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Poly(propylene imine) dendrimers modified with maltose or maltotriose protect phosphorothioate oligodeoxynucleotides against nuclease activity

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ABSTRACT

The antisense oligonucleotides are promising agents for application in anti-HIV therapies. The antiretroviral nucleoside analogues administrated into circulatory system are vulnerable to nuclease degradation and require a vehicle which would not only facilitate therapeutic nucleotides into host cells, but would also provide protection against enzymatic degradation. Such potential is exhibited by poly(propylene imine) dendrimers – the branched cationic polymers easily interacting with oligonucleotides to form complexes called "dendriplexes". The aim of the present study was to evaluate the abilities of the fourth generation poly(propylene imine) dendrimers partially modified with maltose (PPI-Mal G4) or maltotriose (PPI-Mal-III G4) to protect anti-HIV antisense oligonucleotides (ODNs) from nucleolytic degradation. The ODNs (AT, GEM91, SREV) were complexed with dendrimers and subjected to cleavage by serum nucleases or endonuclease S1. The results showed that all examined dendrimers protected ODNs against nucleases contained in FBS. Both PPI-Mal G4 and PPI-Mal-III G4 dendrimers completely prevented ODNs digestion by nuclease S1 at neutral pH. The protective capabilities of investigated dendrimers were significantly weaker in acidic environment. The time stability assay showed that the dendriplexes formed by AT, GEM91, SREV and carbohydrate-modified PPI G4 dendrimers still existed after 12 h incubation both in low and at neutral pH buffers. The conformational change of dendriplexes in acidic environment was proposed as possible phenomenon leading to exposition of ODNs to nuclease attack and significantly diminishing dendriplexes' resistance to nucleolitic digestion.

1. Introduction

The antisense therapy has emerged as an exciting and promising strategy for HIV treatment. The antisense oligodeoxynucleotides AT, GEM91 and SREV, complementary to fragments of HIV virus genome (tat gene and TAR element, gag gene, rev gene, respectively), proved to possess high affinity for their targets which predisposed them to be clinically tested [1,2]. However, one of the problems which still needs to be overcome for effective antisense therapy is the loss of oligonucleotides' integrity following systemic administration due to degradation by nucleases in intercellular matrices. Among many modifications tested towards conferring resistance to nucleases, the most promising one seems to be application of phosphorothioates. These oligonucleotides are characterized by a sulfur atom which replaces a single non-bridging oxygen atom at each phosphorus atom. This modification introduces asymmetry at each internucleotide bond. Hence each linkage can occur as either the Rp- or Sp-diastereomer [3]. The introduction of phosphorothiol modification in linkages between nucleotides makes antisense oligodeoxynucleotides (ODN) markedly more nuclease-resistant than the corresponding phosphodiester oligodeoxynucleotides [4,5]. Therefore, the half-life of these ODNs *in vivo* is increased [6,7]. However, each nuclease reveals stereospecifity to various extent. For example 3′exonuclease from human serum favors the Rp-diastereomers and is significantly less efficient in hydrolyzing linkages containing Sp-diastereomers [8]. Nuclease S1 is similarly sterospecific to phosphodiester bonds. In this case the activity towards phosphorothioates deoxynucleotides is reduced and the enzyme cleaves bonds of Sp-type 10-fold less efficiently than Rp-type [9]. As the process of synthesis of phosphorothiol-modified ODNs produces a mixture of Rp- and Sp- diastereomers, the inherent resistance of phosphorothioate oligodeoxynucleotides to nucleases is not sufficient. Thus, for their medical application, the extracellular and intracellular transport of ODNs requires further means of protection. This necessity should be fulfilled by synthetic vectors which are designed to deli-

Abbreviations: PPI G4, fourth generation poly(propylene imine) dendrimer; PPI-Mal G4, maltose-modified fourth generation poly(propylene imine) dendrimer; PPI-Mal-III G4, maltotriose-modified fourth generation poly(propylene imine) dendrimer.

