Poly(amidoamine)s synthesis, characterisation and interaction with BSA
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Cationic poly(amidoamine)s (PAAs) were synthesised and characterised by NMR and gel permeation chromatography. Their thermal properties were investigated using thermogravimetric analysis and differential scanning calorimetry. Although poly(amidoamine)s have been used as endosomolytic polymers for protein intracellular delivery, the interaction of the polymers with the proteins still need to be investigated. BSA was used as a model protein and complexation with the different poly(amidoamine)s was investigated using gel retardation assays, fluorescence spectroscopy and high sensitivity differential scanning calorimetry. Our results indicate that the thermal stability of BSA was affected upon interaction and complexation with the poly(amidoamine), however these interactions did not seem to modify the structure of the protein. Polymer flexibility seemed to favour polymer/protein complexation and promoted thermal stability.

Introduction

Intracellular delivery of protein is often ineffective. Cellular uptake can be limited due to charge repulsion and cytosolic delivery is generally not efficient due to proteolysis in the lysosomes.⁴ Although many proteins have been used as macromolecular drugs, development of therapeutic proteins has been essentially limited to proteins with an extracellular site of action.⁵ However, there are potential therapeutic applications for proteins with intracellular biological activity and different approaches have been used to promote cytosolic delivery.¹¹ including polymers such as poly(amidoamine)s (PAAs).⁴ The complexes formed from self-assembly of the biomacromolecules with the polymers have generally an overall cationic charge and are able to bind to the cell surface. Alternatively, the carrier will target a specific receptor on the cell membrane and internalisation will be done via receptor mediated endocytosis (RME). Once internalised, via RME or simple pinocytosis, the carrier will need to promote endosomal/lysosomal escape for efficient cytosolic delivery.¹² PAAs are synthesised via Michael polyaddition using primary amines or secondary diamines and bisacrylamides.⁶ They are bioreponsive polyelectrolytes⁷ and have been used as endosomolytic polymers for DNA and siRNA delivery.⁸,⁹ Most poly(amidoamine)s are less cytotoxic in comparison to other polycations and some amphoteric PAAs have displayed invivo stealth properties.¹⁰,¹¹ Recent studies have demonstrated that modulation of the PAAs hydrophobicity influenced binding of the polymer to DNA.¹²,¹³ Although several studies have reported intracellular delivery of proteins with PAAs,¹⁴–¹⁶ there is no report investigating the interaction of the poly(amidoamine)s with proteins. Polyelectrolyte-protein complexation has been studied in the context of protein purification, enzyme immobilisation, sensor development or stimuli responsive systems.¹⁷ Complexation is usually governed by electrostatic interactions and can lead to the formation of soluble complexes, complex coacervates or precipitates.¹⁸ Our aim was to study poly(amidoamine)s-protein complexation. We synthesise and characterise new poly(amidoamine)s and investigated the interaction of the polymers with a model protein, looking at the effect on the thermal stability and structure of the macromolecule.

Experimental section

Materials

Sodium hydroxide, sodium chloride, N,N'-ethylenbisacrylamide, N,N'-bis(acryloyl)pipеразине, 2-methy1piperazine and N,N'-bis[2-hydroxyethyl]ethylenediамине were from Sigma-Aldrich (Gillingham, UK). 4-Amino-1-butanol and 5-amino-1-pentanol were from Alfa Aesar (Heysham, UK). TRIS, hydrochloric acid, HEPES, D-glucose and bovine serum albumin (BSA) were from Fisher (Loughborough, UK). Deuterated water was from Goss scientific (Nantwich, UK). Oregon green 488 was from Life Technologies (Paisley, UK). Agarose was from Roche (Burgess Hill, UK). Disposable cuvettes for zeta potential measurement were from Malvern (Worcestershire, UK). All products were used as received.
Polym. Chem., 2010, 118 000 g mol⁻¹ (Kromekate) were used as narrow standards. Mobile phase was 0.1 M Tris buffer, pH = 8 with 1 M NaCl and the flow rate was 1 mL min⁻¹. Concentration of the samples was 1 mg mL⁻¹. Thermogravimetric analysis was carried out under nitrogen on a Hi-Res TGA 2950 apparatus (TA Instruments) measuring the mass loss on 4 mg samples from 26 °C to 600 °C and at a heating rate of 20 °C min⁻¹. Thermal properties were determined with a DSC822 differential scanning calorimeter (Mettler Toledo). The apparatus was calibrated with indium. 5 mg of polymers were analysed under nitrogen from 0 °C to 200 °C and at a heating rate of 10 °C min⁻¹. Buffer capacities and degree of ionization were determined by acid-base titrations. Polymers (100 mg) were solubilised in NaCl (100 mL, 0.1 M) and pH was adjusted to 2 using 6 M HCl. Titrations were carried out with 0.1 M NaOH. Zeta potentials ([PAA] = 1 mg mL⁻¹ in HEPES 10 mM, pH 7.4, 5 wt% d-glucose) were measured using a Zetasizer ZS nanoseries (Malvern) using DTS 1061 cells.

