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Facilitated nanoscale delivery of insulin across intestinal membrane models

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ABSTRACT

The effect of nanoparticulate delivery system on enhancing insulin permeation through intestinal membrane was evaluated in different intestinal epithelial models using cell cultures and excised intestinal tissues. Multilayered nanoparticles were formulated by encapsulating insulin within a core consisting of alginate and dextran sulfate nucleating around calcium and binding to poloxamer, stabilized by chitosan, and subsequently coated with albumin. Insulin permeation through Caco-2 cell monolayer was enhanced 2.1-fold, facilitated by the nanoparticles compared with insulin alone, 3.7-fold through a mucus-secreting Caco-2/HT29 co-culture, and 3.9-fold through excised intestinal mucosa of Wistar rats. Correlation of Caco-2/HT29 co-culture cells with the animal-model intestinal membrane demonstrates that the mucus layer plays a significant role in determining the effectiveness of oral nanoformulations in delivering poorly absorbed drugs. Albumin was applied to the nanoparticles as outermost coat to protect insulin through shielding from proteolytic degradation. The effect of the albumin layering on insulin permeation was compared with albumin-free nanoparticles that mimic the result of albumin being enzymatically removed during gastric and intestinal transport. Results showed that albumin layering is important toward improving insulin transport across the intestinal membrane, possibly by stabilizing insulin in the intestinal conditions. Transcellular permeation was evidenced by internalization of independently labeled insulin and nanoparticles into enterocytes, in which insulin appeared to remain associated with the nanoparticles. Transcellular transport of insulin through rat intestinal mucosa may represent the predominant mechanism by which nanoparticles facilitate insulin permeation. Nanoformulations demonstrated biocompatibility with rat intestinal mucosa through determination of cell viability via monitoring of mitochondrial dehydrogenases. Insulin permeation facilitated by the biocompatible nanoparticles suggests a potential carrier system in delivering protein-based drugs by the oral route.

1. Introduction

Therapeutic proteins and peptide drugs are of interest in the treatment of several metabolic diseases (Peppas and Carr, 2009). Oral administration is of particular interest as for example, oral insulin is considered to provide greater blood glucose control in type 1 diabetes mellitus than subcutaneous insulin (Raj and Sharma, 2003; Damgé et al., 2007). However, oral delivery is limited by proteolytic degradation of insulin if not shielded, and by poor absorption through the intestinal mucosa if not chemically or physically facilitated (Hamman et al., 2005; Morishita and Peppas, 2006). Strategies for clinical delivery of insulin by the oral route include administration with absorption enhancers or enzyme inhibitors (Mesiha and Sidhom, 1995; Radwan and Aboul-Enein, 2002), chemical modification (Asada et al., 1995) and design of delivery systems (Marschutz and Bernkop-Schnurch, 2000). Nanoparticulate delivery systems are designed to carry and deliver insulin to the site of absorption and facilitate permeation through the intestinal membrane to achieve a pharmacological effect (Thanou et al., 2001).

In the present study, multicomponent nanoparticles encapsulating insulin were prepared by ionotropic gelation of dilute alginate and dextran sulfate through the addition of calcium ions, forming a stable colloidal dispersion by the presence of poloxamer and chitosan, and subsequent coating with albumin. The pH-sensitive alginate network forms an impermeable structure in acidic gastric conditions (Kamiya and Klibanov, 2003; George

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and Abraham, 2006), preventing premature insulin release in combination with dextran sulfate, increasing insulin protection in combination with albumin coating applied as a sacrificial target to gastrointestinal proteases (Reis et al., 2007). Alginate and chitosan, as mucoadhesive polymers are designed to increase the residence time of nanoencapsulated insulin at the site of absorption, facilitating the uptake, permeation and internalization of insulin by enterocytes (George and Abraham, 2006; Chickering and Mathiowitz, 1995; Sarmento et al., 2007). In addition, chitosan transiently opens intercellular tight junctions to improve the paracellular transport (Artursson et al., 1994; Bernkop-Schnurch, 2000).

In vitro permeability studies using cell culture preparations or excised tissue have been used to evaluate the potential effect of delivery systems on enhancing drug permeation. In addition, *in vitro* intestinal models enable the determination of an anatomical site as a route for drug delivery by isolation of the interaction of nanoencapsulated drug with the intestinal membrane independently of other factors such as transit time. Cells from human colon adenocarcinoma (Caco-2 cell monolayers) have been used as an intestinal model to evaluate the effect of delivery systems on drug permeation via transcellular and paracellular transport (Artursson and Karlsson, 1991; Artursson et al., 2001; Shah et al., 2006). Caco-2 cell monolayers are similar to the small intestinal epithelial layer by differentiation into columnar absorptive cells, including tight junctions, brush border, enzymes and carrier-mediated transport systems (Delie and Rubas, 1997; Hilgendorf et al., 2000; Behrens and Kissel, 2003). However, goblet, mucus-secreting and M-cells are absent, thus HT29 subclones have been used in co-culture with Caco-2 cells to mimic the small intestinal epithelial layer by containing both mucus and the columnar absorptive cells (Walter et al., 1996; Lesuffleur et al., 1990; Karlsson et al., 1993; Wikman-Larhed and Artursson, 1995). Tight junctions of cellular models are similar to that presented in the colon, and have been shown to underestimate drug permeability via paracellular transport (Walter et al., 1996; Ichikawa and Peppas, 2003; Schilling and Mitra, 1990). Experiments performed with cell monolayers provide results with lower variability than animal tissues, however cell monolayers lack three-dimensional macrostructure and lack cells of varying degrees of differentiation, as are found in animal tissues (Grass, 1997). Therefore, excised rat intestinal mucosa mounted in Ussing chambers have also been used as an intestinal model to evaluate the permeation enhancing effect of delivery systems, presenting mucus and epithelial layers, and the enzymatic barrier of the human small intestinal mucosa (Aoki et al., 2005; Bravo-Osuna et al., 2007; Ungell et al., 1998).

