

COMPLEXATION OF NOVEL THIOMERS AND INSULIN TO PROTECT AGAINST *IN VITRO* ENZYMATIC DEGRADATION – TOWARDS ORAL INSULIN DELIVERY

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ABSTRACT

A significant barrier to oral insulin delivery is its enzymatic degradation in the gut. Nano-sized polymer-insulin polyelectrolyte complexes (PECS) have been developed to protect insulin against enzymatic degradation. Poly(allylamine) (Paa) was trimethylated to yield QPaa. Thiolation of Paa and QPaa was achieved by attaching either *N*-acetylcysteine (NAC), or thiobutylamidine (TBA) ligands (Paa-NAC/QPaa-NAC and Paa-TBA/QPaa-TBA thiomers). PEC formulations were prepared in Tris buffer (pH 7.4) at various polymer:insulin mass ratios (0.2:1-2:1). PECS were characterised by %transmittance of light and photon correlation spectroscopy. Insulin complexation efficiency and enzyme-protective effect of these complexes was determined by HPLC. Complexation with insulin was found to be optimal at mass ratios of 0.4-1:1 for all

polymers. PECS in this mass range were positively-charged (20-40 mV), nanoparticles (50-200 nm), with high insulin complexation efficiency (> 90 %). Complexation with TBA polymers appeared to result in disulphide bridge formation between the polymers and insulin. *In vitro* enzymatic degradation assays of QPaa, Paa-NAC, and QPaa-NAC PECS showed that they all offered some protection against insulin degradation by trypsin and α -chymotrypsin, but not from pepsin. QPaa-NAC complexes with insulin are the most promising formulation for future work, given their ability to offer protection against intestinal enzymes. This work highlights the importance of optimising polymer structure in the delivery of proteins.

Keywords: Thiomers; Insulin; Polymer-protein complexes; enzymatic degradation; oral protein delivery.

Abbreviations: Poly(allylamine) – Paa; Quaternised Poly(allylamine) – QPaa; PolyElectrolyte Complexes – PECS; *N*-acetylcysteine – NAC; thiobutylamidine – TBA; Gastro-Intestinal Tract – GIT; thiolated polymers – thiomers; *N*-(3-Dimethylaminopropyl)-*N*'-Ethyl Carbodiimide hydrochloride – EDAC; *N*-Hydroxysuccinimide – NHS; Hydrodynamic Diameter – H. diameter; PolyDispersity Index – PDI; Transmission Electron Microscopy – TEM.

INTRODUCTION

Oral delivery of insulin used for the management of Type 1 Diabetes is one of the major long term goals of diabetes research. The limited treatment options for maintenance of blood glucose levels (parenteral insulin), predisposes those with diabetes to physiological stress and pain due to multiple daily injections.¹ Also, parenteral administration of insulin results in abnormal systemic distribution of insulin which has been associated with the occurrence of peripheral hyperinsulinaemia and insulin resistance causing hypoglycaemia, weight gain, neuropathy, retinopathy, atherosclerosis and hypertension.² The need to eliminate these drawbacks as well as offer individuals affected by diabetes a better quality of life has prompted researchers to explore other routes for the delivery of insulin.

The oral route offers an excellent alternative being the easiest and most convenient route of drug administration and also demanding less time and effort from medical personnel and carers.³ Also, on oral administration exogenous insulin distribution mimics the natural physiological fate of insulin in the body thereby closely replicating the direct delivery of endogenous insulin to the liver, where its effects of suppressing or facilitating hepatic glucose production are fundamental in sustaining glucose homeostasis.⁴ However, the bioavailability of orally administered insulin is markedly low (estimated to be less than 1 %) due to proteolytic digestion in the gut and limited absorption of insulin from the gut lumen due to its high hydrophilicity and low log P value.⁵ Retaining the integrity and bioactivity of insulin during formulation and manufacturing procedures presents an additional difficulty in achieving oral administration. Disturbances in the primary, secondary or tertiary structure of insulin

(due to processing conditions) can lead to its deactivation and/or denaturation resulting in loss of pharmacological activity.⁶

Current research in this area has been focused on the development of novel oral formulations of insulin that can overcome these barriers and maximise the bioavailability of oral insulin. Some approaches involve co-administration with known absorption enhancers like fatty acids and protease inhibitors, while others involve the use of smart polymers or carrier systems that can protect insulin from proteolytic digestion while also mediating its absorption across the gastro-intestinal tract (GIT) epithelium.⁷⁻⁹

Recent advances in the design of functional polymeric drug delivery systems have led to the utilisation of polyelectrolyte complexes (PECS) for protein delivery. PECS are formed spontaneously by electrostatic interaction between oppositely charged polyelectrolytes (i.e. a polymer and protein) in aqueous solution.^{10,11} Indeed, PECS containing insulin and other proteins have already been the subject of research by a number of groups.¹¹⁻¹⁶ These complexes are typically positively charged nanoparticles, with hydrodynamic sizes between 100-400 nm in aqueous media. Polyelectrolyte complexation has the advantage of being a more benign process than other methods of manufacturing nanoparticles, which usually require the use of aggressive processing conditions and organic solvents. Formulation of proteins as PECS can also impart improved characteristics, i.e. enhanced absorption and reduced degradation, via modification to the polymeric carrier, rather than to the protein itself; thereby, preserving the original structure of the protein.¹⁵ Hence, rational optimisation of polymer structure to provide a robust network to facilitate optimum complexation, enzymatic protection and GI absorption of proteins is required.

Polyallylamine (Paa) was previously modified by both hydrophobic (palmitoyl, cetyl and cholesteryl ligands) and hydrophilic (quaternary ammonium salt) moieties for use in oral insulin delivery by our group.¹⁷ Quaternised Paa (QPaa) complexation with insulin resulting in spherical nano-sized PECS which exhibited good insulin loading efficiency, protected insulin from tryptic degradation and promoted uptake of insulin by Caco-2 cells.^{16,17} These modified Paa polymers were, however, limited in their protective effect against α -chymotrypsin and pepsin-mediated degradation of insulin.¹⁷ Subsequent work has, therefore, been based on developing a new set of polymers which overcome this limitation: thiolated version of Paa (thiomers).^{16,18}

Thiomers have also been found to be able to maximise the amount of oral insulin available for absorption by chelating the metal ions of endogenous proteases thereby curtailing their activity.^{12,19-21} Thiolated polymers have also been observed to be strong mucoadhesives as they contain reactive thiol groups capable of forming disulphide bonds with cysteine-rich domains of mucus glycoproteins.^{14,22} Mucoadhesive dosage forms also promote oral absorption by adhering tightly to the intestinal mucosa, prolonging the residence time of the active molecule at its absorption site and creating a local concentration gradient that maximises uptake of the drug through the intestinal mucosa.¹⁹

The aim of this study was to develop and evaluate a set of thiolated Paa polymers which were used to form PECS with insulin. This work reports on evaluation of the impact of Paa quaternisation and thiolation on insulin complexation efficiency, particle size and morphology, enzyme-protective capacity and mucoadhesive characteristics of the resultant polymer-insulin PEC. For the first time, we have demonstrated the significance

of the overall polymer structure and the type of thiolation of Paa plays an important role in protecting insulin from enzymatic degradation.

