Uptake and transport of novel amphiphilic polyelectrolyte-insulin nanocomplexes by Caco-2 cells – towards oral insulin

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Header – Uptake and transport of novel polyelectrolyte complexes

Abstract

Purpose: The influence of polymer architecture on cellular uptake and transport across Caco-2 cells of novel amphiphilic polyelectrolyte-insulin nanocomplexes was investigated.

Method: Polyallylamine (PAA) (15 kDa) was grafted with palmitoyl chains (Pa) and subsequently modified with quaternary ammonium moieties (QPa). These two amphiphilic polyelectrolytes (APs) were tagged with rhodamine and their uptake by Caco-2 cells or their polyelectrolyte complexes (PECs) with fluorescein isothiocyanate-insulin (FITC-insulin) uptake were investigated using fluorescence microscopy. The integrity of the monolayer was determined by measurement of transepithelial electrical resistance (TEER). Insulin transport through Caco-2 monolayers was determined during TEER experiments.

Result: Pa and insulin were co-localised in the cell membranes while QPa complexes were found within the cytoplasm. QPa complex uptake was not affected by calcium, cytochalasin D or nocodazole. Uptake was reduced by co-incubation with sodium azide, an active transport inhibitor. Both polymers opened tight junctions reversibly where the TEER values fell by up to 35 % within 30 minutes incubation with Caco-2 cells. Insulin transport through monolayers increased when QPa was used (0.27 $ngmL^{-1}$ of insulin in basal compartment) compared to Pa (0.14 $ngmL^{-1}$ of insulin in basal compartment) after 2 hours.

Conclusion: These APs have been shown to be taken up by Caco-2 cells and reversibly open tight cell junctions. Further work is required to optimise these formulations with a view to maximising their potential to facilitate oral delivery of insulin.

Keywords: amphiphilic polyelectrolytes, Caco-2, transepithelial electrical resistance, insulin, quaternary ammonium moiety.

1. Introduction

Daily subcutaneous injections of insulin are required for the treatment of Type 1 diabetes mellitus and some individuals with Type 2 diabetes mellitus. Patients find this route of delivery undesirable due to the requirement to self-inject and the difficulties involved in maintaining glucose control (1-4). Therefore attempts have been made to deliver insulin via other routes such as nasal (2) and pulmonary (5). Attempts have also been made to deliver insulin orally as this is the most acceptable delivery route for both patients and clinicians (6-8). However there are a number of barriers to overcome in oral protein delivery. The large molecular weight and hydrophilic nature of proteins mean that they cannot diffuse across gastrointestinal tract (GIT) epithelial cells. In addition enzymes present in the GIT degrade therapeutic proteins such that very little of the protein is available for absorption (9-13). Several different technologies have been used to facilitate oral protein absorption and prevent degradation. These include the co-administration of enzyme inhibitors and/or penetration enhancers, pro-drug forms of proteins and colloids such as polyelectrolyte carrier systems (2, 6-8, 12, 14-17).

Cationic polyelectrolytes such as chitosan or its derivatives (i.e. trimethylated chitosan (TMC)) have been shown to form nano-complexes with proteins and facilitate protein transport across gut epithelial cells *in vitro* via both paracellular and transcellular routes (7, 8, 15, 18-22). They promote paracellular transport by interacting with tight junction

proteins via electrostatic interaction and thereby causing a temporary change in cell morphology. This leads to tight junction opening which in turn allows protein passage between cells (19, 23-26). These interactions have been shown to be reversible and less likely to result in permanent cellular damage compared to traditional permeation enhancers, i.e. carbomers and surfactants (27). Transcellular transport is also possible using PEC. It is believed that they are taken up by endocytosis/transcytosis although the exact mechanisms involved in the uptake have not been fully elucidated (2, 7, 8, 26). There are a handful of reports on the use of AP for oral protein delivery (6, 14). These types of systems thus far have been widely explored as hydrophobic drug solubilisers mainly for intravenous administration due to their ability to form polymeric micelles in aqueous solution (28). Unlike polyelectrolytes modified with hydrophilic moieties such as TMC (2), AP consist of both hydrophilic and hydrophobic moieties within the same macromolecules. These hydrophobic moieties enable these systems to interact with proteins via both electrostatic and hydrophobic interactions (6, 14, 29, 30). These additional interactions may reduce the problem of counterion effects *in vivo* which can cause PEC collapse when only electrostatic interactions are present (31).

