanopectin-insulin nanoemulsion had a computed $AUC0_{-\infty}$ of 502.67±15.74 µIU h/mL, indicating a 17.04±1.34% relative bioavailability (Table 3). Finally, the MRT following oral nanopectin-insulin nanoemulsion was much higher than after subcutaneous insulin injection. Improving insulin intestinal permeability is a straight forward way to boost oral insulin bioavailability. The improved cellular and intestinal absorption, as well as biological activity, of the generated nanoemulsion may be attributed to the final component properties and the nanoemulsion size. Nanoemulsions are suitable for mucosal uptake due to their small size. Increasing nanoemulsion absorption in the GI tract by targeting the colon's many Vitamin B_{12} and Folate receptors. Biodegradable nanopectin-insulin nanoemulsion interacts electrostatically with negatively charged cell membranes, allowing nanocargo to enter the cell. Mucoadhesive properties are also linked to nanopectin's hydroxyl groups through hydrogen bonding and the hydrophobic contact of mucin with nanopectin. Physical entanglement of polymer and mucus glycoproteins and increased hydration of OH-carrying matrices for quicker diffusion across the intestinal mucosal layer are 2 often described methods. These connections facilitated nanoemulsion transcellular and paracellular internalisation [22].

8. Conclusion

The current study looked into the feasibility of developing an oral insulin formulation that combined the benefits of nanoencapsulation with lipid emulsion. Protein-stabilized nanoemulsions were discovered to be substantially more stable than surfactant, emulsifier, and cosurfactant-stabilized nanoemulsions. The emulsification / electrostatic self assembly technology was significantly enhanced to overcome the limitations of this method, such as excessive size dispersion in the microscale and low recovery yield. These findings demonstrated that employing ultrasonication to reduce the size of emulsion droplets and so create nanoscale particles was an efficient strategy [23-27]. This method was enhanced by combining the cosurfactant span 20. As a result, the previous finding was most likely due to enhanced protein surface potential. The procedure was simple and required no organic chemicals. In simulated gastric, intestinal, and PBS media, a nanopectin-insulin nanoemulsion effectively retained insulin. Furthermore, gravity separation resistance and biocompatibility in nanopectin-insulin stabilized nanoemulsions were enhanced. Protein concentration, pH, and homogenization pressure all influenced particle size, stability, transmission electron microscopy, morphology, and zeta potential. As a result, we anticipate that by combining insulin, proteins as a model drug for drug delivery, and castor oil as a surfactant, we will be able to build biocompatible and biodegradable nanoemulsion systems that can be used instead of traditional surfactants. This functional nanoemulsion for oral delivery is simply loaded with peptides, nucleotides, and hydrophilic large molecules. The CD-tested secondary structure of insulin demonstrated that it maintained its biological activity during the encapsulation procedure [28]. This electrostatic self-assembled optimised w/o/w nanoemulsion can enter monolayer Caco-2 cells through both para and intracellular channels. The TEER of nanoemulsion encapsulated with insulin is significantly lower than that of nanopectin, showing profound tight-junction opening properties for this newly synthesized nanopectin-insulin conjugated derivative of carbohydrate. The coated nanoemulsion reduced glucose levels in diabetic mice by up to 61% and 51%, respectively, compared to their basal glucose levels at insulin dosages of 25 and 50 IU/kg, while insulin bioavailability was 8.19% and 7.84%, indicating good insulin intestinal absorption. These findings show that a nanopectin-insulin coated nanoemulsion could be created as an oral therapeutic protein delivery method. In contrast, the biological activity of released insulin from nanoparticles following subcutaneous injection into diabetic male albino wistar rats indicated that insulin function was preserved [29,30]. Insulin entrapped in nanoemulsion showed a smaller and less severe drop than insulin released from nanoemulsion due to its interaction with carbohydrates. This optimized methodology, which used mild insulin encapsulation conditions and simple procedures, allowed for the creation of nanopectin-based insulin-loaded nanoemulsions with insulin activity retention in the nanometric range, which will have important pharmaceutical applications in the coming generations.

