Hydrophilic and hydrophobic copolymers of a polyaspartylhydrazide bearing positive charges as vector for gene therapy

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Abstract

BACKGROUND: The design of polymeric vectors for gene delivery provided with specific properties is one of the most critical aspects for a successful gene therapy. These polymers should be biocompatible as well as able to carry efficiently DNA to target tissues and to transfect it into cells.

RESULTS: The formation of complexes of poly[(α,β-asparthylhydrazide)–poly(ethylene glycol)] and poly[(α,β-asparthylhydrazide)–hexadecylamine] copolymers functionalised with glycidyltrimethylammonium chloride (PAHy–PEG–GTA and PAHy–C16–GTA, respectively) with DNA was studied. The effects of the introduction of hydrophilic (PEG) or hydrophobic (C16) moieties on the chains of PAHy–GTA copolymers, such as the stabilising effect on the DNA structure, were evaluated. In particular, we observed a high DNA protection by PAHy–PEG–GTA copolymers. Degradation studies led us to suppose a particular aqueous conformation of the polyionic complex of PAHy–PEG2000–GTA in which DNA should be internalised into an inner core surrounded by a PEG hydrophilic shell; while no significant protection was detected with PAHy–C16–GTA in which DNA should be disposed on the surface of the complex, freely exposed to DNase II action.

CONCLUSION: The insertion of PEG or C16 chains into the polymeric structure of PAHy–GTA copolymers changes significantly the DNA complexing and protecting ability of the PAHy–GTA copolymers, showing that hydrophilic and hydrophobic side chains can play a crucial role in supramolecular arrangements of interpolyelectrolyte complexes between DNA and PAHy copolymers.

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INTRODUCTION

The development of safe and efficient gene carrier systems is the key for the success of gene therapy. DNA complexation, in fact, is necessary to cross the cell membrane, whereas DNA transfection depends on its dissociation from the complex. Therefore, the possibility to control the formation of gene–carrier complex is an essential step to obtain an efficient transfection.

Cationic polyaminoacids represent the most important class of low toxicity, non-viral gene vectors. Moreover, complexation of DNA with copolymers bearing not only hydrophilic cationic groups but also non-ionic residues (e.g. poly(ethylene glycol) (PEG)) could give complexes with interesting properties.

In fact, interaction of hydrophilic moieties with external aqueous medium can lead to the formation of hydrophilic steric palisade, able to reduce interactions with endogenous molecules and macrophages escaping the reticuloendothelial system.\(^1\)

Recently, cationic copolymers containing PEG have been investigated to reduce problems such as formation of large insoluble aggregates, limited stability in the presence of serum and rapid clearance from the bloodstream following systemic administration.\(^2\) Polyion complexes formed between DNA and positively charged copolymers containing PEG chains are totally water-soluble and are narrowly distributed.\(^3\) Moreover, the shell thickness remains constant as long as the molecular weight of the PEG segment remains constant, suggesting that the polyion complex core is surrounded by a palisade of tethered PEG chains with an appreciable stretched conformation.

Complexation of DNA with copolymers bearing lipophilic moieties could ensure a stronger interaction...
of the complex with cell membrane and increase its ability of transfecting cells.4

An aim of the work presented here was the synthesis of new cationic hydrophilic and hydrophobic polyaspartylhydrazide (PAHy) copolymers by introducing PEG (with a number average molecular weight of 2000 g mol⁻¹, PEG₂₀₀₀) or hexadecylamine (C₁₆) residues on the polymeric side chain of polycationic PAHy copolymers such as PAHy with glycidyltrimethylammonium chloride (GTA)⁵ and to preliminarily evaluate the physicochemical properties of the obtained cationic copolymers and those of their interpolyelectrolyte complexes. PAHy–GTA copolymers have already shown interesting properties as copolymers for gene delivery, as they are of low toxicity, non-haemolytic and devoid of trophism for liver.⁵ PAHy–GTA modified with PEG is expected to have higher water solubility and ability to protect DNA against DNase degradation; while PAHy–GTA modified with C₆ is expected not only to protect DNA against DNase degradation but also to potentially enhance cell permeability of polyionic complexes.