To date, there are limited studies on the interaction of these amphiphilic polyelectrolytes-protein nanocomplexes with the intestinal epithelial cells where the studies mainly focused on paracellular transport. Simon et al. (2007) found that AP opened tight cell junctions, but did not investigate whether paracellular or transcellular transport occurred with their AP (6). Our previous work involved the use of novel amphiphilic polyelectrolytes based on 15 kDa PAA to form PECs in Tris buffer. We have previously shown that the nano-complexes with the hydrodynamic size of 200 nm were formed

spontaneously at room temperature upon mixing (29). These complexes have shown varying ability to protect insulin from *in vitro* enzymatic degradation (29, 30). From results from previous studies (29, 30) it was decided to use AP with palmitoyl grafts given that they demonstrated the highest complexation efficiency and protection against a range of gastro-intestinal enzymes compared to AP with other hydrophobic grafts.

The success of oral protein delivery systems is dependant mainly on two factors: the ability of the systems to protect against enzymatic degradation as well as facilitate the transport of protein across the gut epithelial cells. Hence, in this study we have monitored the uptake of two APs, Pa grafted PAA and QPa grafted PAA by Caco-2 cells and determined the uptake mechanisms of the polymers and their PECs. The ability of these polymers to open tight cell junctions and facilitate insulin transport across Caco-2 monolayers has also been undertaken.

2. Materials and Methods

2.1. Materials

Poly(allyl amine hydrochloride) (Mw≈15 kDa), palmitic acid-N-hydroxysuccinimide ester (98 %), tris(hydroxymethyl) aminomethane (Tris base) (≥99 %), insulin (27 U/mg, bovine pancreas), minimum essential media eagle media (EMEM), phosphate buffer tablets (PBS), rhodamine B isothiocyanate (RBITC), FITC-insulin, gluteraldehyde (grade I, 25 % in water), Hank's balanced salt solution with sodium bicarbonate, magnesium and calcium (HBSS) and sodium azide were all purchased from Sigma Aldrich, UK. Methyl iodide and all solvents were purchased from Fisher Scientific Chemicals, UK and were of

HPLC grade. All other reagents used were of analytical grade. Cell culture reagents such as L-Glutamine (200 mM), Trypsin-EDTA (0.05 %), trypan blue, DAPI, nocodazole, cytochalasin D, non-essential amino acids, Penicillin/Streptomycin (+ 10000 UmL^{-1}/+ 10000 μLmL^{-1}) were obtained from Invitrogen, Scotland and foetal bovine serum (activated) (South American) from Biosera, UK.

2.2. Rhodamine-labeling of amphiphilic polymers

The amphiphilic polymer, PAA grafted with 2.5% mole modification of palmitoyl pendant groups (Pa2.5) was produced as previously described (29). RBITC grafting was carried out using a method adapted from Lin and coworkers (2008) (32). Briefly, a 95 mL 0.05 % (w/v) solution of Pa2.5 in double distilled water was prepared. Five mL of a 1 $mgmL^{-1}$ solution of RBITC in DMSO was added to the polymer solution drop wise over 10 mins with gentle stirring on a magnetic stirrer. The solution was stirred for one hour at room temperature before being placed in a dialysis membrane (12-14 kDa cutoff; Medicell, UK) and dialysed against 5 L of double distilled water. The water was changed six times every 24 h and the dialysis was carried out over 48 h to ensure all non-bound RBITC was removed. The contents of the dialysis bag were then lyophilized over 72 h to produce a deep purple, fluffy solid (Pa2.5R). This solid was then quaternised as previously described (33) to produce RBITC-labeled quaternised PAA-palmitoyl graft 2.5% (QPa2.5R).

2.3. Cell culture

Caco-2 cells were cultured in EMEM (supplemented with 10 % foetal bovine serum, 1 % non-essential amino acids and 1% L-glutamine) at 5 % CO_2, 95 % humidity and 37 °C. All testing was carried out on cells of passage 45-60 (29).

