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Interactions among proteins and hydrophobically modified polyelectrolytes

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Abstract

A special class of hydrophobically modified polyelectrolytes was studied wherein poly(acrylic acid) (PAA) was conjugated with Pluronic F127 NF surfactant. The Pluronic-PAA copolymer solutions form gels at low concentrations when exposed to body temperature. Such gels possess enhanced retention in topical applications. Circular dichroism spectra indicate that tertiary structures of human insulin, haemoglobin, and albumin were stabilized in solutions of Pluronic-PAA. Aggregation of insulin in gelled solutions of Pluronic-PAA was impeded as demonstrated in shaking tests. The presence of Pluronic-PAA hindered the insulin degradation by α-chymotrypsin by at least 7-fold. Extraction of calcium ions from trypsin by Pluronic-PAA led to the dramatic changes in the tertiary structure and total loss of enzymatic activity, suggesting that Pluronic-PAA could inhibit tryptic degradation of proteins.

Introduction

Poly(acrylic acid) (PAA) and its cross-linked derivatives can be considered as safe drug carriers, as they are not absorbed through the mucosa and thus are devoid of systemic side effects, when applied in topical drug delivery (Junginger et al 2000). In addition, PAA and its hydrogels are mucoadhesive and show enhanced retention in body cavities (Felt et al 1999). Finally, since PAAs act as adsorption and penetration enhancers they have been suggested for use as facilitators of the paracellular permeation of the hydrophilic peptides (Junginger et al 2000). We have recently discovered a new class of PAA-based copolymers and gels, whereby PAA is modified with a triblock copolymer of poly(ethylene oxide) (PEO) and poly-(propylene oxide) (PPO) (Pluronic, or Poloxamer), which is attached to PAA via carbon–carbon bond (Bromberg 1998a–e; Bromberg & Ron 1998; Bromberg & Barr 1999; Bromberg & Magner 1999; Bromberg & Salvati 1999; Bromberg & Temchenko 1999; Bromberg 2001a, b). The copolymers of PAA and Pluronics (termed Pluronic-PAA herein) possess properties beneficial for topical delivery that complement mucoadhesion of PAA, such as the ability to self-assemble in aqueous solutions forming micelle-like aggregates (Bromberg 1998c, d; Huibers et al 1999). The Pluronic-PAA micelles are capable of solubilizing hydrophobic drugs in aqueous solutions (Bromberg & Temchenko 1999). The copolymers are muco-adhesive (Bromberg 1999), non-irritating (Bromberg & Ron 1998), and enhance bioavailability of drugs (Bromberg 2001a). In this study, we concentrated on Pluronic-PAA as protein formulation aids. It appears that when Pluronic-PAA and model protein solution forms a gel at 37°C, the protein aggregation is impeded, while the tertiary structure of human proteins such as insulin, haemoglobin and

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serum albumin, is not altered. On the other hand, Pluronic-PAA does alter the structure of trypsin by extracting Ca^{2+} (that stabilizes the protein) and diminishes its enzymatic activity. This finding suggests that proteins may be advantageously stabilized against tryptic digestion when formulated with Pluronic-PAA.

Materials and Methods

Materials

Human crystalline insulin (95–98 % by HPLC), human haemoglobin (lyophilized), human albumin (globulin and fatty acid free, ~99 %), trypsin type XIII from bovine pancreas (TPCK treated, essentially salt-free), and α-chymotrypsinogen from bovine pancreas (essentially salt-free, crystallized and lyophilized) were all purchased from Sigma (St. Louis, MO). Pluronic F127 NF was obtained from BASF Corp. (Parsippany, NJ) and used without further treatment. All other chemicals were obtained from commercial sources and were of the highest purity available. Poly(ethylene oxide)-*b*-poly-(propylene oxide)-*b*-(polyethylene oxide)-*g*-poly(acrylic acid) (CAS #186810-81-1) was synthesized by dispersion/emulsion polymerization of acrylic acid along with simultaneous grafting of poly(acrylic acid) onto Pluronic backbone as described in detail elsewhere (Bromberg 1998b). After synthesis, the polymer was purified by dialysis against 10 mM potassium phosphate buffer (pH 7.0) and lyophilized. In this work, we refer to the ensuing copolymer as Pluronic-PAA. The residual concentration of monomers in Pluronic-PAA was below 5 ppm. The polymer had a weight-average molecular mass of 3.5×10^6 Da and consisted of 45 % Pluronic F127 and 55 % poly(acrylic acid) as measured by FTIR and NMR.