In the work reported here, two polycationic copolymers were synthesised by covalent attachment of GTA on PAHy–PEG₂₀₀₀ and PAHy–C₁₆ copolymers. These copolymers were characterised using ¹H-NMR and Fourier transform infrared (FTIR) spectrophotometry. Gel retardation assays were performed to confirm the ability of PAHy–PEG–GTA and PAHy–C₁₆-GTA derivatives to complex DNA and the results were compared to those for their interpolyelectrolyte complexes. PAHy–GTA copolymers have already shown interesting properties as copolymers for gene delivery, as they are of low toxicity, non-haemolytic and devoid of trophism for liver.⁵ PAHy–GTA modified with PEG is expected to have higher water solubility and ability to protect DNA against DNase degradation; while PAHy–GTA modified with C₆ is expected not only to protect DNA against DNase degradation but also to potentially enhance cell permeability of polyionic complexes.

The PAHy–C₁₆ copolymer was synthesised as reported elsewhere.⁹ Briefly, the reaction of polysuccinimide (PSI) with PEG₂₀₀₀-NH₂ was carried out in dimethylformamide (DMF) solution at 60 °C for 15 h. To a solution of PSI–PEG₂₀₀₀ (4.12 mmol) in 5.4 mL of DMF, a mixture of hydrazine monohydrate/DMF was added dropwise under continuous stirring at 22–26 °C. Then the reaction mixture was kept at room temperature for 4 h. It was then centrifuged and the residue washed several times with acetone; after that it was dissolved in water and purified by exhaustive dialysis using Visking dialysis tubing 18/32” with molecular cut-off of 12 000–14 000. After dialysis the solution was lyophilised; the pure product was obtained in 75% yield (w/w) based on starting material (PSI). The copolymer obtained was characterised using FTIR spectrophotometry and ¹H-NMR analysis.

The FTIR spectrum (KBr) shows bands at (cm⁻¹): 3300, 3210, 3055 (–NH₂,–NH–), 1657 (amide I), 1532 (amide II), 1244 (amide III) and 644 (amide IV) belonging to PAHy and at 953 attributed to ether C–O stretching of PEG.

The ¹H-NMR spectrum of PAHy–PEG₂₀₀₀ (D₂O) reveals peaks at δ = 2.68 (m, 2H, –CO–CH–CH₂–CO–NH–), 3.64 (s, 178 H, –CH₂), 4.64 (m, 1H, –NH–CH(CH₂)₂–O–), 4.64 (m, 1H, –NH–CH(CH₂)₂–O–). The reaction mixture was kept at room temperature for 4 h. The reaction mixture was then centrifuged and the residue washed several times with the same solvent, then once with acetone, and dried under reduced pressure; the product was obtained with a yield of 90–95% (w/w) based on starting material (PSI). The obtained product was dissolved in 5.4 mL of DMF and a mixture of hydrazine monohydrate/DMF (28 mmol of hydrazide in 0.52 mL of DMF) was added dropwise under continuous stirring at 22–26 °C. Then the reaction mixture was precipitated in diethyl ether and the residue washed several times with the same solvent, then once with acetone, and dried under reduced pressure; the product was obtained with a yield of 90–95% (w/w) based on starting material (PSI).

Copolymers of a polyaspartylhydrazide for gene therapy
The FTIR spectrum (KBr) shows bands at (cm⁻¹): 3300, 3210, 3055 (–NH₂, –NH⁻) belonging to PAHy, at 2985 and 2854 attributable to stretching C–H of C₁₆ chains and at 1657 (amide I), 1532 (amide II), 1244 (amide III) and 644 (amide IV) belonging to PAHy.

The ¹H-NMR spectrum of PAHy–C₁₆ (D₂O) reveals peaks at δ = 0.79 (t, 3H, –CH₂–CH₃), 1.19 (m, 28H–CH₂–CH₂–CH₂–), 2.68 (m, 2H–CO–CH₂–CO–NH–), 4.64 (m, 1H–NH–CH(CH(CO)CH₂)).

DNA–polymer interaction: gel retardation assay
All copolymers synthesised were solubilised in a sterile NaCl (0.9%, g (100 mL)⁻¹) at room temperature. Calf thymus DNA solutions were prepared in the same NaCl solution with 0.1 mg mL⁻¹ concentration and stored at +4°C. Complexation was performed in 0.9 wt% sterile saline at room temperature with different weight ratios, and left to stand for 30 min prior to analysis. For the assay an agarose gel (0.5% w/v, g (100 mL)⁻¹) was used containing ethidium bromide (0.25 μg mL⁻¹) in tris-acetate/ethylenediaminetetraacetic acid (TAE) buffer run at 80 V for 1 h; the pattern of banding was visualised by UV trans-illuminator and photographed using a Polaroid land camera (667 film). An amount of 500 ng of DNA/well was loaded into agarose gel with a loading buffer (bromophenol blue 0.25% w/v, g (100 mL)⁻¹), xylene cyanole 0.25% w/v, g (100 mL)⁻¹, glycerol 30 vol.% in water.