2.3.1. Polymer, insulin and complex Caco-2 uptake study

Cells were seeded in 24 well polyester plates (Corning, UK) at $0.1x10^6$ cellsmL^{-1}. Cells were grown over 3 days before testing. Polymer and insulin stock solution were prepared separately in a mixture of 0.01 M HCl:0.1 M Tris base (87:13 % (v/v)) (29) and then diluted in EMEM (supplemented with 1 % (v/v) non-essential amino acids and 1 % (v/v) L-glutamine). Pa2.5R solutions were prepared at 6 or 12 µgml^{-1}, while QPa2.5R solutions were prepared at 48 or 96 µgml^{-1}. The polymer concentrations were prepared in accordance to the IC_{50} study conducted previously on Caco2 cells using an MTT assay (Thompson et al., 2009). QPa2.5R was also prepared at 6 µgmL^{-1} for comparison with Pa2.5R. FITC-insulin was prepared at either 3 or 6 ugml^{-1}, while non-tagged insulin was prepared at 3 µgml^{-1}. Selected polymer and FITC-insulin solutions were also prepared in calcium-free EMEM. Polymer and FITC-insulin solutions were mixed together with gentle agitation to produce polyelectrolyte -insulin complexes (PEC) with polymer:insulin concentrations of 6:3 (Pa2.5R) or 48:3 (QPa2.5R) µgml^{-1}. EMEM was then removed from each well and the wells were then washed once with EMEM (containing no serum) prior to the addition of polymer, FITC-insulin or polymer-insulin solutions (1 mL). Sample solutions were incubated with the cells for 0.5, 1 or 2 h at 5 % CO_2, 95 % humidity and 37 °C. Selected samples of cells were stained with DAPI (300

nM for 5 mins) to indicate the relative position of the nuclei. This was followed by the addition of 4% trypan blue (TB) for one min to determine cell viability. Samples were then aspirated from each well and the wells were washed (x3) with (serum free) EMEM. The plate was then incubated under the same conditions as before for 0.5 h prior to imaging with a fluorescence microscope (Leica DMI4000B, Leica Microsystems Ltd., UK). Each experiment was carried out in triplicate.

2.3.2. Determination of PEC uptake mechanism

Cells were grown and polymer, insulin and PEC samples were prepared as above. However wells were pre-incubated with either EMEM (calcium free) or non-tagged insulin for 1 h prior to sample addition.

To further elucidate the mechanism of PEC cell uptake, cells were preincubated with the metabolic inhibitor sodium azide (10 mM) or endocytosis inhibitors cytochalasin D (0.1 mg/mL) or nocodazole (1 mg/mL) for 30 mins prior to the application of the polymer complex and throughout the uptake experiment. Sample solutions were incubated with the cells for 0.5, 1 or 2 h at 5 % CO_2, 95 % humidity and 37 °C. Samples were then aspirated from each well and the wells were washed (x3) with (serum free) EMEM. The plate was then incubated under the same conditions as before for 0.5 h prior to imaging with the Leica DMI4000B fluorescence microscope. Each experiment was carried out in triplicate.

2.3.3. Insulin transport study

Caco-2 cells were seeded into 24 well polycarbonate transwell plates (Corning, UK) at a density of $3.9x10^4$ $cellscm^{-2}$ and grown for 21 days in order to form a confluent monolayer. The conditions used are as described in 2.3 except the media was supplemented with 2 % penicillin/streptomycin (+ 10000 UmL^{-1}/+ 10000 μLmL^{-1}). Fresh media was added to the apical compartment of each well on days 3, 6 and 9 and the media was changed in both apical and basal compartments on days 12, 15, 18 and 20 after seeding.

TEER measurements were taken using a Millicell-ERS potentiometer with chop-stick electrodes (Millipore, USA) before and after media addition to monitor monolayer integrity. The transport study was carried out when all wells had a resistance within the range of 300-500 $\Omega.cm^2$.

The transport study was conducted by addition of polymer and complex solutions applied to apical compartments after 0.5 h pre-incubation with HBSS alone. Polymer and complex solutions were made as in 2.3.1., except that stock solutions were diluted in HBSS instead of EMEM. After polymer and complex solutions were added to apical compartments the plate was incubated for 2 h at 5 % CO_2, 95 % humidity and 37 °C. Solutions were then aspirated and wells were washed with HBSS (x3) before placing the plate back in the incubator in EMEM for a further 2 h.