The aim of this study was to evaluate the effect of multilayered nanoparticles on enhancing insulin permeation through the intestinal membrane, comprising the effect of albumin layer and the poloxamer additive, and evaluating the use of Caco-2 and Caco-2/HT29 cell monolayers, and rat intestinal mucosa as intestinal membrane models. Biocompatibility of nanoparticles was evaluated via cell viability measurements, and internalization of nanoencapsulated insulin was determined in rat small intestinal tissue through confocal microscopy using insulin and nanoparticles, which were independently labeled with fluorescent probes.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt, low molecular weight chitosan, bovine serum albumin (BSA), streptozotocin (STZ), fluorescein isothiocyanate-insulin (FITC-insulin) labeled from bovine pancreas, rhodamine B isothiocyanate (RBITC), FITC-dextran 40 (FD40), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), N-(2-hydroxyethyl)piperazine-N 0-(2-ethane-sulfonic acid) (HEPES) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma–Aldrich Chemie (France). Dextran sulfate sodium salt was purchased from Fluka (Switzerland). Polyvinylpyrrolidone K 30 and poloxamer 188 (Lutrol F68) were kindly supplied by BASF (Germany). Calcium chloride was purchased from Riedel-de-Haën (Germany). Lactic acid was purchased from VWR BDH Prolabo (France). Actrapid® INN-insulin human (rDNA) (Novo nordisk A/S, Denmark) was kindly supplied by Hospitais da Universidade de Coimbra. All other chemicals used were of highest grade commercially available. The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC37-HTB) and used between passage number 53 and 83. The HT29 cell line was obtained from the American Type Culture Collection (ATCC HTB-38) and used between passage number 12 and 25. Small intestinal tissues of Wistar rats for permeation studies were provided by the Center for Pharmaceutical Studies at University of Coimbra. Penicillin, streptomycin, non-essential amino acids, fetal bovine serum, trypsin-EDTA, L-glutamine, and Hank's buffered salt solution (HBSS) were purchase from Invitrogen (Spain). SnakeSkin Pleated Dialysis Tubing 10 K MWCO was purchased from Thermo Fisher Scientific Inc., USA, and SpectralPor molecular porous membrane tubing 8000–10,000 MWCO was purchased from Spectrum Laboratories Inc., USA. Epithelial voltohmmeter EVOM was purchased from World Precision Instruments, USA.

2.2. Methods

2.2.1. Nanoparticle preparation

Nanoparticles with mean diameter of 350–450 nm and polydisperse size distribution were prepared by ionotropic gelation and polyelectrolyte complexation as previously described (Woitiski et al., 2009a). Nanoparticles were prepared by dropwise addition of 7.5 mL of 0.20% calcium chloride in 117.5 mL of pH 4.9 0.06% (w/v) alginic sodium salt, 0.04% (w/v) dextran sulfate, 0.04% (w/v) poloxamer 188 and 0.006% (w/v) insulin solution under stirring. Further stabilization was obtained by dropwise addition of 25 mL of 0.04% chitosan dissolved in 0.04% lactic acid at pH 4.6. Finally, nanoparticles were coated by dropwise addition of 25 mL of 0.46% bovine serum albumin solution at pH 5.1.

Albumin-free nanoparticles were formulated by the same method, as were nanoparticles containing FITC-insulin and RBITC-alginate. Multilayered nanoparticles were concentrated by dialysis using regenerated cellulose membrane tubing with 10 K MWCO in 10% polyvinylpyrrolidone K 30 as dialysis medium for 48 h at 4 °C.

2.2.2. Conjugation of insulin with FITC, and alginate with RBITC

FITC was covalently bound to insulin as previously described (Clausen and Bernkop-Schnurch, 2000). Briefly, 0.2% FITC in dimethylsulfoxide was gradually added in volumes of 25 μL to 40 mg of insulin dissolved in 20 mL of 0.1 M Na_2CO_3. NH_4Cl was added to a final concentration of 50 mM after the solution was incubated for 8 h at 4 °C, then incubated for another 2 h at 4 °C before dialysis and lyophilization. RBITC was attached to alginate as previously described (Strand et al., 2003). Briefly, 25 μL of 0.1% RBITC dissolved in dimethylsufoxide were added to 2% alginate solution at pH of 8.0, and incubated at 40 °C during 1 h, followed by the addition of NH_4Cl to stop the reaction. Alginate and insulin solutions were dialyzed separately against distilled water for 72 h to separate unbound label. The dialysis media were changed every 12 h.

2.2.3. Scanning electron microscopy (SEM)

Multilayered nanoparticle suspension was directly deposited on a polished aluminum sampler holder, dried under vacuum,

and sputter-coated with carbon (Sputter coater SCD 050, Bal-Tec AG, Liechtenstein). Nanoparticle morphology was observed at 5 kV using a field emission gun scanning electron microscope (FEG-SEM) JSM 6330 F (JEOL Ltd., USA).

2.2.4. Caco-2 culture

Caco-2 cells were cultured at 37 °C in an atmosphere of 5% CO_2/95% O_2 and 90% relative humidity in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin, 2 mM L-glutamine, 1% non-essential amino acids, and 10% heat inactivated fetal bovine serum. Cells were subcultured once a week with trypsin-EDTA (0.25%, 0.53 mM), and seeded at a density of 4×10^5 cells per 75 cm^2 flask. Medium was changed every 2 days. It has been well documented that Caco-2 cells form an absorptive polarized monolayer, and develop an apical brush border and secrete enzymes after culture for 21 days (Delie and Rubas, 1997; Behrens and Kissel, 2003). Cells were seeded on transwell diffusion cells (3 μm pores, 1 cm^2 growth area, Corning Life Sciences, Acton, MA, USA) and maintained in incubation medium. Transepithelial electrical resistance (TEER) was measured across cells growing on 1 cm^2 polycarbonate filters of transwell diffusion cells using an epithelial voltohmmeter to evaluate tight junctions.

2.2.5. HT29 culture

HT29 cells were maintained at 37 °C in an atmosphere of 5% CO_2/95% air and 90% relative humidity, in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids, 100 IU/mL penicillin and 100 mg/mL streptomycin. Cells were used at passage numbers 12–25, routinely subcultured once a week with trypsin-EDTA (0.25%, 0.53 mM), and seeded at a density of 4×10^5 cells per 75 cm^2 flask. Medium was changed every 2 days.

2.2.6. Co-culture cells

Caco-2 and HT29 cells were re-suspended at 90:10 ratio, seeded at a density of 100,000 cells/cm^2 on transwell diffusion cells (3 μm pore size, 1 cm^2 growth area, Corning Life Sciences, Acton, MA, USA) and maintained in DMEM medium with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids, and 100 IU/mL penicillin and 100 mg/mL streptomycin. Incubation medium was changed every 2 days. Transepithelial electrical resistance (TEER) was measured across cells growing on 1 cm^2 polycarbonate filters of transwell diffusion cells using an epithelial voltohmmeter to evaluate tight junctions.