MATERIALS AND METHODS

Materials

Poly(allylamine hydrochloride) (average Mw = 15 kDa) was converted to its free base, as previously described.¹³ Tris(hydroxymethyl)aminomethane (Tris base) ($\geq 99\%$), iodomethane, *N*-(3-Dimethylaminopropyl)-*N'*-ethyl carbodiimide hydrochloride (EDAC), *N*-hydroxysuccinimide (NHS), *N*-acetylcysteine (NAC), 2-iminothiolane hydrochloride, sodium borohydride, insulin (27 units per mg/Umg⁻¹, from bovine pancreas), tris(hydroxymethyl)aminomethane (Tris base) ($\geq 99\%$), pepsin (3640 Umg⁻¹, from porcine gastric mucosa), α -chymotrypsin (TLCK treated, Type VII from bovine pancreas, 40 Umg⁻¹), trypsin (TPCK treated, from bovine pancreas, 11,004 Umg⁻¹) and porcine gastric mucin (crude type II) were all purchased from Sigma-Aldrich UK. Mercodia Bovine insulin ELISA kit and sample buffer were obtained from Diagenics Ltd. UK.

Trifluoroacetic acid (TFA) and Acetonitrile (HPLC grade) were purchased from Fischer Scientific, UK. Other chemicals used were of analytical grade.

Polymer synthesis

The method used for the quaternisation and purification of Paa (QPaa) was adapted from the previous work of Thompson *et al.* (n=3).¹³

The methods used for the thiolation of Paa/QPaa were adapted from that used by other groups^{14,19}, but have also been reported and described in detail in a previous publication by our group.¹⁸ Briefly, thiolation of Paa/QPaa using *N*-acetylcysteine (n=3) was carried out by dissolving NAC (250 mg, 1.53 mmol) in 100 ml deionised water containing EDAC/NHS (250 mmol) at pH 4-5 and stirring for 1 hr under nitrogen before adding the Paa (250 mg) and leaving the reaction to proceed for 5 hrs at room temperature. These polymers are designated as Paa-NAC and QPaa-NAC. Thiolation with 2-iminothiolane hydrochloride (n=3) involved reacting the polymer (500 mg) with 2-iminothiolane hydrochloride (400 mg) in 50 ml deionised water and the reaction mixture left stirring under nitrogen for 14 hrs in the dark. These polymers are designated as Paa-TBA and QPaa-TBA.

All thiolated polymers were subsequently recovered by dialysis against dilute (0.1 M) hydrochloric acid at 4°C and subsequently freeze-dried.

The degree of polymer quaternisation was obtained by elemental analysis, while the level of polymer thiolation was determined by iodometric titration of free thiol groups (estimation of total amount of thiol groups added onto the polymer backbone was done after disulphide bond reduction with sodium borohydride) as previously reported.¹⁸

Preparation of polymer:insulin complexes

Formulations of polymer:insulin complexes were prepared in glass vials at room temperature by mixing equal volumes (2 mL) of polymer (polymers used include Paa, Paa-TBA, Paa-NAC, QPaa, QPaa-TBA and QPaa-NAC) with insulin, both previously dissolved in pH 7.4 Tris buffer, as per previous work.²³ For optimal formulations of

PECS to be identified, polymer final concentrations were varied between 0.2-2 mg/mL, while insulin was tested at two final concentrations (0.25 and 1 mg/mL) to give polymer:insulin mass ratios of 0.2:1 up to 2:1 (n=3). Complexes were formed spontaneously after mixing the polymer and insulin solutions. The pH of formulations was checked to ensure it was maintained at 7.4, after which the complexes were left to stand for 2 hours at room temperature before characterisation.

Characterisation of polymer:insulin complexes

Transmittance studies

Transmittance measurements (% transmittance at 630nm) was carried out on 2 ml samples of formulations prepared at all polymer:insulin (P:I) mass ratios using an Agilent G1103A photo diode array (Agilent Technology, China) and the data obtained used for selecting optimal PEC formulations. Tris buffer pH 7.4 and insulin stock solutions were used as controls (n=3).

Particle size analysis

The average hydrodynamic diameter (H. diameter) and polydispersity index (PDI) of insulin complexes formed by each polymer was analysed at 25°C by PCS (Zetasizer Nano-ZS, Malvern Instruments, UK) after 1ml of each complex formulation had been transferred into a plastic cuvette (n=3).

Zeta potential measurements

The zeta potential of complexes formed within each test sample was determined by filling a folded capillary cell with a sample of each complex formulation and analysing at 25°C using the Zetasizer Nano-ZS (n=3).

Complexation efficiency

Determination of insulin complexation efficiency was carried out as described in similar studies conducted previously by our research group.²³ The amount of complexed insulin present in each PEC formulation was quantified by HPLC-fluorescence analysis (Shimadzu HPLC system composed of a DGU-20As degasser attached to an LC-20AD pump with a SIL-20A autosampler, a CTO-10ASvp column oven at 25 °C and a RF-10Axl fluorescence detector: $\lambda_{\text{excitation}} = 276 \text{ nm}$; $\lambda_{\text{emission}} = 600 \text{ nm}$) and the value expressed as a percentage of the amount of insulin detected in a corresponding insulin stock solution.

The stationary phase was an XBridge™ BEH 130 C₁₈ column (150 mm x 4.6 mm) (Waters, U.K.) and the mobile phase was water/acetonitrile (68.5:31.5 %) buffered to pH 2 with TFA at a flow rate of 1 mlmin⁻¹. The insulin peak was detected at 5 minutes and the insulin concentration calculated from a calibration curve prepared from dilutions of a standard stock solution (0.015-1.5 mg/mL; R² = 0.99; n=3).

Complexation efficiency of some complexes was also determined by calculating insulin concentrations using a Merckodia bovine insulin ELISA kit (Diagenics Ltd., UK). Briefly, complexes prepared at 0.8:1 (P:I) ratio were diluted down to an estimated insulin concentration of 2.5 µg/L using ELISA sample buffer and the amount of insulin contained in equivalent samples (1 ml) of complex and control was obtained following

the method outlined by the manufacturer (Diagenics Ltd. UK). The insulin content of both control and complex samples (n=3) was determined using the values obtained from a calibration plot (n=3; $R^2 = 0.98$) prepared from insulin standards provided in the kit (0.05-3 $\mu\text{g/L}$).

Transmission Electron Microscopy (TEM)

The morphology of polymer, insulin complexes was visualised using a LEO 912 energy filtering transmission electron microscope at 100/120kV. Formvar/Carbon-coated 200 mesh copper grids were glow discharged and complex solutions dried down to a thin layer onto a hydrophilic support film. Aqueous methylamine vanadate (1 %) (Nanovan; Nanoprobes, Stony Brook, NY, USA) was applied and the set-up air dried before imaging

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In vitro enzymatic degradation studies

Trypsin (6.4 mg/mL, 2.7×10^{-4} M), test complex suspensions and insulin-alone control (0.25 mg/mL) (each 4.5 ml) solutions were prepared separately in pH 8 Tris buffer and incubated in a water bath at 37°C for 2 hours. Trypsin (0.05 ml) was then added into each complex solution (n=3). Aliquots of the mixture (0.2 ml) were withdrawn every 30 minutes and mixed with ice cold TFA solution (0.015 ml, 0.1 % v/v) to stop enzymatic activity. The experiment was conducted for 4 hours at 37°C with samples being analysed by HPLC, as described earlier. The same process was repeated with α -chymotrypsin (5 mg/mL, 2.0×10^{-4} M) in pH 8 Tris buffer (n=3).