Acknowledgment

We are thankful to the Management of C.Abdul Hakeem College (Autonomous), Melvisharam, Ranipet District, Tamil Nadu – India for their encouragement, providing the necessary facilities and support in carrying out the work.

References

1. McClements DJ. “Encapsulation, protection, and delivery of bioactive proteins and peptides using nanoparticle and microparticle systems: a review”. *Adv Colloid Interface Sci.* 253, (2018),1-22.
2. Lundquist P, Artursson P. “Oral absorption of peptides and nanoparticles across the human intestine: opportunities, limitations and studies in human tissues”. *Adv Drug Deliver Rev.* 106, (2016), 256-276.
3. Yun Y, Cho YW, Park K. “Nanoparticles for oral delivery: targeted nanoparticles with peptidic ligands for oral protein delivery”. *Adv Drug Deliver Rev*. 65, 6, (2013), 822-832.
4. Fuenzalida, J.P.; Weikert, T.; Hoffmann, S.; Vila-Sanjurjo, C.; Moerschbacher, B.M.; Goycoolea, F.M.; Kolkenbrock, S. Affinity protein-based fret tools for cellular tracking of chitosan nanoparticles and determination of the polymer degree of acetylation. *Biomacromolecules.* 2014, 15, 2532-2539.
5. Cournarie F, Savelli MP, Rosilio V, et al. Insulin-loaded W/O/W multiple emulsions: comparison of the performances of systems prepared with medium-chain-triglycerides and fish oil. *Eur J Pharm Biopharm*. 58, (3), (2004), 477-482.
6. Wang JN, Tan JY, Luo JH, et al. “Enhancement of scutellarin oral delivery efficacy by vitamin B12-modified amphiphilic chitosan derivatives to treat type II diabetes-induced retinopathy”. *J Nanobiotechnol*. 15, (2017).
7. Ayame H, Morimoto N, Akiyoshi K. “Self-assembled cationic nanogels for intracellular protein delivery”. *Bioconjugate Chem*. 19, (4), (2008), 882-890.
8. D. Mohnen, Pectin structure and biosynthesis, Curr. Opin. *Plant Biol*. 11, (3), (2008), 266-277.
9. A.N. Round, N.M. Rigby, A.J. MacDougall, V.J. Morris, A new view of pectin structure revealed by acid hydrolysis and atomic force microscopy, *Carbohydr. Res*. 345, (4), (2010), 487-497.
10. H.R. Moreira, F. Munarin, R. Gentilini, L. Visai, P.L. Granja, M.C. Tanzi, P. Petrini, Injectable pectin hydrogels produced by internal gelation: pH dependence of gelling and rheological properties, *Carbohydr. Polym*. 103, (2014), 339-347.
11. D. Saha, S. Bhattacharya, Hydrocolloids as thickening and gelling agents infood: a critical review, *J. Food Sci. Technol*. 47, (6), (2010), 587-597.
12. J. Leroux, V. Langendorff, G. Schick, V. Vaishnav, J. Mazoyer, Emulsion stabilizing properties of pectin, *Food Hydro colloids* 17, (4), (2003), 455-462.
13. P. Sriamornsak, Application of pectin in oral drug delivery, *Expert Opin. Drug Deliv*. 8, (8), (2011), 1009-1023.
14. S. Sungthongjeen, P. Sriamornsak, T. Pitaksuteepong, A. Somsiri, S. Puttipipatkhachorn, Effect of degree of desertification of pectin and calcium amount on drug release from pectin-based matrix tablets. *AAPS PharmSciTech* 5, (2004), (Article 9).
15. P. Sriamornsak, N. Thirawong, S. Puttipipatkhachorn, Emulsion gel beads of calcium pectinate capable of floating on the gastric fluid: effect of some additives, hardening agent or coating on release behavior of metronidazole, *Eur. J. Pharm. Sci*. 24, (2005), 363-373.