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ver nucleic acid drugs in biologically effective doses in a specific and nontoxic fashion [10]. The agents which might satisfy those requirements are cationic dendrimers of different chemical compositions with peripheral amino groups [11–14]. However, the extensive applicability of cationic dendrimers as *in vivo* carrier systems is constrained due to their inherent toxicity. The surface cationic charge of dendrimers dictates their interaction with negatively charged biological membranes, which results in membrane disruption and cell lysis. This is the main process attributed to cationic dendrimer's toxicity [15,16].

In order to alleviate the poly(propylene imine) (PPI) dendrimer's toxicity, various chemical modifications of the surface of PPI dendrimers have been established by the neutralization of the cationic surface charge [17–20]. The significantly reduced toxity was revealed by maltose- and maltotriose-modified fourth generation PPI dendrimers. Those dendrimers with deacresed cationic surface charge provided the desired PPI glycodendrimer's biocompatibility under *in vitro* and *in vivo* conditions [18–20].

Both unmodified and carbohydrate modified PPI dendrimers spontaneously interact with antisense oligonucleotides and form complexes called "dendriplexes" [21,22]. In the case of unmodified PPI dendrimers the driving force for dendriplexes formation is electrostatic interactions between anionic phosphate groups of ODNs and positive charges of amino groups of dendrimers. Even though, the decreased cationic charge of maltose- and maltotriose-modified PPI dendrimers weakens electrostatic interactions with ODNs, the presence of many hydroxyl groups in oligosaccharide molecules enables H-bonds formation with oligonucleotides. However, due to different driving forces that lead to complex formation, modified and unmodified PPI dendrimers-based dendriplexes may vary in stability and susceptibility to nucleases present *in vivo*. The aim of the present paper was to evaluate the ability of carbohydrate-modified fourth generation PPI dendrimers to protect antisense oligodeoxynucleotides from nuclease degradation.

In order to examine susceptibility of dendriplexes to exonucleolytic digestion, we used FBS which mainly reveals 3′ exonuclease activity [23,24]. For investigation of dendriplexes' vulnerability to endonucleolytic cleavage, we used nuclease S1, which exhibits hydrolytic activity for the phosphodiester bonds of single-stranded DNA yielding 5′-phosphomononucleotide and 5′-phosphooligonucleotide end-products [25,26]. The optimum pH for its activity is 4.0–4.6 (in the presence of Zn^{2+} or Co^{2+} ions), but it is also able to digest efficiently at pH 7.5 (if Mg^{2+} ions are the activator of the enzyme) [27]. The activity of nuclease S1 in acidic and neutral pH gave us the possibility to compare protection capabilities of dendrimers in various pH conditions.

2. Materials and methods

2.1. Materials

Fetal Bovine Serum (FBS), DMEM medium, S1 nuclease, heparin and all buffer reagents were purchased from Sigma Aldrich (Poland). Unmodified poly(propyleneimine) dendrimers of the fourth generation (PPI G4, molecular weight (M_w) = 3514 g/mol) were purchased from SyMO-Chem (The Netherlands). Open shell poly(propylene imine) dendrimers were used: (A) possessing maltose shell and mentioned as PPI-Mal G4 (M_w = 10040 g/mol) and (B) possessing maltotriose shell and mentioned as PPI-Mal-III G4 (M_w = 12800 g/mol). They were synthesized and characterized as described previously [22]. The simplified structures of PPI G4, PPI-Mal G4, PPI-Mal-III G4, as well as chemical structure of PPI-Mal-III G4, are presented in Supplementary Materials in Fig. A1.

Synthetic antisense phosphorothioate oligodeoxynucleotides labeled with fluorescein at 5′ ends were purchased as HPLC-purified quality lyophilisate from Metabion (Munich, Germany). The following sequences of oligodeoxynucleotides were used:

AT – 5′GCT CCC GGG CTC GAC C3′ (M_w = 5192 g/mol)
SREV – 5′TCG TCG CTG TCT CCG CTT CTT CCT GCC A3′ (M_w = 8112.5 g/mol)
GEM 91 – 5′CTC TCG CAC CCA TCT CTC TCC TTC T3′ (M_w = 9086 g/mol)
All other reagents were of analytical grade.