Synthesis of the polymers

Poly[(N,N'-bis(acryloyl)piperazine)-co-(2-methylpiperazine)-co-(N,N'-bis(2-hydroxyethyl)ethylenediamine)] (ISA1). ISA1 was synthesised as previously described. Under nitrogen, N,N'-bis(acryloyl)piperazine (1) (2.64 g, 13.7 mmol), 2-methylpiperazine (2) (0.7 g, 6.9 mmol) and N,N'-bis(2-hydroxyethyl)ethylenediamine (3) (1.05 g, 6.9 mmol) were added to 10.3 mL deionised water. The mixture was stirred for 5 days at 30 °C. The polymer was then solubilised in ddH₂O (40 mL) and the pH was adjusted to 2 with 6 M HCl. ISA1 was recovered by ultrafiltration (3 kDa MWCO) and freeze-drying (4.20 g, 96%). Poly[(N,N'-ethylenebisacrylamide)-co-(N,N'-bis(acryloyl)piperazine)-co-(4-amino-1-butanol)] (P1). P1 was synthesised using a protocol similar to ISA1. N,N'-ethylenebisacrylamide (4) (0.21 g, 1.25 mmol) and N,N'-bis(acryloyl)piperazine (1) (1.72 g, 8.75 mmol) were solubilised in ddH₂O (4.06 mL). The solution was stirred under nitrogen until a clear solution was obtained. 4-Amino-1-butanol (5) (0.94 mL, 10 mmol) was added. The mixture was stirred for 6 days at 30 °C. ddH₂O (40 mL) was added and the pH was adjusted to 2. P1 was purified by ultrafiltration (3 kDa MWCO) and recovered by freeze-drying (1.56 g, 54%). Poly[(N,N'-ethylenbisacrylamide)-co-(N,N'-bis(acryloyl)piperazine)-co-(4-amino-1-pentanol)] (P2). P2 was synthesised following the same protocol as for P1 but using N,N'-ethylenbisacrylamide (4) (0.21 g, 1.25 mmol), N,N'-bis(acryloyl)piperazine (1) (1.72 g, 8.75 mmol), 5-amino-1-pentanol (6) (1.13 mL, 10 mmol) and 3.87 mL of ddH₂O (2 g, 67%). Poly[(N,N'-ethylenebisacrylamide)-co-(N,N'-bis(acryloyl)piperazine)-co-(4-amino-1-pentanol)] (P2) was synthesised as previously described.

Results and discussion

Polymer synthesis and characterisation

N,N'-bisacryloylpiperazine (1) has been used successfully to synthesise numerous poly(amidoamine)s. To modulate the interactions with BSA

Agarose gel electrophoresis. Samples of PAA : BSA at weight ratios from 0.1 : 1 up to 100 : 1 were prepared. Briefly, 18.7 µL of PAAs (2.4−14 mg mL⁻¹ in 10 mM HEPES, pH = 7.4, 5 wt% d-glucose) were added to a solution of BSAOG : BSA (1 : 2 (v/v)). The mixtures were left at room temperature for 30 min and 21.5 µL of HEPES was added. Samples were applied onto a 0.7% (w/v) agarose gel. Electrophoresis was run at 60 V for 45 min. BSAOG was visualised using a G:BOX transilluminator (Syngene). Free protein was used as a control. Oregon green (GO) labelling of BSA (BSAOG) was carried out according to Life Technologies protocol (degree of labelling found: 1.75 mol OG/mol BSA). No free dye was detected following thorough purification by ultracentrifugation (MWCO 5 kDa).