Procedures

Insulin was lyophilized from 10 mM phosphate buffer (pH 7.4) and stored at −70°C prior to use. Colloidal stability of insulin was ascertained by a shaking test, whereby insulin was dissolved either in phosphate-buffered saline (PBS, pH 7.4) with 0.01 % sodium azide or in 0.5 or 1 w/v% Pluronic-PAA solutions of Pluronic-PAA in PBS, resulting in 10 μM insulin concentration. The fibrillation kinetics were studied in 5 mL glass HPLC vials (3 mL filling volume) using a horizontal shaker at 37°C and 100 strokes min^{-1}. The vials were intermittently withdrawn and their contents filtered by either a syringe-fitted membrane filter (pore size 0.2 μm) or centrifugation at 37°C using Micropure Filtration Device (pore size 0.45 μm) (Millipore Co., Bedford, MA). Filtered samples were analysed for residual insulin concentration by UV spectrometry using an extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} at 276 nm (Hinds et al 2000) on a Shimadzu 1601 UV-vis spectrophotometer. Sample concentrations were determined by comparison with standard curves in the same PBS buffer. In circular dichroism studies, the insulin-containing stock samples were prepared in PBS buffer (38 mM, pH 7.4) at 0.1 mM and filtered through an Acrodisc 0.2 μm syringe filter. The samples were diluted by PBS buffer or Pluronic-PAA solution in the same PBS buffer to result in 5 μM insulin and 0.5 w/v% Pluronic-PAA concentrations. Enzymatic degradation of insulin by α-chymotrypsin was studied as described by Akiyoshi et al (1998). Namely, a 1 mg mL^{-1} insulin solution in either 38 mM PBS (pH 7.4) or 1 w/v% Pluronic-PAA in the same buffer was equilibrated at 37°C for 1 h and a 1 mL aliquot was added to 1 mL of a 70 μg mL^{-1} α-chymotrypsin solution in the same buffer. The mixture was shaken at 37°C at 100 strokes min^{-1} and at a specified time subjected to the insulin degradation assay using the ninhydrin method (Hovgaard et al 1992). After the completion of the ninhydrin reaction, the solution electronic absorbance was measured at 570 nm and compared with that of the standard glycine solutions. The enzymatic degradation was monitored by the relative increase in the concentration of primary amino groups liberated by the α-chymotrypsinogen cleavage: degradation, $\% = 100 \times (C_t/C_o - 1)$. Herein, C_o and C_t are the total peptide concentrations immediately after (~30 s) addition of α-chymotrypsin and at time t, respectively.

Haemoglobin and albumin were lyophilized from 0.1 M potassium phosphate buffer (pH 7.0) and stored at −70°C prior to use.

Trypsin was lyophilized from 50 mM phosphate buffer (pH 6.8) and stored at −70°C prior to use. Trypsin was dissolved either in 0.5 w/v% of Pluronic-PAA solution in 50 mM phosphate buffer (pH 6.8) or in 50 mM phosphate buffer (pH 6.8) at 1 mg mL^{-1} concentration (Lueßen et al 1995). The solution of trypsin in buffer was stored at −20°C and thawed immediately prior to the CD measurement. The trypsin in Pluronic-PAA solution was kept at 37°C for 0.5 h prior to measurement. The depletion of Ca^{2+} from trypsin as a result of interaction with Pluronic-PAA was ascertained by separation of trypsin and Pluronic-PAA using dialysis. The solution of trypsin (control) or trypsin and Pluronic-PAA was dialysed against calcium-free 50 mM phosphate buffer at 4°C using Spectra/Por cellulose ester membrane (MW cut-off 50 kDa, Spectrum). The buffer

solution was lyophilized and the residual solids were analysed for calcium content using a Spectroflame ICP Model FMD-07 inductively coupled plasma atomic emission spectrometer (Spectro Inc., Littleton, MA). Enzymatic activity of trypsin in solution and in the presence of 1 w/v% Pluronic-PAA was measured as described in detail elsewhere (Lueßen et al 1995) using N-α-benzoyl-L-arginine ethylester (Sigma) as a substrate.

The CD spectra were recorded, without noise reduction, in the 180–250 nm range in quartz cuvettes with a 1 mm light path length (JASCO 165-01) with a thermostatted cell holder incorporating a Peltier device (JASCO PTC-343) using a JASCO J-720 Spectropolarimeter (JASCO Inc., Easton, MD). Ten scans were averaged before the final spectrum was acquired. The data for insulin (ellipticity in mdeg) were transformed to mean residue ellipticity (Θ) using the expression (Goldman & Carpenter 1974) $\Theta = \Theta_o M/C\lambda$, where Θ_o is the observed ellipticity (mdeg), M is the mean residue molecular weight (g mol^{-1}), λ is the optical path length (cm), and C is the protein concentration (g mL^{-1}).

