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SHORT COMMUNICATION

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Novel polyaminoacidic copolymers as nonviral gene vectors

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Abstract Human gene therapy is one of the most promising methods developed in recent years, providing great potential for the treatment of a variety of diseases. Complexes formed between DNA and cationic polymers are attracting increasing attention as novel synthetic vectors for the delivery of genes. We have synthesized polycations with quaternary ammonium groups in their side chains for self-assembly with calf thymus DNA. This paper describes the functionalization of α,β-polyasparthydrazide (PAHy), a synthetic macromolecule having many potential applications in the field of biomedical sciences, with glycidyltrimethylammonuim chloride (GTA) in order to introduce positive charges into their chains. Derivatized PAHy with various GTA contents have been obtained and characterized. Highly functionalized copolymers have been used for condensing DNA, yielding discrete complexes. The complex formation has been confirmed by gel electrophoresis and the surface charge of interpolyelectrolyte complexes has been assessed by the zeta potential.

Key words α,β-Polyasparthydrazide · Gene therapy · DNA complexes · Gene delivery

Introduction

A new generation of drugs is being designed based on molecular biology and recombinant DNA technology. Gene therapy, which utilizes expression vectors encoding genes, is being considered for replacement or correction of aberrant genes, whereas antisense oligonucleotides, relatively small synthetic DNA, are designed to hybridize to mRNA sequences in target cells. The Achilles heel of gene therapy is gene delivery. Thus far, the problem has been the inability to deliver genes efficiently and to obtain sustained expression. To date, a number of techniques have been developed for DNA introduction into cells, the most common being precipitation with calcium phosphate [1, 2], DEAE-dextran (diethylamino)ethyl-dextran [3] or polybrene [4, 5], direct introduction of DNA using cell electroporation [6, 7] or DNA microinjection [8, 9], and DNA incorporation in virus coats [10–12] and cationic liposomes.

Viruses are the most widely used vectors, although immunogenicity and inflammatory effects, coupled with safety concerns and difficulties of modifying intrinsic tissue tropism, may restrict their usefulness [13].

Poor definition, safety concerns and difficulties of scale-up production of viruses have led to the development of synthetic vectors (nonviral vectors) for DNA delivery. Such a vector must be safe, fully defined, small enough for efficient extravasation, nonimmunogenic, noninflammatory, and capable of mediating efficient delivery of genes to the nucleus of target cells in transfection-active form.

Among synthetic vectors, cationic liposomes have already seen extensive use in vitro and in vivo; however they also exhibit poor biocompatibility and rapid degradation [14] and effective application in vivo is limited to local delivery mechanisms, such as inhalation [15, 16], capillary blockade [15], or injection directly into tumors, lesions or tumor-feeding arteries [17].

An alternative approach to the development of synthetic vectors has been proposed based on soluble cationic polymers designed to self-assemble DNA expression vectors [18]. The cationic polymers that form the basis of these self-assembling systems interact electrostatically with the phosphates on DNA to form a compact particle [19]. Interpolyelectrolyte complexes (IPECs) are self-assembling objects that are thermodynamically stable under a certain set of conditions, i.e., pH, ionic strength, temperature, medium composition, etc. This facilitates their preparation, storage, and application if compared, for instance, with liposomes that are usually unstable and cannot be stored for a long time.

The physicochemical characteristics of IPECs, in particular their solubility, dimensions, and surface charge, can be varied by altering the composition of the complex and the chemical structure of its constituents. Incorporation in an IPEC results in significant changes in DNA properties, specifically in its compaction. Because it is tightly packed in the IPEC species the DNA chain is protected from contact with the external medium, which, in particular, leads to DNA stabilization against digestion by nuclease [20, 21].

It has been demonstrated that incorporation in an IPEC leads to an enhancement of DNA uptake into cells and an increase in its transfection activity with respect to both procariotic [20] and animal cells [22, 23].

Specific targeting or membrane-permeabilizing groups can be easily incorporated into such structures and significantly enhance their transport into cells via the receptor-mediated pathway [24–26], as well as their in vivo delivery into target cells [27, 28].

In recent years intensive studies have been performed in order to develop new materials for biomedical applications [29, 30]. In this context, α,β-polyasparthydrazide (PAHy) (Fig. 1) is a linear polymer [31] with interesting properties, such as water solubility, absence of toxicity, antigenicity, and teratogenicity.