2.2.7. Permeation of insulin through Caco-2 and Caco-2/HT29 cell monolayers

Nanoencapsulated insulin suspension or insulin alone dispersed in 0.5 mL of distilled water to final insulin concentration of 100 μg/mL was placed on Caco-2 or Caco-2/HT29 cell monolayer grown on a 1 cm^2 polycarbonate filter that separates the upper and lower compartments of the transwell diffusion cell. Nanoencapsulated insulin or insulin that permeated the cell monolayer was sampled from the lower compartment which was filled with 4 mL of pH 7.4 Hank's buffered salt solution (HBSS). Samples of 200 μL were taken at different times, and replaced by the same volume of HBBS medium at 37 °C. In HBSS, nanoparticles are unstable, releasing insulin that is quantified in the supernatant by HPLC after centrifugation at 20,000 × *g* for 15 min (Sarmento et al., 2006). The transwell diffusion cells were incubated at 37 °C in atmosphere of 5% CO_2/95% air for maintaining cell viability during permeation studies. Insulin nanoformulations studied were multilayered nanoparticles, or albumin-free nanoparticles and comparisons to insulin alone without nanoparticles were carried out to evaluate the nanoparticle effect on insulin permeation across cell monolayers. Permeation assays were performed in triplicate.

Apparent permeability coefficient (P_{app}) and enhancement ratios (P_{app} nanoparticles/P_{app} insulin control) were calculated from the measurement of the transfer rate of insulin across Caco-2 cells from upper to lower compartments of the transwell diffusion cells:

$$P_{app} \quad (\text{cm/s}) = \frac{Q}{A \times C \times t}$$

where Q is the total amount of permeated insulin (μg), A is the 1 cm^2 diffusion area, C is 100 μg/cm^3 insulin in the upper compartment at time zero, and t is the time of experiment (s). The coefficient Q/t represents the steady-state flux of insulin across the monolayer.

2.2.8. Permeation of insulin through rat intestinal mucosa

Fresh excised jejunum (2–3 cm) from Wistar rats was mounted in a 0.64 cm^2 Ussing chamber, and the donor compartment was filled with 1.0 mL of pH 6.8 40 mM HEPES buffer, and the acceptor chamber was filled with pH 7.4 phosphate buffered saline solution (PBS). Ussing chambers were placed in a water bath at 37 °C during permeation studies. After 15 min, the solution in the donor chamber was replaced by incubation medium containing 100 μg/mL of nanoencapsulated insulin or insulin alone. Insulin nanoformulations studied were as multilayered nanoparticles or albumin-free nanoparticles. During 3 h incubation, 200 μL samples were taken from the acceptor chamber, and the volume replaced by the incubation medium equilibrated at 37 °C. The permeation enhancing effect of nanoparticles was evaluated by measuring the amount of insulin in the acceptor chamber using HPLC (Sarmento et al., 2006). Nanoparticles that permeate through intestinal cells release insulin in the pH 7.4 incubation medium with high amount of phosphate ions, enabling HPLC detection of insulin. Cumulative corrections were made for the previously removed samples to determine the total amount of permeated insulin. Apparent permeability coefficient (P_{app}) and enhancement ratios (P_{app} nanoparticles/P_{app} control) were calculated to determine the effect of nanoparticles on insulin permeation and to compare results from different intestinal models.

2.2.9. TEER measurements

An epithelial voltohmmeter was used to measure TEER of Caco-2 and Caco-2/HT29 cell monolayers and rat intestinal mucosa every 15 min during 3 h of permeation of insulin to evaluate viability and opening of tight junctions. In order to determine the resistance across the cellular monolayer or rat intestinal mucosa (Rtissue), a blank resistance measurement was taken in the presence of medium without cells or tissue (Rblank) subtracted from the experimental TEER value.

2.2.10. Integrity of intestinal mucosa

FITC-dextran (FD40: average mol wt 40,000) was added at 10 mg/mL to the donor chamber after the permeability study and quantified in the receptor chamber after 1 h by fluorescence spectroscopy (PTI QuantaMaster QM-1 fluorometer) at an excitation wavelength of 495 nm and emission wavelength of 515 nm. Integrity of intestinal mucosa is maintained if FD40 in the donor chamber is < 0.01% since it is impermeable to intact cells.

2.2.11. Biocompatibility of nanoformulations

Biocompatibility of nanoparticles was evaluated according to cell viability determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The assay detects living cells via mitochondrial dehydrogenases, and the signal generated is dependent on the degree of cell activation (Slater et al., 1963). Intestinal mucosa of Wistar rats were cut in 2–3 cm length

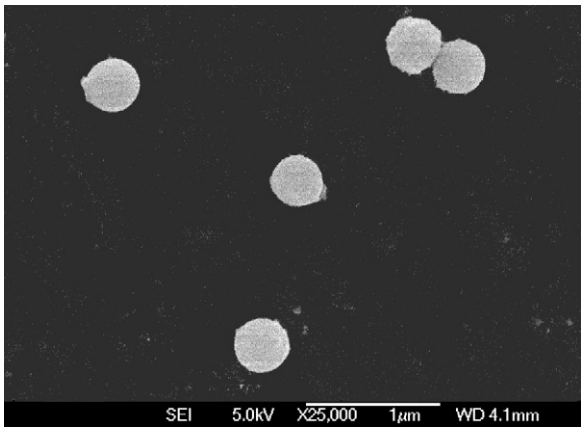

Fig. 1. Scanning electron micrograph of multilayered nanoparticles encapsulating insulin.

and incubated with multilayered nanoparticles containing insulin, and compared with intestinal mucosa exposed to HEPES buffer (untreated cells), 2% (v/v) Triton, insulin alone, empty nanoparticles, chitosan and albumin-free nanoparticles, and albumin-free nanoparticles. Intestinal tissues were incubated for 4 h at 37 °C and 100 rpm, and 1 mL of 2 mg/mL MTT filtered through 0.45 μm membrane was added to each sample for further 2 h. The medium was removed after incubation, and tissue was rinsed with 2 mL of HEPES buffer and minced with surgical scissors. Formazan was dissolved with 2 mL dimethylsulfoxide and stirred at 300 rpm for 80 min at 37 °C. The amount of formazan was quantified spectrophotometrically at 540 nm (Thermo Scientific Multiskan EX, Thermo Fisher Scientific, Inc., USA). Tests were performed in triplicate.

