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The influence of polymer architecture on the protective effect of novel comb shaped amphiphilic poly(allylamine) against *in vitro* enzymatic degradation of insulin—Towards oral insulin delivery

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ABSTRACT

Nanocomplexes formed between amphiphilic poly(allylamine) (PAA) and insulin were prepared, characterised and the impact of polymer architecture on the protection of insulin against three enzymes was investigated. PAA previously modified with either cetyl or cholesteryl pendant groups at two levels of hydrophobic grafting and its quaternised derivatives were used to produce polymer–insulin nanocomplexes. Transmittance study, differential scanning calorimetry, hydrodynamic size and zeta potential measurement were conducted and the morphology of the complexes were visualised using transmission electron microscopy. All polymers were found to have an optimal polymer to insulin ratio of 0.4:1 mg mL^{-1} with particle size ranging from 88 to 154 nm. Polymer architecture has an impact on the morphology of the complexes produced but has little influence on the complexation efficiency (CE). Almost all polymers were unable to produce complexes with a CE of above 50%. Most polymers demonstrated an ability to reduce insulin degradation by trypsin while the polymer architecture plays a pivotal role against α-chymotrypsin and pepsin degradation. Quaternised cholesteryl polymers were able to significantly limit insulin degradation by α-chymotrypsin while cetyl polymers were particularly effective against pepsin degradation. These results indicated that a combination of polymers might be required to enhance protection against all three proteolytic enzymes for efficacious oral delivery of insulin.

1. Introduction

Today, the advancement of biotechnology has resulted in the discovery of many therapeutic proteins and peptides. Although oral administration is the most favourable choice due to ease of administration and patient compliance (Hamman et al., 2005; Qian et al., 2006; Sarmento et al., 2007), oral delivery of proteins such as insulin remains one of the greatest challenges in modern pharmaceutical technology (Saffran et al., 1997; Jung et al., 2000; Soppimath et al., 2001; Lambkin and Pinilla, 2002; Shen, 2003; Hamman et al., 2005; Cui et al., 2006; Mahkam et al., 2006; Qian et al., 2006; des Rieux et al., 2006; Liu et al., 2006; Sarmento et al., 2007; Simon et al., 2007). Due to the large molecular size and hydrophilicity, these proteins generally have poor permeability through the intestinal membrane. In addition, they are easily degraded in the gastrointestinal tract in the presence of various proteolytic enzymes (Schilling and Mitra, 1991; Saffran et al., 1997; Jung et al., 2000; Soppimath et al., 2001; Lambkin and Pinilla, 2002; Shen, 2003; Hamman et al., 2005; Cui et al., 2006; Mahkam et al., 2006; des Rieux et al., 2006). Various conventional strategies have been employed in order to deliver proteins orally, including: liposomes (Hamman et al., 2005), solid micro-/nanoparticles (Fan et al., 2006; des Rieux et al., 2006; Soppimath et al., 2001) and prodrug forms of the proteins (Hamman et al., 2005). One of the emerging technologies is the use of amphiphilic polymers for oral protein delivery (Simon et al., 2007; Jintapattanakit et al., 2007).

Amphiphilic polymers spontaneously form nano-aggregates in aqueous environment where the hydrophobic core is formed upon aggregation of hydrophobic pendant groups and is stabilised by the hydrophilic corona (Gaucher et al., 2005). We previously reported on the use of novel comb shaped palmitoyl-grafted amphiphilic polyallylamines (PAA), for complexation with insulin (Thompson et al., 2009). When the pH is above its iso-electric point of 5.3, insulin is negatively charged and thus able to interact with cationic PAA via electrostatic interaction (Simon et al., 2004; Thompson et al., 2009). In addition, due to the amphiphilic nature of insulin, it is thought to associate hydrophobically with the amphiphilic polymers (Simon et al., 2004, 2007; Thompson et al., 2009). As a

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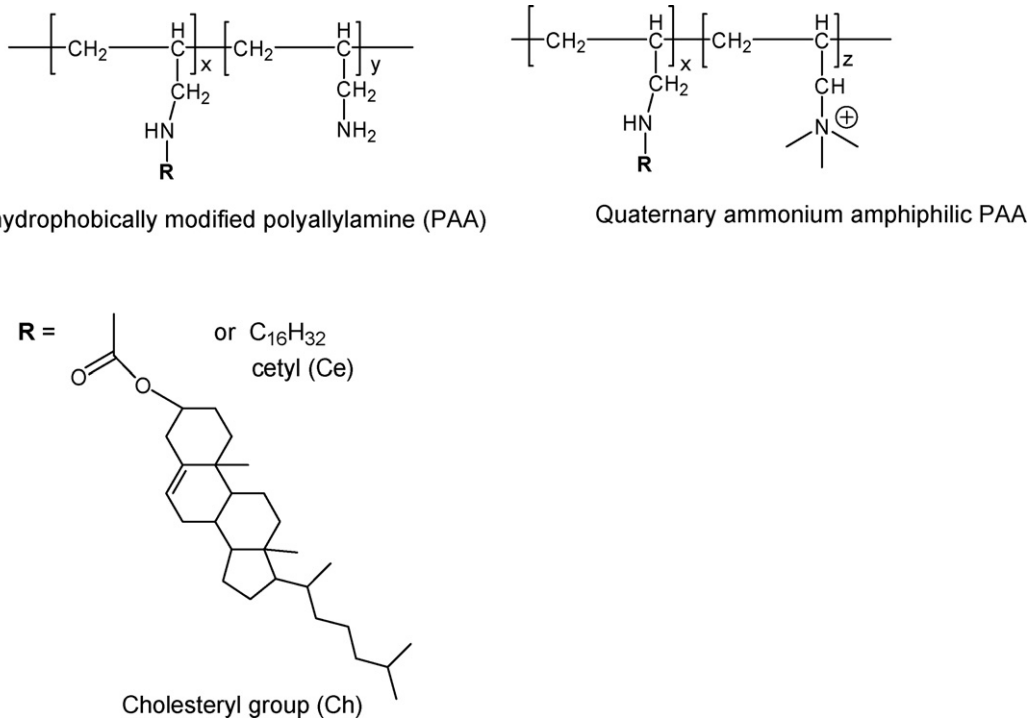

Scheme 1. Structure of PAA backbone and hydrophobic grafts.

result, spontaneous formation of polymer–insulin complexes was observed in pH 7.4 Tris buffer with high complexation efficiency. These palmitoyl-grafted polymers were shown to limit degradation by both pepsin and trypsin *in vitro*. Interestingly these polymers proved ineffective in reducing α-chymotrypsin degradation and in some cases actually increased the degradation. Although it is thought that the amphiphilic polymer architecture has a significant impact on the properties of the polymer–insulin complexes, there are very limited studies on these factors. To our knowledge, there was one study conducted by Simon et al. (2007) demonstrating increasing the hydrophobic load increased the protection against degradation by trypsin and enhanced paracellular transport across CaCo-2 cell monolayer.

Therefore, the aim of this study is to elucidate the impact of three key parameters of the polymer architecture: (1) type of hydrophobic pendant group, (2) the level of hydrophobic substitution and (3) the presence of quaternary ammonium moieties on the polymer–insulin complexation efficiency, cytotoxicity and the protection of insulin against three major proteolytic enzymes in the gastrointestinal tract. Trypsin, α-chymotrypsin and pepsin are major proteolytic enzymes in the gastrointestinal tract and therefore it is imperative that an efficacious oral insulin delivery system should be able to offer protection against these enzymes. To achieve this aim, PAA was modified with two levels of cholesteryl (Ch) or cetyl (Ce) substitutions, which have been previously synthesised and characterised (Thompson et al., 2008). Quaternisation (Q) was also carried out on these amphiphilic polymers to obtain quaternary ammonium moieties (Scheme 1).

2. Materials and methods

2.1. Materials

Poly(allylamine hydrochloride) (PAA-HCl) (average M_w = 15 kDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), eagle minimum essential medium (EMEM), Triton X-100, PBS buffer, triethylamine (99%), cholesteryl chloroformate (98%), 1-bromododecane (97%), Tris(hydroxymethyl) aminomethane (Tris) (≥99%), insulin (27 U/mg, bovine pancreas), trypsin (TPCK treated, bovine pancreas, 7500 U/mg), pepsin (3260 U/mg) and α-chymotrypsin (TLCK treated, Type VII from bovine pancreas, ≥40 U/mg) were all purchased from Sigma–Aldrich, UK. Trifluoroacetic acid (TFA) (HPLC grade) was purchased from Fisher Scientific Chemicals, UK. Fetal calf serum was purchased from Biosera, UK. Non-essential amino acids, trypsin/EDTA (0.05%, v/v) and L-glutamine (200 nM) were purchased from Invitrogen, UK. All solvents were purchased from Fisher Scientific Chemicals, UK and were of HPLC grade. All other reagents used were of analytical grade.