2.2. Formation of dendriplexes

Phosphorothioate oligodeoxynucleotides and dendrimers were dissolved in water and mixed in molar ratios (established previously by the use of fluorescence polarization [22] and showed in Supplementary data as Table 1). The components were incubated at 37 °C for 15 min to enable spontaneous formation of dendriplexes.

2.3. Nuclease protection assay

Dendriplexes or ODNs (as control) were incubated with S1 nuclease (5U per 200 pmols of ODN used for the formation of dendriplexes) in digestion buffer pH 4.6 (containing 30 mM CH_3COONa, 50 mM NaCl, 1 mM $ZnSO_4$) or alternatively in digestion buffer pH 7.4 (containing 50 mM Tris–HCl, 20 mM $MgCl_2$). All digestions were performed by incubation of samples at 37 °C for 12 h. Digestions with serum nucleases were obtained by incubation of dendriplexes or oligodeoxynucleotides in 75% FBS in DMEM for 12 h at 37 °C. After digestion both S1 and FBS nucleases were inactivated by samples incubation at 70 °C for 30 min. All samples were treated with heparin (100 μg/200 pmol ODN) for 15 min at 37 °C to dissociate the ODNs from dendriplex by competitive displacement. The samples were spun down, the supernatant was mixed with loading buffer (20% Ficoll 400 in water) and component ODNs were separated by electrophoresis (130 μmols of ODN per line). The electrophoresis was performed at 20 V/cm for 4.5 h on 25% polyacrylamide (Acrylamide:Bisacrylamide 19:1) gel containing TBE buffer and 7 M urea. After electrophoresis the gel was illuminated in UV-light to visualize the location of (ODN with fluorescein attached to the 5′ends) and digitally photographed.

2.4. Dendriplexes stability assay

The dendriplexes formed by PPI dendrimer and ODN were incubated in digestion buffer pH 4.6 or alternatively in digestion buffer pH 7.4 for 1 h or 12 h. Subsequently, selected samples were treated with heparin (100 μg/200 pmol ODN) for 15 min at 37 °C. All samples were loaded with 20% Ficoll 400 and subjected to electrophoresis on 20% polyacrylamide (Acrylamide:Bisacrylamide 19:1) gel containing TBE buffer and 7 M urea, at 20 V/cm for 3.5 h.

3. Results and discussion

The results of gel electrophoresis showed that nucleases contained in FBS were not able to cut AT, GEM91 or SREV bound in complexes with unmodified (Fig. 1A–C lines 4) and maltotriose-modified PPI G4 dendrimers (Fig. 1A–C lines 8). The maltose-modified PPI G4 dendrimer completely protected AT (Fig. 1A line 6), but exerted slightly weaker protection towards GEM91 (Fig. 1B line 6) and SREV (Fig. 1C line 6) because shades of degraded oligonucleotides could be observed. However, in the case of degradation of ODNs in bovine serum, all studied dendrimers played the protective role. Taking into account that oligodeoxynucleotides introduced into serum (fetal bovine serum as well as human serum)