Peptic degradation studies was carried out by dissolving pepsin (0.1 mg/mL, 2.8×10^{-6} M) in 0.01 M HCl after which complex, insulin-alone control (0.25 mg/mL) and enzyme solutions were then buffered to pH 2 with a drop of 5 M HCl and incubated in a water bath at 37°C for 2 hrs (n=3). Pepsin (0.016 ml) was added into the complex solutions and samples (0.15 ml) were drawn from each mixture every 30 minutes and put into ice cold Tris base (0.15 ml; 0.1 M) to halt enzyme activity.

Results obtained for PECS in all enzymatic degradation studies were expressed as % of non-degraded insulin present in each sample over time, in order to compare with the insulin-alone controls as per a previous study.¹⁷

In vitro evaluation of mucoadhesive capacity of complexes

Assessment of the mucoadhesive capacity of each polymer, insulin PEC formulation was determined by measurement of the amount of mucin adsorbed by the complexes using an established mucin adsorption assay method.¹⁸ Experiments were carried out by mixing 1 ml of mucin solution (Tris buffer pH 7.4; 1 mgml⁻¹) with a 0.25 ml sample of each PEC formulation. Controls were prepared by mixing 0.25 ml of Tris buffer pH 7.4 with 1 ml (1 mgml⁻¹) mucin solution. An additional control sample containing mucin and 0.25 ml of insulin in Tris buffer pH 7.4 (0.25 mgml⁻¹) was also prepared. These mixtures were subsequently incubated at 37°C on a shaking water bath for 5 hrs.

All control and test samples were subsequently transferred into separate Eppendorf tubes and centrifuged at 10,000 rpm for 30 minutes, and the concentration of mucin in each supernatant measured by UV spectrometry at 251 nm. Percentage (%) of total mucin adsorbed to complexes was obtained as shown below¹⁸:

Percentage (%) of total mucin adsorbed to each sample of polymer was calculated as shown below:

$$\% \text{ mucin adsorption } (M_{ad}) = [M_o - M_s] / M_o \times 100$$

Where,

M_o = concentration of free mucin in control supernatant

M_s = concentration of free mucin in the sample supernatant

RESULTS AND DISCUSSION

Polymer synthesis

Quaternisation of Paa was confirmed by ¹H-NMR and average degree of Paa quaternisation determined by elemental analysis and found to be 72 ± 2 mol%, as per previous publications.^{13,16,18} Subsequent thiolation of both Paa and QPaa yielded two types of thiolated derivatives-the thiobutylamidine (TBA) conjugates (Paa-TBA and QPaa-TBA) and N-acetylcysteine (NAC) conjugates (Paa-NAC and QPaa-NAC). Based on results of iodometric titration the thiol content of each thiomers was obtained and is displayed in table 1. Further characterisation of these polymers including IR analysis, thermal analysis, mucoadhesive characteristics and biocompatibility have been described in these publications.^{16,18}

Characterisation of polymer:insulin complexes

Transmittance studies

For each polymer, turbidimetric analysis was used alongside particle size data to select optimal polymer:insulin mass ratios and insulin stock concentration levels for the formulation of stable PECS. Greater clarity (higher % transmittance) and lack of precipitation was deemed to be indicative stable complexation.^{17,23} The optimisation process was done for all polymers tested, but only the data for the parent polymers, Paa and QPaa is shown as this is representative of their thiolated derivatives.

Results of the formulation optimisation process carried out at various P:I mixing ratios showed that at low P:I mass ratios (specifically 0.2:1 at either insulin stock

concentration) PEC formulations had low transmittance values (Figure 1) and were observed to be turbid suggesting the attractive forces between polymer and insulin were weak at this P:I mixing ratio. After 4 hrs, these formulations were observed to be unstable separating into a bottom layer composed of flocculated precipitates and a clear supernatant layer. This was likely due to the excess of insulin in solution causing a marked charge neutralisation of the polymer on complexation which in turn resulted in these complexes precipitating.

Increasing the P:I mass ratio to 0.4:1 or 0.8:1 stabilised the formulations facilitating the formation of clear PECS containing soluble, non-aggregated complexes which displayed higher transmittance values and showed no visible signs of precipitation.

However, at higher P:I mass ratios of 2:1, PEC formulations were observed to be translucent displaying a corresponding decrease in transmittance as can be observed in Figure 1. This suggests that at the higher P:I mass ratios (2:1) where there is an excess of the polymer, compression of the electrical double layer around suspended particles may reduce the magnitude of the repulsive barrier between dispersed particles promoting PEC aggregation and causing a corresponding decrease in transmittance values.

Raising the concentration level of the insulin stock solution used from 0.5 to 2 mg/mL did not noticeably change the dynamics of the complexation process across the different P:I mass ratios combinations used. The stability profile of complexes followed a similar trend with that obtained at 0.5 mg/mL: PECS at the upper and lower margins of the mass ratio combinations (0.2:1 and 2:1) having relatively lower transmittance values than other formulations and PECS prepared at 0.2:1 containing visible aggregates. More importantly, each formulation prepared using the higher insulin stock concentration (2 mg/mL) was relatively more turbid than the corresponding formulation prepared using

the lower insulin stock (0.5 mg/mL) resulting in a relative decrease in transmittance values with increase in insulin stock concentration (Figure 1). Hence, although stable formulations (0.4-1:1) prepared using this higher insulin stock concentration (2 mg/mL) were free of aggregation/precipitation the PEC suspension was translucent. This translucent appearance of complexes could be a direct effect of the higher concentration of complexes in the suspension and/or as a result of intercomplex associations as interparticulate distances fall allowing attractive forces to predominate promoting PEC aggregation.

These results indicate that in Tris buffer pH 7.4, P:I mass ratios between 0.4-1:1 were optimal formulations for the preparation of polymer, insulin PECS. This is in agreement with the results of similar work which reports the formation of unstable aggregates at low P:I mass ratios presumed to be as a result of excess of the interacting protein.²⁴

Particle size analysis

Results showed that mixing each polymer solution with insulin (in Tris buffer pH 7.4) resulted in the spontaneous formation of nanoparticles (Figure 2). PEC formulations prepared at low P:I ratio of 0.2:1, which were observed to be turbid and precipitated after 4 hrs, displayed large particle sizes and high PDI values at 2 hrs.

The formation of small, compact nano-sized complexes of relatively narrow size distribution at P:I mass ratios of 0.4:1 shows that interactive forces were sufficiently strengthened by an increase in polymer concentration facilitating optimal P:I complexation. Particle size data also showed that complexes were relatively larger at 2:1 P:I ratio, confirming that the fall in transmittance observed at this mass ratio could be related to increase in PEC sizes as suspected. The excess concentrations of either

interacting polyelectrolyte results in increase in PEC sizes which may be attributed to the screening effect of excess charged polyelectrolyte on the repulsive charges exhibited by dispersed particles compressing the double layer and reducing the charge barrier to the point at which aggregation sets in.²⁵ This could also be a direct result of an increase in particle count as polyelectrolyte concentration increases resulting in a reduction in interparticulate distances (since suspension volume was kept constant) which causes attractive forces to predominate leading to adjacent complexes associating to form larger aggregates.

