International Journal of Pharmaceutics 376 (2009) 46–55

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The complexation between novel comb shaped amphiphilic polyallylamine and insulin—Towards oral insulin delivery

C.J. Thompson[a], L. Tetley[b], I.F. Uchegbu[c], W.P. Cheng[d,*]

[a] School of Pharmacy and Life Sciences, The Robert Gordon University, Schoolhill, Aberdeen AB10 1FR, UK
[b] Division of Infection & Immunity, IBLS, Integrated Microscopy Facility, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK
[c] School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK
[d] School of Pharmacy, University of Hertfordshire, College Lane Hatfield AL10 9AB, UK

ARTICLE INFO

Article history:
Received 2 February 2009
Received in revised form 7 April 2009
Accepted 8 April 2009
Available online 16 April 2009

Keywords:
Oral delivery
Proteins and peptides
Polymeric self-assemblies
Nano-complexes
Enzymatic degradation

ABSTRACT

Novel amphiphilic polyallylamine (PAA) were previously synthesised by randomly grafting palmitoyl pendant groups and subsequent quaternising with methyl iodide. The ability of these self-assembled polymers to spontaneously form nano-complexes with insulin in pH 7.4 Tris buffer was evaluated by transmittance study, hydrodynamic size and zeta potential measurements. The transmission electron microscopy images showed that non-quaternised polymer complexes appeared to form vesicular structures at low polymer:insulin concentrations. However, at higher concentrations they formed solid dense nanoparticles. The presence of quaternary ammonium moieties resulted in insulin complexing on the surface of aggregates. All polymers exhibited high insulin complexation efficiency between 78 and 93%. Incubation with trypsin, α-chymotrypsin and pepsin demonstrated that most polymers were able to protect insulin against enzymatic degradation by trypsin and pepsin. Quaternised polymers appeared to have better protective effect against trypsinisation, possibly due to stronger electrostatic interaction with insulin. Interestingly, non-quaternised polymers significantly enhanced insulin degradation by α-chymotrypsin. All polymers were less cytotoxic than PAA, with the quaternised polymers exhibiting up to 15-fold improvement in the IC_{50} value. Based on these results, quaternised palmitoyl graft polyallylamine polymers showed promising potential as oral delivery systems for insulin.

1. Introduction

Oral delivery of proteins has been attempted for many years as an alternative to conventional injectable delivery systems (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; Simon et al., 2007). It is considered the most favourable route due to ease of administration and patient convenience (Hamman et al., 2005). However proteins cannot be administered orally in their native state due to degradation in the gastro-intestinal tract (GIT). Enzymes such as trypsin and pepsin as well as the wide pH range in the GIT inactivate proteins and restrict the amount of active protein reaching the systemic circulation (Schilling and Mitra, 1991; Saffran et al., 1997; Jung et al., 2000; Soppimath et al., 2001; Lambkin and Pinilla, 2002; Shen, 2003; Calceti et al., 2004; Hamman et al., 2005; Cui et al., 2006; Mahkam et al., 2006). In the case of insulin this can be less than 0.1% of the administered dose (Qian et al., 2006). Proteins are also hydrophilic macromolecules and hence they are unable to diffuse passively through the GIT epithelium unaided (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).

Therefore, different technologies have been employed to protect proteins in the GIT as well as promote their absorption through the GIT epithelium. These include entrapment in liposomes (Soppimath et al., 2001), solid micro/nanoparticles (Soppimath et al., 2001; Fan et al., 2006), chemical modification of proteins to limit enzymatic attack (Hamman et al., 2005), prodrugs (Hamman et al., 2005) and co-administration with absorption enhancers/enzyme inhibitors (Hamman et al., 2005). However, there are limitations with the current technologies. Liposomes are unstable on storage and in the GIT (Soppimath et al., 2001). The fabrication of micro/nanoparticles often requires the use of organic solvents and high temperatures/pressures which can denature proteins. In addition their use in oral protein delivery has lead to highly variable levels of protein in the circulation (Hamman et al., 2005; Mao et al., 2006). Altering protein structures or the use of prodrugs have proven to be highly complex and might affect the protein's biological activity if structural modification alters its binding to receptor sites (Schilling and Mitra, 1991; Hamman et al., 2005). The administration of absorption enhancers or enzymatic inhibitors has been shown to damage GIT epithelium and

* Corresponding author. Tel.: +44 1707 285060; fax: +44 1707 284870.
E-mail address: w.p.cheng3@herts.ac.uk (W.P. Cheng).

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doi:10.1016/j.ijpharm.2009.04.014

adversely affect absorption of digestive nutrients (Hamman et al., 2005).

Amphiphilic polymers consist of hydrophobic and hydrophilic moieties and they are commonly produced by copolymerisation of hydrophilic and hydrophobic monomers (Gaucher et al., 2005). Block copolymers have been widely explored as drug delivery systems and comb shaped amphiphilic polymers formed by grafting of hydrophobic pendant groups to a hydrophilic backbone are starting to gain attention in the pharmaceutical field (Wang et al., 2001, 2004a; Cheng et al., 2006). In an aqueous environment, these amphiphilic polymers form self-assemblies consisting of a hydrophobic core stabilised by a hydrophilic corona. Different therapeutic agents such as hydrophobic drugs, plasmid DNA, oligodeoxyribonucleotides (ODN) can be incorporated in the core by means of hydrophobic or electrostatic interactions (Gaucher et al., 2005). The self-assemblies formed can have significantly lower critical aggregation concentrations compared to conventional surfactants and so are less likely to be dissipated in large volumes of distribution (Gaucher et al., 2005). Hence, they have been widely used for intravenous administration. Recently, this unique characteristic has also been exploited in oral delivery. Previous work using amphiphilic polyethylenimine (PEI) demonstrated the ability of these amphiphiles to facilitate the oral absorption of a hydrophobic peptide drug, cyclosporine, in vivo (Cheng et al., 2006). Others also reported on the use of block amphiphilic polymers for oral delivery of anticancer drugs (Bromberg, 2008). The attempt to deliver protein orally using these systems has not been reported until recently by Kissel and colleagues. Using insulin as a model protein drug, they have shown the ability of amphiphilic polyesters to form nanocomplexes with insulin (Simon et al., 2004, 2007).

In this paper, the use of novel comb shaped amphiphilic polymers based on polyallylamine (PAA) in the oral delivery of a model protein drug, insulin, was investigated. The amphiphilic polymers were previously synthesised by attaching pamitoyl pendant groups to PAA and subsequently reacted with methyl iodide to obtain quaternary ammonium palmitoyl PAAs (Thompson et al., 2008). We evaluated the cytotoxicity of the amphiphiles. The characterisation of polymer, insulin nano-complexes were conducted and finally, the protective capability of these complexes against trypsin, α-chymotrypsin and pepsin, the three major proteolytic enzymes responsible for insulin degradation in GIT, was examined.

2. Materials and methods

2.1. Materials

Poly(allyl amine hydrochloride) (Mw = 15 kDa), palmitic acid-N-hydroxysuccinimide ester (98%), tris(hydroxymethyl) aminomethane (Tris) (≥99%), insulin (27 U mg^{-1}, bovine pancreas), minimum essential media eagle (EMEM, phosphate buffer tablets (PBS), trypsin (TPCK treated, bovine pancreas, 7500 U mg^{-1}), pepsin (3260 U mg^{-1}) and α-chymotrypsin (TLCK treated, Type VII from bovine pancreas, ≥40 U mg^{-1}) were all purchased from Sigma–Aldrich, UK. Trifluoroacetic acid (TFA) (HPLC grade) was purchased from Fisher Scientific, UK. All solvents were purchased from Fisher Scientific Chemicals, UK and were of HPLC grade. All other reagents used were of analytical grade. Cell culture reagents such as L-glutamine (200 mM), trypsin–EDTA (0.05%), non-essential amino acids were obtained from Invitrogen, Scotland, and foetal bovine serum (South American) from Biosera, UK.