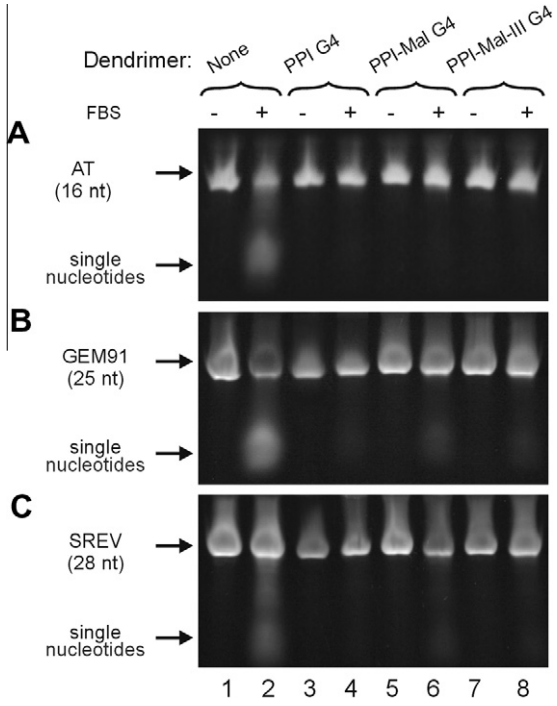

Fig. 1. Degradation of AT (A), GEM91 (B), SREV (C) by nucleases contained in FBS. In all photographs of the gels (A, B, C) lines 1, 3, 5 and 7 represent controls (not cleaved samples) and lines 2, 4, 6 and 8 represent products of cleavage after treatment with nucleases-containing FBS. Some negatively charged proteins of FBS were observed to migrate throughout the gel which results in background fluorescence. In order to exclude that source of differences in fluorescence intensity between FBS treated samples and controls, inactivated FBS was added to all control samples. The results shown are representative of three separate experiments.

are predominantly degraded by 3′-exonucleases [23,24], our results clearly indicate that the interactions between dendrimers and ODNs exclude exonucleolytic degradation of complexed ODNs.

Although all examined dendrimers provided protection of ODNs from exonucleolytic cleavage, further experiments showed that the modification of outer shell of dendrimer highly influenced the susceptibility of bound ODNs to degradation by endonuclease S1. Fig. 2A–C show that at neutral pH cleavage of AT, GEM91 and SREV by nuclease S1 was not effective if ODNs were complexed with PPI-Mal G4 (Fig. 2A–C lines 6) or PPI-Mal-III G4 (Fig. 2A–C lines 8). Contrary, complexation of ODNs with uncoated dendrimer PPI G4 did not protect from nuclease S1 degradation (Fig. 2A–C lines 4). These results might suggest that the oligosaccharide shell establish additional covering, which isolates freely accessible parts of ODNs from attacks of nucleases. However, hardly any protective abilities of modified dendrimers were observed when degradation by nuclease S1 was performed at pH 4.6. Trace amounts of not degraded ODNs were reported only in the case of dendriplexes composed of AT and PPI-Mal-III G4 (Fig. 2D line 8) and dendriplexes formed by SREV and PPI-Mal-III G4 (Fig. 2F line 8).

In order to estimate whether observed discrepancy in the protective capabilities of dendrimers in low and neutral pH derive from the differences in stability of dendriplexes, we compared the stability of complexes under these various conditions. As Fig. 3A show dendriplexes formed by uncoated PPI G4 dendrimer and AT were not detected on electrophoresis gel. However, dissociated forms of ODNs were not observed either. Those results indicate that the resultant charge of PPI G4-containing dendriplexes is too positive and prevents migration of dendriplxes towards anode.

Further results of the electrophoresis showed that dendriplexes formed by modified dendrimers (PPI-Mal G4 and PPI-Mal-III G4) and all ODNs were stable for 12 h in low and neutral pH conditions. Furthermore, any unbound forms of ODNs were not detected (lines 5 and 7 in Fig. 3B and C). Similar results of complex stability were obtained for dendriplexes containing GEM91 and SREV oligonucleotides, and are shown in Figs. A2 and A3 in Supplementary Materials.

Although above results indicate that ODNs are strongly bound to all types of dendrimers, nuclease S1 protection assay conducted at pH 4.6 clearly showed that PPI G4 dendrimers did not prevent ODNs from degradation and oligosaccharide-modified dendrimers almost completely lost their protective capabilities. The elucidation of this phenomenon require taking into consideration that uncoated and carbohydrate-coated PPI G4 dendrimers differ in chemical nature of interactions between dendrimer's surface and ODNs. While formation of dendriplex from ODNs and PPI G4 dendrimer is driven by electrostatic interactions, the attachment of maltose or maltotriose units to the PPI G4 surface reduces the positive charge of the dendrimer [11,12]. As a consequence, the electrostatic interaction between oligosaccharide-modified dendrimers and negatively charged phosphate groups of ODNs is reduced. Nevertheless, oligosaccharide-modified dendrimers maintain their precursors ability to form stable complexes with ODNs due to residual electrostatic and H-bonds-driven interactions. The complexity of non-covalent interactions presumably determine the degree of dendriplexes' integrity and putative structural changes in various conditions, which in turn influence the accessibility of ODNs to endonucleolytic attack.