2.2.12. Internalization of nanoencapsulated insulin in small intestinal cells

Nanoparticles formulated with FITC-insulin and RBITC-alginate were added at 0.1 mL/cm (5 IU/mL insulin) to 3–5 cm of isolated segments of duodenum, jejunum and ileum of Wistar rats. Small intestinal tissues not exposed to fluorescent-labeled nanoparticles were used as control to exclude the possibility of autofluroescence associated with tissues. After 2 h incubation in DMEM at 37 °C in an atmosphere of 5% CO_2, samples were rinsed with 5 mL physiological solution, fixed with 10% neutral buffered formalin solution during 24 h and placed into 30% sucrose solution for 12 h for cryoprotection. Tissue samples were embedded in a cryostat medium (Tissue Tek, Miles Diagnostics, USA), and 10 μm cross-sections sliced using a cryostat (Thermo Shandon, Thermo Fisher Scientific Inc., USA). Nanoencapsulated insulin internalized in the intestinal mucosa was observed using a LSM 710 NLO Confocal Microscope (Carl Zeiss Inc., USA) with 488 and 524 nm excitation wavelengths for FITC and RBITC, respectively.

2.2.13. Statistical analysis

Mean and standard deviation (SD) of permeated insulin were calculated for each intestinal membrane model, and a one-way analysis of variance (ANOVA) followed by Bonferroni and Scheffe post hoc test (SPSS 17.0, Chicago, USA) used to determine significance ($p < 0.05$).

3. Results and discussion

Multilayered nanoparticles were formed by alginate and dextran sulfate in association with insulin, nucleating around calcium ions and interacting with poloxamer, stabilized by layering with chitosan, and subsequent coating with albumin. As seen in Fig. 1, nanoparticles appear rough, spherical and discrete. Observed particle size of around 400 nm is comparable with results obtained using Photon Correlation Spectroscopy (PCS) as summarized in Table 1, together with zeta potential measured by Laser Doppler Velocimetry.

Table 1
Characterization of insulin nanoformulations.

Insulin nanoformulations	Mean diameter ± SD (nm)	Zeta potential ± SD (mV)
Multilayered nanoparticles	317 ± 139	−37.1 ± 0.5
Albumin-free nanoparticles	239 ± 92	−34.5 ± 0.6

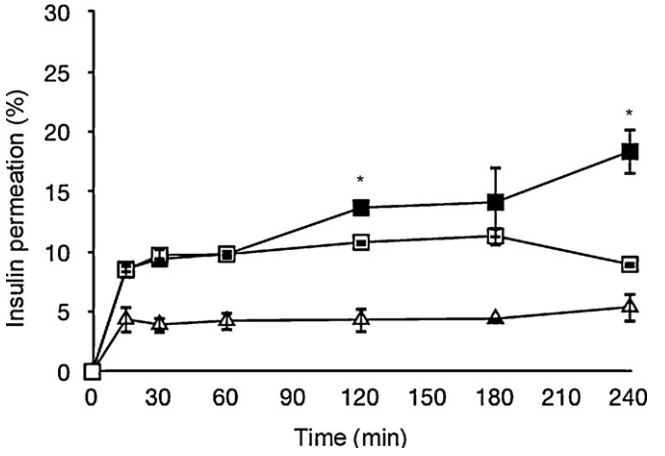

Fig. 2. Transport of insulin across Caco-2 cell monolayer. Data (means ± SD; $n = 3$) are expressed as percentage of the total dose of insulin in formulations applied to the upper compartment of transwell diffusion cells: (■) multilayered nanoparticles, (△) albumin-free nanoparticles and (□) insulin alone. (*) Indicates multilayered nanoparticles provide response that differs statistically from insulin alone, $p < 0.05$.

Multilayered nanoparticles had a mean diameter of 317 nm with polydisperse distribution. Nanoformulation free of albumin showed smaller (193 nm) mean size and polydisperse distribution. It was the objective to formulate nanoparticles under 500 nm to promote contact with intestinal cells and to facilitate permeation via different mechanisms (Florence, 2005). Nanoparticles under 500 nm are more likely to be absorbed by the transcellular pathway, while particles greater than 500 nm are more likely to be taken up by M cells of the Peyer's patches (Jani et al., 1990; Desai et al., 1997).

Nanoformulations had zeta potential less than −30 mV as seen in Table 1, representing high negative charge density that would promote colloidal stability in suspensions (Woitiski et al., 2009b). Negatively charged nanoparticles have been reported to interact with enterocytes, and therefore permeation across the intestinal barrier is more likely to occur (Rieux et al., 2005; Yin Win and Feng, 2005; Mathiowitz et al., 1997).

3.1. Effect of nanoparticles on insulin permeation across intestinal cell models

Three intestinal cell models comprising Caco-2 and Caco-2/HT29 cell monolayers, and rat intestinal mucosa were selected to study the effect of multilayered nanoparticulate carriers on insulin permeation. Caco-2/HT29 co-culture provided the ability to examine the effect of mucus layer in a cell monolayer model contributed by the mucus-secreting cells of HT29 subclones. Permeation of insulin through the intestinal models is presented as cumulative transport over time in Figs. 2–4. For each model, insulin was administered in 3 formulations. The first formulation was insulin encapsulated in multilayered nanoparticles. The nanoparticles described are complex structures essentially consisting of three layers. The inner core material contains the insulin, and that

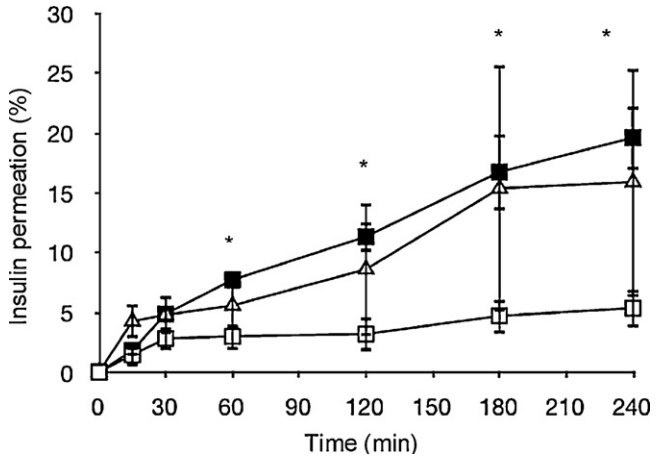

Fig. 3. Transport of insulin across Caco-2/HT29 cell monolayer. Data (means ± SD; $n = 3$) is expressed as percentage of the total dose of insulin applied to the upper side of the transwell diffusion cells: (■) multilayered nanoparticles, (△) albumin-free nanoparticles, (□) insulin alone. (*) Indicates insulin permeation enhanced by multilayered nanoparticles differ statistically from permeation of insulin alone, $p < 0.05$.