2.2. Polymer synthesis

The methods have been previously described (Thompson et al., 2008). Briefly the PAA was reacted with palmitic acid-N-hydroxysuccinimide ester based on molar feeds of 1:0.25 or 1:0.5 (PAA monomer to palmitoyl group) to obtain $Pa_{2.5}$ and Pa_5. The subscript numerals of the polymer abbreviations indicate the expected mole% of palmitoylation based on the initial molar feeds. Quaternisation (Q) was carried out by reacting $Pa_{2.5}$ and Pa_5 with over a 1000 molar excess of methyl iodide to obtain quaternary ammonium compounds, $QPa_{2.5}$ and QPa_5. The four novel amphiphilic polymers were characterised by elemental analysis and ^{1}H NMR.

2.3. Preparation of polymer, insulin complexes

This was carried out by adapting a method reported by Simon et al. (2004). 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)). Various polymer solutions (0.1–4 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 (0.5 or 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. The solutions were then left at room temperature for 2 h and 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 (Simon et al., 2004) (n = 2). Polymer, insulin complexes were prepared as described above and 2 h after preparation, photographs of complexes were taken 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 were 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 polymers, freeze-dried complexes and insulin (2–6 mg each) were analysed for melting point (T_m) and glass transition (T_g) temperatures 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 optimal polymer:insulin mass ratio of 2:1 mg mL^{-1} as described above as well as at 0.2:0.1 and 0.002:0.001 mg mL^{-1} and freeze dried over 48 h in a VirTis adVantage freeze drier (Biopharma Process Systems, UK). A physical mixture of each polymer and insulin (2:1 mass ratio) was also analysed in the same way.

2.4.5. Transmission electron microscopy (TEM)

Formvar/carbon-coated 200 mesh copper grids were glow discharged and polymer, insulin complexes solutions were dried down to a thin layer onto the hydrophilic support film. 1% aqueous methylamine vanadate (Nanovan; Nanoprobes, Stony Brook, NY, USA) stain was applied and the mixture air-dried. The negatively stained complexes were imaged with a LEO 912 energy filtering transmission electron microscope at 80 or 100 kV.

2.4.6. Determination of complexation efficiency

This study was carried out by adapting the method described by Simon et al. (2007). Polymer, insulin complexes were prepared at polymer/insulin ratio = 4:2 mg mL^{-1}. The insulin content in the insulin stock solution (diluted 1 in 1 with pH 7.4 Tris buffer) and complex solution as made were determined using an HPLC with fluorescence detector ($\lambda_{excitation}$ = 276 nm; $\lambda_{emission}$ = 600 nm). The Shimadzu HPLC system consisted 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. The stationary phase was a Gemini C_{18} column (25 mm × 4.6 mm; 110 Å) (Phenomenex, UK) and the mobile phase was water/acetonitrile (68.5:31.5) buffered to pH 2 with TFA at a flow rate of 1 mL min^{-1}. 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 the following equation:

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

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

2.5. Cytotoxicity assay

The cytotoxicity of PAA and amphiphiles was determined using an MTT assay. Caco-2 cells were cultured in EMEM (supplemented with 10% foetal bovine serum, 1% non-essential amino acids and 1% L-glutamine) at 5% CO_2, 95% humidity and 37 °C. 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 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).

2.6. Enzymatic degradation study

Trypsin (6.4 mg mL^{-1}, 2.7 × 10^{-4} M) and polymer, insulin complexes (polymer/insulin ratio = 4 mg mL^{-1}/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 triplicates 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.

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.

3. Results and discussion

3.1. Polymer synthesis and characterisation

The presence of palmitoyl pendant groups and quaternary ammonium moieties was confirmed by ^{1}H NMR (Thompson et al., 2008). For Pa_5: $\delta_{0.8} = CH_3$ (palmitoyl), $\delta_{0.9-1.8} = C_{14}H_{28}$ (palmitoyl) and CH_2 (PAA), $\delta_{2.2}$ = CH–CO–N (palmitoyl), $\delta_{2.6} = CH_2$–NH_2 (PAA); QPa_5: $\delta_{0.9} = CH_3$ (palmitoyl), $\delta_{1.1-1.8} = C_{14}H_{28}$ (palmitoyl) and CH_2 (PAA), $\delta_{1.9} = CH_2$–NH (PAA), $\delta_{2.2}$ = CH–CO–N (palmitoyl), $\delta_{3.3-3.8} = CH_2$ and CH_3 (quaternary ammonium moiety). The level of palmitoylation was estimated by both ^{1}H NMR and elemental analysis while the degree of quaternisation was calculated based on the elemental analysis data, as previously described (Table 1) (Thompson et al., 2008).

3.2. Characterisation of polymer, insulin complexes

3.2.1. Transmittance studies

All polymers produced isotropic liquids on probe sonication in pH 7.4 Tris buffer. Addition of insulin solution in similar buffer resulted in the spontaneous formation of polymer, insulin complexes. Proteins are polyampholytes and depending on the pH, it will have either a net negative or positive charge, which is balanced at its isoelectric point (Wittemann et al., 2007). Insulin has an isoelectric point of 5.3 and above this pH, it is negatively charged and hence it interacts strongly with polymers of positive charge (Simon et al., 2004). Transmittance study is a simple and non-invasive method to provide basic information on the complex formation and the optimum polymer to insulin mass ratios (Simon et al., 2004).

The turbidity curves produced over a range of polymer concentrations using stock insulin concentration of 0.5 mg mL^{-1} or 2 mg mL^{-1} are shown in Fig. 1. As demonstrated by the photos, complex solutions below 10% transmittance indicated precipitation, between 10 and 30% turbidity, 30–90% translucence and above 90% were considered clear (Fig. 1). On the whole, most of these complexes followed the same turbidity pattern with the lowest polymer concentrations having the lowest transmittance and increasing the polymer concentration resulting in increase clarity of the complex solution. This suggests that large and unstable complexes with an excess of insulin were formed at low polymer concentrations corresponding to precipitation or turbid formulations (Simon et al., 2004). However, stable and compact complexes were formed at increasing polymer concentration resulting in translucent or clear formulations (Simon et al., 2004). Therefore based on transmittance data alone the optimal polymer:insulin mass ratio for all polymers was ≥0.1:0.25 (Fig. 1A) or ≥0.8:1 (Fig. 1B). An interesting trend was observed whereby polymer and insulin concentrations seem to have an impact on the resulting complexes despite using similar mass ratios. For example, at the same polymer to insulin mass ratio of 2:1, $Pa_{2.5}$ gave rise to a clear formulation after complexation with stock insulin of 0.5 mg mL^{-1} (Fig. 1A) while at a higher stock insulin concentration (2 mg mL^{-1}), the appearance of the formulation was translucent (Fig. 1B). This phenomenon might be due to the formation of different types of complexes, which was confirmed later by the TEM images (Fig. 2).

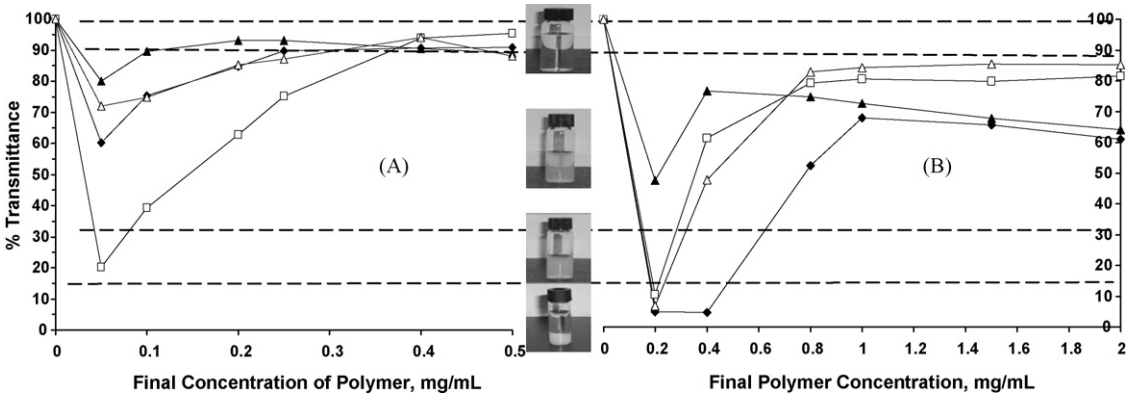

Fig. 1. (A) Transmittance (%) of polymer, insulin complexes produced using 0.5 mg mL^{-1} insulin stock. (B) Transmittance (%) of polymer, insulin complexes produced using 2 mg mL^{-1} insulin stock. (♦) $Pa_{2.5}$; (▲) Pa_5; (□) QPa_5; (△) QPa_5.