This macromolecule has attractive peculiarities allowing its possible use in the pharmaceutical field. Due to its physicochemical characteristics and biocompatibility, PAHy has already been proposed as a plasma expander [32], a carrier for macromolecular prodrugs [32], and for macromolecular networks after cross-linking [33, 34].

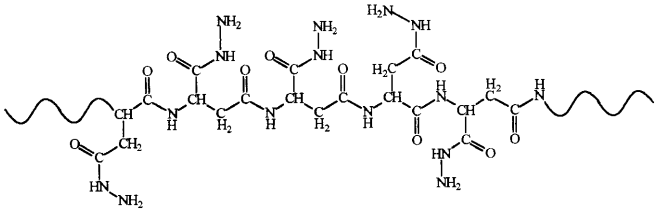

Fig. 1 Chemical structure of α,β-polyasparthydrazide (*PAHy*)

Partial chemical modification of a macromolecule is a strategy currently pursued in order to improve its reactivity towards a particular reaction, such as cross-linking, grafting or linkage with a biologically active agent. Structure modification can be carried out by introducing suitable reactive groups into the backbone or into the side chains of the polymer.

Our objective is to modify partially the structure of this macromolecule by introducing groups bearing cationic charge to obtain polycations. Such structures can bind with negatively charged polynucleotide backbone to give IPECs for DNA delivery.

The present paper focuses on the functionalization of PAHy with glycidyltrimethylammonium chloride (GTA). The influence of reaction conditions on the degrees of derivatization (DD) of the macromolecule has been studied.

Highly functionalized copolymers have been used for condensing DNA, yielding discrete complexes. The complex formation has been confirmed by gel electrophoresis and the surface charge of IPECs has been assessed by the zeta potential.

Materials and methods

Materials

All the reagents used were of analytical grade, unless otherwise stated. D,L-aspartic acid, hydrazine hydrate, GTA and DNA from calf thymus were from Fluka (Switzerland). Anhydrous *N*,*N*-dimethylformamide and D_2O (isotopic purity 99.9%) were purchased from Aldrich Chemical Co. (St. Louis, Mo., USA).

PAHy was prepared and purified according to a previously reported procedure [35]. The spectroscopic data, Fourier transform (FT) IR and NMR, were in agreement with the literature values [35]. The PAHy weight-average molecular weight was 21240 (M_w/M_n = 1.88) determined according to a previously reported procedure [36].

Analytical methods

Elemental analysis (C,H,N) was carried out using a Carlo Erba model 1106 analyzer. Compounds were quantitatively dried before analysis under reduced pressure (10^{-3} mmHg) at room temperature for 48 h on P_2O_5. FT-IR spectra were recorded using a Perkin-Elmer 1720 FT spectrometer. The ^{1}H NMR spectra were obtained with a Bruker AC-250 instrument operating at 250.13 MHz. Molecular weights and polydispersity indices of both starting and functionalized polymers were determined by light-scattering measurements, using a Spectra Physics Dawn DSP-F laser spectrometer. The zeta potential was assessed using a Zetamaster system (Malvern Instruments).

Reaction of PAHy with GTA

PAHy (300 mg) was dissolved in 6 ml phosphate buffer solution (PBS) (KH_2PO_4, K_2HPO_4, pH 8.5). GTA was slowly added to achieve the molar ratios given in Table 1.

The reaction was kept at room temperature under continuous stirring for 24 h. After this time, the reaction solution was neutralized and dialyzed using Visking dialysis tubing (18/32 in.)

Table 1 Reaction conditions in the synthesis of α,β-polyasparthydrazide (*PAHy*)–glycidyltrimethylammonium chloride (*GTA*) derivatives

Sample	Solvent	GTA/PAHy molar ratio	Reaction time (h)
PAHy-GTA_1	phosphate buffer solution	0.37	24
PAHy-GTA_2	phosphate buffer solution	0.75	24
PAHy-GTA_3	phosphate buffer solution	1.12	24

with a molecular-weight cut-off of 12 000–14 000. After dialysis the solution was concentrated under vacuum and lyophilized. PAHy–GTA derivatives were obtained with a yield of 91–92% (w/w), based on the starting PAHy.

Analysis

PAHy–GTA_1: calculated for $C_{4.78}H_{8.82}N_{3.13}O_{2.13}Cl_{0.13}$ (relative to 13% mol substitution) C, 38.57; H, 5.97; N, 29.46; Cl, 3.09; found: C, 38.67; H, 6.01; N, 29.54; Cl. 3.11.