2.2. Polymer synthesis and characterisation

The methods have been previously described (Thompson et al., 2008). Briefly, the PAA was reacted with cholesteryl chloroformate based on molar feeds of 1:0.25 or 1:0.5 (PAA monomer to hydrophobic group) to obtain Ch2.5 or Ch5, respectively. The numerals of the polymer abbreviations indicate the expected %mole of hydrophobic grafting based on the initial molar feeds. PAA was also reacted with 1-bromododecane at similar molar feeds to obtain Ce2.5 and Ce5. Quaternisation (Q) was carried out by reacting amphiphiles with over a 1000 molar excess of methyl iodide to obtain quaternary ammonium compounds, QCe2.5/QCh2.5 and QCe5/QCh5. The novel amphiphilic polymers were characterised by elemental analysis and ^{1}H NMR.

2.3. Preparation of polymer–insulin complexes

This was carried out as previously described (Thompson et al., 2009). Briefly, polymer and bovine insulin solutions were prepared separately in Tris buffer, pH 7.4. The buffer was made up of 0.1 M Tris:0.01 M HCl (87:13%, v/v). Polymer solutions ranging from 0.4 to 4 mg mL^{-1} were prepared by sonicating the polymer in the Tris buffer using a Soniprep 150 sonicator (MSE Ltd., UK) for 5 min at the maximum amplitude. Insulin stock solutions

(2 mg mL^{-1}) were prepared using gentle magnetic stirring. Equal volumes (1 mL each) of polymer and insulin solutions were added together and the polymer–insulin complexes were formed spontaneously after mixing. After 2 h at room temperature, the pH of the final complex solutions was re-checked to confirm that it was at pH 7.4 before characterising the complexes as described in Section 2.4.

2.4. Characterisation of polymer–insulin complexes

2.4.1. Transmittance studies

Determination of the optimum polymer:insulin mass ratios was conducted using turbidity measurements (Thompson et al., 2009). Polymer–insulin complexes were prepared as described above and the transmittance studies were carried out on insulin alone and the complexes using an Agilent G1103A photo diode array (Agilent Technology, China) at 630 nm. Transmittance values (%) were recorded from the attached computer.

2.4.2. Particle size analysis: photon correlation spectroscopy (PCS)

Hydrodynamic diameters and polydispersity indices (PDI) of polymer–insulin complexes and insulin alone in pH 7.4 Tris buffer were determined using photon correlation spectroscopy (PCS) (Zetasizer Nano-ZS, Malvern Instruments, UK) at 25 °C.

2.4.3. Zeta potential

The zeta potential of polymer–insulin complexes was analysed using PCS (Zetasizer Nano-ZS, Malvern Instruments, UK). Prior to complex zeta potential measurement, standards (−50 mV, Malvern Instrument, UK) were analysed and the data obtained agreed with that stated by the manufacturer.

2.4.4. Differential scanning calorimetry (DSC)

The melting point (T_m) and glass transition (T_g) temperatures of freeze-dried complexes and polymer–insulin physical mixtures (2–6 mg each) were analysed using a Q100 differential scanning calorimeter (TA Instruments, UK), precalibrated with indium. Samples were heated from −90 °C up to 350 °C at 20 °C min^{-1}. The freeze-dried complexes were made up at polymer:insulin mass ratios of 0.004:0.01, 0.4:1 and 2:1 mg mL^{-1} as described above and freeze-dried over 48 h in a VirTis adVantage freeze drier (Biopharma Process Systems, UK).

2.4.5. Transmission electron microscopy (TEM)

Transmission electron microscopy was perfomed by applying polymer–insulin complexes solution onto glow discharged carbon coated 200 mesh copper grids. Aqueous methylamine vanadate (Nanovan; Nanoprobes, Stony Brook, NY, USA) stain (1%) was applied and the mixture was air-dried. The negatively stained complexes were imaged with a LEO 912 energy filtering transmission electron microscope at 120 kV.

2.4.6. Determination of complexation efficiency

This study was carried out as previously described (Simon et al., 2004; Thompson et al., 2009). Briefly, polymer–insulin complexes at polymer:insulin ratio of 0.8:2 mg mL^{-1} or 4:2 mg mL^{-1} were prepared. The insulin content in the insulin stock solution (diluted 1 in 1 with pH 7.4 Tris buffer) and complex solutions were analysed using a Shimadzu HPLC system consisted of a LC-20AD pump, a SIL-20A autosampler and a RF-10Axl fluorescence detector ($\lambda_{excitation}$ = 276 nm; $\lambda_{emission}$ = 600 nm) with a mobile phase flow rate of 1 mL min^{-1}. The mobile phase was water:acetonitrile (68.5:31.5) buffered to pH 2 with TFA and the stationary phase was a Gemini C_{18} column (25 mm × 4.6 mm; 110 Å) (Phenomenex, UK) maintained at 25 °C by a CTO-10ASvp column oven. The insulin peak was detected at 5.5 min and the insulin concentration was calculated using a calibration curve prepared from various standard solutions (10–1000 μg mL^{-1}, R^2 = 0.993). Complexation efficiency was calculated using Eq. (1):

$$\text{complexation efficiency} = \left(\frac{Ca}{Cb/2}\right) \times 100 \quad (1)$$

where *Ca* is the insulin concentration detected by HPLC in the complexes and *Cb* is the insulin stock concentration.

2.5. Cytotoxicity assay

Caco-2 cells (ECACC, Wiltshire, UK) with the passage numbers of 45–60 were grown in EMEM supplemented with 10% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids and 1% (v/v) L-glutamine at 5% CO_2 and 95% humidity at 37 °C. The cells were seeded in 96-well plates at a density of 10,000 cells/well and grown in the same condition for 24 h. Polymer solutions ranging from 0.0001 to 1 mg mL^{-1} in the cultured media (200 μL) were added and incubated with cells for 24 h. Polymer solutions were then aspirated and replaced with fresh media (200 μL). After further 24 h incubation, 50 μL of MTT solution (5 mg mL^{-1} in PBS) was added and the plates were incubated in dark for 4 h. The solutions were aspirated and DMSO (200 μL) was added. The plates were read spectrophotometrically at 570 nm using a VERSA$_{max}$ tunable microplate reader (Molecular Devices, UK) after the addition of 25 μL glycine buffer (7.5 g L^{-1} glycine, 5.9 g L^{-1} NaCl, pH 10.5). Cell viability (%) was expressed relative to the absorbance values found for the negative control (untreated cells) and positive control (Triton X in PBS) (n = 3). The IC_{50} value for each polymer was taken as the polymer concentration which inhibits growth of 50% of cells.

2.6. Enzymatic degradation study

All complex solutions (4.5 mL) were prepared at either polymer:insulin ratio = 0.8:2 mg mL^{-1} or 4:2 mg mL^{-1}. Trypsin (6.4 mg mL^{-1}, 2.7 × 10^{-4} M) and polymer–insulin complexes (polymer:insulin ratio = 4:2 mg mL^{-1}, 4.5 mL) were prepared separately in pH 8 Tris buffer (0.01 M HCl:0.1 M Tris = 84:16%, v/v). They were incubated at 37 °C for 2 h before addition of trypsin (0.05 mL) to the complex solutions. Aliquots of the mixture (0.2 mL) were withdrawn at regular intervals and added to ice cold TFA solution (0.015 mL, 0.1%, v/v) to stop enzymatic activity. Samples were analysed using the same HPLC system detailed as above and the experiment was performed in triplicate and conducted at 37 °C for 4 h. The same procedure was repeated using α-chymotrypsin (5 mg mL^{-1}, 2.0 × 10^{-4} M) in pH 8 Tris buffer. For the pepsin degradation study, the enzyme (0.1 mg mL^{-1}, 2.8 × 10^{-6} M) was prepared in 0.01 M HCl and both complexes and enzyme solutions were buffered to pH 2 with a drop of 10 M HCl prior to adding the pepsin (0.016 mL) into complex solutions. Aliquots of the mixture (0.15 mL) were removed at regular intervals and added to ice cold Tris base (0.15 mL, 0.1 M) to stop pepsin activity and analysed as previously detailed.

2.7. Statistical analysis

Statistical significance was assessed using two-way analysis of variance ANOVA and Dunnett multiple comparison *t*-test via the statistical software package SPSS 13.0 for Windows.

Table 1
Grafting, quaternisation (%) and IC_{50} (mg mL^{-1}) values of graft polymers ($n = 3$; ±SD).