Samples of media were collected from the basal compartment of each transwell plate and replaced with fresh HBSS at 0.5, 1, 1.5 and 2 h. The insulin concentrations of these samples were determined using a Mercodia bovine insulin ELISA kit (Diagenics Ltd, UK) and the final values presented have been adjusted to reflect the change in volume at each successive sampling point. TEER measurements were taken at 0 h (before sample

addition) and at 0.5, 1, 1.5 and 2 h (before samples were removed) and at 3 and 4 h (after sample aspiration and plate washing). After the final TEER measurement, HBSS was removed from all wells and 0.1 mL of a 2.5 % (v/v) gluteraldehyde in PBS solution was added to each apical compartment. The plate was stored overnight in a refrigerator prior to further testing. Each experiment was carried out in triplicate. Results of the TEER and the ELISA were then analysed using a one-tailed Student's t test and presented as followed; $p<0.05$ *; <0.01** or $p<0.001$***.

2.3.4. Transmission electron microscopy (TEM)

Cell monolayers, were stored overnight in gluteraldehyde (2.5 % (v/v) in pH 7.4 phosphate buffer) and then prepared for TEM analysis. Samples were treated with buffer for 45 mins, osmium oxide for 1 h and then washed with distilled water three times over 30 mins. Samples were then exposed to ethanol solutions of 30-100 % (v/v) over 5.5 h. Membranes were then treated with ethanol:Spurr's resin mixtures of 7:1 to 1:6 ratios over 21 h. Membranes were then sliced in half and embedded in Spurr's resin in a flat polythene embedding capsule. Cross-sections of the membranes (80-100 nm) were then obtained on a Leica UC6 and collected on 200 mesh grids. Cross-sections were stained for contrast using the Leica AC20. The grids were viewed under a Philip's CM10 Transmission Electron Microscope.

3. Results

3.1. Caco-2 uptake of polymers and polymer-insulin nanocomplexes

Caco-2 cells were treated with rhodamine labeled polymers, free FITC-insulin and rhodamine labeled polymer-FITC insulin nanocomplexes over 0.5-2 h periods. The IC_{50} concentrations of Pa and QPa based on MTT assay were 0.021 and 0.114 $mgmL^{-1}$, respectively (29) and hence Pa at 6 μgmL^{-1} and QPa at 48 μgmL^{-1} were used, which are non-cytotoxic at these concentrations (IC_{50}). Insulin concentration was fixed at 3 μgmL^{-1}. The microscopy images showed that free FITC-insulin was found in the cytoplasm within 30 mins (Figure 1A). (These images are representative of each sample.) In contrast polymer and PEC uptake was slower and localisation within cells varied with polymer architecture. Pa was found localized in the cell membrane (after 2 h), while QPa was present in the cytoplasm/perinuclear region after 1 h (Figure 1B and C). Similar results were observed for Pa and QPa-insulin complexes. Figures 1D and 1E indicate that when insulin was complexed with Pa, it was only present around the cell membrane (after 2 h) while insulin was co-localised with QPa in the cell cytoplasm (after 1 h). Figure 1F confirms the co-localisation of QPa-insulin in the perinuclear area using a DAPI nuclear stain.

The cells were also post-treated with trypan blue (TB) after the experiment to check for the integrity of the cell membrane (2). Non-viable cells or cells with damaged cell membranes were stained blue/black due to the diffusion of TB into these non-viable cells. As can be seen in bright field image of figure 2A, there was minimal staining present and

the lack of staining of cells within the monolayer indicated the viability of the monolayer after treatment with PEC.

In addition it was found that QPa PEC were located in cell cytoplasm after an hour even at a QPa:FITC insulin concentration of 6:3 μgmL^{-1} (Figure 2B). Therefore it appeared that the difference in QPa and Pa PEC uptake was not concentration dependent.

3.1.1. Uptake mechanism of polymers and PEC into Caco-2 cells

The uptake mechanisms were subsequently elucidated to check if calcium dependant processes or insulin receptor mediated uptake were involved. Pretreatment with a high concentration of insulin (3 μgmL^{-1}) will result in the down regulation/blocking of insulin receptors. As expected, no free insulin uptake was observed following insulin pre-treatment confirming the uptake of free insulin is likely to be mediated by insulin receptors (data not shown). However neither the absence of calcium nor down regulation of insulin receptors had any noticeable effect on QPa PEC uptake, while Pa PEC uptake was prevented in the absence of calcium in the media (Figure 3).