Table 1
Properties of PAA amphiphiles, self-assemblies and complexes at polymer:insulin ratio (2:1 mg mL^{-1}) in pH 7.4 Tris buffer (n = 3; mean ±S.D.).

Polymer	Monomer:palmitoyl molar ratio	Grafting, %[a]	Quaternisation, %[a]	IC_{50}, mg mL^{-1}[b]	Hydrodynamic diameter, nm[c]		Zeta potential, mV		Complexation efficiency at polymer:insulin ratio (2:1 mg mL^{-1})
					Before[d]	After[d]	Before[d]	After[d]	
$Pa_{2.5}$	1:0.025	4.2 ± 4.4	n/a	0.021 (2)	185 ± 1 (0.26)	123 ± 1 (0.25)	37 ± 1	58 ± 7	78 ± 8
$QPa_{2.5}$	1:0.025	4.2	73.0 ± 2.0	0.114 (11)	221 ± 26 (0.40)	118 ± 1 (0.24)	68 ± 1	23 ± 1	83 ± 2
Pa_5	1:0.05	6.6 ± 7.8	n/a	0.025 (3)	165 ± 0 (0.43)	210 ± 15 (0.3)	44 ± 2	66 ± 10	78 ± 8
QPa_5	1:0.05	6.6	65.0	0.145 (15)	151 ± 8 (0.49)	105 ± 0 (0.23)	55 ± 3	27 ± 1	92 ± 10

[a] Number of palmitoyl groups and quaternary ammonium moieties per 100 monomer units determined by elemental analysis.
[b] The bracket values indicate the number of folds higher than the IC_{50} of PAA (0.01 mg mL^{-1}).
[c] The bracket values indicate the polydispersity index (PDI) of the dispersion.
[d] Polymer self-assembly characteristics before and after complexation.

3.2.2. Particle size analysis

The hydrodynamic size of all complexes exceeded 1 μm at lower polymer concentrations and declined with increasing polymer concentration (Tables 1 and 2). This phenomenon correlated well with the transmittance data demonstrating the formation of stable, compact complexes at higher polymer concentrations. At polymer to insulin mass ratio of 2:1, all polymers were able to spontaneously form complexes in the range of 100–200 nm. Pa_5–insulin complexes behaved differently whereby the particle size remained below 300 nm at all polymer:insulin ratios with a narrow size distribution indicated by a low polydispersity index (PDI) value (Table 2). This again was in agreement with the transmittance result which indicated that all Pa_5 complexes had relatively high transmittance values at all polymer concentrations. It is reported that self-assembled polyelectrolytes were able to interact with proteins via electrostatic interaction and hydrophobic association (Simon et al., 2004; Mao et al., 2006). Therefore, an increase in the hydrophobic load of Pa_5 would probably result in stronger hydrophobic association with insulin and thus more defined and compact nano-complexes were formed even at low polymer concentrations.

3.2.3. DSC

Polymer, freeze-dried complexes and insulin thermal profiles are displayed in Fig. 3A and B. Both Pa_5 and QPa_5 insulin complexes exhibited different thermal behaviour from that of the free insulin and polymers. On dissolution these polyelectrolytes lose any order in their structure present in their solid state and form self-assemblies upon aggregation of palmitoyl pendant groups in aqueous medium. When these polymers are then freeze dried it allows for a restoration of order to the system. However the presence of insulin would appear to affect the reordering of the polymer. In the case of QPa_5 complexes there is an extra endothermic peak at 132 °C which cannot be assigned at this time. The DSC data reveals that there are interactions between the polymer and insulin which altered the thermal properties of the polymer. It also shows that the nature of these interactions appear to differ between the quaternised and non-quaternised polymers.

Altering polymer and insulin concentrations also shown to have an effect on both T_m and apparent decomposition of the freeze-dried complexes (Fig. 3C and D). Increasing the polymer:insulin concentrations from 0.002:0.001 to 2:1 mg mL^{-1} caused the suppression of an endotherm at 100 °C, leaving only a single melting

Table 2
Hydrodynamic size (nm) of polymer, insulin complexes with various polymer to insulin mass ratios using insulin stock solution of 0.5 mg mL^{-1} or 2 mg mL^{-1} (n = 3; mean ±SD). The brackets indicate polydispersity index (PDI) of samples.

Polymer:insulin	Insulin stock 0.5 mg mL^{-1}				Insulin stock 2 mg mL^{-1}			
	$Pa_{2.5}$	Pa_5	$QPa_{2.5}$	QPa_5	$Pa_{2.5}$	Pa_5	$QPa_{2.5}$	QPa_5
0.2:1	682 ± 14 (0.69)	291 ± 2 (0.3)	>1 μm	>1 μm	>1 μm	219 ± 20 (0.35)	>1 μm	>1 μm
0.4:1	387 ± 5 (0.91)	188 ± 2 (0.23)	>1 μm	280 ± 3 (0.30)	>1 μm	125 ± 2 (0.20)	177 ± 7 (0.20)	196 ± 6 (0.36)
0.8:1	190 ± 0.3 (0.38)	144 ± 1 (0.23)	>1 μm	249 ± 4 (0.31)	149 ± 3 (0.17)	165 ± 26 (0.32)	111 ± 2 (0.16)	112 ± 0 (0.17)
1:1	165 ± 0.6 (0.27)	151 ± 2 (0.24)	560 ± 25 (0.56)	247 ± 3 (0.35)	110 ± 10 (0.21)	161 ± 5 (0.26)	109 ± 2 (0.17)	108 ± 1 (0.18)
1.5:1	149 ± 1 (0.26)	180 ± 1 (0.33)	157 ± 2 (0.33)	160 ± 4 (0.28)	113 ± 7 (0.24)	209 ± 27 (0.30)	110 ± 1 (0.22)	105 ± 0 (0.20)
2:1	159 ± 1 (0.29)	176 ± 2 (0.24)	102 ± 1 (0.26)	161 ± 2 (0.32)	123 ± 1 (0.25)	210 ± 15 (0.30)	118 ± 1 (0.24)	105 ± 0 (0.23)