High sensitivity differential scanning calorimetry. Thermal stability of BSA was determined using a nano DSC microcalorimeter (TA Instruments) equipped with capillary cells (300 µL). BSA : PAA samples at ratio 10 : 1 (w/w) were prepared by mixing BSA (400 µL, 2.5 mg mL⁻¹, 10 mmol L⁻¹ HEPES pH = 7.4, 0.15 M NaCl) with PAA solutions (1.6 mL, 6.25 mg mL⁻¹ HEPES pH 7.4, 0.15 M NaCl). Final concentration of BSA was 0.5 mg mL⁻¹. The samples were left at room temperature for 30 min and were degassed for 10 min, at 10 °C and under vacuum (23 mmHg). Stirring was set up at 230 rpm. During the analysis, a pressure of 4 atm was applied over the reference and sample cells. Thermograms were recorded from 10 °C to 100 °C with a scanning rate of 1 °C min⁻¹. Data were analysed using the launch nanoanalyse software from TA Instrument. No thermal transition were detected for the PAAs alone (Fig S1†).

Steady-state fluorescence spectroscopy. Fluorescence measurements were carried out using a Cary Eclipse fluorescence spectrophotometer (Varian). PAA : BSA solutions were prepared as previously described. The excitation wavelength was at 280 nm and emission spectra were recorded from 290 to 500 nm. The slit for the excitation and emission were adjusted to 5 and 2.5 nm, respectively. Fluorescence spectra of the PAAs alone were also recorded to confirm that they did not interfere with the protein fluorescence (Fig. S2†).

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flexibility of the polymers, we synthesised two new PAAs (P1 and P2) using N,N'-ethylenebisacrylamide (4) in combination with 1 (Fig. 1). Two amino alcohol with different chain length (4-amino-1-butanol (5) and 5-amino-1-pentanol (6)) were used to modulate the hydrophobicity of the polymers. Following synthesis by Michael-type polyaddition, the structures of P1 and P2 were confirmed by NMR (Fig. 2).

For P1 and P2, the feed ratio of the bisacrylamides was 12.5 mol% for 4 and 87.5 mol% for 1. Experimental ratios, determined by 1H-NMR via comparison of the integrated intensity of the peak at ~3.6 ppm (1) and peak at ~3.4 ppm (4), correlated well with the feed (Table 1). Although ISA1 has been previously used to deliver gelonin, a non permeant protein, the nature of the physico-chemical interaction with the proteins has never been investigated. Consequently we synthesised ISA 1 as previously described and used it as a reference polymer. The molecular weight of all the PAAs was determined in aqueous environment, relative to poly(ethylene glycol) (PEG) standards (Fig. S3†) (Table 1). Higher molecular weights have been previously reported for ISA1 but these were determined relative to poly(N-vinylpyrrolidone).

Thermal stability of the polymers was evaluated under nitrogen (Fig. S4†). All thermograms displayed an initial mass loss (~5%) corresponding to the desorption of water. Degradation of the polymers started above 200 °C (Table 1). Early studies reported decomposition of PAAs at 140 °C in air and 170–180 °C under inert atmosphere. Analysis of a series of poly(amidoamines) synthesised with α,ω-bis-(methyl-amino)alkanes and N,N'-bis(acryloyl)piperazine or 1,12-bisacryloyl-n-diaminododecane also indicated that the polymers were stable up to 200–220 °C. More recent studies demonstrated that thermal decomposition of PAAs containing N,N'-methylenebisacrylamide and piperazine (Pip–MBA) was also observed around 210 °C. However, analogues of PAAs such as poly(glycoamidoamine)s and Pip-MBA containing N-vinylpyrrolidone have displayed increased thermal stability (Tg > 300 °C). DSC demonstrated that the polymers were semicrystalline with similar melting temperatures (Tm) and endothermic enthalpies (Table 1 and Fig S5†). However, the glass transition temperatures (Tg) of P1 and P2 were below 37 °C and reflected increased chain flexibility in comparison to ISA1. This might facilitate interaction of the polymers with proteins and could suggest the complexation of proteins with P1 and P2 may be facilitated at room temperature.