Polymer	Monomer:graft molar ratio	Grafting (%)[a]	Quaternisation (%)[a]	Zeta potential (mV)	IC_{50} (mg mL^{-1})[b]
PAA	n/a	n/a	n/a	22.6 (1.04)	0.010 (0)
Ch2.5	1: 0.025	2.2	n/a	31.7 (0.30)	0.025 (0) [2.5]
QCh2.5	1: 0.025	2.2	68.0	74.8 (1.10)	0.042 (0.0028) [4.2]
Ch5	1: 0.05	6.3 (1.48)	n/a	46.9 (1.17)	0.038 (0.003) [3.8]
QCh5	1: 0.05	6.3	71.0	62.6 (2.63)	0.050 (0.006) [5]
Ce2.5	1: 0.025	4	n/a	48.1 (3.52)	0.008 (0.0002) [0]
QCe2.5	1: 0.025	4	78.0	57.8 (0.11)	0.081 (0.006) [8.1]
Ce5	1: 0.05	7.5 (0.9)	n/a	37.2 (2.30)	0.017 (0.0017) [1.7]
QCe5	1: 0.05	7.5	70.5	48.5 (0.73)	0.053 (0.003) [5.3]

[a] Number of cetyl/cholesteryl groups and quaternary ammonium moieties per 100 monomer units determined by elemental analysis.
[b] The square bracket values indicate the number of folds higher than the IC_{50} of PAA (0.010 mg mL^{-1}).

3. Results and discussion

3.1. Polymer characterisation

The polymer synthesis was confirmed with ^{1}H NMR and elemental analysis where the grafting values for all polymers were in close agreement with the theoretical values (Thompson et al., 2008). Quaternisation values were also all around 70% (Table 1) (Thompson et al., 2008).

Chemical shifts for the polymers were: Ch5—$\delta_{0.75}$, $\delta_{0.9}$, $\delta_{1.0}$, $\delta_{1.1} = CH_3$ (cholesteryl), $\delta_{1.1-2.1} = CH_2$ (cholesteryl and PAA), $\delta_{2.3} = CH_2$ (cholesteryl), $\delta_{2.4-3.2} = CH_2$ (PAA), $\delta_{4.4} = CH–O$ (cholesteryl), $\delta_{5.4} = CH$ (cholesteryl); QCh5—$\delta_{0.75}$, $\delta_{0.9}$, $\delta_{1.0}$, $\delta_{1.1} = CH_3$ (cholesteryl), $\delta_{1.1-2.7} = CH_2$ (cholesteryl), $\delta_{3.1} = CH_2$ (PAA), $\delta_{3.3-4.1} = CH_2$ and CH_3 (quaternary ammonium moiety), $\delta_{4.2} = CH–O$ (cholesteryl), $\delta_{5.4} = CH$ (cholesteryl). For Ce5—$\delta_{0.9} = CH_3$ (cetyl), $\delta_{1.1-2.0} = C_{14}H_{28}$ (cetyl) and CH_2 (PAA), $\delta_{2.6} = CH_2–NH$ (cetyl); QCe5 (Fig. 1D)—$\delta_{0.9} = CH_3$ (cetyl), $\delta_{1.3} = C_{14}H_{28}$ (cetyl), $\delta_{1.6-2.0} = CH_2$ (PAA), $\delta_{2.0-2.3} = CH_2–NH$ (cetyl) and $CH_2–NH$ (PAA), $\delta_{3.0-3.6} = CH_2$ and CH_3 (quaternary ammonium moiety).

3.2. Characterisation of polymer–insulin complexes

3.2.1. Transmittance study

All polymers produced isotropic solutions upon sonication in pH 7.4 Tris buffer except Ch2.5 and Ch5 where the solutions were opaque. Ch5 polymer is the most hydrophobic polymer given that cholesteryl is a longer chain than cetyl (Gaucher et al., 2005). However, Ch2.5 and Ch5 polymer solutions became clear after filtration. Addition of insulin in the same buffer resulted in the spontaneous formation of polymer–insulin complexes. Fig. 1A and B indicates the appearance of polymer–insulin complexes produced over a range of polymer concentrations at a fixed initial insulin concentration of 2 mg mL^{-1}; it is a simple method to provide basic information on the complexation and the optimal polymer to insulin mass ratios (Simon et al., 2004; Thompson et al., 2009). The lower the transmittance the more turbid the solutions, with anything below 10% transmittance indicating precipitation, between 10 and 30% turbid, 30 and 90% translucent and above 90% clear (Thompson et al., 2009).

As expected, excess insulin resulted in the formation of unstable complexes at low polymer to insulin mass ratios for most amphiphilic polymers. This was demonstrated by precipitation or the turbid appearance of the formulations. An increase in polymer concentration resulted in translucent and clear formulations suggesting the presence of stable and compact complexes (Fig. 1). This trend was similar to the palmitoyl PAAs which we previously reported (Thompson et al., 2009). The exception to this was QCh5 at concentrations above 0.8 mg mL^{-1}. This could be explained by the polymer solution appearing more turbid at higher concentrations and so when added to the clear insulin solution they produced more translucent/turbid formulations than polymer solutions at lower concentrations.

The level of hydrophobic grafting seems to have an impact on the complex formation whereby translucent formulations were formed at the optimal polymer to insulin ratio of ≥0.4:1 mg mL^{-1} for all polymers apart from Ch2.5 and Ce2.5. Both Ce2.5 and Ch2.5 had the highest transmittance values at polymer concentrations of 0.2 mg mL^{-1} indicating formation of stable complexes. It appears that a lower hydrophobic load favours insulin complexation at low polymer concentrations irrespective of the type of hydrophobic pendant groups.

3.2.2. DLS

In general, the hydrodynamic sizes of complexes were greatest at low polymer concentrations and declined with increasing poly-

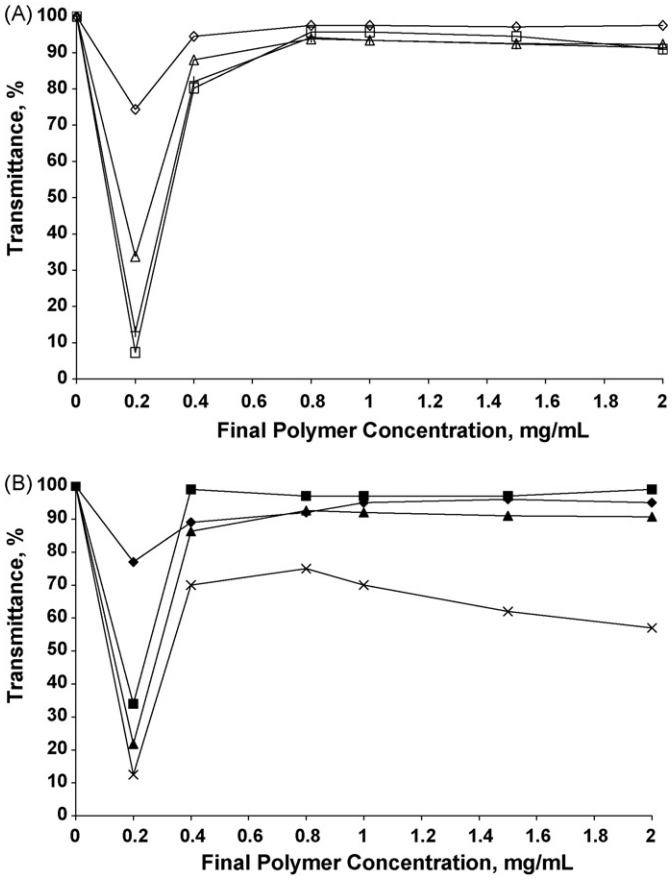

Fig. 1. Transmittance (%) of (A) cetyl graft polymer–insulin complexes (◊) Ce2.5; (□) Ce5; (△) QCe2.5; (+) QCe5 and (B) cholesteryl graft polymer–insulin complexes (♦) Ch2.5; (■) Ch5; (▲) QCh2.5; (×) QCh5 produced using 2 mg mL^{-1} insulin stock.

Table 2
Hydrodynamic size (nm) of polymer, insulin complexes with various polymer to insulin mass ratios using insulin stock solution of 2 mg mL^{-1} (n = 3; mean ± SD). The brackets indicate polydispersity index (PDI) of samples.