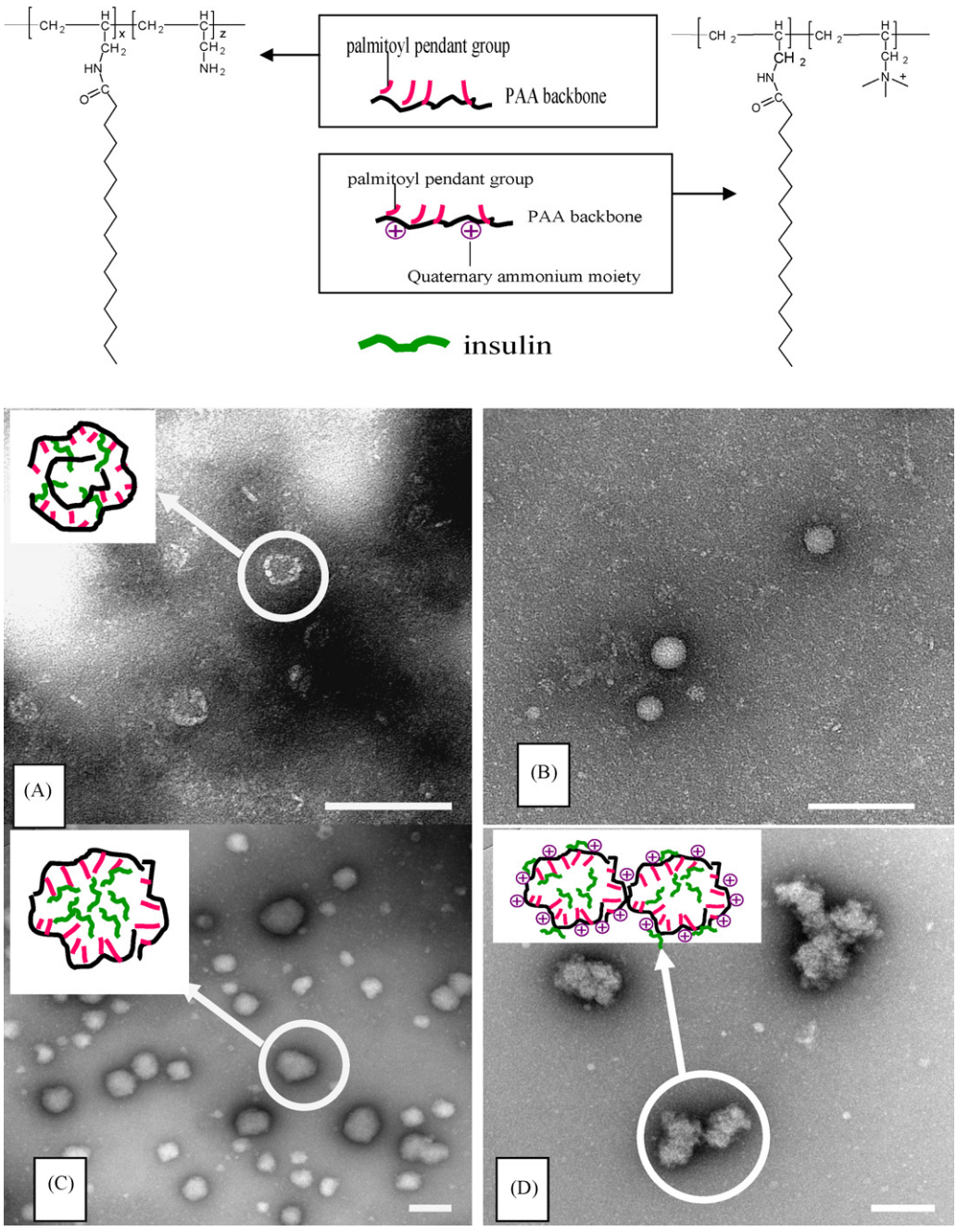

Fig. 2. TEM images of polymer, insulin complexes in pH 7.4 Tris buffer. Scale bar = 200 nm (A) $Pa_{2.5}$ complexes of polymer to insulin ratio 0.5:0.25 mg mL^{-1}; (B) $QPa_{2.5}$ complexes of polymer to insulin ratio 0.5:0.25 mg mL^{-1}; (C) $Pa_{2.5}$ complexes of polymer to insulin ratio 2:1 mg mL^{-1}; and (D) $QPa_{2.5}$ complexes of polymer to insulin ratio 2:1 mg mL^{-1}.

peak at 130 °C at 2:1 mg mL^{-1}. In addition, increasing concentrations also resulted in the disappearance of the shoulder peak at 90 °C. This would suggest that there is a difference in polymer–insulin interaction depending upon the concentrations used. The ratio at 0.002:0.001 mg mL^{-1} is below the polymer critical aggregate concentration (CAC) of 0.08 mg mL^{-1} (Thompson et al., 2008) and hence any interactions present are likely between single polymer chains and insulin. The sample at 0.2:0.1 mg mL^{-1} is above the polymer CAC and we hypothesise that the interactions possibly involve insulin interacting with multiple chains within a particle core. Once the concentrations reached 2:1 mg mL^{-1} the interactions may be stronger and changes in complex morphology over a range of polymer:insulin ratios would seem to confirm this (see Section 3.2.5). It is possible that the interaction between polymers and insulin resulted in the conformational change of insulin and hence altering the thermal profiles observed (Kang et al., 2002). For quaternised polymer QPa5, similar trend was observed where there is a gradual suppression of the endotherms between 90 and 100 °C with the increase of polymer:insulin concentrations, and at 2:1 mg mL^{-1} only one endotherm remaining at 132 °C. This again may be due to differences in interaction (and particle morphology) as the concentrations are increased. In the case of both Pa_5 and QPa_5 the physical mixtures with insulin give different thermal profiles than the freeze-dried complexes (Fig. 3C and D). This would indicate that the interactions between polymer and insulin differ depending on whether the samples are physical mixture or molecular interactions and also the concentrations of polymer and insulin used to form complexes.