PECS prepared using the modified Paa derivatives (quaternised and/or thiolated) were observed to be smaller (<100 nm) than unmodified Paa complexes (Table 2). This suggested that the processes of quaternisation and thiolation enhanced P:I interaction resulting in more tightly bound, compact complexes. This was expected as quaternisation alters the positive charge on the polymer facilitating the process of electrostatically induced P:I complexation. Thiolation may result in the formation of polymeric intra-/inter-molecular disulphide bonds, which creates hydrophobic regions within the polymer capable of mediating hydrophobic interactions with the insulin molecule; hence, providing additional forces for P:I complexation.

This improvement in complexation was further reflected by thiolated QPaa (QPaa-NAC and QPaa-TBA) complexes which benefit from the cumulative effects of quaternisation and thiolation. These PECS displayed smaller sizes than both QPaa and their thiolated Paa counterparts (Table 2). Further evaluation of particle size data also indicated that complexes prepared from TBA-based thiomers were smaller than complexes from NAC-based thiomers (Table 2). This could be related to the extra positive charge present on

the amidine bond of TBA-conjugates providing a site for additional electrostatic interaction with insulin and hence condensing PECS even more.

The size of nanocomplexes for oral delivery is very important in optimising their intestinal uptake as smaller-sized particles (< 300 nm) have been found to be favoured in the processes like transcellular uptake by Peyer's patches and paracellular uptake through tight junctional spaces.^{26,27} Particle size has also been found to affect the process of nanoparticle clearance by macrophages; as complexes with smaller sizes (<150 nm) have been shown to exhibit a higher level of exocytosis.²⁸

Based on results of the formulation optimisation process in 3.2.1 and 3.2.2., PECS prepared at 0.8:1 P:I mass ratio, using the 0.5mg/mL insulin stock solution, were identified as optimal formulation formulations for further testing. This was based on their relatively small sizes and PDI values, as well as their relatively high %transmittance. These values were, also, shown to be relatively stable over 3 days incubation at room temperature (data not shown).

Zeta potential

Complexation of the polymers with insulin resulted in the production of positively charged PECS at optimal P:I mass ratios (Table 2). The zeta potential of polymer, insulin complexes was observed to range between approximately 28-38 mV. The positive surface charge on complexes is beneficial in facilitating processes like particle uptake/transport and mucoadhesion through electrostatic interaction with tight junction proteins and the intestinal mucosa. Surface charge of nanoparticles also affects their biodistribution.²⁸ Nanoparticles with a slight negative charge have been shown to

accumulate better in tumour sites. While an elevation of positive surface charge was found to be associated with an increased affinity for the negatively charged cell membrane enhancing cellular uptake of cationic nanoparticles.²⁸ The differences in the zeta potential of separate polymer and insulin solutions from that of their complexes indicate the role of electrostatic forces in the formation of these polymer-insulin complexes (Table 2).¹⁸ NAC conjugates displayed lowest zeta potential values, Paa and QPaa showed intermediate zeta potential values, while TBA conjugates had the highest magnitude of surface charge. This may be due to the fact that there was a reduction in surface charge of NAC-based thiomers due to substitution with the neutral amide bond.¹⁸ However, complexes from TBA-based thiomers exhibited the highest zeta potential probably due to the extra-cationic charge conferred by the permanently protonated amidine bond.

Complexation efficiency

Insulin complexation efficiency was determined by HPLC analysis of insulin contained within optimal PEC formulations (Table 2). This method was developed based on the assumption that insulin detected in the PEC formulation was complexed with the polymer. This theory was supported by results from the optimisation process which showed that for unstable PEC formulations prepared at 0.2:1, the insulin content as well as the zeta potential of the clear supernatant layer was negligible (data not shown) indicating that polymer and insulin molecules were held together as loose complexes, visible as large flocs settled at the bottom of the vial.

Comparing HPLC analysis of insulin contained in all PEC formulations prepared at 0.8:1 P:I mass ratio using the 0.5mg/mL insulin stock and different Paa/QPaa thiolated derivatives, insulin complexation efficiency was above 90% for all formulations as shown in Table 2. However, for Paa-TBA/QPaa-TBA, insulin complexes, insulin complexation efficiency results were obtained by an alternate ELISA method. This was because the insulin peak detected by HPLC was absent in these PEC formulations (Figure S1) leading to the assumption that the structure of insulin was altered on complexation with the polymer thereby affecting the normal interaction of the protein with the stationary phase of the column. The insulin content of these complexes was observed to gradually decline from 100% after the complexes were made (t=0 hrs) to approximately 10% after 2 hrs, proving that as insulin complexes with the polymer its ability to be detected by HPLC is altered resulting in a fall in insulin concentration recorded with time.

However, it was observed that acidification of Paa/QPaa-TBA insulin complexes using 2 M HCl resulted in a distinct insulin peak similar to that obtained on acidification of a similar insulin control solution (Figure S2). This implies that the insulin complexed with TBA-based thiomers could be recovered by acidification, hence insulin complexation efficiency of these complexes was obtained by analysing this acidified peak and comparing the results of the insulin complex with that of the acidified insulin control (Figure S2). The fact that the effect of TBA conjugates on insulin may be reversed by acidification suggests that this alteration in insulin characteristics may be caused by covalent (thiol-disulphide) bonding between the thiol groups on the polymer and insulin at pH 7.4, reversed by protonation of reactive thiolate ions on acidification.¹⁹ The increased reactivity of the thiol groups on TBA conjugates compared to NAC conjugates

may be due to the cationic substructure of the amidine thiol-bearing moiety enhancing the affinity of the polymer thiols for the cysteine groups on insulin. This sort of P:I interaction could have deleterious effects on the conformation of the insulin chains and may affect the ability of the protein to interact with its receptor.²⁰ Techniques like circular dichroism could be used in the future in investigating the nature of conformational change in insulin complexed with TBA-conjugates.²⁴

Confirmation of this reduction of insulin content of Paa-TBA insulin complexes was carried out using an insulin ELISA kit. Results confirmed that after 2 hours, only $11.8 \pm 4.2\%$ of insulin was available as opposed to $97.8 \pm 0.2\%$ of insulin present in the control.

TEM

The morphology of different PECS prepared at 0.8:1 P:I mass ratio using the 0.5 mg/mL insulin stock was elucidated using TEM (Figure 3). The TEM micrographs show that quaternised polymers (QPaa, QPaa-NAC and QPaa-TBA) appeared to be capable of forming nanoparticles having a single layer of polymer chains as their outer corona (3B), while non-quaternised thiomers (Paa-NAC/Paa-TBA) appeared to form nano-vesicles with a distinctive bilayered outer corona (3A). These nano-vesicle bilayers appear to show darkened regions which suggests that these areas may have resisted staining indicating they are likely more hydrophobic than other regions of the complex.¹³

The formation of bilayer vesicles has been observed to be directly related to the hydrophobic content of the polymer, with nano-vesicular self-assembly being initiated by an attempt to minimise the high energy interaction between hydrophobic groups of

the polymer and the aqueous disperse phase while also maximising interfacial area by sustaining low level interactions between hydrophilic groups and the disperse phase.^{29,30} Water-soluble oppositely charged polyelectrolytes have also been found to promote vesicle formation in non-vesicle forming water-soluble amphiphiles. This phenomenon arises due to partial charge neutralization by the adsorption of one polyelectrolyte onto the bilayer of the polymer of opposite charge, influencing the balance of opposing forces of electrostatic repulsion and hydrophobic interaction in favour of vesicle formation.³¹ Considering that modification of Paa by thiolation imparts some level of hydrophobicity to the resultant constructs may suggest that interaction with anionic insulin molecules can promote the formation of the vesicles observed in the TEM micrographs through the aforementioned mechanism. TEM results further emphasize the role of hydrophobic interactions arising from the intramolecular disulphide bonds formed during the thiolation of the polymers in complexation with insulin.