Polymer:insulin	Ce2.5	Ch2.5[a]	Ce5	Ch5[a]	QCe2.5	QCh2.5	QCe5	QCh5	QCh5[a]
0.2:1	140(0.250)	140(0.243)	>1 μm	290(0.665)	298(0.526)	465(0.809)	>1 μm	>1 μm	>1 μm
0.4:1	88(0.271)	117(0.367)	131(0.239)	154(0.392)	99(0.224)	110(0.214)	125(0.249)	144(0.275)	143(0.259)
0.8:1	53(0.519)	162(0.464)	41(0.560)	160(0.328)	56(0.426)	44(0.542)	30(0.515)	96(0.483)	61(0.313)
1:1	58(0.446)	163(0.587)	32(0.698)	157(0.315)	79(0.506)	69(0.429)	30(0.579)	245(0.657)	54(0.565)
1.5:1	58(0.520)	122(0.578)	42(0.586)	162(0.310)	84(0.509)	78(0.305)	53(0.566)	470(0.696)	46(0.808)
2:1	74(0.461)	148(0.532)	43(0.752)	114(0.562)	104(0.561)	88(0.285)	41(0.694)	661(1.000)	80(0.622)

[a] Polymer solutions filtered with 0.45 μm filter prior to complexation with insulin

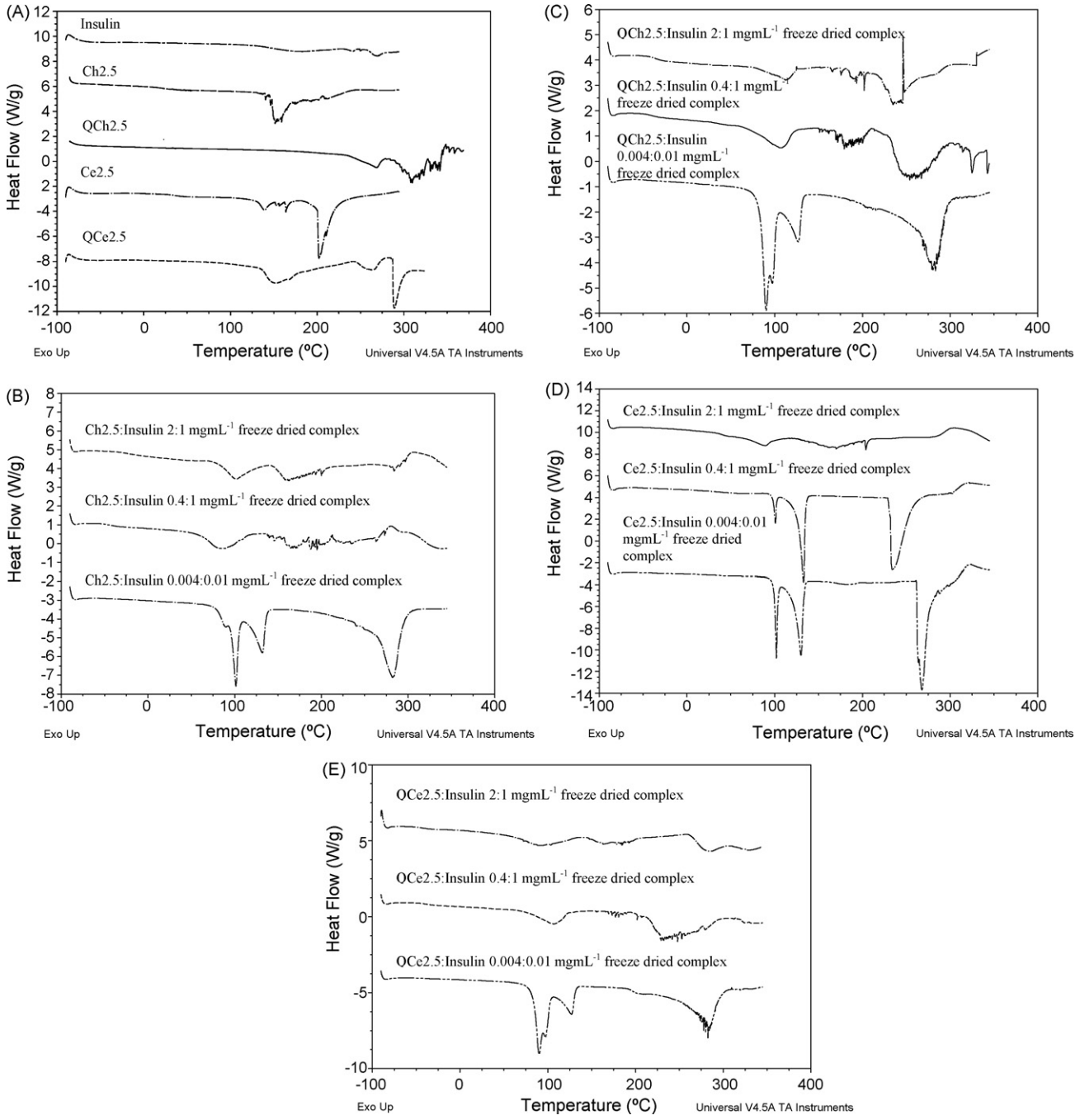

Fig. 2. DSC profile of (A) insulin, Ch2.5, QCh2.5, Ce2.5, QCe2.5; (B) Ch2.5:insulin complex at 2:1 mg mL^{-1}, 0.4:1 mg mL^{-1} and 0.004:0.01 mg mL^{-1}; (C) QCh2.5:insulin complex at 2:1 mg mL^{-1}, 0.4:1 mg mL^{-1} and 0.004:0.01 mg mL^{-1}; (D) Ce2.5:insulin complex at 2:1 mg mL^{-1}, 0.4:1 mg mL^{-1} and 0.004:0.01 mg mL^{-1}; (E) QCe2.5:insulin complex at 2:1 mg mL^{-1}, 0.4:1 mg mL^{-1} and 0.004:0.01 mg mL^{-1}.

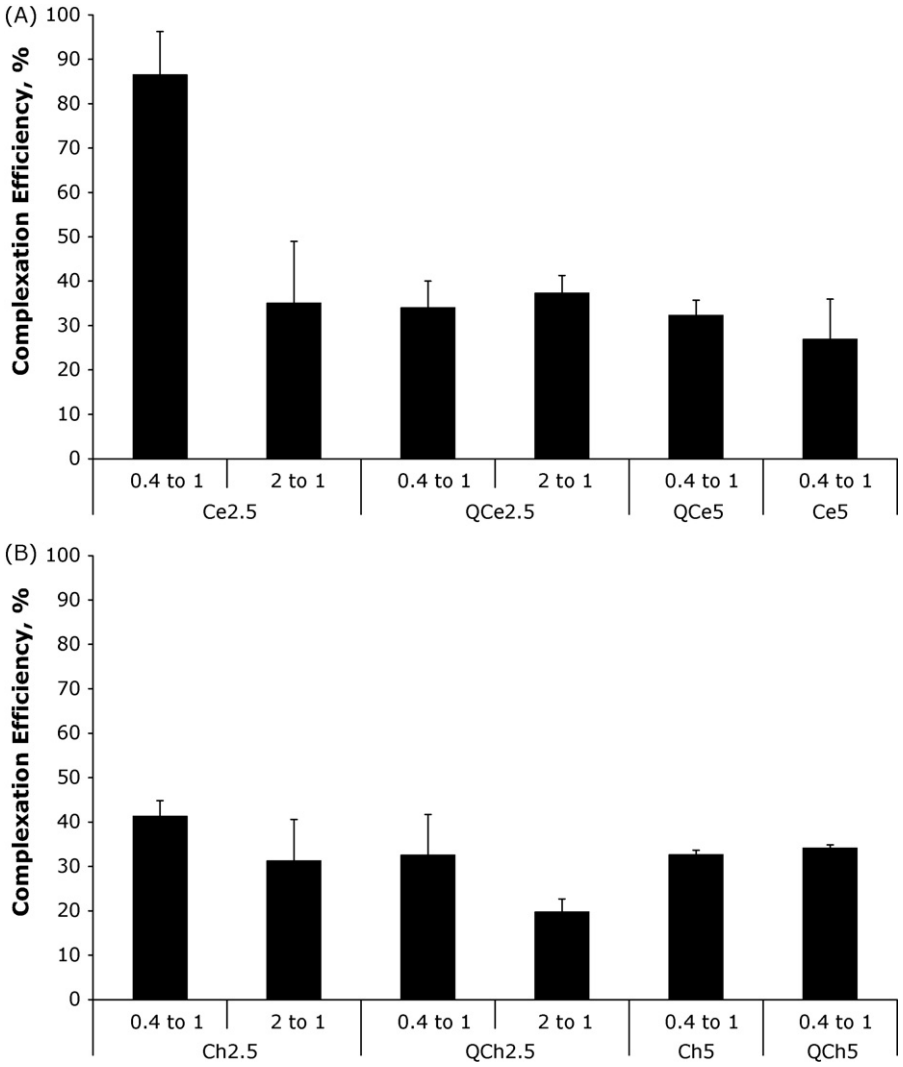

Fig. 3. Complexation efficiency (CE) of complexes (A) at cetyl graft polymer:insulin ratio of 0.4:1 or 2:1 mg mL^{-1} and (B) at cholesteryl graft polymer:insulin ratio of 0.4:1 or 2:1 mg mL^{-1} ($n = 3$; ±SD).

mer concentration (Table 2). This result appears to be in agreement with transmittance data whereby the larger the particles the more translucent/turbid complex solutions become. As a whole, most of the quaternised polymers appeared to form larger complexes at lower polymer to insulin mass ratios compared to their non-quaternised counterparts. With the exception of QCh5, increasing polymer to insulin mass ratios resulted in a considerable reduction of the hydrodynamic size where most of the quaternised polymers produced relatively small complexes (less than 100 nm) above polymer:insulin mass ratios of 0.8:1. Interestingly, QCh5 did not follow the same trend and above 0.8:1 mg mL^{-1} polymer:insulin mass ratio, the size increased to over 600 nm with a corresponding drop in transmittance (Table 2, Fig. 1).