PAHy–GTA_2: calculated for $C_{5.62}H_{10.78}N_{3.27}O_{2.27}Cl_{0.27}$ (relative to 27% mol substitution) C, 39.69; H, 6.39; N, 26.93; Cl, 5.63; found: C, 39.88; H, 6.51; N, 27.12; Cl. 5.77.

PAHy–GTA_3: calculated for $C_{6.76}H_{13.44}N_{3.46}O_{2.46}Cl_{0.46}$ (relative to 46% mol substitution) C, 40.83; H, 6.81; N, 24.37; Cl, 8.20; found: C, 41.01; H, 6.99; N, 24.44; Cl, 8.33.

FT-IR spectra (KBr disks) showed a broad band centered at 3300 cm^{-1} (-NH, $-NH_2$, -OH) and bands at 1655 cm^{-1} (broad, amide I), 1540 cm^{-1} (amide II) with a shoulder at 1488 cm^{-1} (symmetric bending $-CH_3$) and at 1124 cm^{-1} (stretching alcoholic C-O).

^{1}H-NMR (D_2O, δ): 2.6–3.1 (m, br, 5H, -CH-$\underline{CH_2}$-CO-NH-, -NH-$\underline{CH_2}$-CH(OH)-CH_2-, -CH_2-$\underline{CH}$(OH)-CH_2-), 3.25 (s, 9H, -$N^{\oplus}(\underline{CH_3})_3$, 3.4–3.7 (m, 2H, -CH(OH)-$\underline{CH_2}$-$N^{\oplus}(CH_3)_3$), 4.74 (m, 1H, -NH-$\underline{CH}$(CO)-CH_2-).

Assembly of complexes at different charge ratios

Complexes were induced to self-assemble in water by mixing DNA (20 or 40 mg/l) with the appropriate volume of polymer solution at different polymer concentrations and were left for at least 2 h at room temperature before use.

Agarose gel electrophoresis

Polymer–DNA complexes prepared in water using a polymer solution at different concentrations were electrophoresed on agarose gel (0.5% wt/vol) for 60 min at 50 V. Ethidium bromide (50 mg/l) was included in the gel to show the localization of DNA using a UV transilluminator.

Analysis of the zeta potential

The system was routinely calibrated using a −55 mV standard. Experimental samples (4 ml) contained a final DNA concentration of 20 mg/l and were measured three times for 20 s at 1.000 Hz with zero field correction.

Dynamic light-scattering measurement

Dynamic light-scattering (DLS) measurements were carried out by using a Brookhaven Sm 200 spectrometer equipped with a Brookhaven BI2030 autocorrelator with 128 channels and a HeNe laser (λ = 632.8 mm). Samples were prepared using dust-free water and were filtered through a 0.45-μm filter (Waters) immediately before analysis. Experiments were performed at 25 ± 0.1 °C using 0.1 M NaCl as a solvent.

Results and discussion

Synthesis and characterization of PAHy–GTA copolymers

The present study deals with synthesis and characterization of polyaminoacids containing side cationic groups which can be used as synthetic vectors in the preparation of IPECs.

The chemical structure of the PAHy–GTA derivatives is shown schematically in Fig. 2.

The GTA derivatization of PAHy was performed in PBS solution at pH 8.5 with a reaction time of 24 h and GTA/PAHy molar ratios of 0.37, 0.75 and 1.12 (Table 1).

The purified functionalized polymers were characterized by FT-IR and ^{1}H NMR analysis. FT-IR spectroscopy of the copolymers revealed the presence of bands due to the glycidyltrimetylammonium group in the PAHy–GTA derivatives which are absent in the starting polymers.

^{1}H NMR spectra of PAHy-GTA confirmed the introduction of trimethylammonium groups into the side chain of PAHy.

The DD were determined by ^{1}H NMR and were calculated using the following ratio:

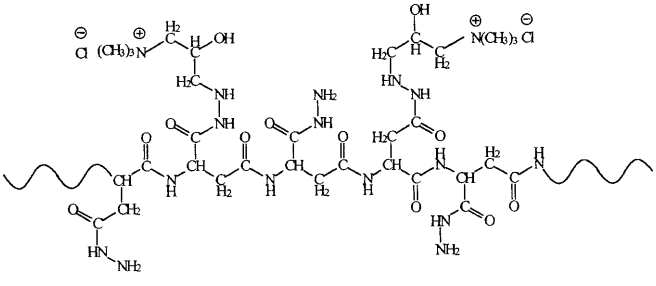

Fig. 2 Chemical structure of PAHy–glycidyltrimethylammonium chloride (*GTA*) derivatives