In vitro enzymatic degradation studies

The difficulties in analysing insulin concentration of TBA-conjugates, meant that enzymatic degradation studies were only carried out on NAC-conjugate complexes. The ability of QPaa, Paa-NAC and QPaa-NAC complexes (optimal formulations at 0.8:1, P:I, mass ratio-0.5mg/mL insulin stock) to protect complexed insulin from degradation by the serine proteases trypsin, α -chymotrypsin and pepsin was assessed. The results were compared to that obtained for a similar control solution of free insulin and are illustrated in Figure 4.

Results of exposure of polymer, insulin complexes to tryptic degradation showed that > 90% of insulin contained within the thiolated complexes was non-degraded after 4 hrs; in comparison to less than 70% for the insulin control. The fact that 88% of undegraded insulin was present in QPaa complexes after 4 hrs and 96% insulin available in a similar formulation of QPaa-NAC after exposure to trypsin for the same time interval indicated that thiolation further enhanced enzymatic protection. Paa-NAC and QPaa-NAC complexes exhibited only slight variations in protection of insulin from the effects of trypsin.

Thiomer, insulin complexes showed a different insulin degradation profile on exposure to α -chymotrypsin. Thiolated QPaa (QPaa-NAC) insulin complexes showed increased resistance to degradation by α -chymotrypsin than either QPaa or Paa-NAC complexes. The amount of undegraded insulin available in QPaa-NAC complexes after 4 hrs was observed to be 10% more than QPaa complexes, 15% more than Paa-NAC complexes, and 30% more than was present in the insulin control. This may show a synergistic effect was obtained from thiolation and quaternisation of the parent polymer, Paa, in terms of protection of insulin from degradation by α -chymotrypsin. Bonds prone to tryptic cleavage in insulin include the carboxyl terminus of B29-Lys and B22-Arg (in the B-chain), these carboxyl groups are deprotonated and hence negatively charged at pH 7.4. It may, therefore, be that the electrostatic binding of positively charged polymer molecules at this negative terminal shields susceptible bonds at this site from tryptic attack.³² This sort of enzymatic protection/shielding of insulin by polymer-insulin interactions have previously been reported by researchers working with modified chitosan and Paa.^{33,34} Hence, this protective effect would be limited to sites on insulin which are capable of coulombic interactions with compatible polymer chains. However,

two bonds prone to cleavage by α -chymotrypsin are enclosed within the hydrophobic core of the insulin molecule³¹ and complexation with a less hydrophobic polymer like QPaa may not allow for interaction of these regions with the polymer chains due to differences in polarity. Hence, these areas may not be protected from the attacking protease.

Conversely, polymer thiolation creates hydrophobic regions within the thiomers as seen with the TEM photos displayed in Figure 3 due to intra-/intermolecular disulphide bond formation. Hence, the additional protection of complexed insulin from proteolytic attack by α -chymotrypsin after complexation with NAC-conjugates may be because these thiomers are capable of protecting the bonds located within the hydrophobic core of insulin. (Hydrophobic interactions between the thiomers and insulin occurring at this region.) The ability of hydrophobic interactions to offer improved protection of insulin associated within a PEC delivery system to degradation by serine proteases has been previously reported.^{17,33}

Consequently, QPaa-NAC which may incorporate charge-mediated and hydrophobic interactions with insulin on complexation yields a cumulative protective effect being able to shield more susceptible sites on the insulin molecule from proteolytic degradation by α -chymotrypsin than either QPaa or Paa-NAC. This result is in agreement with previous publications which showed that the ability of PECS formulated from Paa-based polymers to protect complexed insulin from the effects of α -chymotrypsin was enhanced when Paa was functionalised with both hydrophobic and quaternary groups.¹⁷

No insulin PEC formulation protected insulin from degradation by pepsin, as insulin present in complexes was observed to be completely degraded after 30 minutes of

exposure to pepsin. This is probably as a result of the numerous sites on the insulin molecule susceptible to degradation by pepsin. Examples of sites susceptible to peptic degradation include sites before leucine, phenylalanine, tyrosine, and tryptophan (except if preceded by proline).³⁵ Rapid peptic degradation of complexed insulin may also be accelerated by acidification of the complexes: simulation of normal physiological pH conditions for peptic activity was required, thereby making insulin positively charged (below its pI of 5.5) and destabilizing electrostatic polymer-insulin interaction. Amphiphilic Paa-insulin nanocomplexes which benefited from both hydrophobic and electrostatic interactions were observed to show some protective effect from peptic degradation.¹⁷ However, exposure of oral insulin formulations to the effect of pepsin can be prevented by the use of enteric formulations which target the release of insulin to the small intestine and distal parts of the GIT, hence curtailing insulin proteolysis by enzymes operating within the gastric region of the gut.²⁴

In vitro evaluation of mucoadhesive capacity of complexes

Assessment of mucin adsorption properties of polymer, insulin complexes prepared in Tris buffer pH 7.4 at 0.8:1 P: I mass ratio using the 0.5 mgml⁻¹ insulin stock solution (Figure 5).

The mucin adsorption profile of insulin PECS showed that modified Paa-based complexes showing better mucoadhesive properties than complexes prepared from the unmodified Paa backbone. Thiolated Paa (Paa-NAC and Paa-TBA) PECS displayed the greatest mucoadhesive properties, while thiolation of QPaa did not offer any substantial improvement in the mucoadhesive profile of the quaternised backbone or its

complexes. High levels of quaternisation have been associated with decreased mucoadhesivity due to reduced chain flexibility, interpenetration and steric hindrance while lower levels of quaternisation reduce interactions with mucus, also causing corresponding decreases in mucoadhesion.^{36,37} Optimisation of the level of quaternisation has been found to be vital in facilitating paracellular transport and mucoadhesion using quaternised chitosan.^{24,38} In summary, all complexes exhibited good mucoadhesive properties with modified polymers performing better than the unmodified Paa backbone.

CONCLUSION

The data collected from the above processes support the spontaneous formation of spherical, nano-sized PECS with good insulin complexation efficiency on mixing optimal mass ratios of Paa/QPaa and their thiolated derivatives with insulin in Tris buffer solutions. Optimisation of the complexation process varying polyelectrolyte stoichiometry showed that complexes prepared at 0.4-1:1 P:I mass ratio were consistently stable for all polymers, with 0.8:1 showing the best overall stability profile. Electrostatic-induced polymer, insulin complexation carried out using Paa-based thiomers was found to result in the formation of nano-sized, positively-charged complexes which were either in the form of nanoparticles or bilayered nanovesicles. Complexation efficiency data obtained showed that interaction of TBA-based thiomers with insulin in Tris buffer pH 7.4 may have altered the conformation of insulin limiting its detection by HPLC. The complexation efficiency of TBA PECS was also confirmed using an insulin ELISA assay. Assessment of the ability of QPaa, QPaa-NAC and Paa-NAC PECS to shield complexed insulin from proteolytic degradation by trypsin, α -chymotrypsin and pepsin showed that all formulations are effective against tryptic

degradation with about 30% more undegraded insulin being recovered from complexes than an equivalent sample of free insulin. QPaa-NAC complexes were observed to offer the best protection of complexed insulin from the effects of α -chymotrypsin containing approximately 30% more undegraded insulin than the insulin control solution after 4 hours. PECS were, however, unable to protect insulin from the effects of pepsin. Overall, this work will assist other groups in the rational design of polymers for insulin delivery. Future work will involve further optimisation of the QPaa-NAC polymer, by increasing the degree of thiol substitution to increase protection against intestinal enzymes, and enteric coating of formulations.