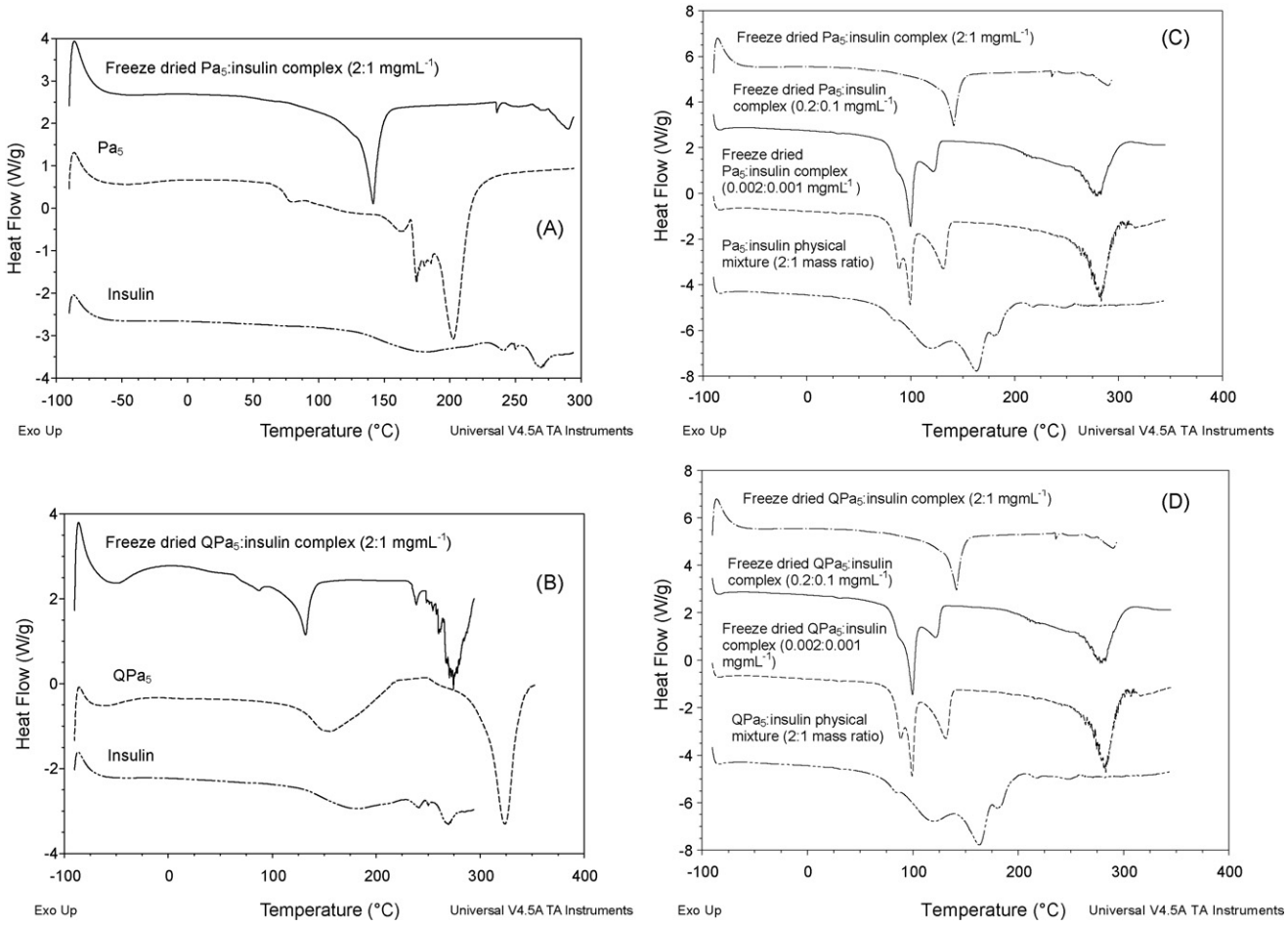

Fig. 3. DSC profile of (A) Pa_5, insulin and freeze dried Pa_5:insulin complex (2:1 mg mL^{-1}). Pa_5 —; Pa_5:insulin freeze dried 2:1 mg mL^{-1} -·-·; insulin - - -. (B) QPa_5, insulin and freeze dried QPa_5:insulin complex (2:1 mg mL^{-1}). QPa_5 —; QPa_5:insulin freeze dried 2:1 -·-·; insulin - - -. (C) Freeze dried Pa_5:insulin complex at 2:1 mg mL^{-1} -·-·; complex at 0.2:0.1 mg mL^{-1} —; Complex at 0.002:0.001 mg mL^{-1} - - -; physical mixture at 2:1 mass ratio —. (D) Freeze dried QPa_5:insulin complex at 2:1 mg mL^{-1} -·-·; complex at 0.2:0.1 mg mL^{-1} —; complex at 0.002:0.001 mg mL^{-1} - - -; physical mixture at 2:1 mass ratio —.

3.2.4. Complexation efficiency (CE)

The determination of CE commonly involves centrifugation to separate polymer, insulin complexes from free insulin in the supernatant (Simon et al., 2004; Mao et al., 2006; Jintapattanakit et al., 2007; Simon et al., 2007). We conducted a series of experiments on the supernatant after centrifugation at 13,000 rpm for 30 min to determine the changes in the zeta potential, hydrodynamic size and the PDI. It was expected that the supernatant should consist of free insulin and possibly polymeric self-assemblies alone. Therefore, two distinct size populations accompanying with a high PDI was predicted. Since the zeta potential of insulin is −22 mV in pH 7.4 Tris buffer while the polymeric self-assemblies have a positive zeta potential (Table 1), two peaks of positive and negative zeta potential should be observed in the supernatant. However, the zeta potential of the supernatant after centrifugation remained positive.

It is similar to the zeta potential of quaternised polymer, insulin complexes as made while there was only a marginal reduction of zeta potential in non-quaternised polymers (Fig. 4A). In addition, the zeta potential graph indicated that there was one distinct peak at positive value demonstrating complexes were present in the supernatant after centrifugation (Fig. 4B). A sharp fall in the hydrodynamic size and PDI in the supernatant suggests that this change was due to the separation of two or more populations of complexes of different sizes rather than the separation of complexes and free insulin (Fig. 4C and D). This correlates to the change of the translucent solution to a clear solution after centrifugation, with the corresponding reduction of insulin concentration in the supernatant (data not shown). The determination of pellet characteristics was not possible due to the centrifugation force resulting in the caking of colloidal complexes. Therefore, based on these observations, CE of complexes was determined without centrifugation as it did not separate free insulin from complexed insulin.

Non-quaternised polymers ($Pa_{2.5}$ and Pa_5) exhibited similar CE of 78% while quaternised polymers, $QPa_{2.5}$ and QPa_5 had higher CE of 83% and 92%, respectively (Table 1). It was reported that hydrophobic and electrostatic interactions are the two most important parameters governing polymer/protein association in water (Simon et al., 2004). Hence, the high CE observed in quaternised polymers indicated that the addition of quaternary ammonium moieties enhanced the electrostatic interaction between insulin and the polymers and thus resulted in a higher CE.

3.2.5. TEM

The negatively stained TEM images of $Pa_{2.5}$–insulin complexes at 2:1 polymer:insulin mass ratios indicate differing morphologies at high and low polymer and insulin concentrations (Fig. 2). Interestingly at 0.5:0.25 mg mL^{-1}, $Pa_{2.5}$:insulin complexes appeared to form vesicular structures consisting of lipid bilayer (resists staining) and stained interior (aqueous core) (Fig. 2A). At higher polymer:insulin concentrations (2:1 mg mL^{-1}) they form solid dense nanoparticles (Fig. 2C). This phenomenon is not well understood. Previous work showed that the critical aggregation concentration (CAC) for $Pa_{2.5}$ is 0.18 mg mL^{-1} and it formed nanoparticles at 1 mg mL^{-1} (Simon et al., 2004). Therefore, it was expected that

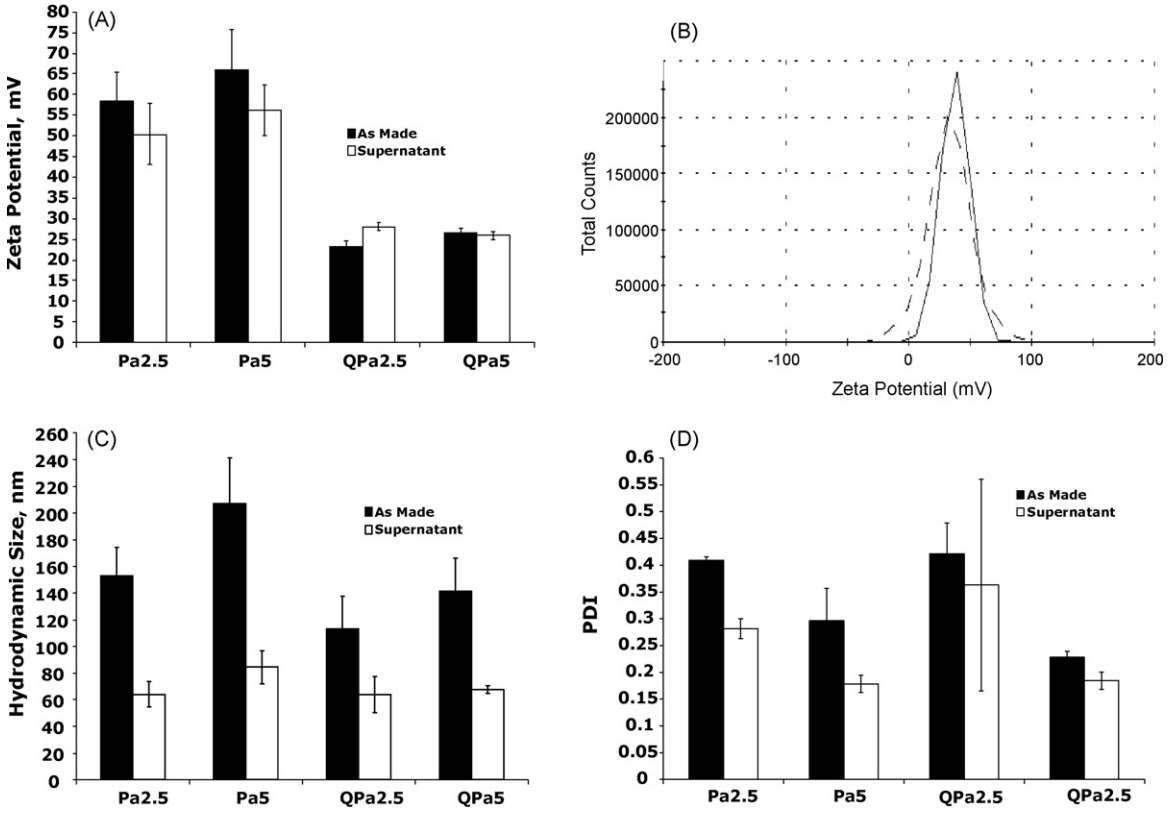

Fig. 4. Characteristics of polymer, insulin complexes as made and after centrifugation ($n = 3$; ±S.D.) (A) Zeta potential (mV); (B) Zeta potential distribution (mV) of $Pa_{2.5}$ complexes with a polymer to insulin ratio of 2:1 mg mL^{-1}. As made —; supernatant - - -; (C) hydrodynamic size (nm); and (D) polydispersity index (PDI).

at 0.5 mg mL^{-1}, $Pa_{2.5}$ would form nanoparticles as well. The difference in morphology was possibly due to the integration of insulin between palmitoyl chains and thus increases the fluidity of polymer chains and the formation of bilayer vesicles. The same trend was observed when cholesterol, a membrane stabilising agent, was incorporated into amphiphilic polymers (Wang et al., 2004a). Unlike cholesterol, 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 et al., 1990; Chang et al., 2003). It is possible that the aliphatic side chains increased the hydrophobic interaction of palmitoyl chains while the polar residues of the insulin were contained within the aqueous core.