Poly(amidoamine)s are polyelectrolytes and their ability to interact with proteins will also depends on their acido-basic behaviour. The degree of ionisation (α) and buffer capacity (β) of the PAAs were therefore investigated as a function of pH (Fig. 3). The buffer capacities (β) were determined from the titration curves according to von Harpe (Fig. 3a). P1 displayed the highest maximum buffer capacity (βmax = 0.15 mmol, pH 8) in comparison to P2 (βmax = 0.08 mmol, pH 8.2) and ISA1 (βmax = 0.11 mmol, pH 7.6). In the context of oligonucleotide delivery with cationic polymeric carriers, the “proton sponge effect” has become a standard theory to explain the release of DNA from the endosomes into the cytosol. The hypothesis relies on the buffer capacity of the polymers, inducing osmotic destabilisation of the endosomal membrane, higher buffer capacity corresponding to higher transfection efficiency. However, several studies have demonstrated that this mechanism is not always prevalent. For example, destabilisation of the endosomal membrane and hence cytosolic delivery may occur due to the adsorption of the charged polycations to the inner surface of the endosome or via induced insolubilisation of polymers with controlled hydrophobicity. Under physiological condition (pH 7.4) all the polymers were highly ionised (80% < α < 90%) with full ionisation below pH 6 (Fig. 3b). This corresponded to the sequential protonation of the tertiary amines in the polymer backbone and correlated with the positive values of the zeta potentials (ζ) measured at pH 7.4 (Table 1).

**Interaction with BSA**

Although poly(amidoamine)s have been used to promote intracellular delivery, the interactions between the polymers and the protein and their effect on the protein stability are poorly understood. Protein-polyelectrolyte complexation may result from different types of interactions, including ionic and hydrophobic interactions, hydrogen bonding or coulombic forces.
The poly(amidoamine)s used in this study are all cationic at physiological pH (Table 1 and Fig. 3), hence we decided to use BSA\(^a\) as a model protein. BSA is a 66 kDa protein with an isoelectric point (IEP) around 4.7.\(^b\) At pH 7.4, the protein should be essentially anionic and interactions with the polymers should be favoured.

Evidence of complex formation was first examined using gel retardation assays (Fig. 4). Fluorescently labelled BSA was used in order to avoid detection of the PAAs using classical coomassie staining for protein detection. Samples were prepared at different PAA : BSA weight ratios, no coacervation or precipitation was observed for any of the samples. A decrease of the mobility of BSA was noticed with increasing weight ratios. For P1, strong retardation of BSA was noticed from weight ratio 5 : 1 with no residual protein detectable above 10 : 1. P2 displayed a similar trend. Although very faint bands were observed for ISA1 above 10 : 1, we could still observe some interaction with BSA. Complexation between cationic PAAs and anionic proteins, mostly due to electrostatic interactions, has been observed previously with β-galactosidase (β-gal, IEP = 4.6).\(^b\) However, p(CBA/HIS) was able to fully complex β-gal at weight ratio as low as 1 : 1. This might be due to additional interactions between disulfide bonds from the \(N,N'\)-cystaminebisacrylamide (CBA) or imidazole groups from the histamine (HIS) and the protein.