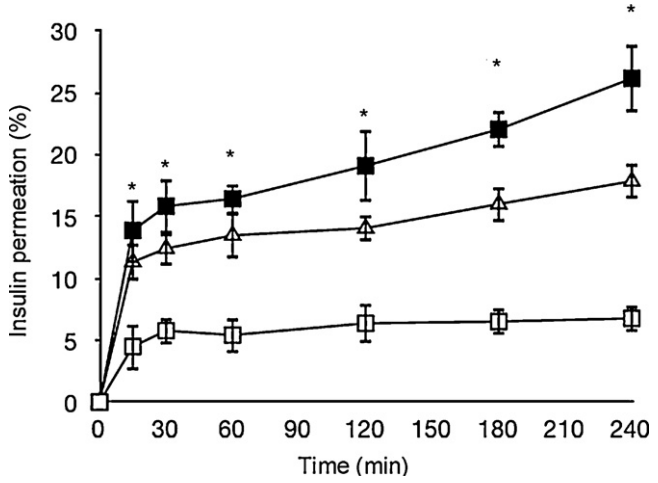

Fig. 4. Transport of insulin across rat intestinal mucosa. Data (means ± SD; $n = 3$) are expressed as percentage of the total dose of insulin applied to the luminal side of the intestinal membrane mounted in a Ussing chamber: (■) multilayered nanoparticles, (△) albumin-free nanoparticles, (□) insulin alone. (*) Indicates insulin permeation enhanced by multilayered nanoparticles differs statistically from permeation of insulin alone, $p < 0.05$.

core is subsequently coated with chitosan and then with albumin. Insulin in nanoparticles lacking the outermost albumin layer was also examined, since nanoparticles are designed to be stripped of the albumin layer during gastric and intestinal transport, thus an examination of the effect of underlying chitosan on insulin permeation is an important consideration. The function of the albumin is to serve as a sacrificial target to gut proteases, and *in vitro* evidence demonstrates that the albumin layer is being stripped away during simulated gastric/enzymatic exposure (Reis et al., 2008). This was observed by measuring the surface charge of the nanoparticles from negative to positive, when the polycationic chitosan was exposed during enzymatic removal of the outermost albumin layer. The final form of insulin tested was insulin alone, without nanoparticles. While it is unlikely that insulin administered orally in free form would remain intact to be absorbed by the intestinal mucosa, there is evidence that some insulin is prematurely released from nanoparticulate carrier in the intestine (Woitiski et al., 2009a), thus the permeation of insulin alone is an important consideration.

Starting with the simplest intestinal model, Caco-2 cell monolayer was exposed to insulin in the delivery forms described. Transport of insulin is plotted in Fig. 2, where it can be seen that the largest amount of insulin transport was facilitated by the multilayered nanoparticles. Most transport of insulin occurred in the first 15 min, with little additional transport to 240 min, except for the multilayered nanoparticles that continued to facilitate insulin transport. Insulin permeation through Caco-2 cells was significantly increased by multilayered nanoparticles in comparison to insulin alone, representing 2.1-fold enhancement of the insulin permeation coefficient. Examining results for albumin-coated nanoparticles in comparison with albumin-free nanoparticles, the albumin layer appeared to play a role in insulin protection against enzymatic degradation, maintaining insulin stability during permeation through Caco-2 cell monolayer. In addition, the paracellular permeation of insulin through Caco-2 cells can be 100 times less in comparison to human small intestine, therefore the effect of underlying chitosan layer on opening the tight junctions is difficult to determine and can be difficult to identify using this intestinal model (Wikman-Larhed and Artursson, 1995; Silva et al., 2006).

The permeation study was carried out with nanoparticles lacking the albumin layer to examine permeation when the albumin coating is largely removed. The benefit in this type of simulation is that the initial conditions including the precise structure of the nanoparticles is known, including the amount of intact insulin within the particles. The benefit of this approach then is in being able to study the role of the mucoadhesive polymer exposed following albumin removal, without the complication of needing to assess exactly how the particles have been affected in the gastric environment. It is expected that the particles remain intact during gastric transit, since insulin is not released in gastric simulation, however insulin is released upon swelling of the nanoparticles entering the small intestine as shown in a previous study (Woitiski et al., 2009a).

Insulin permeation through Caco-2/HT29 cell monolayer as shown in Fig. 3, continued steadily throughout 240 min, in contrast to permeation through the Caco-2 cell monolayer (Fig. 2), with approximately the same cumulative amount of insulin ultimately delivered by the multilayered nanoparticles (about 20%). Less insulin in free form permeated through the co-culture compared to the Caco-2 cell model possibly because the mucus layer represents a barrier to insulin permeation. The transport of insulin through Caco-2/HT29 cell monolayer increased by 3.7-fold when delivered by multilayered nanoparticles as summarized in Table 2. Albumin-free nanoparticles increased transport by 3-fold in com-

Table 2
Apparent permeability coefficient (P_{app}) and enhancement ratio (P_{app} nanoformulations/P_{app} insulin alone) of insulin permeability across Caco-2 cells, Caco-2/HT29 cells and Wistar rat intestinal mucosa.

Insulin formulations	$P_{app} \times 10^{-6}$ (cm/s) Caco-2 cells	$P_{app} \times 10^{-6}$ (cm/s) Caco-2/HT29 cells	$P_{app} \times 10^{-6}$ (cm/s) rat intestinal mucosa	Enhancement ratio Caco-2 cells	Enhancement ratio Caco-2/HT29 cells	Enhancement ratio rat intestinal mucosa
Multilayered nanoparticles	12.74 ± 0.14	13.62 ± 0.15	28.40 ± 0.30	2.1	3.7	3.9
Albumin-free nanoparticles	3.74 ± 0.08	11.04 ± 0.55	19.38 ± 0.15	0.6	3	2.7
Insulin free-form	6.21 ± 0.02	3.71 ± 0.01	7.35 ± 1.18	1	1	1

parison with permeation of insulin alone, slightly less compared to multilayered nanoparticles. In contrast, substantially more insulin was transported across the co-culture in albumin free nanoparticles, in contrast to what was observed in Fig. 2, with Caco-2 cells. Higher permeation of insulin in multilayered nanoparticles compared to albumin-free nanoparticles across Caco-2/HT29 cells is possibly related to the mucus layer that may act as an enzymatic barrier to insulin absorption, thus albumin coating is important to protect insulin while in transit through the intestine (Reis et al., 2007). An intimate contact of nanoencapsulated insulin to the intestinal mucosa can be described by the diffusion theory of mucoadhesion as defined by Peppas and Carr (2009), related to interpenetration and entanglement of polymer chains within the mucus layer. Thus, the addition of mucus-producing cells to the monolayer provided a better simulation of natural conditions, which contribute to nanoparticles facilitating insulin permeation.