Rheological measurements were performed using a controlled stress Rheolyst Series AR1000 Rheometer (TA Instruments, New Castle, DE) with a cone and plate geometry system (cone: diameter, 4 cm; angle, 2°, truncation, 57 μm) equipped with a solvent trap. The zero shear viscosity (η_o) of the Pluronic-PAA solutions was estimated from the creep experiments according to (Bromberg & Barr 2000):

$$\gamma(t)/\sigma_0 = J_e^0 + t/\eta_0$$

where t is the time, $\gamma(t)$ is the shear strain, σ_o is the shear stress and J_e^0 is the steady state shear compliance.

Results and Discussion

Pluronic-PAA in aqueous solutions shows a tendency to self-assemble via formation of micelle-like aggregates. Such aggregates can dramatically change rheological properties of the solution (Bromberg 1998d, e). The effect of temperature on the property of 1 w/v% Pluronic-PAA solution to flow under shear is illustrated in Figure 1. The sol–gel transition is reflected in the steady shear (creep) experiments. The Pluronic-PAA solution pre-equilibrated at 20°C was about 20-fold more compliant (capable of flowing) than at 30°C and recovered to a much higher degree upon cessation of stress. Further increases in temperature up to 45°C lead to still more rigid gels, as the lesser compliance shows. The gelation of Pluronic-PAA is also reflected in the increase of the complex viscosity in oscillatory shear experiments above certain critical gelation thresholds (Figure 2). Note that Pluronic-PAA solutions are gels at 37°C, at which temperature the polymer–protein interactions are studied.

In order to characterize the effects of Pluronic-PAA, if any, on the tertiary structure of proteins, circular dichroism spectroscopy was employed (Figure 3).

Notably, while the presence of Pluronic-PAA did not induce any significant changes in insulin, haemoglobin,

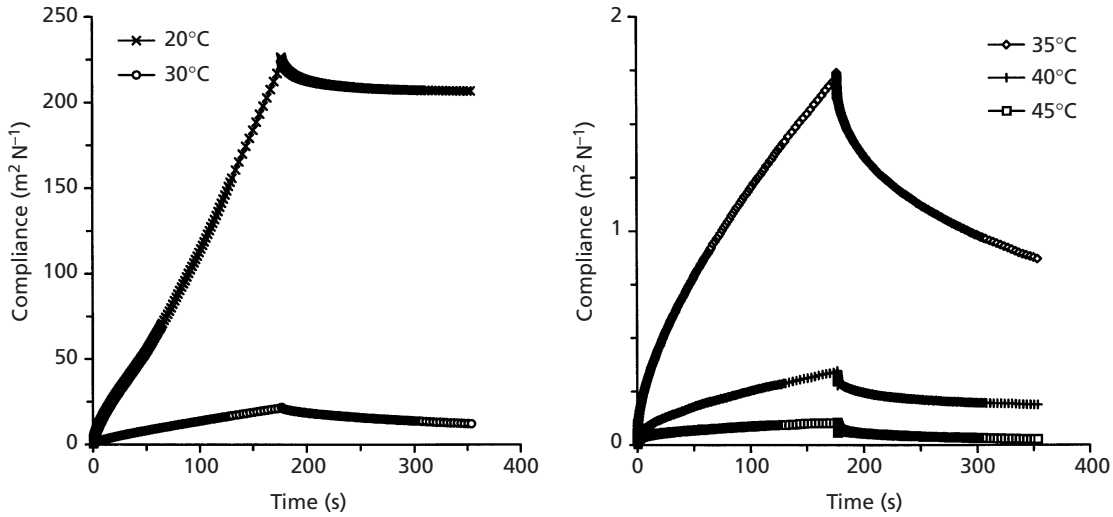

Figure 1 Retardation and recovery of 1 w/v% Pluronic-PAA solution in 10 mM phosphate buffer/0.01% sodium azide (pH 7.4) at different temperatures. Applied stress 0.2 Pa.