Hydrodynamic sizes for all cetyl complexes at 0.4:1 mg mL^{-1} were around 100 nm or less with a low polydispersity index (PDI) indicating narrow size distribution (Table 2). Increasing polymer concentrations resulted in a reduction in hydrodynamic size and an increase of PDI. This phenomenon suggests the possibility of the complexes became unstable at higher concentrations and broke down into multiple different size populations. However, we have shown that cetyl polymers formed multicore micelles at higher polymer concentrations resulting in heterogeneity of the sample, which might also explain the increase of PDI in this instance (Thompson et al., 2008).

Unlike cetyl polymers, non-quaternised cholesteryl polymers produced larger complexes with sizes of between 100 and 150 nm over the range of polymer:insulin ratios tested. This shows that the type of hydrophobic pendant group has an impact on the complex formation. Similar to cetylated polymers, the lowest PDI values were found at polymer to insulin ratio of 0.4:1 mg mL^{-1}.

It had been expected that a higher level of hydrophobic grafting would allow for a reduction in the polymer concentration required to complex with insulin, which we found with palmitoyl-grafted PAAs (Thompson et al., 2009). However this was not the case with cetyl-grafted polymers. At 0.2:1 mg mL^{-1}, both Ce2.5 and QCe2.5 formed complexes with insulin of less than 300 nm while Ce5 and QCe5–insulin solutions were precipitated. It is thought that the increased in hydrophobic load would lead to stronger hydrophobic interactions with insulin while maintain the complex stability (Wang et al., 2004; Simon et al., 2004). It appears that a lower level of cetylation is more favourable although this trend is not well understood.

Both transmittance and DLS data indicated that the optimal polymer:insulin ratios were 0.4:1 mg mL^{-1} for all cetyl and cholesteryl complexes. Therefore this ratio was chosen to carry out TEM, complexation efficiency and enzymatic degradation studies. Some experiments were also conducted other ratios for comparison purposes.

3.2.3. DSC

From Fig. 2, all freeze-dried complexes demonstrated a reduction in endotherm peak size as the polymer:insulin ratio was increased. Indeed in some cases there appeared to be little or no endothermic activity for complexes prepared at 2:1 mg mL^{-1}. This would suggest that at low polymer:insulin ratios there was a higher degree of crystallinity than at 2:1 mg mL^{-1}. This may be due to the higher polymer:insulin concentrations preventing re-ordering of the complexes on freeze drying. The complexes at 2:1 mg mL^{-1} may be mostly amorphous whereas the lower polymer and insulin concentrations at 0.004:0.01 mg mL^{-1} may allow for re-ordering of the polymer/insulin structure as there are fewer molecules present to reorient themselves during freeze drying. All polymer:insulin sample thermal profiles were also different from the polymer and insulin samples alone in terms of the peak/onset temperature, and number, of endotherms. This would indicate a different crystalline structure on combination of the polymers and insulin.

The change in thermal profiles is supported by the changes in morphology of the samples at different polymer:insulin ratios (Section 3.2.6). Changes in sample morphology between 0.4:1 and 2:1 mg mL^{-1} would seem to confirm that the polymers and insulin interact differently depending on the polymer concentration. This could explain the attendant changes in thermal profile between 0.4:1 and 2:1 mg mL^{-1} (Kang et al., 2002).

3.2.4. Complexation efficiency (CE)

Overall, there was little difference in CE values of the complexes between cetyl-grafted polymers, except Ce2.5 at 0.4:1 mg mL^{-1} (Fig. 3). Ce2.5 exhibits highest CE of 86.5% at polymer:insulin ratio of 0.4:1 mg mL^{-1} while the CE was only 44.9% at a higher polymer:insulin ratio of 2:1 mg mL^{-1} (Fig. 3A). This corresponds well with the transmittance and size analysis data demonstrating a lower polymer concentration is more favourable for complexation. However, all cholesteryl-grafted polymers had low CE values regardless of the polymer concentrations (Fig. 3B).

Unlike palmitoyl-grafted polymers, which had CE above 80% (Thompson et al., 2009), both cetylated polymers and cholesteryl-grafted polymers were not efficient at complexing with insulin. Therefore it seems that the polymer architecture, especially the type of hydrophobic pendant group has a major influence on the ability of these polymers to interact with insulin. This could be due to the microviscosity within the polymer aggregates' cores (Thompson et al., 2008). The long, rigid planar transfused ring structure of cholesteryl pendant groups conferred a greater core rigidity to the nanoparticles they produced (Thompson et al., 2008). This increase in microviscosity may limit the ability of insulin to interact with cholesteryl-grafted polymers when compared to the more fluid nature of palmitoyl-grafted polymer aggregates. Interestingly, cetyl is not dissimilar to palmitoyl pendant groups and the reasons why most of cetylated polymers had low CE values was unclear. It is possible that the presence of a carbonyl group facilitated the interaction of insulin with the palmitoyl polymers.

3.2.5. Zeta potential

Insulin is negatively charged (−22 mV) in pH 7.4 Tris buffer while polymers were positively charged (Table 1). Overall, complexes formed by cetylated polymer had a higher zeta potential than their cholesteryl counterparts (Fig. 4). Apart from Ch2.5 at polymer to insulin ratio of 0.4:1 mg mL^{-1}, a consistent trend was observed where addition of insulin into polymer solutions resulted in the reduction of polymer zeta potential, indicating formation of electrostatic interactions (Fig. 4B). The greatest drop of zeta potential was observed in quaternised cholesteryl polymers suggesting stronger electrostatic interactions with quaternary ammonium moieties, similar to our findings in quaternised palmitoyl-grafted polymers (Fig. 4B, Table 1) (Thompson et al., 2009). However, cetylated polymers behaved differently, with all quaternised cetylated polymers and Ce2.5 showing an increase in zeta potential after complexing with insulin (Fig. 4A, Table 1). This phenomenon might be due to charge displacement (Simon et al., 2004; Thompson et al., 2008), but again this trend is inconsistent with the quaternised palmitoyl-grafted polymers, where the pendant groups were also straight alkyl chains.

3.2.6. TEM

Electron micrographs of the complexes at 0.4:1 and 2:1 mg mL^{-1} polymer:insulin ratios indicate differing morphologies at high and low polymer concentrations (Fig. 5). For Ce2.5, at lower polymer concentrations, irregular shapes of the complexes were found (Fig. 5A). However, at higher polymer concentrations, discrete spherical nanoparticles with the size of approximately 50–70 nm were found, which correlates well with the hydrodynamic size (Fig. 5B). This could be due to the increase in polymer concentration as well as the intercalation of the hydrophobic segments of the insulin promoting greater chain interaction (Simon et al., 2004; Thompson et al., 2009; Wang et al., 2004). A similar trend was observed in previous work where the polymer and insulin concentrations affected the morphologies of the palmitoyl-grafted polymer–insulin complexes (Thompson et al., 2009).

Similar to Ce2.5 at 2:1 mg mL^{-1}, solid nanoparticles were found in Ch2.5 complexes at a polymer:insulin ratio of 2:1 mg mL^{-1} (Fig. 5D). Unlike Ce2.5 complexes, these nanoparticles have a stained coat surrounding a dense core which resists the ingress of the heavy metal staining (Fig. 5D). These structures are similar to those found when free cholesterol was dissolved together with cholesteryl polymers (Thompson et al., 2008). Cholesterol has been widely used as a membrane stabilising agent and has been shown to encourage the formation of bilayer vesicles by integrating between polymer alkyl chains (Wang et al., 2001, 2004). We hypothesised that the closed packing of the rigid planar transfused ring structure of cholesteryl pendant groups prevented the integration of cholesterol into the polymer, resulting in cholesterol residing in the core of these structures (Thompson et al., 2008). It is possible that insulin interacts in the same way as cholesterol does with cholesteryl-grafted polymers indicating that the insulin might be residing in the core(s) with PAA as the outer coating. However, insulin is a macromolecule with three-dimensional structure and is completely different from cholesterol molecule. Hence further investigations are required to confirm this hypothesis.