It would appear that Pa PEC uptake required the presence of calcium ions in solution, while QPa PEC uptake involved some other mechanism(s). Therefore further studies involving the use of inhibitors of specific cellular processes were carried out on QPa-insulin nanocomplexes Sodium azide (metabolic inhibitor) or cytochalasin D and nocodazole (inhibitors of the endocytotic trafficking pathway (34)) were used separately to block intracellular trafficking prior to QPa PEC addition (2). Cells were exposed to the respective inhibitors for 0.5 h prior to PEC addition. All PEC samples were also spiked with the inhibitors to maintain inhibition over the 1 h exposure to QPa-insulin samples.

QPa was still taken up by cells even after blockage of endocytosis by nocodazole and cytochalasin D (Figure 4A and 4B).

Endocytosis requires the presence of calcium (35) and QPa PEC demonstrated uptake despite the absence of calcium in the media. Together with the result showing that QPa PEC uptake was unaffected by endocytosis inhibitors, this indicates that perhaps these nano-complexes were taken up by an active process, which is demonstrated by figure 4C. The intensity of fluorescent emission of QPa-PEC in the cells appears weaker after treatment with sodium azide compared to normal conditions (Figure 1E). This would suggest a reduced degree of uptake when cellular energy dependent processes are blocked. Further work is required to quantify this change.

3.2. Transport of insulin across Caco-2 monolayers

The ability of these two amphiphilic polyelectrolytes (Pa and QPa) to facilitate paracellular transport of insulin across a cell monolayer was investigated. Caco-2 cells were grown in transwell plates over 21 days and then treated with: Pa (6 μgmL^{-1}), QPa (48 μgmL^{-1}), insulin (3 μgmL^{-1}) or polymer-insulin complexes (6:3 or 48:3 μgmL^{-1} respectively for Pa and QPa PEC). The TEER was measured at various time points over 4 h after sample addition to the cells. TEER is a measure of cell junction integrity and will fall if the junctions are opened or damaged (6). The samples were removed after 2 h to check if the effect of opening tight junctions is reversible. This is important as it signifies that the polymers did not cause permanent damage to the tight junctions. Our result indicates that both polymers were able to open Caco-2 cell tight junctions after 30 mins (Figure 5).

The TEER values dropped by around 15-35 % in the first 30 mins after polymer or PEC addition and then gradually climbed back up. After 1h, all samples containing either QPa or Pa, with or without the insulin (QPa $p<0.001$; QPaI $p<0.05$; Pa $p<0.05$; PaI $p<0.01$) showed a significant decrease in the TEER value suggesting that the presence of the polymer had facilitated the opening of junctions. After sample removal at 2 h the TEER values for all monolayers returned to around 90 % of their original value demonstrating that this was a reversible process, Neither the control sample nor insulin alone displayed a discernable drop in TEER at any time point. This shows that insulin alone is unable to open tight junctions and that the fall in TEER was not due to a physical effect on addition/removal of samples/buffer.

It was evident that the extent of TEER reduction is less for corresponding polymer-insulin PEC. After 0.5 h, Pa PEC had lowered TEER by around 15 %, while Pa alone caused 30 % TEER reduction. Similarly QPa PEC lowered TEER by around 25 %, while QPa alone lowered TEER by 35 % after 0.5 h. It would appear that the presence of insulin reduced the interaction between the polymers and the tight junctions and hence resulting smaller reduction in TEER.

The transport of insulin across Caco-2 cells was increased when complexed with Pa or QPa (Figure 6). Insulin concentration was greater for QPa PEC than Pa PEC, which corresponded well with the TEER values. QPa PEC produced a cumulative insulin concentration of 0.27 $ngmL^{-1}$ in the basal compartment of plates, while Pa complexes only reached 0.14 $ngmL^{-1}$ after 120 mins incubation, which is 52 % less compared to QPa PEC. After 30 minutes the effect of the complexation of the insulin with either Pa or QPa was significant ($p<0.001$ ***) while samples taken from 60 minute showed evidence of

insulin in the lower chamber of the non-complexed insulin. Transverse cross sections of the cells were also analysed using TEM to determine any differences in cell morphology after treatment with the polymer samples (Figure 7).

Following the addition of the polymers the monolayers appeared intact and the microvilli were still present following the addition of the polymers demonstrating that the polymer did not damage the cells. It would have been expected that if the polymers are cytotoxic then the microvilli would have been destroyed (36). This figure is representative for all polymer and complex samples.