The solid dense nanoparticles observed at higher polymer and insulin concentrations were possibly due to the presence of insulin within the hydrophobic core. It was reported that the charge on the interior of the micelle may be displaced to the surface by the presence of hydrophobic moieties (Mao et al., 2005). We have demonstrated in our previous work that addition of hydrophobic grafts increased the zeta potential of PAA (Thompson et al., 2008). The same trend was observed when insulin was added as evidenced by the higher positive zeta potential values (Table 1). This indicates the presence of insulin in the core forcing the amino groups on the PAA backbone out from the hydrophobic domains, which resulted in a higher zeta potential value.

Using bovine serum albumin (BSA) as a model protein, Wittemann reported part of BSA is encapsulated within the interior of the vesicles formed by biamphiphilic triblock copolymer poly(ethylene oxide)-*b*-poly(caprolactone)-*b*-poly(acrylic acid) while a further fraction of the protein is located on the surface of the vesicles (Wittemann et al., 2007). However, association with bovine serum albumin did not alter the morphology of the aggregates. On the other hand, increasing the hydrophobic loads of acetylated polyethylenimine (PEI) resulted in the formation of different self-assemblies such as micelles, vesicles and nanoparticles (Wang et al., 2004a). However, to our knowledge, the association of a protein resulting in the change of polymeric self-assemblies morphology is seldom reported. At low polymer and insulin concentrations, $QPa_{2.5}$ complexes formed spherical nanoparticles while at higher concentrations, they had a 'fluffy appearance' and appear to be aggregated (Fig. 2B and D). The electrostatic interaction of the permanently charged quaternary ammonium moieties with the negatively charged insulin at pH 7.4 led to more than 50% reduction of zeta potential values of quaternised polymers (Table 1). This would suggest the presence of insulin on the surface of $QPa_{2.5}$ complexes resulting in the different morphologies to the non-quaternised counterpart (Fig. 2C).

We are aware that these systems are complex and the interaction between insulin and the polymers might be more complicated as illustrated in our schematic diagrams. It is possible that the complexation involves a network of polymers and insulin which requires further investigation to confirm our hypothesis.

3.3. Cytotoxicity assay

The cytotoxicity of amphiphiles and PAA were assessed using IC_{50} values which determine the polymer concentration required to reduce the cell survival by 50%. Overall, all modified polymers have higher IC_{50} values compared to the unmodified PAA after 24 h incubation with Caco-2 cells, indicating they are less cytotoxic than the parent polymer (Table 1). Palmitoylation of PAA has reduced the cytotoxicity up to 2–3-fold. Interestingly, an opposite trend was observed by Thomas and colleagues who showed that cytotoxicity of polycations such as PEI was enhanced after the addition of alkyl chains (Thomas and Klibanov, 2002). Compared to PAA, the presence of quaternary ammonium moieties has further improved the

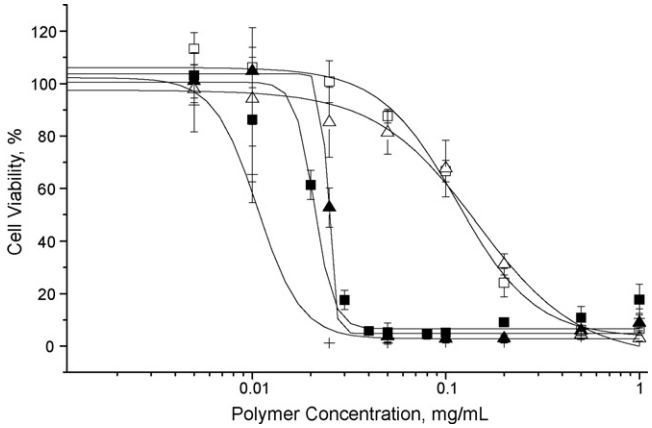

Fig. 5. Caco-2 cell viability (%) after 24 h incubation with PAA and PAA amphiphiles as measured by MTT assay ($n = 3$; ±S.D.). (+) PAA; (■) $Pa_{2.5}$; (▲) Pa_5; (□) QPa_5; (△) QPa_5.

cell viability, with the increase of the IC_{50} by 11-fold and 15-fold, respectively, for $QPa_{2.5}$ and QPa_5 (Table 1 and Fig. 5). It is thought that the cytotoxicity of polycations is related closely to the structure of charge groups and the charge density (Hill et al., 1999; Fischer et al., 2003). Primary amines are known to have the highest cytotoxicity among other charge groups (Wang et al., 2004b). Therefore, conjugation of hydrophobic moieties to the PAA backbone reduced the number of primary amino groups which could interact with the negatively charged membrane. Consequently, the reduction of the positive charge density in the modified polymers increased the cell viability. Conversion of primary amines to quaternary ammonium moieties reduces the overall basicity of the polymer, making the quaternised polymer less cytotoxic (Hill et al., 1999; Wang et al., 2004b). The lower cytotoxicity of PAA amphiphiles, especially $QPa_{2.5}$ and QPa_5 indicates the suitability of these biomaterials as delivery systems for proteins.

3.4. Enzymatic degradation studies

3.4.1. Trypsin

Fig. 6A demonstrates about 24% of free insulin was degraded after 4 h while all complexes were able to protect insulin from trypsin degradation ($p < 0.001$). Trypsin cleaves insulin at two sites, the carboxyl side of residues B29-Lys and B22-Arg (Schilling and Mitra, 1991). These bonds are relatively exposed at the carboxyl terminus of B chains in the native three-dimensional structure of insulin. Simon et al. (2007) reported that these two sites are both in the hydrophobic segment of B chain and therefore hydrophobic association with amphiphilic polyesters resulted in the reduction of insulin degradation after 1 h exposure (Simon et al., 2007). It was also reported that electrostatic interaction of trimethyl chitosan with insulin provides protection against trypsin activity. As a result, the complexes formed are more efficient in protecting insulin from trypsin enzymatic degradation than the conventional chitosan nanoparticles (Jintapattanakit et al., 2007). The tight binding between the positively charged chitosan and negatively charged insulin limits trypsin access to its target sites (Jintapattanakit et al., 2007). It is therefore thought that both the hydrophobic and electrostatic interactions between insulin with PAA amphiphiles restricted trypsin from accessing its target sites and hence resulted in a reduction of insulin degradation.

The degree of protection seems to depend on the polymer architecture. $QPa_{2.5}$ and QPa_5 complexes had a higher level of non-degraded insulin after 4 h than non-quaternised complexes. This was unexpected since insulin is thought to be present on the surface of complexes and therefore would lead to a higher degree of insulin degradation. It may be that quaternary ammonium moieties have inhibitory effects on trypsin which resulted in the observed