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Table 1: Total thiol content, free thiol and disulphide bond content of thiomers (indicated values are mean \pm S.D.) (n = 3). (as in Ibie et al., 2015b)

Polymer	Free SH content ($\mu\text{mol/g}$)	S-S bond content ($\mu\text{mol/g}$)	Total thiol Substitution ($\mu\text{mol/g}$)
Paa-NAC	60 \pm 1.2	280	340 \pm 4.1
QPaa-NAC	60 \pm 4.3	220	280 \pm 3.3
Paa-TBA	490 \pm 18	590	1080 \pm 28
QPaa-TBA	440 \pm 21	560	1000 \pm 31

Table 2: Comparative analysis of PEC properties showing polymer IC₅₀ (mg/mL) values and transmittance, %T (%), particle size (nm), PDI, zeta potential (mV) and complexation efficiency (%) of different polymer, insulin PEC formulations prepared at 0.8:1 P: I mass ratio with the 0.5mg/mL insulin stock (Mean \pm S.D.; n=3).

Polymer	%T (%)	Particle Size (nm)	PDI	Zeta potential (mV)	Complexation efficiency (%)
Paa	93 \pm 2	104.0 \pm 4	0.45 \pm 0.03	31.2 \pm 2	84 \pm 4
QPaa	94 \pm 3	75.0 \pm 9	0.24 \pm 0.05	30.4 \pm 1	94 \pm 5
Paa-NAC	95 \pm 2	74.8 \pm 3	0.28 \pm 0.03	29.2 \pm 3	99 \pm 9
QPaa-NAC	98 \pm 2	71.1 \pm 9	0.27 \pm 0.01	28.7 \pm 3	92 \pm 12
Paa-TBA	98 \pm 3	64.6 \pm 9	0.43 \pm 0.03	35.1 \pm 6	95 \pm 5
QPaa-TBA	99 \pm 1	54.0 \pm 7	0.26 \pm 0.04	37.0 \pm 2	98 \pm 10

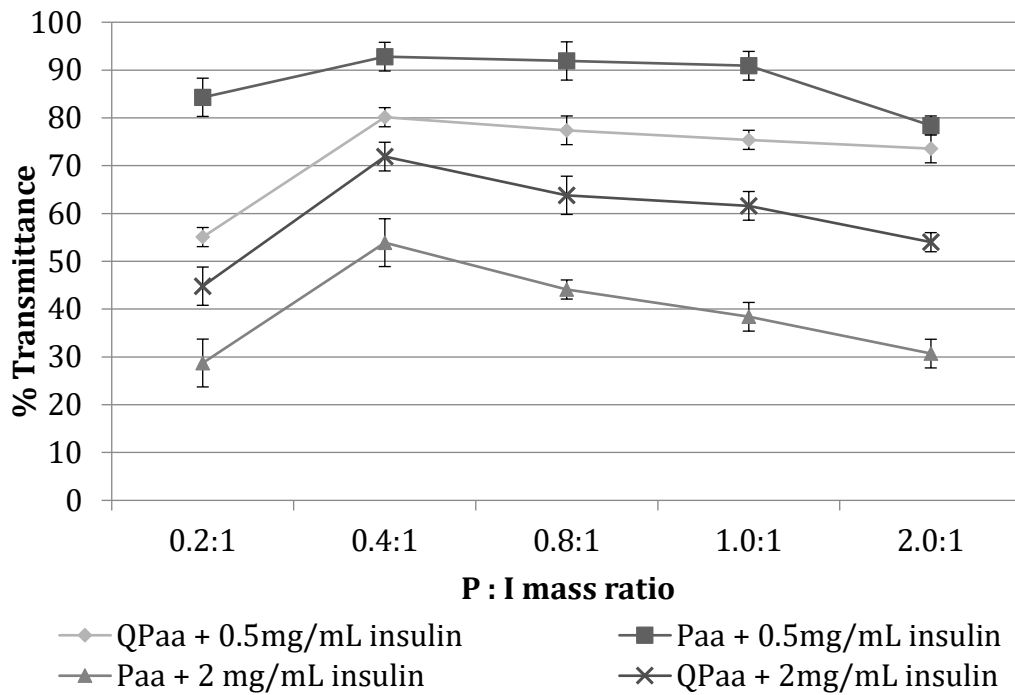


Figure 1: Transmittance (%) of Paa and QPaa insulin complexes prepared at varied P:I mass ratios with 0.5 or 2 mg/mL insulin stock concentrations (mean \pm S.D.; n=3).