Quaternised polymers appeared to form nanoparticles on complexation with insulin (Fig. 5E and F). This would suggest in the case of cholesteryl-grafted polymers that the introduction of quaternary ammonium moieties altered the way in which the polymer and insulin interacted. The permanent positive charge could allow for greater electrostatic interaction between the polymer and insulin (Thompson et al., 2009). The greater electrostatic repulsion between quaternised moieties and the bulky nature of the quaternary moieties could also produce changes in cholesteryl chain stacking and core viscosity/rigidity during polymer aggregation resulting in the change of morphology observed (Thompson et al., 2008).

To date, the association of a protein with amphiphilic polymers has seldom been studied. One study conducted by Sandanaraj et al. (2007) examine the effect of an amphiphilic polymer on the properties of cytochrome. The authors demonstrated the electrostatic interaction is the major driving force for the polymer–protein complexation which led to a reversible conformational change of cytochrome. Insulin has a three-dimensional structure with two polypeptide chains linked by disulfide bonds and a non-polar core is formed by buried aliphatic side chains from both chains (Manosroi

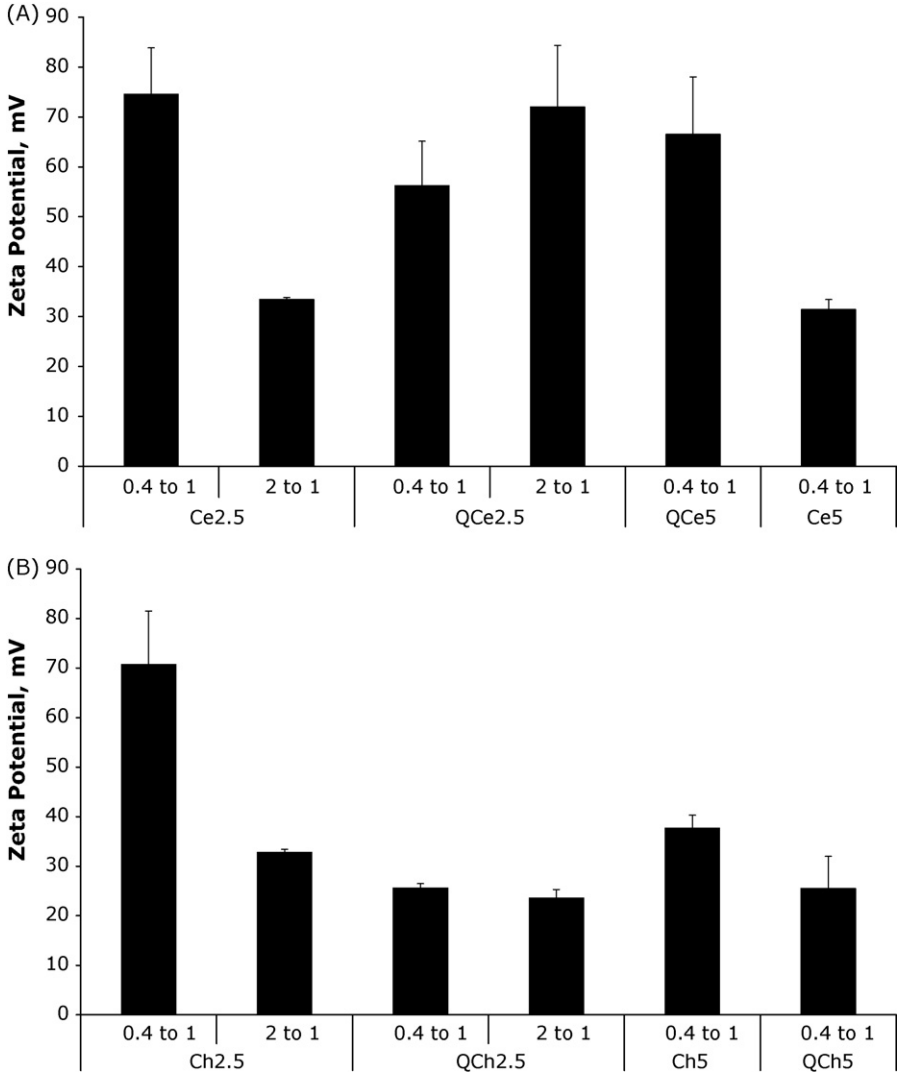

Fig. 4. Zeta potential of complexes at (A) cetyl graft polymer:insulin ratio of 0.4:1 or 2:1 mg mL^{-1} and (B) at cholesteryl graft polymer:insulin ratio of 0.4:1 or 2:1 mg mL^{-1} ($n = 3$; ±SD).

et al., 1990; Chang et al., 2003). Interaction between polymers and proteins involve complex mechanisms and currently a complete understanding of these mechanisms is seriously lacking (Cooper et al., 2005).

3.3. Cytotoxicity assay

All graft polymers had higher IC_{50} values than PAA (except Ce2.5) (Table 1). This would suggest that hydrophobic grafting has increased the biocompatibility of these polymers. In addition the quaternised polymers all had higher IC_{50} values than their equivalent non-quaternised couterparts. This was expected given that others have found that reducing the number of primary amines increases IC_{50} values (Fischer et al., 2003; Unger et al., 2007; Vroman et al., 2007). Altering the charge density and type of cationic functionality of the polymer backbone by quaternisation has made the polymers less cytotoxic. The backbone contains a high number of primary amines, which are considered cytotoxic (Fischer et al., 2003; Mao et al., 2005; Unger et al., 2007). After methylation, all were converted to tertiary or quaternary amines which led to the reduction of IC_{50} (Thompson et al., 2008).

This could also explain the increase in IC_{50} of the non-quaternised polymers in relation to PAA. It has been postulated that a three-point attachment to a cell membrane is required for induction of a biological response (Fischer et al., 2003; Unger et al., 2007). Therefore when the space between reactive primary amines is increased by, e.g. hydrophobic substitution, the ability of the amines to interact with the membrane is reduced. It is thought that the presence of hydrophobic pendant groups restrict the PAA polymer chain flexibility that may limit the exposure of the cell surface to the remaining primary amines (Fischer et al., 2003). This may also explain the IC_{50} values of the 5% mole substituted polymers are greater than those produced at 2.5% mole substitution.

3.4. Enzymatic degradation studies

3.4.1. Trypsin

Fig. 6A and B shows that complexation with both cetyl and cholesteryl graft polymers protected insulin from enzymatic degradation by trypsin ($p < 0.001$). The levels of non-degraded insulin are comparable to that of palmitoyl even though the polymer to insulin mass ratio is much lower (0.4:1 compared to 2:1 for palmitoyl) (Thompson et al., 2009). From the data, it seems that polymer architecture, i.e. the type and the level of hydrophobic pendant groups or the presence or absence of a quaternary ammonium moiety had little influence in the degree of protection. This result is