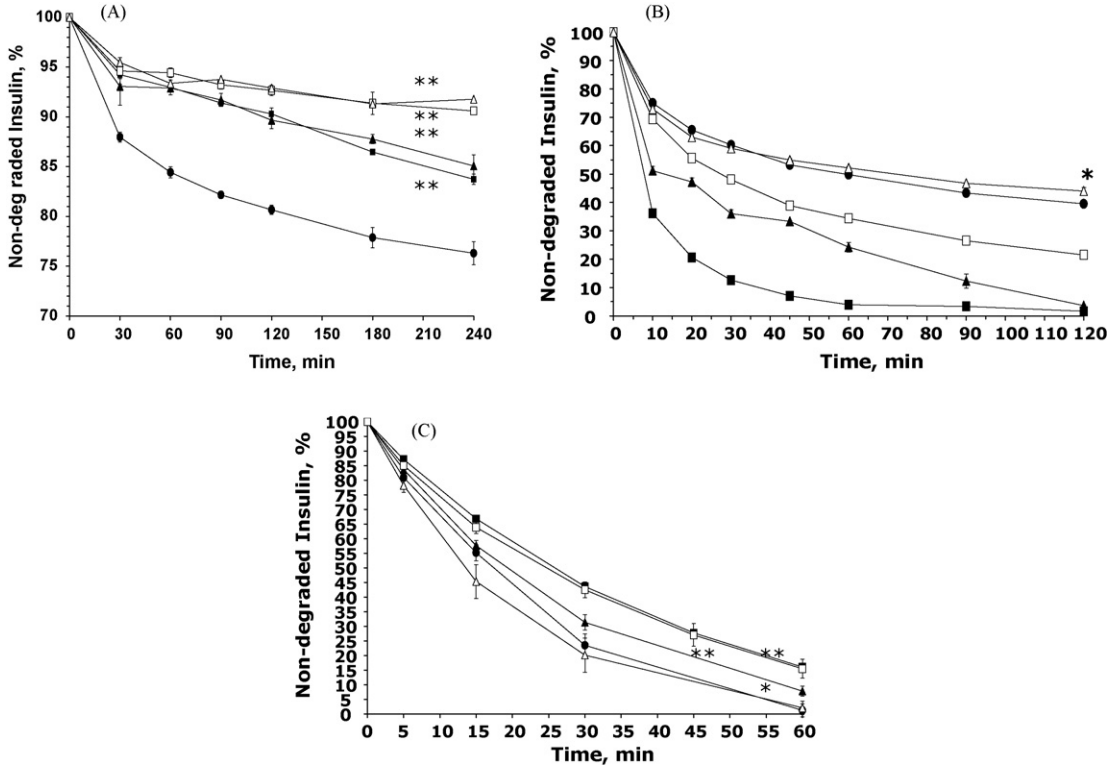

Fig. 6. Degradation profiles of free insulin and complexes of polymer to insulin ratio 2:1 mg mL^{-1} after incubation with (A) trypsin; and (B) α-chymotrypsin; (C) pepsin ($n = 3$; ±S.D.). (●) Free insulin; (■) $Pa_{2.5}$; (▲) Pa_5; (□) $QPa_{2.5}$; (△) QPa_5; $^{**}p < 0.001$, $^{*}p < 0.05$ compared to free insulin.

phenomenon although more investigations have to be conducted to confirm this hypothesis.

3.4.2. α-Chymotrypsin

α-Chymotrypsin has five initial cleavage sites the bonds of which are all exposed on the surface of insulin molecules. On long-term incubation, the less accessible bonds (B15-Leu and A11-Cys residues) in the hydrophobic core of the insulin molecule will be hydrolysed (Schilling and Mitra, 1991). Its effect is much more rapid than trypsin as it destroys 100% of free insulin within an hour whereas trypsin degrades less than 10% of insulin when both are incubated in equimolar quantities (Schilling and Mitra, 1991). An unusual trend was observed when non-quaternised polymers $Pa_{2.5}$ and Pa_5 were incubated with chymotrypsin. There was a significant insulin degradation compared to free insulin demonstrating $Pa_{2.5}$ and Pa_5 complexation did not offer any protection but in fact enhanced the degradation process. This phenomenon was possibly due to the unfolding of insulin to allow the hydrophobic association of the aliphatic chains in the hydrophobic core of insulin with palmitoyl chains. Indeed the degradation peak profile seen during HPLC analysis indicated that the degradation products differed in the presence on $Pa_{2.5}$ and Pa_5 when compared to insulin alone or in the presence of $QPa_{2.5}$ and QPa_5 (data not shown). An extra peak appeared initially on the chromatograph which contributed to a much more rapid degradation seen in Fig. 6B. This peak could be attributed to cleavage of two target sites in the hydrophobic segment of insulin which are normally protected when insulin is in its folded state (Schilling and Mitra, 1991).

The dynamic nature of the polymer aggregates may also have an effect whereby the unimer exchange between the aggregates and bulk may actually facilitate chymotrypsin activity. As these aggregates would not exist as solid particles the chymotrypsin may then have an opportunity to attack the exposed target sites of insulin. To date, there is limited data on the effect of polymeric self-assemblies against α-chymotrypsin degradation of insulin since most work focus on the effect of these systems on trypsin activities only (Jintapattanakit et al., 2007; Simon et al., 2007).

From Fig. 6B, after 2 h of incubation, $QPa_{2.5}$ did not protect insulin but accelerated insulin degradation although not as much as the non-quaternised polymers. The degradation profile of QPa_5 is similar to free insulin but interestingly after 1 h of incubation, the rate of insulin degradation slowed down where the level of non-degraded insulin was statistically significant higher than free insulin at 2 h ($p < 0.05$). Given that insulin appeared to be on the surface of quaternised polymer aggregates it is probable that they do not prevent cleavage at the five hydrophilic target sites as with insulin in its native state. However if they do not cause insulin molecules to unfold to a greater extent, it is possible that the two hydrophobic target sites remained protected.

3.4.3. Pepsin

Unlike trypsin and α-chymotrypsin, pepsin in the gastric juice attacks all peptide bonds in insulin and hence is very destructive even at very low concentrations (Calceti et al., 2004). Therefore it is thought that the amphiphiles would not able to protect insulin against pepsin activities based on the phenomenon observed in the α-chymotrypsin degradation study. Interestingly, from the degradation profile, $Pa_{2.5}$ and $QPa_{2.5}$ were able to protect insulin from pepsin degradation and slowed down insulin degradation ($p < 0.001$) and, to a lesser extent ($p < 0.05$), Pa_5 also exhibit protective effect against pepsin enzymatic degradation of insulin (Fig. 6C). This phenomenon is not well understood. It could be due to the working pH of the enzymes whereby the optimum pH for trypsin and α-chymotrypsin is pH 8 while pepsin is pH 2. It is probable that protonation of primary amines in PAA of non-quaternised amphiphiles resulted in a stronger electrostatic interaction with insulin and hence a tighter binding restricted the access of pepsin to its target sites. We observed that on addition of concentrated HCl, all complexes solutions became clear regardless of translucent appearance at pH 7.4. However, measurement of size, PDI and zeta potential indicated that there was little difference in any of their values at low pH (data not shown). This shows that complexes are stable in acidic environment but their stability in the stomach is not known since the presence of food, bile salts and other typical stomach contents might have an impact as well. The demonstration of any protection against pepsin activity is crucial if these systems are to be used orally. Again very few workers have tested similar systems with pepsin. Indeed, as with α-chymotrypsin, this may be the first time these types of polymeric aggregates have been tested against pepsin activity on insulin. To date, most studies involved conjugation of insulin to a carrier (Calceti et al., 2004) or entrapment of insulin in a solid particle (Zhenqing et al., 2004; Trotta et al., 2005). These studies showed the ability of these systems to protect insulin at varying degrees. It was reported that the protective effect via encapsulation of insulin in nano/microparticles was achieved by having a solid, physical barrier between the bulk solution and the insulin, which made enzyme penetration very difficult (Zhenqing et al., 2004; Trotta et al., 2005).

4. Conclusion

This work showed that these novel comb shaped amphiphilic polymers were able to spontaneously form nano-complexes with insulin in pH 7.4 buffer at room temperature. This mild fabrication process offers an alternative formulation to micro/nanoparticles, which the conventional encapsulation technique might cause protein degradation. The study revealed that polymer architecture, polymer and insulin concentrations have a major impact on the formation and morphology of polymer, insulin complexes. Overall these polymers have been shown to produce nano-complexes with high complexation efficiency and were able to protect insulin from enzymatic degradation to differing degrees. Trypsin, α-chymotrypsin and pepsin are major proteolytic enzymes in the GIT and therefore, it is crucial that the delivery system should offer protection against these enzymes for efficacious insulin delivery via the oral route. From the data, no one polymer was able to provide protection against all three enzymes. The protection capability is dependant on polymer architecture and the type of enzyme. The exact mechanism(s) for how polymers' structural differences control the level of protection requires further study. However quaternised polymers appear to offer the widest range of protection. Future work will determine the ability of these polymers to facilitate paracellular transport across Caco-2 cell monolayers and the *in vivo* efficacy as oral delivery systems for insulin.