4. Discussion

In this study we have demonstrated that cellular uptake of free insulin is dependant on insulin-receptor interaction while insulin complexed with PAA amphiphilic polyelectrolytes uptake and transport are affected by the polymer architecture. Changes in polymer structure resulted in differences in cellular uptake rate, localisation within cells and mechanism of uptake as well as facilitation of insulin transport across cell layers. Free insulin was taken up via insulin receptors within 30 min while QPa and Pa PEC uptake took 1 h and 2 h respectively. Fluorescent microscopy images indicated co-localisation of Pa-insulin PEC around cell membranes and the use of DAPI stain indicated a co-localisation of QPa-insulin PEC around the cell nuclei. This co-localisation suggests PEC were taken up as a whole and PEC did not dissociate into free insulin and AP before being taken up by the cells.

To rule out the uptake mechanisms between Pa and QPa PEC was not due to the difference in polymer concentration, a lower concentration similar to Pa PEC was used.

Given that QPa PEC uptake was still evident at low polymer concentrations (Figure 3B), it indicates that the differences in cellular localization and uptake mechanism was is likely to be due to the presence of quaternary ammonium moieties in QPa. Quaternary ammonium moiety confers a permanent, pH independent, positive charge which allows stronger electrostatic interaction with insulin (29). There are organic cationic transporters in the small intestine wall and Caco-2 cells which are used to mediate uptake of both endogenous cations, e.g. thiamine, cationic small drug molecules, e.g pancuronium bromide, and 'model' compounds, e.g. tetraethyl ammonium all of which have quaternary ammonium moieties (37). Therefore QPa may be able to interact with these transporters as well to facilitate its uptake into cells. However the cations taken up by these receptors are much smaller in molecular weight than QPa; all of them have a molecular weight of less than 800 Da (37) compared to QPa which has a molecular weight of around 15000 Da (33). Therefore further work involving saturating these receptors with known ligands would be required to demonstrate that such a large molecule could be taken up by these receptors.

Depletion of intracellular stores of ATP using sodium azide appeared to reduce, but did not prevent, QPa PEC uptake. This was not due to cell death or damage as illustrated by the lack of trypan blue uptake. This would suggest that QPa PEC uptake was partly energy dependent (2). Neither the use of cytochalasin D nor nocodazole, as inhibitors of the endocytotic trafficking pathway appeared to have any effect on QPa PEC uptake. It is possible that the uptake of QPa may be similar to that of another polycation, poly-L-lysine. It was found that internalisation of poly-L-lysine was reduced when cell energy was depleted (26). However this is in contrast to Mao and coworkers who found that

uptake of polyethylene glycol (PEG) grafted TMC was reduced in the presence of sodium azide as well as nocodazole and cytochalasin D, which they hypothesized the uptake was due to adsorptive endocytosis. The difference in the mechanism of uptake may be due to the absence of hydrophobic pendant groups in PEG grafted TMC. It is likely that hydrophobic groups may interact with cell membranes, but this has not yet been explored.

The differences in polymer structure were also found to alter the extent of insulin transport across Caco-2 monolayers in transwell plates. QPa PEC resulted in a 50% increase of insulin concentration in the basal chambers compared to Pa PEC (Figure 6). This would again correlate well with cell uptake studies demonstrating the presence of quaternary ammonium moieties had an effect on PEC cell interaction. Free insulin was also present in the basal compartments although it was markedly lower in concentration compared to when the insulin was complexed with both Pa and QPa (Figure 6). Free insulin cannot passively diffuse across epithelial cells as it is a large (5800 D) molecule with a low lipophilicity (16). Since free insulin did not reduce TEER, it indicates that insulin itself is able to cross cells possibly via insulin receptors, as shown in our cellular uptake study. It is likely that insulin receptors in the GIT wall and in Caco-2 cells initiate receptor-mediated endocytosis (active transcellular transport) (16) where insulin can then be carried from apical to basal membranes via the Golgi-apparatus (16, 38). It might have been expected that any insulin entering cells would have been degraded by intracellular proteases (16, 39). However the relatively large concentration of insulin used (3 μgmL^{-1}) could have resulted in enzyme saturation resulting in a small amount of insulin passing