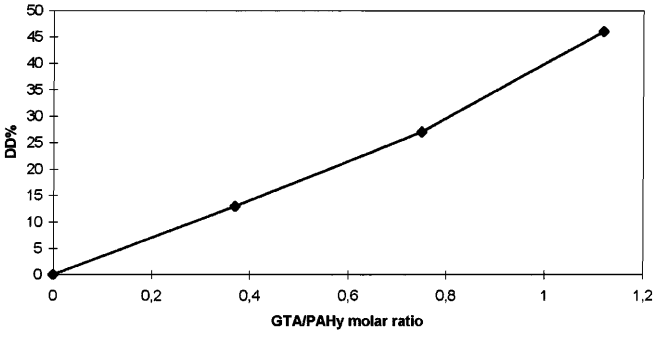

Fig. 3 Degree of PAHy derivatization (*DD*) in pH 8.5 phosphate buffer solution by GTA as a function of the GTA/PAHy molar ratio (reaction time = 24 h)

DD

= (trimethylammonium groups/polymer repeating unit) × 100 (mole)

The DD versus the GTA/PAHy molar ratios are reported in Fig. 3.

Referring to PAHy–GTA conjugates, as previously reported, the spectra were recorded in D_2O and the DD was calculated by comparing the integral of the peak related to protons at 4.74 δ assigned to -NH-CH(CO)-CH_2- (belonging to PAHy) with the integral of the peak related to protons at 3.25 δ assigned to -$N^{\oplus}(CH_3)_3$ (belonging to linked GTA). The DD were expressed as mean values. Each determination was carried out three times and the maximum estimated error was 3%.

PAHy reacted rapidly and extensively and the DD ranged from 13 to 46% (samples PAHy–$GTA_{1–3}$). The high reaction rate of GTA with PAHy can reasonably be attributed to the presence of hydrazine functions.

Agarose gel electrophoresis

Complexes were formed between DNA and PAHy–$GTA_{2–3}$ by modifing the polymer concentration from 0.1 to 3.0 g/l, and were analyzed by electrophoresis on agarose gel containing ethidium bromide (Fig. 4).

Agarose gel analysis shows that the polyelectrolyte complexes do not migrate into the gel and were retained at the origin.

Analysis of the zeta potential

An important parameter for the control of the aggregation behavior in classical colloidal theory is the charge on the surface of the particle [37]. The surface charge of the cationic polymer–DNA complexes was estimated from measurements of the zeta potential. Complexes formed using calf thymus DNA water solutions and PAHy–GTA_2 or PAHy–GTA_3 at concentrations ranging between 0.05 and 2.4 g/l. The results of the zeta potential analysis are depicted in Fig. 5.

The complexes formed with both polycations show a zeta potential that increases with rising cationic polymer concentrations in the medium.

DLS measurements

DLS experiments were carried out in order to confirm the formation of IPECs just on the samples obtained by mixing DNA at 20 mg/l with an appropriate volume of PAHy–GTA_3 (final polymer concentration 0.4 and 0.6 g/l); the complexes obtained were also characterized by gel electrophoresis and zeta-potential studies.

Data analysis indicated an interaction between DNA and PAHy–GTA copolymer since the diameter of the