Insulin transport examined across rat intestinal mucosa is shown in Fig. 4. Within 15 min, 14% of the insulin had been transported across the intestinal barrier facilitated by multilayered nanoparticles, increasing to over 25% after 240 min. This level of permeation is considerably higher than that observed for Caco-2 cell monolayer (Fig. 2). Nanoparticles lacking the albumin layer had less effect on insulin transport, and only 6% of the free-form insulin permeated the rat intestinal mucosa. Insulin transport was enhanced by multilayered nanoparticles 3.9-fold compared to insulin alone as shown in Table 2, and 1.4-fold higher than albumin-free nanoparticles. Rate-limiting steps that control extent of insulin absorption through the intestinal membrane include the rate that nanoparticles and insulin are metabolized by enzymes, and the diffusion across the mucosal layer, unstirred water layer and enterocytes. Permeation of insulin enhanced by multilayered nanoparticles is therefore related to different mechanisms that facilitate insulin intestinal permeability (Aungst et al., 1996). Thus, the nanoparticle composition shows to play an important role in enhancing insulin permeation through the intestinal mucosa. Differences in nanoparticle size comparing particles with and without albumin coating, and after various periods in buffer solutions, were not significant. In fact, nanoparticles in the size range of 50–1000 nm have shown to enable effective delivery of insulin through the gastrointestinal tract (Sarmento et al., 2007). Thus, nanoparticle composition is the key factor for insulin protection against harsh conditions through the gastrointestinal tract and in modulating insulin permeation across the intestinal mucosa.

Table 2 summarizes insulin P_{app} through the different intestinal models. Multilayered nanoparticles had higher effect on insulin transport across Caco-2 cells demonstrated by higher value of P_{app} compared to albumin-free nanoparticles and insulin alone. Insulin permeation was also significantly improved, by multilayered nanoparticles across Caco-2/HT29 co-culture and rat intestinal mucosa, as may be seen by comparing the P_{app} values of all insulin formulations (differences are statistically significant at $p < 0.05$). Results of insulin permeation through the co-culture and the intestinal mucosa facilitated by nanoparticles would indicate that co-cultures of Caco-2 cells and HT29 mucus-producing goblet cells provided an *in vitro* drug absorption model more closely related to the rat intestinal mucosa. Apparent permeability coefficients for nanoencapsulated insulin across Caco-2/HT-29 cell monolayer correlated well with fractions of insulin in nanoformulation permeating Wistar rat intestinal mucosa. The *in vitro* experimental models can therefore be designed to isolate the barrier of interest to permit relatively rapid and mechanistic evaluation of nanoformulations with enhanced absorption properties. Indeed, it was shown that once the co-culture system mimics the intestinal barriers for evaluation of the effect of the drug delivery system on insulin permeation, Caco-2/HT29 cells monolayers may be chosen over rat intestinal mucosa.

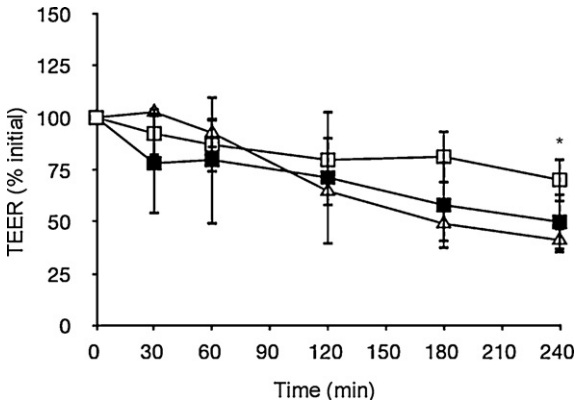

Fig. 5. Effect of insulin formulations on TEER across Caco-2 cell monolayer measured for (■) multilayered nanoparticles; (△) albumin-free nanoparticles and (□) insulin alone. Variations are based as percentage of the initial TEER value. Each point represents the mean ± SD of 3 replicate experiments. (*) Albumin-free nanoparticles differ from insulin alone, $p < 0.05$.

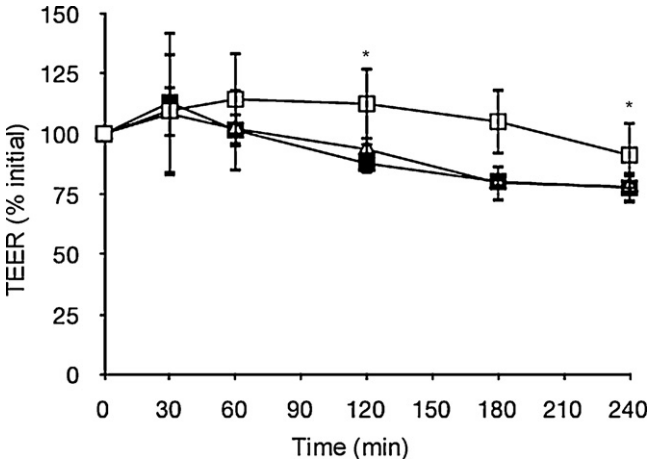

Fig. 6. Effect of insulin formulations on TEER across Caco-2/HT29 cell monolayer measured for (■) multilayered nanoparticles; (△) albumin-free nanoparticles and (□) insulin alone. Variations are based on the initial TEER values. Each point represents the mean ± SD of at least 3 replicate experiments. (*) Nanoparticles differ from insulin alone, $p < 0.05$.

3.2. Transepithelial electrical resistance (TEER)

TEER of intestinal membrane models was measured during the permeation studies since decrease in TEER represents increased ion permeability through the tight junctions (Silva et al., 2006). TEER measurements across Caco-2 cells, co-culture and rat intestinal mucosa incubated with nanoformulations or insulin alone for 240 min are shown in Figs. 5–7, respectively. Insulin is not expected to affect TEER, since it is not known to affect tight junctions, therefore insulin is the control as basis for comparison.

Incubating Caco-2 cell monolayers with multilayered nanoparticles or albumin-free nanoparticles decreased the initial TEER value by 50% and 60% respectively, after 240 min as shown in Fig. 5. Nanoparticles caused increased TEER reductions in comparison with insulin control after 240 min incubation ($p < 0.05$), where decrease in TEER values may be explained by decreased cell viability after long period of incubation. It is noted in Fig. 5 that TEER of Caco-2 cell monolayer incubated with insulin had smaller variation from the initial values in comparison with nanoparticles that showed a stronger effect in reducing the TEER.