through the cells undegraded. Further work on intracellular enzymes and the rate and mechanisms of transcellular and exocytosis of insulin would be required to clarify this. The increase in insulin transport across Caco-2 monolayers on complexation with both Pa and QPa would indicate that AP were able to facilitate paracellular transport via tight cell junction opening. It was evident from the data that the presence of either Pa or QPa facilitated transport of insulin across the epithelial barrier after 30 minutes. While non-complexed insulin transport was only apparent after 60 minutes suggesting a difference in transport mechanism utilized. This is similar to other polycations including poly-L-lysine and chitosan (19, 23-26). They are able to reversibly open tight cell junctions due to electrostatic interaction with F-actin and tight junctional proteins (ZO1 and occludin). During these interactions, changes in the F-actin cytoskeleton and cell localisation of ZO1 and occludin alter cell morphology and thus allow the opening of the junctions (19, 23-26).

However the APs ability to lower TEER appeared to be reduced by the formation of PEC. Reductions in TEER values were 10-15 % lower after 30 mins when PEC were incubated with Caco-2 cells compared to AP alone. It has been shown previously that the charge on the quaternised AP is roughly halved when they form PEC with insulin (29). A similar effect has been found with chitosan and heparin (15) and TMC with insulin (8). In both cases the zeta potential and subsequent ability to open tight cell junctions and facilitate transport across Caco-2 monolayers was reduced in comparison to solutions of polymer co-incubated with the protein. The formation of PEC is driven by Coulombic interactions between the positively charged moieties on the polymer and the negatively charged groups on the protein. Therefore charge reduction on the polymer chains will take place

upon PEC formation resulting in a reduced ability to form electrostatic interactions with cell junction proteins (8).

The relatively low concentration of insulin (1% of total insulin concentration) present in basal chambers for QPa PEC could be due to either insulin still remained in the apical chamber and/or the inherent limitations of paracellular protein transport (20) or incomplete transcellular transport (7, 8) It has been shown that paracellular transport is limited by the molecular weight of proteins and peptides due to the narrow size of junctions (2, 20). Kotzé et al. (1997b) found that the ability of TMC solutions (2.5 % (w/v)) to facilitate hydrophilic molecule transport across a Caco-2 monolayer was limited by the molecular weight of the molecule (20). The transport of [^{14}C]-mannitol (182.2 D) was greater than that of buserelin (1299.5 D) which in turn was greater than dextran (4400 D); 12 %, 2.25 % and less than 1 % of the total dose in basal compartment of transwell plate after 180 mins, respectively. This occurred even though the TMC solution reduced TEER by around 80 % of its initial value within the first 20 min and the reduction was maintained up till 120 mins. Their work demonstrates clearly that facilitation of paracellular transport is largely dependent on the molecular weight of the hydrophilic molecule regardless of the extent of TEER reduction. Insulin has a larger molecular weight (5800 Da) compared to those model compounds and hence it is expected that its ability to go through tight junctions will be extremely limited.

It has been shown that when formulated as part of a PEC, polyelectrolytes may be able to facilitate both paracellular and transcellular transport of proteins (7, 8). When formulated as PEC, polyelectrolytes have been shown to interact electrostatically with cell membrane and taken up by non-specific endocytosis/transcytosis as long as they have a positive

charge and are less than 200 nm (7). This correlates well with our result since QPa and Pa PEC have an overall positive charge of between 23 to 58 mV with particle size of 118-123 nm (29). As discussed above, Pa and QPa PEC were shown to be taken up by cells within 2 h and 1 h, respectively (Figure 1). Therefore facilitation of cellular uptake of PEC may have occurred within a similar time frame to tight junction opening. The slower uptake of PEC compared to free insulin may indicate some insulin was indeed trapped within cells during the transwell experimental work which could in part account for the low insulin concentrations in basal compartments. This slow uptake could mean that PEC may not have passed across monolayers transcellularly within the 2 h of the study and could have become trapped inside the Caco-2 cells in their Golgi apparatus or endoplasmic reticulum (18). In addition, it is not known if the dissociation of insulin from PEC occurs during the intracellular trafficking. Future studies involving tracking the dissociated insulin and analysis of intracellular insulin concentration will be required to confirm these hypotheses.