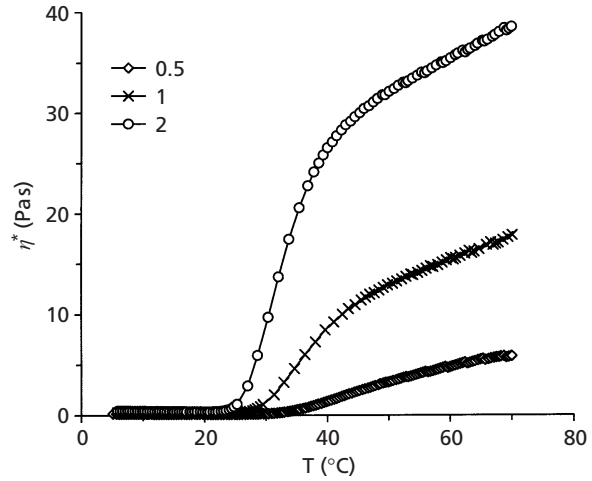

Figure 2 Complex viscosity (η^*) of Pluronic-PAA solution in 10 mM phosphate buffer/0.01 % sodium azide (pH 7.4) as a function of temperature. Numbers designate polymer concentration in w/v%. Oscillatory stress 0.6 Pa, temperature ramp 3°C/min, angular frequency 6.28 rad/s.

and albumin, very pronounced changes were observed in trypsin, analogous to the ones observed upon autodegradation (Lueßen et al 1995). It is interesting to observe that Pluronic-PAA and all proteins under study, except for trypsin, had a negative net charge (isoelectric point of insulin, albumin and haemoglobin is 4.7–5.3 (Bromberg & Klibanov 1995), and pI of trypsin is 10.1–10.8 (Tsuboi et al 1996)). Thus the formation of polyelectrolyte complexes between Pluronic-PAA and insulin, albumin, or haemoglobin due to attractive electrostatic interactions can be ruled out. However, hydrophobic interactions must be prevalent. Indeed, Pluronic-PAA interacts strongly with co-ionic surfactants such as alkyl sulfates (Bromberg et al 2000). It has been shown that alkyl chains of surfactant can be incorporated into the micelles of Pluronic-PAA, cores of which consist primarily of poly(propylene oxide) (PPO) (Huibers et al 1999). The micelles form in Pluronic-PAA solutions at temperatures above 20°C (Bromberg 1998a–e) and serve as junctions of physical cross-links bridging