DNase II degradation assay
DNase II degradation of calf thymus DNA was assayed as previously described. Calf thymus DNA (100 μg mL⁻¹) and the DNA–polymer complexes were incubated with DNase II (300 units mL⁻¹) in sodium acetate–acetic acid buffer (0.2 mol L⁻¹) at 37°C. Immediately two 0.5 mL samples were taken and used as blanks. At various time intervals up to 1 h, samples were precipitated with 10% (w/v, g (100 mL)⁻¹) perchloric acid (0.5 mL⁻¹). After 20 min at 4°C, the samples were centrifuged at 12 000 × g for 20 min and the absorbance of the supernatant was measured at 260 nm. Results were expressed as percentage control degradation at 60 min.

RESULTS AND DISCUSSION
Synthesis of PAHy–PEG₂₀₀₀ copolymers
The synthesis of PAHy–PEG₂₀₀₀ was performed by a two step procedure (Scheme SI in the supplementary information):

1. Partial aminolysis of PSI by O-(2-aminoethyl)-O'-methylpolyethylene with Mₐ = 2000 g mol⁻¹ to obtain PSI–PEG₂₀₀₀ copolymers.
2. Total aminolysis of PSI–PEG₂₀₀₀ by hydrazine to obtain PAHy–PEG₂₀₀₀ copolymers.
The copolymer so obtained, purified by exhaustive dialysis and lyophilised, was characterised using FTIR spectrophotometry and \(^1\)H-NMR analysis.

The molar degree of derivatisation by PEGs (Table 1) was determined by \(^1\)H-NMR analysis, comparing the integral of peaks corresponding to protons at 3.69 ppm assigned to \((-\text{CH}_2-\text{CH}_2-O-)_n\) belonging to PEG with the integral of the peaks related to protons at 4.64 ppm assigned to \(-\text{NH}-\text{CH}(\text{CO})\text{CH}_2\) belonging to PAHy.

The analysis shows a slight decrease of molecular weight for PAHy–PEG\(_{2000}\) in comparison with reference PAHy. This effect can be attributed to a partial degradation of the polymeric backbone, due possibly to the contemporaneous reaction of PEG\(_{2000}-\text{NH}_2\) on both carboxylic groups on the succinimide ring of PSI.

**Synthesis of PAHy–C\(_{16}\) copolymers**

The synthesis of PAHy–C\(_{16}\) was performed by the following two-step procedure (Scheme SII):

1. Partial aminolysis of PSI by hexadecylamine to obtain PSI–C\(_{16}\) copolymers.
2. Total aminolysis of PSI–C\(_{16}\) by hydrazine to obtain PAHy–C\(_{16}\) copolymers.

The copolymer obtained, purified by exhaustive dialysis and lyophilised, was characterised using FTIR spectrophotometry and \(^1\)H-NMR analysis.

The molar degree of derivatisation by C\(_{16}\) (Table 1) was determined by \(^1\)H-NMR analysis comparing the integral of the peak corresponding to protons at 0.79 ppm assigned to \(-\text{CH}_2-\text{CH}_3\) (or the integral of peaks related to protons at 1.19 ppm assigned to \(-\text{CH}_2-\text{CH}_2-\text{CH}_3-\) ) belonging to linked C\(_{16}\) with the integral of the peaks related to protons at 4.64 ppm assigned to \(-\text{NH}-\text{CH}(\text{CO})\text{CH}_2\) belonging to PAHy.

For PAHy–C\(_{16}\) it was evidenced the same effect on molecular weight recorded for PAHy–PEG\(_{2000}\) in comparison with parent PAHy (see Table 1 in comparison with parent PAHy).

**Synthesis and characterisation of PAHy–PEG\(_{2000}\)-GTA and PAHy–C\(_{16}\)-GTA derivatives**

The synthesis of PAHy–PEG\(_{2000}\)-GTA and PAHy–C\(_{16}\)-GTA copolymers is schematically reported in Schemes SIII and SIV of the supplementary material.

The GTA derivatisation of PAHy–PEG\(_{2000}\) and PAHy–C\(_{16}\) copolymers was performed in DMSO at 20 °C with a reaction time of 24 h and \(X\) (moles of derivatising agent/moles of PAHy repeating units) values of 1.1 and DMAP as catalyst agent using a ratio \(Y\) (moles of DMAP/moles GTA) equal to 1. The purified functionalised polymers were characterised using FTIR and \(^1\)H-NMR analyses.