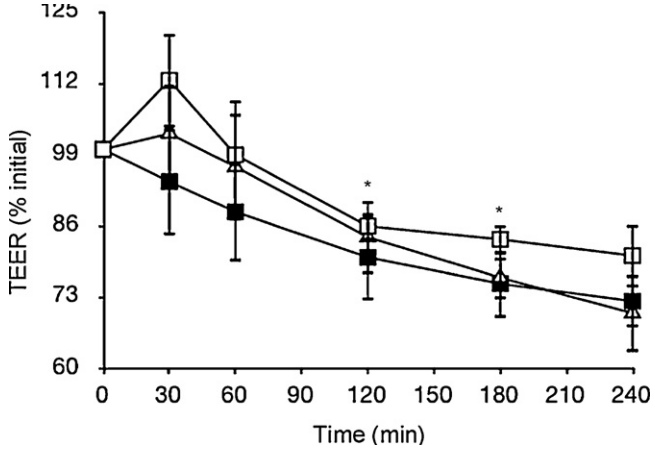

Fig. 7. Effect of insulin formulations on TEER across Wistar rat intestinal mucosa measured for (■) multilayered nanoparticles; (△) albumin-free nanoparticles and (□) insulin alone. Variations are based on the initial TEER values. Each point represents the mean ± SD of at least three replicate experiments. (*) Multilayer nanoparticles differ from insulin alone, $p < 0.05$.

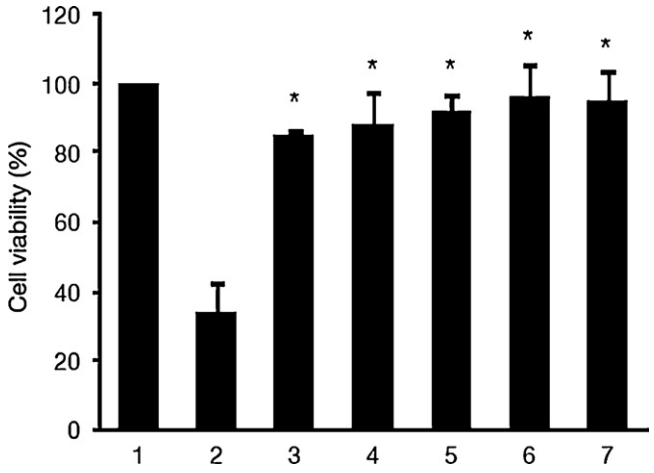

Fig. 8. Cell viability of rat intestinal tissue after 4 h, expressed as the mean ± SD, $n = 3$. Intestinal tissue incubated with (1) HEPES buffer 40 mM; (2) Triton 2% (v/v); (3) insulin alone; (4) alginate/dextran/poloxamer core encapsulating insulin; (5) albumin-free nanoparticles containing insulin; (6) empty multilayered nanoparticles; (7) multilayered nanoparticles encapsulating insulin. (*) All tissue samples are not statistically different from the intestinal tissue exposed to HEPES, $p < 0.05$.

Incubating Caco-2/HT29 cell monolayers with nanoparticles decreased TEER values by 12% after 120 min in comparison with insulin control, and a maximum reduction of 20% was measured after 180 min, sustained after 240 min incubation as seen in Fig. 6. A different pattern on reducing TEER of the co-culture when incubated with nanoparticles compared to insulin alone is showed in Fig. 6. Incubation with insulin showed little or no decrease in TEER, however decrease in TEER caused by the nanoparticles may be related to opening of tight junctions.

Decrease in TEER across the rat intestinal mucosa incubated with multilayered nanoparticles was statically significant in comparison to insulin alone, decreasing by 20% and 25% after 120 and 180 min in comparison with insulin alone, and by 27% after 240 min as shown in Fig. 7 As mentioned, decrease in TEER in the control, may be explained by decrease of cell viability. Multilayered nanoparticles caused TEER of rat intestinal mucosa to decrease to a statistically significant extent compared to the control, possibly by opening the intercellular junctions, however it may not represent the predominant mechanism of transport.

For all intestinal models, chitosan layer in both multilayered and albumin-free nanoparticle structure may interact with the tight junction proteins, promoting protein re-organization and opening of the intercellular junctions, which may facilitate drug permeation via paracellular pathway (McEwan et al., 1993; Ranaldi et al., 2002; Schipper et al., 1999).

3.3. Integrity of intestinal membranes after permeation studies

Integrity of intestinal models after permeation studies was evaluated by measuring the concentration of the fluorescent probe FD40 that may pass to the lower/acceptor compartment of the diffusion cell or through the rat mucosa if the intestinal membrane is damaged (Figueiras et al., 2009). Integrity studies were performed to rule out membranes that resulted in free passage of formulations through the intestinal membrane. Intestinal membrane models were incubated with 10 mg/mL FD40 for 1 h following completion of the permeation study. Fluorescence measured in lower/acceptor compartments showed concentration of FD40 below 0.01% for all intestinal models, indicating that membrane integrity was maintained since FD40 as a large molecule, cannot permeate the intestinal membrane and pass to the acceptor chamber unless the membrane is damaged.

3.4. Biocompatibility of nanoencapsulated insulin to rat intestinal tissue

The MTT assay has been used to evaluate the viability of tissues, where MTT is converted in viable cells to formazan by tetrazolium reductase in active mitochondria (Nicolazzo et al., 2003). The amount of formazan generated is directly proportional to the number of viable cells, and thus a measure of biocompatibility. Percentage of cell viability was in reference to full cell viability of untreated tissues incubated in HEPES buffer. Viability of the rat intestinal tissue incubated in suspensions of different nanoparticulate formulations remained between 90 and 100% after 4 h and showed no statistical difference from untreated tissues as shown in Fig. 8. Viability of tissues incubated with 2% Triton showed cell viability decreasing to 40% due to toxicity. Thus, nanoformulations showed biocompatibility to the intestinal mucosa, indicating no toxicity to the membrane.