5. Conclusion

Our work is the first attempt to elucidate the cellular uptake mechanisms of amphiphilic polyelectrolyte- insulin complexes based on polyallylamine modified with hydrophobic and hydrophilic moieties. We have shown that Pa- and QPa-insulin PEC are taken up by Caco-2 cells via different mechanisms to free insulin. Insulin alone is taken up by insulin receptors and enters cell cytoplasm within 30 mins. Pa PEC are located around cell membranes after 2 h while QPa complexes are taken up into the cell after 1 h. Pa PEC appears to be taken up by a calcium dependent process while QPa PEC seems to be taken

up by an active process(es). Both Pa and QPa are able to facilitate insulin transport across a Caco-2 cell monolayer by reversibly opening tight cell junctions (paracellular transport) as well as possibly promoting insulin transport through cells (transcellular transport). This work shows the potential of these PECs in oral protein delivery and future work using an *in vivo* model will be needed to determine the potential use of these novel polymers in promoting oral absorption of insulin.

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Notations

Q – quaternary ammonium moiety

AP – amphiphilic polyelectrolyte

Pa – palmitoyl

PEC – polyelectrolyte complex

TEER – transepithelial electrical resistance

PAA – polyallylamine

EMEM – eagle minimum essential media

TB – trypan blue

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Legends to Figures

Figure 1. Fluorescent microscopy images of Caco-2 cells (representative of each sample) after incubation with different samples at 37 ^{o}C. (A) FITC-insulin after 30 min incubation (3 μgml^{-1}); (B) Pa after 2 h (6 μgml^{-1}); (C) QPa after 1 h (48 μgml^{-1}); (D) Pa PEC after 2 h (polymer:insulin of 6:3 μgml^{-1}); (E) QPa PEC after 1 h (polymer:insulin of 48:3 μgml^{-1}); (F) QPa PEC (polymer:insulin of 48:3 μgml^{-1}) post-treated with DAPI stain (Mag. x400 (to eyepiece)).

Figure 2. Microscopy images of incubation of Caco-2 cells with polymer-insulin PEC for 1 h at 37 ^{o}C. Exposure of Caco-2 cells to (A) bright field of QPa-insulin complex (polymer:insulin of 48:3 μgml^{-1}) post-treated with trypan blue (representative of all PEC samples); (B) fluorescent image of QPa-insulin complex (polymer:insulin of 6:3 μgml^{-1}) (scale bar 50μm).

Figure 3. Fluorescent microscopy images of incubation of Caco-2 cells with polymer-insulin PEC at 37 ^{o}C. Cells pre-treated with calcium-free media for 1 h then exposed to Pa-insulin for 2 h (A) or QPa PEC for 1 h (B); Cells pre-treated with 3 μgmL^{-1} insulin for 1 h then exposed to Pa PEC for 2 h (C) or QPa PEC for 1 h (D) (scale bar 50μm).

Figure 4. Fluorescent microscopy images of incubation of Caco-2 cells with QPa-insulin PEC for 1 h at 37 ^{o}C. Cells exposed to QPa-insulin for 1 h after pre-treatment with (A)

nocodazole; (B) cytochalasin D; (C) sodium azide for 0.5 h prior to QPa-insulin exposure (scale bar 50μm).

Figure 5. TEER of Caco-2 cells exposed to: HBSS buffer; Insulin (3 μgmL^{-1}); Pa (6 μgmL^{-1}); QPa (48 μgmL^{-1}); Pa:Insulin (6:3 μgmL^{-1}) or QPa:Insulin (48:3 μgmL^{-1}) for 2 h. Samples were removed at 2h, cells washed (x3) and replaced with fresh media (n=3; ± SD). After 1h, all samples containing either Qpa or Pa with and without insulin showed significant decrease in the TEER value when compared to control. Insulin alone showed no significant decrease.

Figure 6. Cumulative insulin concentration in the basal compartment of transwell plates after addition of: Insulin (3 μgmL^{-1}); Pa-Insulin (6:3 μgmL^{-1}) or QPa-Insulin (48:3 μgmL^{-1}) to apical compartments (n=3; ± SE). (*<0.05; **<0.01; ***<0.001 effect of polymer when compared with insulin alone at respective time point). Insulin values at 30 minutes were below assay detection limits.

Figure 7. TEM cross sections of Caco-2 cells after exposure to: (A) Pa (6 μgmL^{-1}); (B) Pa-Insulin (6:3 μgmL^{-1}) for 2 h. Scale bar = 2 μm.