Generally, the pH values, salt concentrations and ionic strength of solvent significantly influence the stability of dendriplexes [21]. In acidic pH environment PPI dendrimers gain the extanded form due to high protonation of interior tertiary amines which causes electrostatic repulsion between the positively charged ammonium groups [28]. Futhermore, at low pH PPI G4 dendrimers reach higher cationic surface charge and the electrostatic interactions with ODNs are suppressed due to more protonated state of phosphate groups. Moreover, the residual electrostatic interactions of dendriplexes are additionaly weakened by interference with sodium ions present in digestion buffer at pH 4.6. Thus, theorethically, the stability of PPI-Mal G4 and PPI-Mal-III G4 dendriplexes partialy based on H-bonds-driven interactions should be realtively less susceptible to changes of pH conditions than PPI G4 dendriplexes based almost completely on electrostatic interactions. In accordance with this assumption, at pH 4.6 we reported a protection effect only in the case of PPI-Mal-III-based dendriplexes possessing the highest number of H-bonds. Taking into account all those potential pH-dependent changes in dendriplexes' structure, we suggest that although at pH 4.6 ODNs are bound to PPI dendrimers those interactions seem to be loosened. Therefore, we assume that not the dendriplexes dissociation but changes of dendrimer conformation lead to ODNs exposition to nucleases and are responsible for the observed degradation. It is noteworthy that our results showed that AT and SREV were relatively more protected by modified PPI G4 in comparison to GEM91. These observations imply that not only the dendrimer type but also a sequence specific properties of ODNs play a role in the mechanism of dendriplex resistance to nucleases. This notion is supported by the computational analysis of ODNs sequences which revealed that self-complementarity of AT and SREV sequences' predestinated those ODNs to produce secondary structures (duplexes and hairpins) [21] which might impede digestion performed by nucleases. In a recent study, further theoretical calculations were conducted to describe the combination of non-covalent interactions which enable the self-assembly process of dendriplexes [22]. Maly and co-workers showed that complexes of ODNs with PPI-Mal G4 and PPI-Mal-III G4 form higher hierarchically organized supramolecular structures in solution as well as on solid substrates. In the case of dendriplexes containing AT and GEM91 fibril-like structures were reported while com-