3.5. Internalization of nanoencapsulated insulin in small intestinal cells

Internalization of nanoencapsulated insulin was studied to evaluate the absorption mechanism related to transcellular transport to facilitate insulin permeation across the intestinal membrane. Fig. 9 shows fluorescence of FITC-insulin and RBITC-alginate distributed in the small intestinal mucosal cross-sections consisting of (a) duodenum, (b) jejunum and (c) ileal Peyer's patches of follicle-associated epithelium observed using confocal microscopy. FITC-insulin emitting in green (a.1, b.1, c.1) and RBITC-alginate emitting in red (a.2, b.2, c.2) are observed internalized in the enterocytes of the different cross-sections of the rat small intestinal mucosa, since no fluorescence is observed in the lumen or mucus layer. (For interpretation of the references to color in text, the reader is referred to the web version of the article.) Fluorescently labeled insulin and alginate are mostly co-localized as observed by comparing figures .1 to .2, and through examination of figures .3, suggesting that most insulin is associated with alginate based nanoparticles passing through the intestinal membrane. The three-dimensional figures a.4, b.4 and c.4 are formed by reconstruction of scans of plane sections of the intestinal cross-section, and show that the FITC-insulin and RBITC-alginate are internalized in the intestinal cells by the random distribution of fluorescence

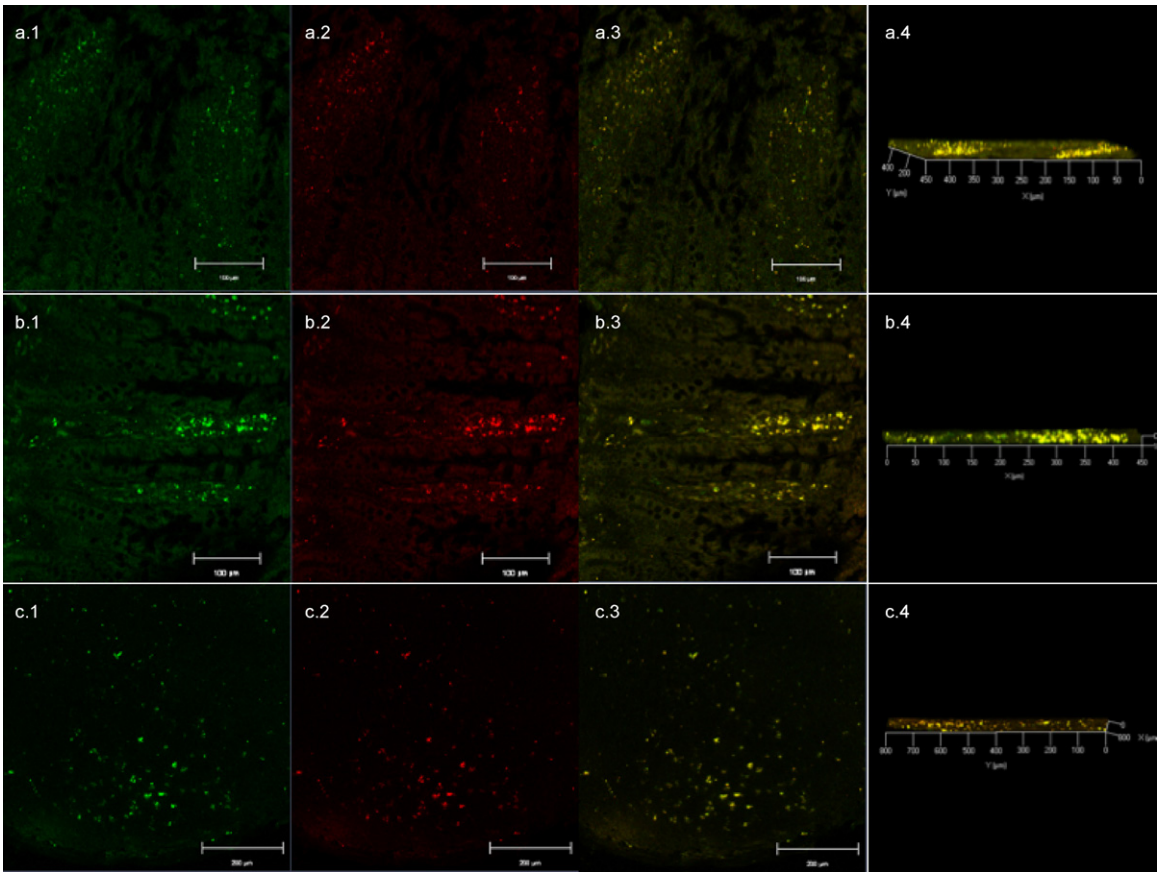

Fig. 9. Cross-sections of intestinal mucosa of Wistar rats: (a) duodenum (scale bar 100 μm), (b) jejunum (scale bar 50 μm), and (c) ileal Peyer's patches (scale bar 200 μm) showing internalization of nanoencapsulated insulin by observing FITC-insulin (a.1, b.2, c.3), RBITC-alginate (a.2, b.2, c.2), both in combination (a.3, b.3, c.3), and both in combination in three-dimensional image showed as the lateral view of the cross section (a.4, b.4, c.4).

probes in the intestinal section. It has been shown previously that nanoparticles <100 nm permeate through transcellular pathway, and >500 nm are taken up by M cells of the Peyer's patches in the ileum distal portion of the small intestinal mucosa (Florence, 2005; Jani et al., 1990; Desai et al., 1996; Florence, 1997). Transcellular transport then may be the predominant mechanism by which the multilayered nanoparticles facilitate insulin absorption. Insulin appears to remain associated with the multicomponent nanostructure while permeating through the intestinal mucosa, indicating that nanoparticles play an important role in facilitating insulin permeation through the intestinal cells.

4. Conclusion

Multilayered nanoparticles facilitated the permeation of insulin evaluated by using different models of intestinal mucosa, including cell monolayers and rat intestinal tissues. Insulin permeation across Caco-2/HT29 cell monolayer was closely related to permeation observed using animal model intestinal mucosa, showing the presence of mucus-secreting cells are important in the evaluation of the effect of delivery systems on drug permeation. Absorption of insulin facilitated by nanoparticles may be explained by different mechanisms. Alginate and chitosan components of nanoparticles, with mucoadhesive features provided intimate contact of the nanoencapsulated insulin with absorptive intestinal membrane as suggested by enhanced insulin nanoparticle permeation through Caco-2/HT29 cells and rat intestinal mucosa, in comparison to insulin alone. Chitosan may facilitate insulin permeation by potentially opening the tight junction between enterocytes, and the albumin coating may protect insulin against enzymatic degradation. In addition, permeation of the fluorescently labeled nanoencapsulated insulin across the intestinal mucosa showed that insulin is transported in association with the nanoparticles likely through the transcellular route. Thus combinations of biocompatible polymers forming a newly designed nanoparticulate delivery system, represent a promising strategy to increase *in vivo* efficacy of peroral delivery of insulin and potentially other protein drugs.

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