Microcalorimetry can be used to understand how proteins unfold and how they can be stabilised. The variation of heat capacity recorded results from changes in the hydration of side-chains that were buried in the native state of the protein and that became exposed to the solvent in the denatured state.\(^b\) The maximum temperature of transition \((T_{\text{max}})\) can be used as an indicator of thermostability. Generally, the higher is the \(T_{\text{max}}\) the more stable is the protein. The thermal stability of BSA in different environment has been well characterised by microcalorimetry. \(T_{\text{max}}\) for the native protein depends on factors such as the ionic strength and the pH of the samples\(^c\) and values ranging from 56 °C to 86 °C have been reported.\(^d\) Under our experimental conditions, \(T_{\text{max}}\) was around 66 °C (Fig. 5 and Table 2) with a corresponding unfolding enthalpy of \(\Delta H_{\text{cal}} = 756.78 \text{ kJ mol}^{-1}\). Further analysis assuming an approximation of a two-states model of denaturation\(^\ddagger\) gave a

![Fig. 2 1H-NMR spectrum of the poly(amidoamine).](Image 69x477 to 527x729)

### Table 1 Physico-chemical properties of the poly(amidoamine)

<table>
<thead>
<tr>
<th></th>
<th>EbA(^a) (mol%)</th>
<th>bPip(^a) (mol%)</th>
<th>(M_n)(^b) (g mol(^{-1}))</th>
<th>(M_w)(^b) (g mol(^{-1}))</th>
<th>PDI(^b)</th>
<th>(T_d)(^c) (°C)</th>
<th>(T_g)(^c) (°C)</th>
<th>(T_m)(^c) (°C)</th>
<th>(\Delta H_{\text{cal}})(^c) (J g(^{-1}))</th>
<th>(\zeta)(^c) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>13.9</td>
<td>86.1</td>
<td>10 400</td>
<td>5100</td>
<td>2.0</td>
<td>211.1 ± 3.0</td>
<td>25.9 ± 0.7</td>
<td>190.5 ± 1.2</td>
<td>46.7 ± 0.7</td>
<td>27.6 ± 0.2</td>
</tr>
<tr>
<td>P2</td>
<td>11.7</td>
<td>88.3</td>
<td>5700</td>
<td>3000</td>
<td>1.9</td>
<td>209.2 ± 1.9</td>
<td>23.1 ± 0.7</td>
<td>192.2 ± 0.6</td>
<td>46.1 ± 2.0</td>
<td>26.6 ± 0.4</td>
</tr>
<tr>
<td>ISA1</td>
<td>—</td>
<td>—</td>
<td>16 300</td>
<td>8200</td>
<td>2.0</td>
<td>219.3 ± 8.3</td>
<td>50.2 ± 0.6</td>
<td>207.0 ± 3.1</td>
<td>51.4 ± 0.4</td>
<td>27.6 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) EbA: \(N,N'\)-ethylenbisacrylamide; bPip: \(N,N'\)-bis(acryloyl)piperazine; feed ratio for the bisacrylamides was: 12.5 mol% for EbA and 87.5 mol% for bPip. Reported experimental ratios were determined by \(^1\)H-NMR via comparison of the integrated intensity of bPip peak (3.6 ppm) and EbA peak (3.4 ppm).\(^b\) Determined using PEG standards (2010–118 000 g mol\(^{-1}\)) (Fig. S3); \(M_n\): weight average molecular weight; \(M_w\): number average molecular weight; PDI: polydispersity index; \(T_d\): degradation temperature; \(T_g\): glass-transition temperature, \(T_m\): melting temperature, \(\Delta H_{\text{cal}}\): melting enthalpy, \(\zeta\): zeta potential.\(^c\) Mean value of two replicates ± SEM.
value for the van’t Hoff enthalpy of $\Delta H_{\text{vH}} = 376.12 \text{ kJ mol}^{-1}$. As $\Delta H_{\text{vH}}$ was smaller than $\Delta H_{\text{cal}}$ ($\Delta H_{\text{vH}} < \Delta H_{\text{cal}}$) this suggested the existence of one or more intermediates states of significance and implies a non-two-states unfolding process for BSA as reported in the literature.$^{32,34}$ Although conjugation of PEG to proteins such as BSA$^{35}$ or interferon$^{38}$ has improved the thermal stability of the proteins, PEG seemed to be inefficient when used to form non-covalent complexes.$^{39}$ However, other studies have shown that polyelectrolyte-protein complexation can improve the thermal stability of the protein.$^{30,40}$

To determine the effect of BSA complexation with our poly(amidoamine)s, stability of the protein was further investigated at PAA : BSA weight ratio of 10 : 1 (Fig. 4). All the peaks for the complexes were broader (Fig. 5) in comparison to the native protein and indicate inter-molecular interactions.$^{18}$ Furthermore, P1 and P2 BSA complexes displayed a $T_{\text{max}}$ which was 4 °C higher in comparison to BSA alone whereas ISA1 induced a decrease of 6 °C (Table 2). Similarly, $\Delta H_{\text{cal}}$ and $\Delta S_{\text{cal}}$ increased in the presence of P1 and P2 and decreased for ISA1. This suggested that P1 and P2 improved the thermal stability of BSA while ISA1 decreased it.