FTIR spectrophotometry of the copolymers indicated the presence of bands due to the hydroxypropyltrimethylammonium groups in PAHy–PEG\(_{2000}\)-GTA and PAHy–C\(_{16}\)-GTA derivatives which are absent in the starting copolymers. \(^1\)H-NMR confirmed the introduction of hydroxypropyltrimethylammonium groups in the side chain of PAHy–PEG\(_{2000}\) and PAHy–C\(_{16}\) (see experimental section).

The degree of derivatisation (DD) was determined using \(^1\)H-NMR, and was calculated from the following ratio: \(\text{DD} = \frac{\text{linked trimethylammonium residues/polymer repeating units}}{100}\times\). \(\text{DD}\) was calculated by comparing the integral of the peak related to protons at 3.22 ppm assigned to \(-\text{N}^+\text{(CH}_3)_3\) belonging to linked GTA with the integral of the peak related to protons at 4.71 ppm assigned to \(-\text{NH}-\text{CH}(\text{CO})-\text{CH}-\) (belonging to PAHy derivatives). The DD values (Table 2) were expressed as mean values. Each determination was carried out in triplicate and the maximum estimated error was ±3%.

The lower molecular weights of PAHy–PEG\(_{2000}\)-GTA and PAHy–C\(_{16}\)-GTA in comparison with PAHy–PEG\(_{2000}\) and PAHy–C\(_{16}\), respectively, can be attributed to a partial degradation of the polymeric backbone of both copolymers during the reaction with GTA (Table 2).

**Gel retardation assay**

DNA gel retardation has been traditionally used to study interpolyelectrolyte complex (IPEC) formation\(^1\)\(^\text{12}\) and DNA charge neutralisation. Gel retardation examines both the apparent change in molecular weight of the DNA molecule\(^1\)\(^\text{13}\) as well as the neutralisation of the net charge of the DNA molecule as a function of IPEC formation.\(^1\)

In this study we evaluated the interaction between calf thymus DNA and PAHy–PEG\(_{2000}\)-GTA, PAHy–C\(_{16}\)-GTA, PAHy–PEG\(_{2000}\) or PAHy–C\(_{16}\) derivatives observing the shift of DNA migration on agarose gel.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>DD(_GTA) (mol%)</th>
<th>Yield, w/w (%)</th>
<th>(M_w) (g mol(^{-1}))</th>
<th>(M_w/M_n)</th>
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<tr>
<td>PAHy–PEG(_{2000})-GTA</td>
<td>90.8</td>
<td>95</td>
<td>18300</td>
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<tr>
<td>PAHy–C(_{16})-GTA</td>
<td>76.2</td>
<td>67.5</td>
<td>16500</td>
<td>1.5</td>
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</table>

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>DD, PEG (mol%)</th>
<th>DD, C(_{16}) (mol%)</th>
<th>DD, PEG (wt%)</th>
<th>DD, C(_{16}) (wt%)</th>
<th>Yield, w/w (%)</th>
<th>(M_w) (g mol(^{-1}))</th>
<th>(M_w/M_n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAHy–PEG(_{2000})</td>
<td>0.96</td>
<td>–</td>
<td>15.0</td>
<td>–</td>
<td>75</td>
<td>20000</td>
<td>1.3</td>
</tr>
<tr>
<td>PAHy–C(_{16})</td>
<td>–</td>
<td>4.6</td>
<td>–</td>
<td>2.6</td>
<td>65</td>
<td>17500</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Table 1. Degree of derivatisation (DD), yield, weight average molecular weight and polydispersity index (\(M_w/M_n\)) of copolymers obtained**

**Table 2. Degree of derivatisation (DD), yield and molecular parameters of PAHy–PEG\(_{2000}\)-GTA and PAHy–C\(_{16}\)-GTA derivatives**
Copolymers were mixed with calf thymus DNA (1 μg) with specific weight ratios and DNA complexation was carried out in sterile NaCl (0.9 wt%) solution at room temperature for 30 min before analysis. IPEC formation, evaluated by electrophoresis on agarose gel, is indicated by the shift of DNA electrophoresis migration due to neutralisation of its net anionic charge as a consequence of incubation with increasing amounts of polycations.