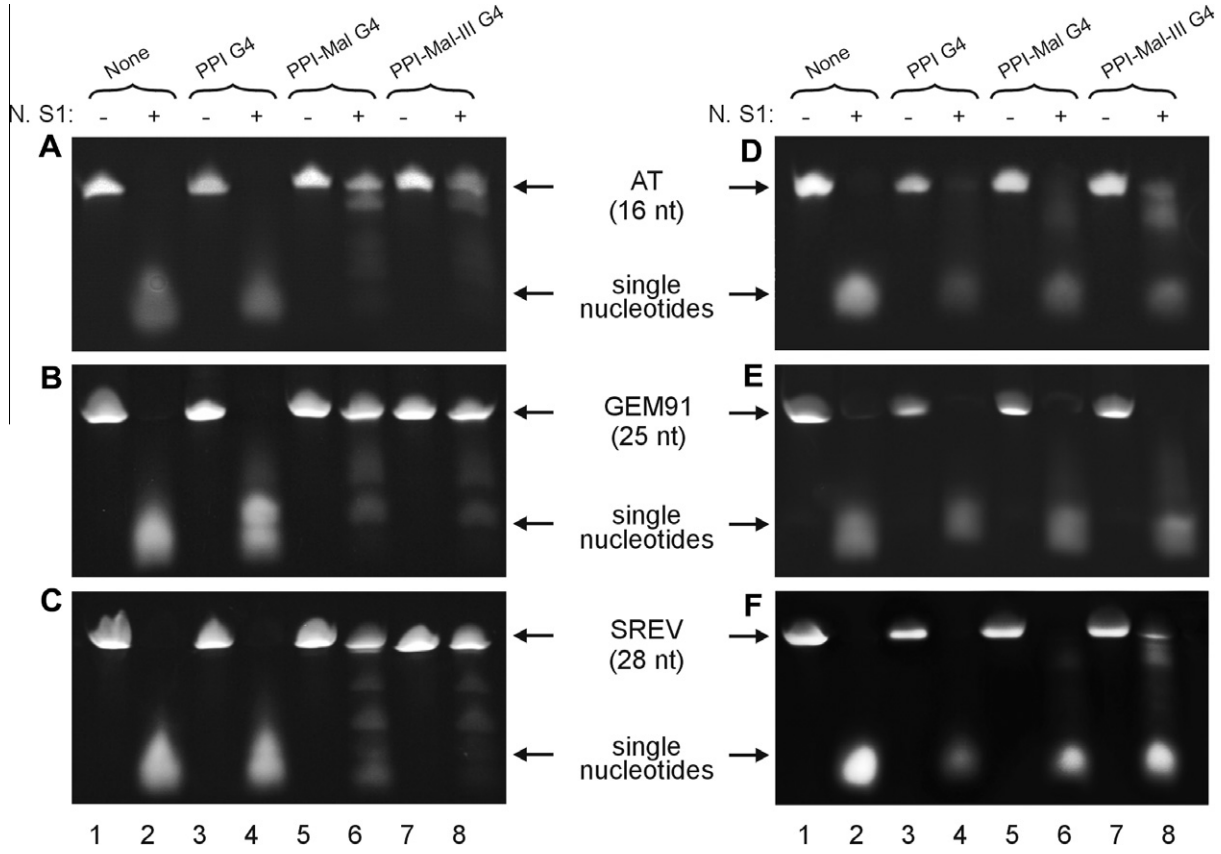

Fig. 2. Degradation of AT, GEM91 and SREV by nuclease S1 at pH 7.4 (A, B and C, respectively) and at pH 4.6 (D, E and F, respectively). In all photographs of the gels lines 1, 3, 5 and 7 represent controls (not cleaved samples), while lines 2, 4, 6 and 8 represent products of cleavage after treatment with N.S1 (nuclease S1).

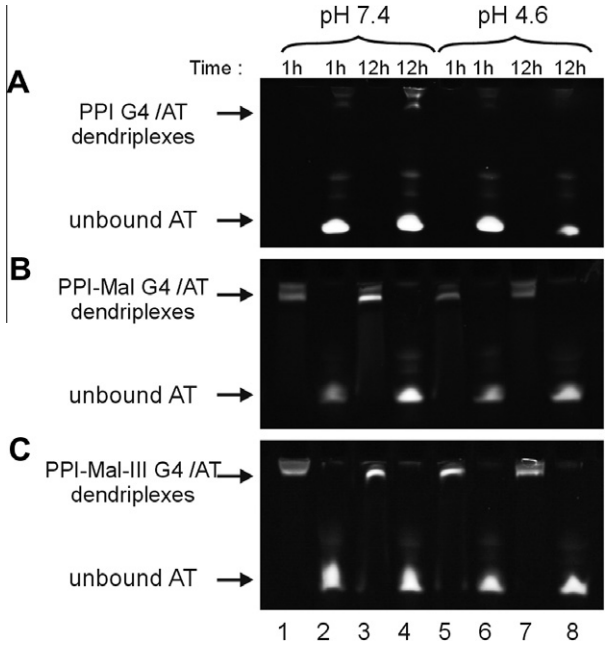

Fig. 3. Stability of dendriplexes composed of AT/GEM91/SREV and (A) PPI G4, (B) PPI-Mal G4, and (C) PPI-Mal-III G4. Line 1 and 2 - dendriplexes were incubated in buffer at pH 7.4 for 1 h; lines 3 and 4 - dendriplexes were incubated in buffer at pH 7.4 for 12 h; lines 5 and 6 - dendriplexes were incubated in buffer at pH 4.6 for 1 h; lines 7 and 8 - dendriplexes were incubated in buffer at pH 4.6 for 12 h. Lines 2, 4, 6 and 8 contain ODNs released from dendriplexes by heparin-mediated dissociation and represent negative controls of dendriplexes stability. The results shown are representative of three separate experiments.

plexes of PPI-Mal G4 and PPI-Mal-III G4 with SREV formed quadrilateral-like 3D nanostructures [22]. Those results give us a clue that not only conformational changes in dendriplexes but also their ODN-specific organization in higher structure might be the important factor determining ODNs' accessibilty for nucleases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.043.

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