Acknowledgement

The authors would like to thank The Cunningham Trust for the financial support of this project.

References

Bromberg, L., 2008. Polymeric micelles in oral chemotherapy. J. Control. Release 128 (2), 99–112.
Calceti, P., Salmaso, S., Walker, G., Bernkop-Schnurch, A., 2004. Development and in vivo evaluation of an oral insulin–PEG delivery system. Eur. J. Pharm. Sci. 22, 315–323.
Chang, S.G., Choi, K.D., Jang, S.H., Shin, H.C., 2003. Role of disulfide bonds in the structure and activity of human insulin. Mol. Cells 16, 323–330.
Cheng, W.P., Gray, A.I., Tetley, L., Hang, T.L.B., Schatzlein, A.G., Uchegbu, I.F., 2006. Polyelectrolyte nanoparticles with high drug loading enhance the oral uptake of hydrophobic compounds. Biomacromolecules 7, 1509–1520.
Cui, F., Shi, K., Zhang, L., Tao, A., Kawashima, Y., 2006. Biodegradable nanoparticles loaded with insulin–phospholipid complex for oral delivery: preparation, in vitro characterization and in vivo evaluation. J. Control. Release 114, 242–250.

Fan, Y.F., Wang, Y.N., Fan, Y.G., Ma, J.B., 2006. Preparation of insulin nanoparticles and their encapsulation with biodegradable polyelectrolytes via the layer-by-layer adsorption. Int. J. Pharm. 324, 158–167.
Fischer, D., Li, Y.X., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and haemolysis. Biomaterials 24, 1121–1131.
Gaucher, G., Dufresne, M.-H., Sant, V.P., Kang, N., Maysinger, D., Leroux, J.-C., 2005. Block copolymer micelles: preparation, characterization and application in drug delivery. J. Control. Release 109, 169–188.
Hamman, J.H., Enslin, G.M., Kotze, A.F., 2005. Oral delivery of peptide drugs—barriers and developments. Biodrugs 19 (3), 165–177.
Hill, I.R.C., Garnett, M.C., Bignotti, F., Davis, S.S., 1999. In vitro cytotoxicity of poly(amidoamin)s: relevance to DNA delivery. Biochim. Biophys. Acta 1427, 161–174.
Jintapattanakit, A., Junyaprasert, V.B., Mao, S., Sitterberg, J., Bakowsky, U., Kissel, T., 2007. Peroral delivery of insulin using chitosan derivatives: a comparative study of polyelectrolyte nanocomplexes and nanoparticles. Int. J. Pharm. 342 (1–2), 240–249.
Jung, T., Kamm, W., Breitenbach, A., Kaiserling, E., Xiao, J.X., Kissel, T., 2000. Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? Eur. J. Pharm. Biopharm. 50, 147–160.
Kang, F., Jiang, G., Hinderliter, A., DeLuca, P.P., Singh, J., 2002. Lysozyme stability in primary emulsion for PLGA microscphere preparation: effect of recovery methods and stabilizing excipients. Pharm. Res. 19, 629–633.
Lambkin, I., Pinilla, C., 2002. Targeting approaches to oral drug delivery. Exp. Op. Bio. Ther. 2 (1), 67–73.
Mahkam, M., Allahverdipoor, M., Mohammadi, R., Ranaei-Siadat, S.O., Rashidi, M.R., Davaran, S., Barshan, M., Ranaei-Siadat, S.E., 2006. An oral delivery system for insulin. J. Bioact. Compat. Polym. 21, 135–148.
Manosroi, A., Blume, A., Manosroi, J., Bauer, K.H., 1990. Thermodynamic characteristics of a human insulin–DEAE–dextran complex entrapped in liposomes. Drug Dev. Ind. Pharm. 16, 837–854.
Mao, S., Bakowsky, U., Jintapattanakit, A., Kissel, T., 2006. Self-assembled polyelectrolyte nanocomplexes between chitosan derivatives and insulin. J. Pharm. Sci. 95, 1035–1048.
Mao, S., Shuai, X., Unger, F., Wittmar, M., Xie, X., Kissel, T., 2005. Synthesis, characterization and cytotoxicity of poly(ethylene glycol)–graft–trimethyl chitosan block copolymers. Biomaterials 26, 6343–6356.
Qian, F., Cui, F., Ding, J., Tang, C., Yin, C., 2006. Chitosan graft copolymer nanoparticles for oral protein drug delivery: preparation and characterization. Biomacromolecules 7, 2722–2727.
Saffran, M., Pansky, B., Budd, G.C., Williams, F.E., 1997. Insulin and the gastrointestinal tract. J. Control. Release 46, 89–98.
Schilling, R.J., Mitra, A.K., 1991. Degradation of insulin by trypsin and alpha-chymotrypsin. Pharm. Res. 8 (6), 721–727.
Shen, W.-C., 2003. Oral peptide and protein delivery: unfulfilled promises? Drug Discov. Today 8 (14), 607–608.
Simon, M., Behrens, I., Dailey, L.A., Wittmar, M., Kissel, T., 2007. Nano-sized insulin-complexes based on biodegradable amine-modified graft polyesters poly[(vinyl-3-(diethylamino)-propylcarbamate-co-(vinyl acetate)-co-(vinyl alcohol)]–graft–poly(L-lactic acid): protection against enzymatic degradation, interaction with Caco-2 cell monolayers, peptide transport and cytotoxicity. Eur. J. Pharm. Biopharm. 66 (2), 165–172.
Simon, M., Wittmar, M., Bakowsky, U., Kissel, T., 2004. Self-assembling nanocomplexes from insulin and water-soluble branched polyesters, poly[(vinyl-3-(diethylamino)-propylcarbamate-co-(vinyl acetate)-co-(vinyl alcohol)]–graft–poly(L-lactic acid): a novel carrier for transmucosal delivery of peptides. Bioconj. Chem. 15, 841–849.
Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., Rudzinski, W.E., 2001. Biodegradable polymeric nanoparticles as drug delivery devices. J. Control. Release 70, 1–20.
Thomas, M., Klibanov, A.M., 2002. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 99, 14640–14645.
Thompson, C.J., Ding, C.X., Qu, X., Yang, Z., Uchegbu, I.F., Tetley, L., Cheng, W.P., 2008. The effect of polymer architecture on the nano self-assemblies based on novel comb-shaped amphiphilic poly(allylamine). Coll. Polym. Sci. 286 (13), 1511–1526.
Trotta, M., Cavalli, R., Carlotti, M.E., Battaglia, L., Debernardi, F., 2005. Solid lipid micro-particles carrying insulin formed by solvent-in-water emulsion–diffusion technique. Int. J. Pharm. 288, 281–288.
Wang, J., Gao, S.J., Zhang, P.C., Wang, S., Mao, H.Q., Leong, K.W., 2004b. Polyphosphoramide gene carriers: effect of charge group on gene transfer efficiency. Gene Ther. 11, 1001–1010.
Wang, W., Qu, X., Gray, A.I., Tetley, L., Uchegbu, I.F., 2004a. Self-assembly of cetyl linear polyethylenimine to give micelles, vesicles, and dense nanoparticles. Macromolecules 37, 9114–9122.
Wang, W., Tetley, L., Uchegbu, I.F., 2001. The level of hydrophobic substitution and the molecular weight of amphiphilic poly-L-lysine-based polymers strongly affects their assembly into polymeric bilayer vesicles. J. Colloid Interf. Sci. 237, 200–207.
Wittemann, A., Azzam, T., Eisenberg, A., 2007. Biocompatible polymer vesicles from biamphiphilic triblock copolymers and their interaction with bovine serum albumin. Langmuir 23, 2224–2230.
Zhenqing, H., Zhenxi, Z., Chuanxin, Z., Mei, H., 2004. Use of plant exudates (Sanguis Draxonis) for sustained oral insulin delivery with dramatic reduction of glycemic effects in diabetic rats. J. Control. Release 97, 467–475.