As expected, PAHy–PEG$_{2000}$ and PAHy–C$_{16}$, as neutral polymers, did not induce retardation at 1:1, 1:10 and 1:100 weight ratios (λ Hind III DNA:copolymer) (Fig. S1 of the supplementary material), while the cationic derivatives (PAHy–PEG$_{2000}$–GTA and PAHy–C$_{16}$–GTA) showed the ability to retard DNA migration as a function of weight ratio used to form complexes. Examination of DNA/polycation weight ratios ranging between 1:0 and 1:10 (Fig. S2) showed that calf thymus DNA retardation is proportional to the amount of PAHy–PEG$_{2000}$–GTA in the complex. This suggests that PAHy–PEG$_{2000}$–GTA forms complexes with DNA neutralising its anionic charges. A complete retardation was achieved at 1:1.25 (DNA:PAHy–PEG$_{2000}$–GTA) weight ratio.

Figure S3 shows the effect of PAHy–C$_{16}$–GTA on DNA electrophoresis. This copolymer shows a complete shift at 1:3.75 weight ratio according to the lower cationic degree of derivatisation in comparison with PAHy–PEG$_{2000}$–GTA.

The efficacy of both copolymers was, in terms of weight ratios, higher with respect to previously studied PAHy–GTA. These results confirm that a high density of positive charge is necessary to obtain a complete DNA complexation; moreover the presence of hydrophilic and/or hydrophobic portions in the polymeric chains can contribute to the complex formation.

**DNApolymers and DNase II activity**

Rapid degradation of plasmid DNA by serum and cytosolic nucleases prevents the successful administration of free plasmid DNA intravenously. Thus, for efficient in vivo delivery, a synthetic vector must stabilise DNA during transport to the target tissues or cells. DNase II was used as a model system to study the ability of PAHy–PEG$_{2000}$–GTA and PAHy–C$_{16}$–GTA to protect DNA. Figure 1 shows that at 1:3 (DNA:copolymer) weight ratio with PAHy as complexing agent 87.65 ± 8.15% degradation occurred after 60 min. An analogous behaviour was observed using PAHy–PEG$_{2000}$ at the same weight ratio (73.21 ± 4.51% degradation). In both cases, since no positive charges are present, DNA protection depends on other physical interactions between copolymers and DNA that are able only to partially protect DNA from enzymatic degradation but that are not sufficient to reduce DNA migration on agarose gel (Fig S1 of the supplementary material).

As far as the two cationic copolymers are concerned, PAHy–C$_{16}$–GTA showed no significantly higher protection (69.3 ± 5.81%) in comparison with starting polymers even though 76% mol/mol of positive charges are present in the side chains. In contrast, PAHy–PEG$_{2000}$–GTA at the same weight ratio (1:3) was able to efficiently protect DNA from enzymatic degradation, probably as a consequence not only of the high positive charge density, but also of the presence of PE chains. The results obtained support the hypothesis that PAHy–PEG$_{2000}$–GTA and PAHy–C$_{16}$–GTA form different supramolecular systems with DNA. In particular, in DNA/PAHy–PEG$_{2000}$–GTA complexes, DNA is probably associated with cationic side chains of the copolymers while PE chains could interact with the external medium reducing the access of enzyme to substrate, and thus reducing its degradation activity (Fig. 2(a)). This condition should involve an improved condensing effect of DNA, and gives reasonably small and low polydispersity IPECs.

In contrast, in DNA/PAHy–C$_{16}$–GTA complexes, lipophilic chains would be organised into a micellar core, and consequently DNA disposed at the complex surface, resulting in more susceptibility towards enzymatic degradation (Fig. 2(b)). This interaction could give systems with minor condensation effect on DNA and then bigger dimensions. Preliminary dynamic light scattering measurements of the size and polydispersity of these complexes support this hypothesis, giving, at polycation/DNA weight ratio of 3:1, DNA/PAHy–C$_{16}$–GTA complexes with a size of 400 ± 25 nm (polydispersity index = 0.5) against 250 ± 17 nm (polydispersity index = 0.3) for DNA/PAHy–PEG$_{2000}$–GTA.

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**Figure 1.** Effect of polymer/DNA interaction on degradation by DNase II. Degradation of DNA and polymer/DNA mixture at a weight ratio of 3:1 is shown. Values are mean ± SD (n = 3).

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CONCLUSIONS
The effect of the introduction of PEG or C16 chains into DNA complexes and the protecting ability of PAHy copolymers containing GTA groups have been studied. The cationic copolymers obtained showed a sufficient ability to complex DNA at low polymer/DNA weight ratio. Moreover, results of DNase II degradation led us to suppose a particular supramolecular organisation of polyionic systems (like polyinonic micelles) with a superior ability of PAHy–PEG2000–GTA to protect DNA that should be associated with inner micellar cores. No significant protection was recorded for PAHy–C16–GTA in which DNA should be disposed on the surface of the systems, freely exposed to DNase II action.

Supplementary material
Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/ipages/0959-8103/suppmat/.

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