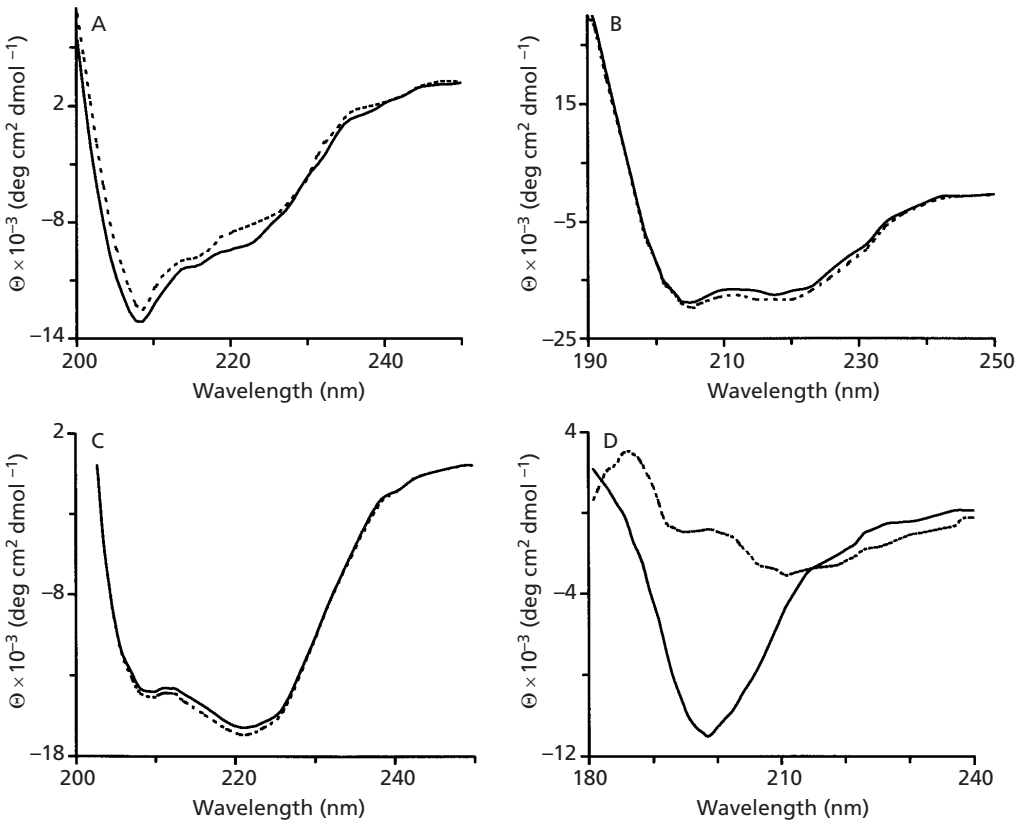

Figure 3 Far-UV circular dichroism spectra of human insulin (A), albumin (B), haemoglobin (C), and bovine trypsin (D) at 37°C. Spectra were measured in buffers (dashed line) or in 0.5 w/v% Pluronic-PAA solutions in the same buffers (solid line). Concentrations of the proteins were 5 μM (A), 10 μM (B), 2 μM (C) and 42 μM (D). Buffers were PBS (38 mM, pH 7.4) (A), 10 mM potassium phosphate (pH 7.0) (B,C), and 50 mM phosphate (pH 6.8) (D).

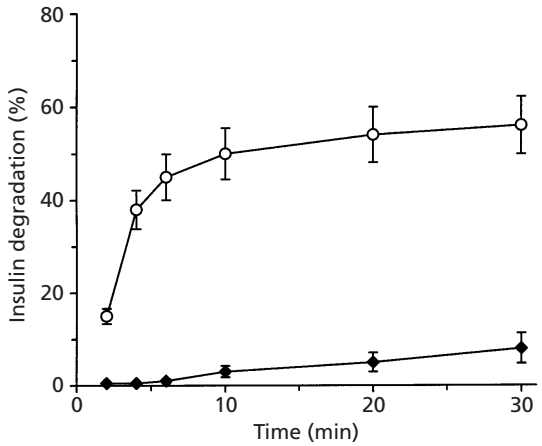

Figure 4 Kinetics of insulin degradation at 37°C in the presence of α-chymotrypsin with (diamonds) and without (circles) Pluronic-PAA. For experimental detail, see text.

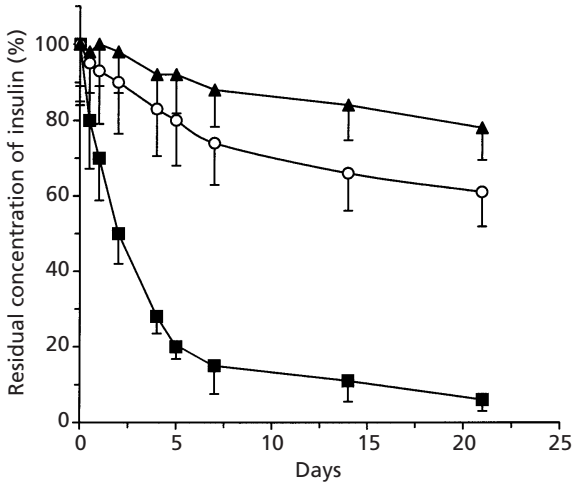

Figure 5 Kinetics of aggregation of insulin at 37°C as measured in a shaking test. Squares, circles, and triangles designate insulin in a buffer solution, in 0.5 w/v% Pluronic-PAA solution, and 1 w/v% Pluronic-PAA solution, respectively. See experimental section for further detail.