16. P. Sriamornsak, S. Sungthongjeen, S. Puttipipatkhachorn, Use of pectin as a carrier for intragastric floating drug delivery: carbonate salt contained beads, *Carbohydr. Polym*. 67, (2007), 436-445.
17. P. Sriamornsak, N. Thirawong, Y. Weerapol, J. Nunthanid, S. Sungthongjeen, Swelling and erosion of pectin matrix tablets and their impact on drug release behavior, *Eur. J. Pharm. Biopharm*. 67, (2007), 211-219.
18. J.H. Guo, G.W. Skinner, W.W. Harcum, P.E. Barnum, Pharmaceutical application of naturally occurring water-soluble polymers, *Pharm. Sci. Technol. Today* 1 (1998), 254-261.
19. P. Sriamornsak, N. Thirawong, S. Puttipipatkhachorn, Morphology and buoyancy of oil-entrapped calcium pectinate gel beads, *AAPS J.* 6, (2004), (Article 24).
20. K. Burapapadh, M. Kumpugdee-Vollrath, D. Chantasart D, P. Sriamornsak, Fabrication of pectin-based nanoemulsions loaded with itraconazole for pharmaceutical application, *Carbohydr. Polym*. 82, (2010), 384-393.
21. P. Sriamornsak, N. Thirawong, S. Puttipipatkhachorn, Emulsion gel beads of calcium pectinate capable of floating on the gastric fluid: effect of some additives, hardening agent or coating on release behavior of metronidazole, *Eur. J. Pharm. Sci*. 24, (2005), 363-373.
22. D.J. McClements, E.A. Decker, J. Weiss, Emulsion-based delivery systems forlipophilic bioactive components, *J. Food Sci*. 72, (8), (2007), R109-R124.
23. D.J. McClements, Food Emulsions: Principles, Practices, and Techniques, *CRC press*, (2015).
24. A.F. Esfanjani, S.M. Jafari, E. Assadpoor, A. Mohammadi, Nano-encapsulation of saffron extract through double-layered multiple emulsions of pectin and whey protein concentrate, *J. Food Eng*. 165, (2015), 149-155.
25. A. Mohammadi, S.M. Jafari, A.F. Esfanjani, S. Akhavan, Application of nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil, *Food Chem*. 190, (2016), 513-519.
26. S.M. Jafari, E. Assadpoor, Y. He, B. Bhandari, Re-coalescence of emulsion droplets during high-energy emulsification, *Food Hydro colloids*. 22, (7), (2008),1191-1202.
27. M. Nakauma, T. Funami, S. Noda, S. Ishihara, S. Al-Assaf, K. Nishinari, G.O.Phillips, Comparison of sugar beet pectin, soybean soluble polysaccharide, and gum arabic as food emulsifiers. 1. Effect of concentration, pH, and salts on the emulsifying properties, *Food Hydro colloids.* 22, (7), (2008), 1254-1267.
28. Lai MH, Wang JN, Tan JY, et al. “Preparation, complexation mechanism and properties of nano-complexes of Astragalus polysaccharide and amphiphilic chitosan derivatives”. *Carbohyd Polym*. 161, (2017), 261-269.
29. Mukhopadhyay, P.; Chakraborty, S.; Bhattacharya, S.; Mishra, R.; Kundu, P.P. pH-sensitive chitosan/alginate core-shell nanoparticles for efficient and safe oral insulin delivery. *Int. J. Biol. Macromol*. 72, (2015),640-648.
30. Kleine-Brüggeney, H.; Zorzi, G.K.; El-Gueddari, N.E.; Moerschbacher, B.M.; Goycoolea, F.M. A rational approach towards the design of chitosan-based nanoparticles obtained by ionotropic gelation. *Colloid Surf. B.* 135, (2015), 99-108.