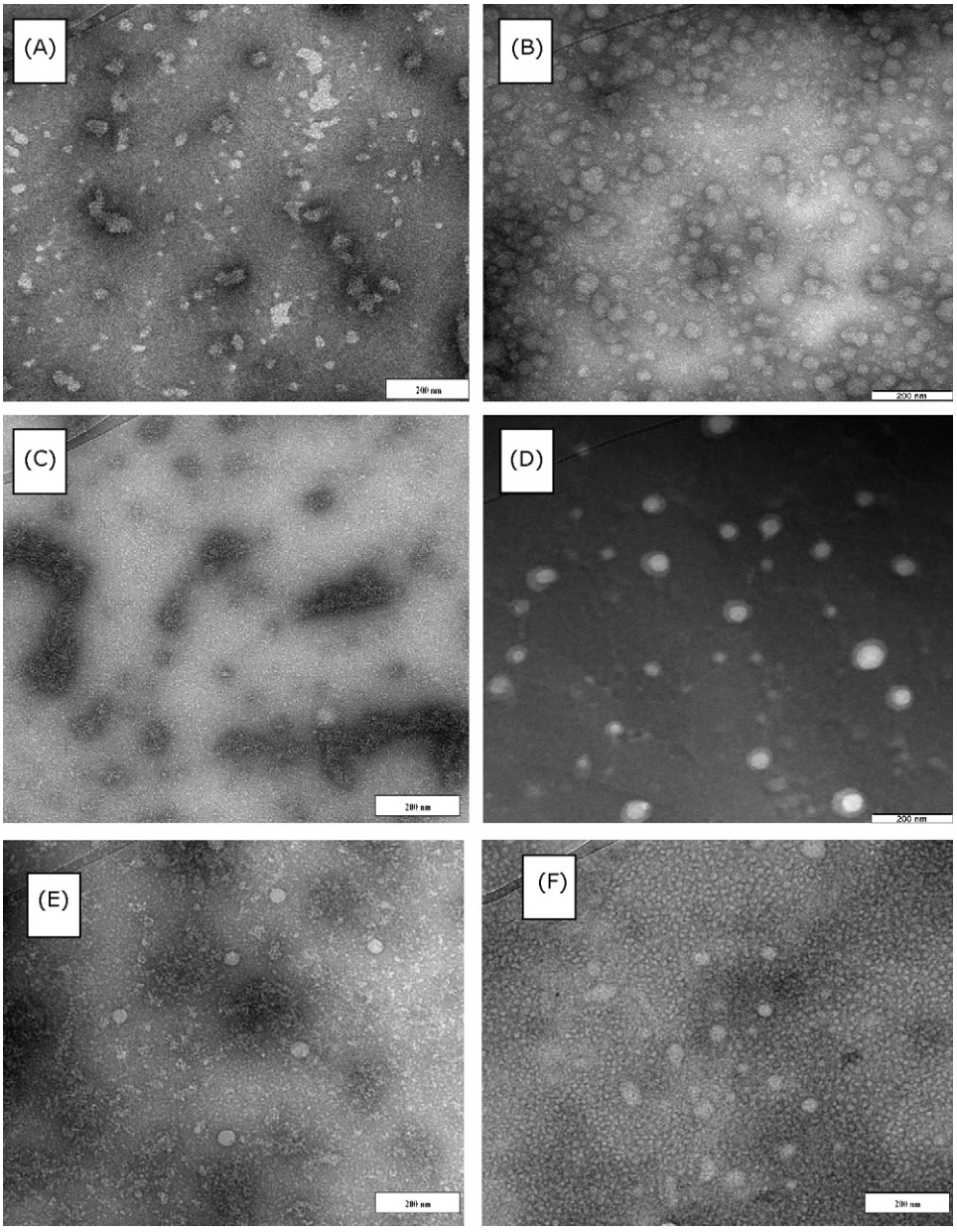

Fig. 5. TEM micrographs of (A) Ce2.5:insulin (0.4:1 mg mL^{-1}); (B) Ce2.5:insulin (2:1 mg mL^{-1}); (C) Ch2.5:insulin (0.4:1 mg mL^{-1}); (D) Ch2.5:insulin (2:1 mg mL^{-1}); (E) QCe2.5:insulin (0.4:1 mg mL^{-1}); (F) QCh2.5:insulin (0.4:1 mg mL^{-1}). Scale bar = 200 nm.

different from our previous work and others. Simon et al. (2007) reported that higher level of hydrophobic grafting of polyesters resulted in better protection against trypsin degradation of insulin. Trypsin attacks only two residues in insulin (B29-Lys and B22-Arg) which are found in its hydrophobic domain (Schilling and Mitra, 1991). It is possible that both cetyl and cholesteryl groups are able to associate more strongly with the hydrophobic region of insulin as compared to the lactide chains described by Simon et al. (2007). As a result, the level of hydrophobic pendant groups did not have an impact on the degree of protection. Previously we have shown that quaternised palmitoyl-grafted PAA complexes had a higher level of non-degraded insulin after 4 h than non-quaternised complexes (Thompson et al., 2009), suggesting the presence of quaternary ammonium moieties played an important role due to electrostatic interaction with insulin. However, the results obtained here seem to demonstrate that hydrophobic association is more important than electrostatic interaction in preventing the trypsin from accessing the target sites.

3.4.2. α-Chymotrypsin

Apart from Ch5, non-quaternised cetyl and cholesteryl-grafted polymers were not able to protect insulin from enzymatic degradation but instead enhanced the degradation by α-chymotrypsin over 2 h (Fig. 7A and B). This finding correlates well with our previous work on palmitoyl-grafted PAA which showed similar results. However, the extent of the degradation is less compared to palmitoyl-grafted polymers. After 20 min incubation with α-chymotrypsin, there was only 20% non-degraded insulin remaining for palmitoyl-grafted polymers (Thompson et al., 2009) while in this study there was 56% (Ce2.5), 62% (Ce5) and 60% (Ch2.5) non-degraded insulin. The addition of quaternary ammonium moieties offered significant protection against α-chymotrypsin degradation compared to insulin alone after 2 h ($p < 0.001$). Based on the last time point at 2 h, the order of polymers that provide the highest level of protection is QCh2.5 (52% non-degraded insulin) > QCh5 (51%) > QCe5 (48%) > QCe2.5 (42%) > Ch5 (42%) > Ch2.5 (36%) > Ce5 (27%) > Ce2.5 (26%). Unlike the trypsin degradation study, these

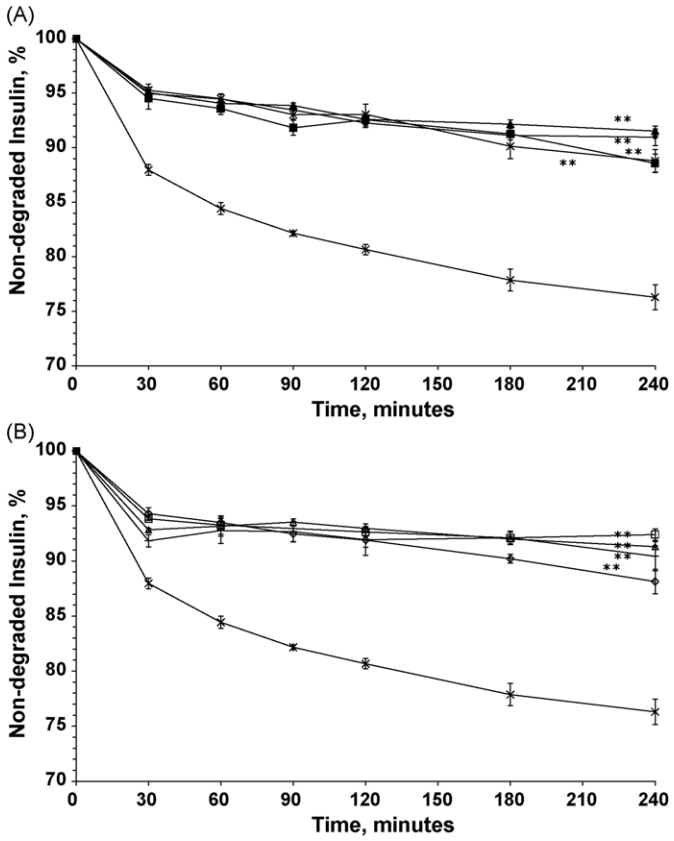

Fig. 6. Determination of the protective effect of (A) cetyl graft polymers against trypsin degradation of insulin—(∗) insulin; (♦) Ce2.5; (■) Ce5; (▲) QCe2.5; (×) QCe5 and (B) cholesteryl graft polymers against trypsin degradation of insulin—(∗) insulin; (◊) Ch2.5; (□) Ch5; (▲) QCh2.5; (+) QCh5 ($n = 3$; ±SD). $^{**}p < 0.001$ compared to free insulin. The polymer to insulin ratio for all polymers was 0.4:1 mg mL^{-1}.

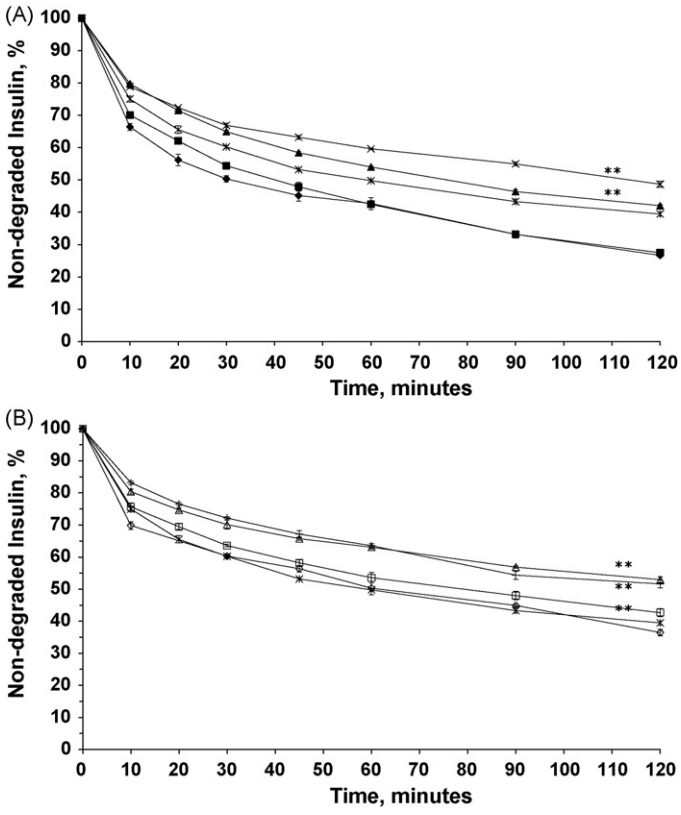

Fig. 7. Determination of the protective effect of (A) cetyl polymers against α-chymotrypsin degradation of insulin—(∗) insulin; (♦) Ce2.5; (■) Ce5; (▲) QCe2.5; (×) QCe5 and (B) cholesteryl polymers against α-chymotrypsin degradation of insulin—(∗) insulin; (◊) Ch2.5; (□) Ch5; (△) QCh2.5; (+) QCh5 ($n = 3$; ±SD). $^{**}p < 0.001$ compared to free insulin. The polymer to insulin ratio for all polymers was 0.4:1 mg mL^{-1}.

results demonstrate that the type of hydrophobic pendant groups and the presence of quaternary ammonium moieties do play an important role on the degree of protection against α-chymotrypsin.