polymer domains thus creating gels. However, as surfactant molecules complex with PPO segments, the Pluronic-PAA gels weaken, indicating that the formation of the intermolecular associations, which form cross-links, is hindered (Bromberg et al 2000). Analogous effects can be expected upon addition of insulin (the most hydrophobic out of the proteins studied) to Pluronic-PAA solutions. Indeed, addition of insulin at 5 mg mL^{-1} and 11 mg mL^{-1} lowered the zero-shear viscosity of 1 w/v% Pluronic-PAA measured at 37°C and pH 7.4 by 6 and 12%, respectively. Similarly, hydrophobic interactions between other (anionic) proteins and glycoproteins and PPO segments of Pluronic-PAA have been observed to reduce viscosity of Pluronic-PAA solutions (Bromberg 2001a). CD spectra of insulin in buffer and in the Pluronic-PAA solution were very similar (Figure 3A), indicating comparable α-helix and β-sheet contents. The magnitudes of the negative minima at 208 and 223 nm (α-helix and β-sheet, respectively) reflect upon self-association of insulin. The far-ultraviolet CD-band at 208 nm is essentially due to α-helices formed from residues between B10–B19, A2–A6, and A13–19. Structure is the primary component of the far ultraviolet CD-band at 223 nm, and the ratio between the intensities ($\Theta_{208}/\Theta_{223}$) is a qualitative measure of the amount of insulin self-association (Pocker & Biswas 1980). Although both CD-bands (208 and 223 nm) decrease in intensity with decreased concentration/association, the ratio $\Theta_{208}/\Theta_{223}$ increases. This is because an antiparallel β-structure is formed when two monomers dimerize resulting in an increase of Θ_{223} without an increase of Θ_{208} (Hinds et al 2000). In a very dilute solution (0.1 μM), almost all insulin exists in its monomeric form ($\Theta_{208}/\Theta_{223} = 1.52$), while concentrations of 1–10 μM result in dimers ($\Theta_{208}/\Theta_{223} = 1.43$) (Pocker & Biswas 1980). In our experiments, the $\Theta_{208}/\Theta_{223}$ values of 1.49 and 1.45 were measured in 5 μM solutions of insulin in the buffer and 0.5 w/v% Pluronic-PAA, respectively. Note that at 0.5 w/v% and 37°C Pluronic-PAA forms micellar aggregates in aqueous solutions (Bromberg 1998c, d). Therefore, it can be concluded that the presence of Pluronic-PAA does not alter the overall conformation (tertiary structure) of insulin. Figure 4 shows the kinetics of insulin degradation in the presence of α-chymotrypsin with and without Pluronic-PAA. As is seen, the presence of Pluronic-PAA hinders the insulin degradation by at least 7-fold.

These observations are indicative of the formation of mixed Pluronic-PAA-protein micelles with proteins embedded inside the PPO-rich cores. Such an arrangement would enhance stability of the proteins, analogously to stabilization observed in micellar solutions of low-molecular weight surfactants (Constantino et al 1997), and decelerate enzymatic degradation of the proteins.

The ability of Pluronic-PAA to prevent insulin from aggregation was estimated in the shaking test by assaying the amount of residual insulin after filtration (Figure 5).

Without Pluronic-PAA, the insulin self-aggregates via nucleation (formation of active centres), growth, and precipitation (Constantino et al 1997). Without the polymeric additive, the precipitates are removed by

filtration, so that over 95% of the initial insulin is precipitated (Figure 5). In contrast, Pluronic-PAA prevents the insulin precipitation, lowering the protein removal to about 40 and 20% after 3 weeks with 0.5 and 1 w/v% Pluronic-PAA, respectively. This effect can be attributed to the gelling and surface activity of the Pluronic-PAA. Viscosification of the solution lowers the convectional flows and protein diffusion in Pluronic-PAA solutions (Ho et al 2001), thus reducing the frequency of encounters of the protein molecules necessary for aggregation. Furthermore, since Pluronic-PAA is surface active it occupies the hydrophobic air–water interface (Bromberg et al 2000). This prevents insulin from aggregating on the interface and hinders the aggregation. Similar effects on insulin aggregation have been reported with Pluronics, but a higher concentration of Pluronic is required to stabilize insulin (Chawla et al 1985; Barichello et al 1999).

Interaction of the trypsin with Pluronic-PAA caused the appearance of the minimum around 199 nm (Figure 3D), indicating changes in the tertiary structure of the enzyme. Analogous changes have been observed upon interaction of trypsin with lightly cross-linked poly(acrylic acid) (Noveon AA1 and Carbopol 934P from B. F. Goodrich company) (Lueßen et al 1995). It has been shown that the changes in the trypsin structure occur due to the extraction of Ca^{2+} ions, which play an important role in maintaining the thermodynamic stability of the enzyme (Delaage et al 1967). Extraction of calcium ions from trypsin by 1 w/v% Pluronic-PAA at 37°C leads to the total loss of the enzymatic activity, in agreement with the results with Carbopol and Noveon polymers (Lueßen et al 1995). In our experiments, a 1.7-fold depletion of Ca^{2+} from trypsin due to interaction with 0.5 w/v% Pluronic-PAA was observed, in agreement with the depletion of Ca^{2+} by polyacrylates. These results suggest that if a Pluronic-PAA and peptide formulation were to be administered orally, the peptide could be protected from the tryptic degradation.

In summary, in this communication we report on interactions of Pluronic-PAA copolymers with proteins that appear to be favourable from the drug delivery and formulation standpoint. Tertiary structure of human insulin, albumin, and haemoglobin is stabilized by such interactions, while trypsin is degraded and tryptic activity is suppressed.

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