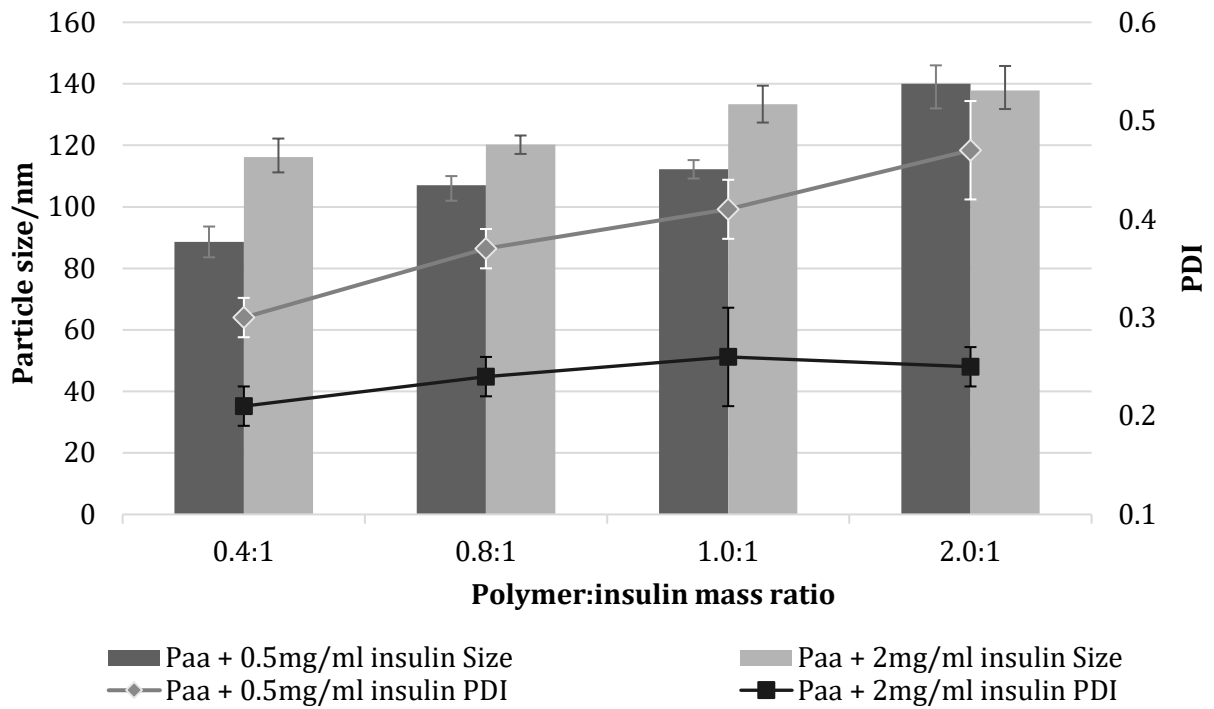
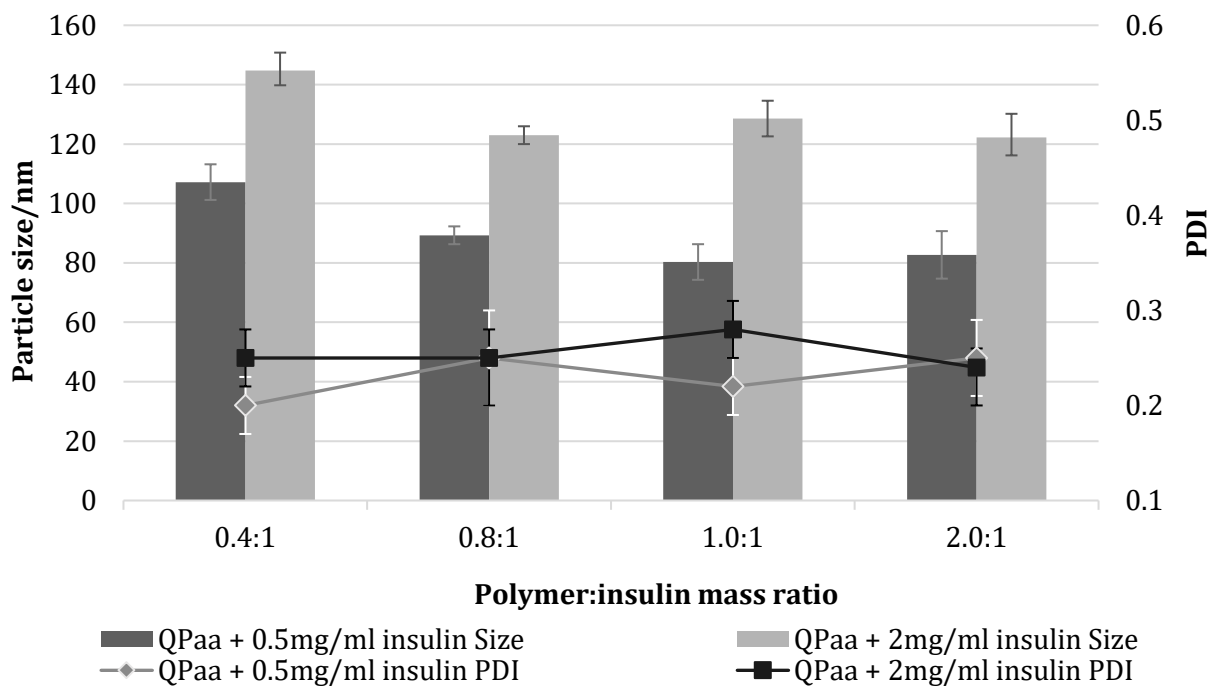
A**B**

Figure 2: Particle size analysis of polymer, insulin complexes showing hydrodynamic diameter and PDI of A) Paa, insulin and B) QPaa, insulin PECS prepared at varied P:I mass ratios using 0.5 and 2 mg/mL insulin stock (n=3; mean \pm S.D.).

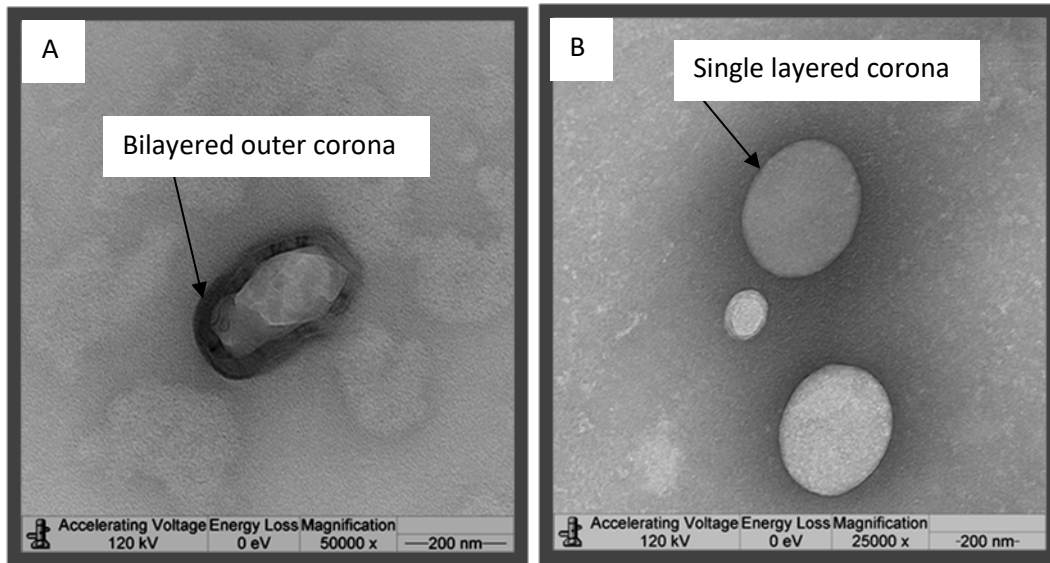
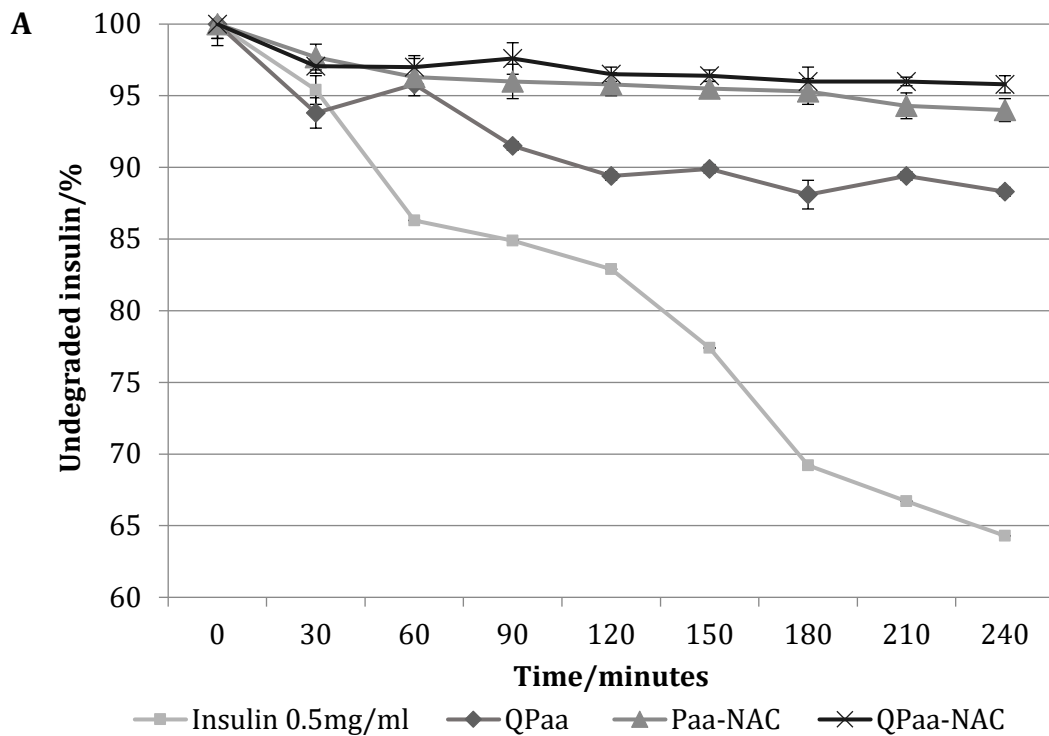