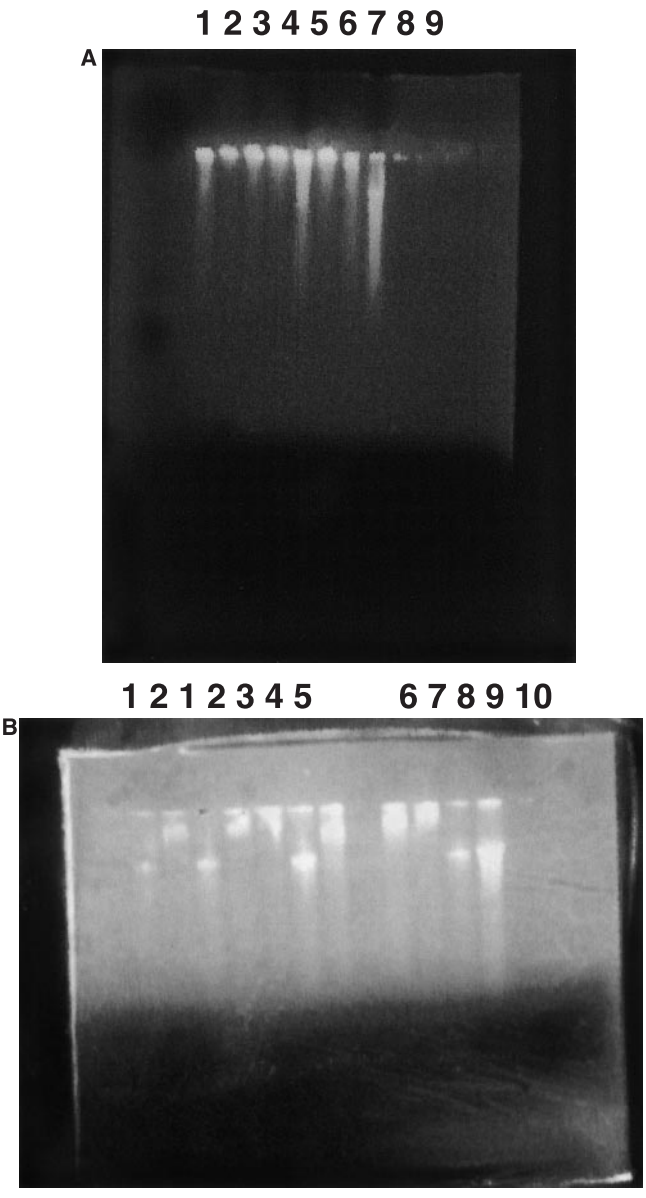

Fig. 4A Agarose gel analysis of complexes formed between DNA and PAHy–GTA_2 at different DNA and PAHy–GTA_2 concentrations. *Lanes 1–4* show DNA/PAHy–GTA_2 complexes at a DNA concentration of 20 mg/l and PAHy–GTA_2 concentrations of 0.6, 1.8, 2.4, 3.0 g/l. *Lanes 5–6* show DNA/PAHy–GTA_2 complexes at a DNA concentration of 40 g/l and PAHy–GTA_2 concentrations of 1.8 and 3.0 g/l. *Lane 7*: DNA (20 mg/l). *Lane 8*: DNA (40 mg/l). *Lane 9*: PAHy-GTA_2 (1.2 g/l). **B** Agarose gel analysis of complexes formed between DNA and PAHy–GTA_3 at different DNA and PAHy–GTA_3 concentrations. *Lanes 1–3* show DNA/PAHy–GTA_3 complexes at a DNA concentration of 20 mg/l and PAHy–GTA_3 concentrations of 0.1, 0.2, 0.4, 0.6 g/l. *Lane 8*: DNA (20 mg/l). *Lane 9*: DNA (40 mg/l). *Lane 10*: PAHy–GTA_3 (0.1 g/l)

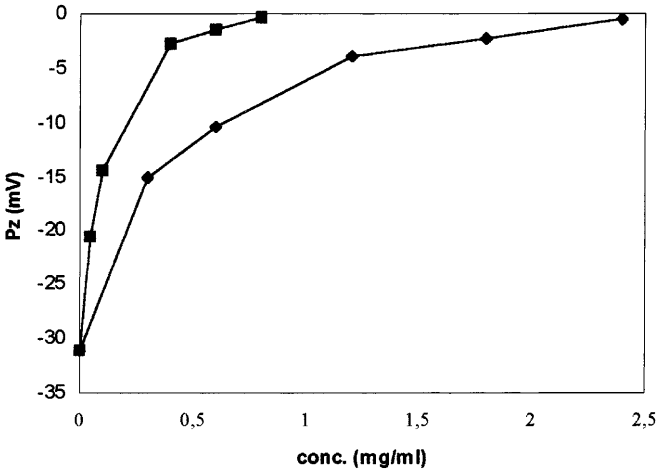

Fig. 5 Influence of PAHy–GTA_2 (◆) and PAHy–GTA_3 (■) concentration on the zeta potential of complexes formed between DNA and each of these polycations

complexes was different and was greater than that of DNA alone (about 2000 and 500 nm, respectively).

Conclusions

The results of this study indicate that the derivatization of PAHy with GTA is a profitable method to introduce cationic groups into polymeric chains. It is possible to obtain PAHy–GTA derivatives containing the expected number of positive charges by choosing a suitable amount of derivatizing agent. The functionalized polymers give rise to IPECs by electrostatic interaction with DNA.

The complex formation of these polymers with DNA was confirmed by gel retardation assay, and zeta-potential studies show that the surface charge of these complexes can be decreased with increasing polymer concentration. A decrease in the surface charge of IPECs would improve the interaction of DNA with the cellular membrane, which is negative in water solution [38].

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