Tryptophan (Trp) fluorescence of proteins is sensitive to environmental changes such as polarisability$^{41}$ and can be used as an optical probe to analyse the effect of a co-solute on the protein tertiary structure.$^{36}$ Conformational changes of the protein can then be monitored due to changes of fluorescence parameters, such as the maximum emission wavelength ($\lambda_{\text{max}}$)

| Table 2 | Thermodynamic properties$^a$ of BSA in the absence and presence of the PAAs |
|-----------------|-----------------|-----------------|-----------------|
|                | $T_{\text{max}}$ (°C) | $\Delta H_{\text{cal}}$ (kJ mol$^{-1}$) | $\Delta S_{\text{cal}}$ (kJ mol$^{-1}$ K$^{-1}$) |
| BSA            | 65.8 ± 0.9       | 757.9 ± 46.3    | 2.24 ± 0.13     |
| P1–BSA         | 69.8 ± 0.1       | 829.8 ± 48.0    | 2.42 ± 0.14     |
| P2–BSA         | 69.9 ± 0.6       | 836.0 ± 13.2    | 2.44 ± 0.03     |
| ISA1–BSA       | 59.7 ± 0.5       | 258.4 ± 23.0    | 0.77 ± 0.07     |

$^a$ Mean value of two independent replicates ± SEM; $T_{\text{max}}$: temperature of denaturation. $T_{\text{max}}$ was determined as the temperature corresponding to the maximum heat capacity ($C_p$); $\Delta H_{\text{cal}}$: enthalpy; $\Delta S_{\text{cal}}$: entropy.
BSA complexes were prepared at ratio 10 : 1 (w/w). The fluorescence spectra indicated that the tertiary structure of BSA was not affected upon complexation. Although, P2 was more hydrophobic in comparison to P1, due to longer amino alcohol side chains, we did not notice any significant difference between the two polymers. These preliminary results suggested that at pH 7.4, the PAA–BSA complexes were prepared at ratio 10 : 1 (w/w).

and the maximum fluorescence intensity \( (I_{\text{max}}) \). BSA possesses two tryptophan residues. To determine the effect of complexation on the tertiary structure of the protein, the fluorescence of BSA was measured in the absence and presence of the poly(amidoamine)s at the same weight ratio (Fig. 6). The maximum fluorescence intensity of BSA was observed at 348 nm and decreased in presence of all the poly(amidoamine)s which suggests that the microenvironment of the tryptophan residue was modified. This may reflect some structural changes in the protein, however as we did not observe any shift (red or blue) of the maximum emission wavelength these conformational changes are probably not significant and the structure of BSA remains almost unaltered at PAA : BSA 10 : 1 (w/w) ratio.

Conclusion

The effect of the interaction between the synthesised poly(amidoamine)s and bovine serum albumin (BSA) was investigated at PAA : BSA (10 : 1) weight ratio. P1, P2 and ISA1 were able to form water soluble complexes with BSA. Microcalorimetry analysis demonstrated that the thermal stability of BSA was affected upon interaction with the poly(amidoamine)s. The unfolding temperature \( (T_{\text{max}}) \) of BSA increased in the presence of P1 and P2. This may be due to increased flexibility of these poly(amidoamine)s in comparison to ISA1. However, fluorescence spectra indicated that the tertiary structure of BSA was not affected upon complexation. Although, P2 was more hydrophobic in comparison to P1, due to longer amino alcohol side chains, we did not notice any significant difference between the two polymers. These preliminary results suggested that at pH 7.4, the PAA–BSA complexes were prepared at ratio 10 : 1 (w/w).

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