Figure 3: TEM micrographs of polymer, insulin complexes A) nanovesicular Paa-NAC complex with a bilayered outer corona B) QPaa-NAC nanoparticles with a single layer outer corona.



B

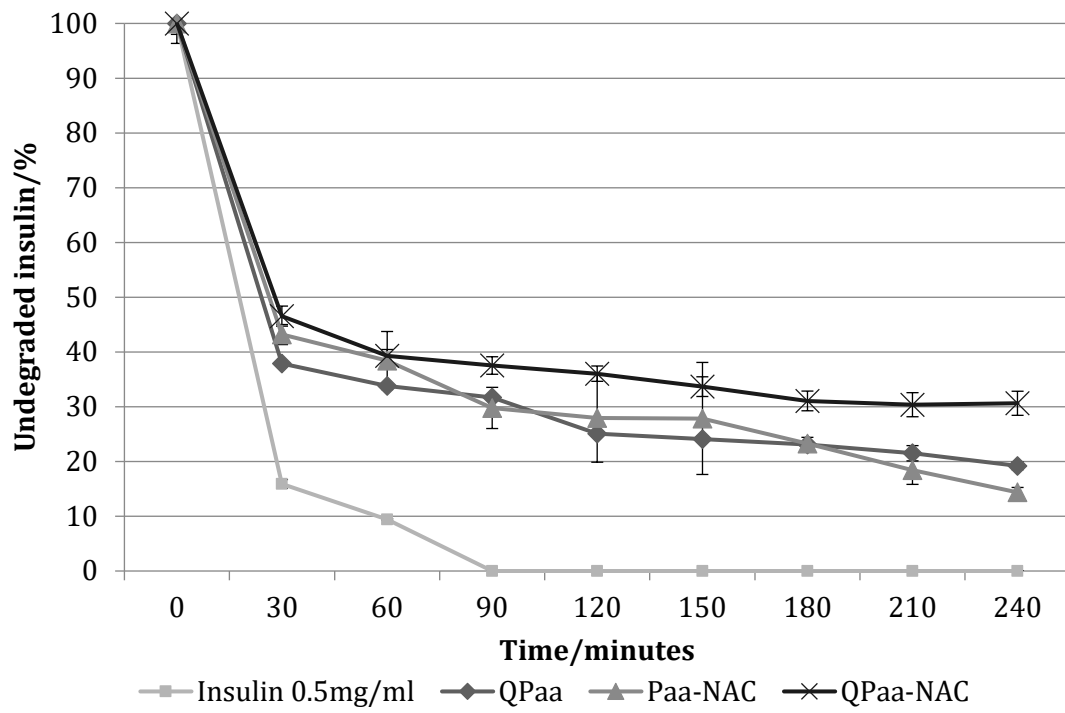


Figure 4: Degradation curves of insulin and insulin PECs prepared from QPaa, Paa-NAC or QPaa-NAC complexes exposed to A) trypsin and B) α -chymotrypsin (mean \pm S.D.; n=3).

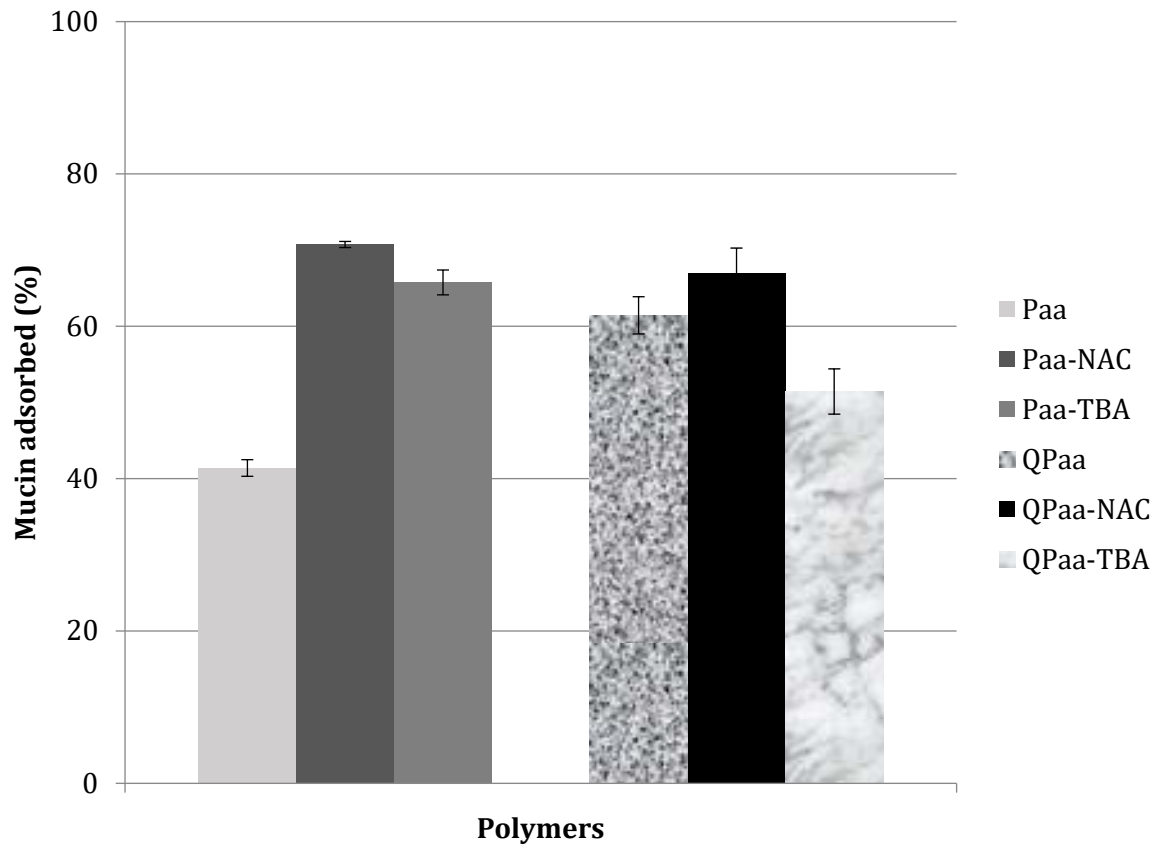


Figure 5: Mucin adsorption profile of polymer, insulin complexes (mean \pm S.D.; n=3).