α-Chymotrypsin has seven target sites on insulin molecules where the first five target sites on the surface of insulin molecules will be cleaved rapidly and followed by the two sites which are embedded in the hydrophobic region of insulin (Schilling and Mitra, 1991). With the addition of quaternary ammonium moieties, these polymers were able to form electrostatic interaction with insulin. The tight binding between the positively charged quaternised polymers and negatively charged insulin may limit α-chymotrypsin access to its initial five target sites. As a result, all the quaternised cetyl and cholesteryl polymers were able to significantly reduce the enzymatic degradation compared to their non-quaternised counterparts. Like the palmitoyl chains, cetyl and cholesteryl pendant groups were able to interact with the hydrophobic region of the insulin and thus it is thought that the unfolding of insulin would expose the remaining two target sites hidden in the hydrophobic region and hence enhances the insulin degradation (Thompson et al., 2009). However, the degree of insulin degradation for non-quaternised cholesteryl-grafted polymers was less than non-quaternised cetyl-grafted polymers. Our previous work showed that Ch5 self-assemblies have the highest core rigidity due to the planar, rigid structure of the cholesterol pendant groups (Thompson et al., 2008). As a result, it is thought that the hydrophobic association between insulin and the more ordered, close packing of the Ch5 hydrophobic core will restrict the access of α-chymotrypsin to the two target sites despite the unfolding of the insulin. It is also possible that the bulky nature of cholesteryl pendant groups provided steric hindrance and prevented α-chymotrypsin from attacking the two sites in the hydrophobic region of insulin.

3.4.3. Pepsin

From Fig. 8A, statistical analysis showed that both QCe5 and Ce2.5 were able to protect insulin from pepsin degradation and slow down the degradation of insulin ($p < 0.001$) and to a lesser extent QCe2.5 also exhibited protection against pepsin degradation ($p < 0.005$). However, apart from QCh5 ($p < 0.001$), none of the choesteryl-grafted polymers offered any discernable protection against pepsin activity (Fig. 8B). This indicates that cetyl pendant groups seem to confer better protection against pepsin degradation than cholesteryl pendant groups. Like cetyl-grafted polymers, similar results were obtained for palmitoyl-grafted PAA showing protection against pepsin degradation (Thompson et al., 2009).

Unlike trypsin and α-chymotrypsin, pepsin randomly cleaves peptide bonds between all amino acid residues of insulin and hence it is very effective in destroying insulin even at a very low concentration (Calceti et al., 2004). To our knowledge, there are no reports on the interaction between amphiphilic polymer–insulin complexes and pepsin. Based on the reports on nanoparticles which demonstrated that a solid physical barrier is essential to prevent the enzymes from accessing the target site (Zhenqing et al., 2004; Trotta et al., 2005), it is thought that for any compound to protect insulin against pepsin degradation, it must encapsulate/complex with the whole insulin molecule at all times. The cetyl pendant group is not dissimilar to the palmitoyl pendant group as they both are alkyl chains with 16 carbon atoms. As a result, these pendant

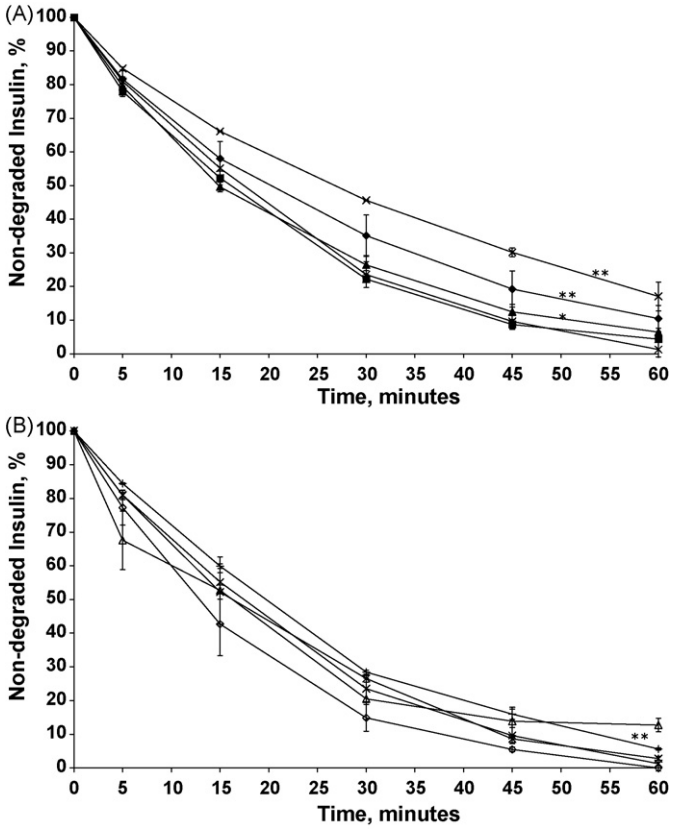

Fig. 8. Determination of the protective effect of (A) cetyl polymers against pepsin degradation of insulin (polymer:insulin ratio 0.4:1 mg mL^{-1})—(∗) insulin; (♦) Ce2.5; (■) Ce5; (▲) QCe2.5; (×) QCe5 and (B) cholesteryl polymers against pepsin degradation of insulin (polymer:insulin ratio 0.4:1 mg mL^{-1})—(∗) insulin; (◊) Ch2.5; (□) Ch5; (△) QCh2.5; (+) QCh5 ($n = 3$; ±SD). $^{**}p < 0.001$, $^{*}p < 0.05$ compared to free insulin. The polymer to insulin ratio for all polymers was 0.4:1 mg mL^{-1}.

chains are much more flexible than cholesteryl pendant groups. It could be that these polymers interact with specific regions of the insulin molecule and some parts of the insulin molecule are still exposed. Upon incubation with pepsin, cleavage of these exposed bonds occur but due to the flexibility of the alkyl chains, cetylated polymers could associate with insulin at different sites and hence prevented complete degradation after 1 h. Alternatively the presence of alkyl pendant groups such as cetyl chains might have an impact on the activity of pepsin since it has been reported that the attachment of alkyl chains to a phosphorous atom could inhibit the activity of gastric lipases (Miled et al., 2003) although further investigation is required to confirm this hypothesis.

In comparison to palmitoyl-grafted PAA all of the cetyl and cholesteryl graft PAA offered less protection against pepsin activity (Thompson et al., 2009). It was thought that the protective effect demonstrated by palmitoyl graft polymers was in some way related to the change in pH involved in pepsin activity experiments when compared to trypsin and α-chymotrypsin (pH 2 compared to pH 8). There may be a morphological change in the palmitoyl complexes at pH 2 which does not occur with cetyl or cholesteryl complexes. However it has not been possible to image these complexes as the pH is incompatible with the staining dye used in TEM.

4. Conclusion

These novel comb shaped amphiphilic PAAs have demonstrated their ability to complex with insulin and were able to protect insulin from enzymatic degradation to differing degrees. From the results, it was shown that both hydrophobic and electrostatic interaction between insulin and amphiphilic polymers play a significant role in the protection against enzymatic degradation. The type of hydrophobic pendant groups is pivotal in reducing insulin degradation by pepsin and α-chymotrypsin while the presence of quaternary ammonium moieties is essential against α-chymotrypsin. The addition of quaternary ammonium moieties to cetyl and cholesteryl-grafted polymers has resulted in a significant enhancement of protection against α-chymotrypsin, which was not observed in previously reported palmitoyl-grafted polymers. However, no polymer was able to provide significant protection against all three proteolytic enzymes tested although quaternised polymers appear to offer the widest range of protection. Together with the fact that these polymers all had relatively poor complexation efficiency values compared to the previously published data on palmitoyl-grafted PAAs, this suggests that a combination of quaternised palmitoyl and cetyl or cholesteryl polymers will be required to offer protection against all three enzymes. We are aware that drug absorption from the gastrointestinal tract is a complex process. Factors such as presence of food, the variation in age, genomic factors and the physiological conditions will affect drug absorption and therefore future work will investigate the transport mechanism across CaCo-2 cell monolayer and *in vivo* pharmacokinetic studies using a diabetic rat model.

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