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Research paper

Efficiency of polyethylenimines and polyethylenimine-graft-poly (ethylene glycol) block copolymers to protect oligonucleotides against enzymatic degradation

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Abstract

Enzymatic instability of oligonucleotides (ON) is one of the major drawbacks of this new class of therapeutic agents. The development of safe, efficient delivery systems capable of stabilizing and protecting these molecules within the formulation, as well as during application, is a challenge in modern gene therapy. In the present study, polyethylenimine (PEI) of different molecular weights and PEGylated PEI block copolymers (PEI-g-PEG) were investigated with regard to their protective properties when complexes with chemically unmodified DNA (d-ON) and RNA (r-ON) oligonucleotides. PEI/ON complexes were incubated with different amounts of serum or nucleases. The influence of pH on the stability was studied and the integrity of the ON was determined by gel electrophoresis. The amount of stable ON within the gels was quantified via densitometric analysis. PEI homopolymers ranging from 800 to 2 kDa protected both types of ON very efficiently, whereas PEI 0.8 kDa demonstrated a slight decrease in protection. The PEGylated PEI derivatives generally protected ON as efficiently as the PEI homopolymers. In particular, the PEI-g-PEG derivative containing 100 PEG chains of 550 Da yielded the highest protection efficiency for both d-ON and r-ON. In general, the highest protection could be achieved at pH 6.7. The ratio of polymer and ON (N/P ratio) also had a great impact on ON stability with higher N/P ratios achieving a better protection. In conclusion, PEIs showed advantageous protective properties for ON. The results of this study offer indications for a rational design of PEI derivatives for the protection and the delivery of ON.

Keywords: Polyethylenimine; PEGylation; Stabilization; Oligonucleotides; RNA

1. Introduction

Oligonucleotides (ON) are a promising class of therapeutic agents, due to their ability to inhibit gene expression in a sequence specific manner. However, their rapid cleavage by nucleases and poor cellular uptake lower their efficiency [1]. To overcome these problems different strategies such as chemical modifications of the ON molecule [2] or the use of non-viral carrier systems are currently under investigation. Several cationic lipid and polymer formulations, which are capable of spontaneous complex formation with the negatively charged nucleic acid backbones, were found to stabilize ON and to enhance their cellular uptake [3]. Polyethylenimine (PEI) is regarded as an efficient non-viral vector demonstrating high transfection rates under in vitro and in vivo conditions [4]. The strong buffer capacity, described by the 'proton sponge theory', seemed to be responsible for the fact that PEI based delivery systems do not require endosome disruptive agents for lysosomal escape. Additionally, it was shown that an excess of polymer protects the complexed nucleic acid against enzymatic degradation [4].

Recently, hydrophilic block copolymers were introduced as ON delivery vehicles. These copolymers are composed of a cationic segment, e.g. PEI, polylysine (PLL) or polyspermine (PS), and a non-ionic hydrophilic component, such as poly(ethylene glycol) (PEG). It was suggested that interpolyelectrolyte complexes of ON and such block copolymers form structures in which the ON is trapped within a cationic core surrounded by a hydrophilic PEG shell. The increased complex solubility, enhanced

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enzymatic resistance and modified interactions with cell membranes support the hypothetical core-shell structure of these self-assembling complexes [5].

Several studies have been published regarding the physicochemical aspects of complex formation, as well as antisense activity and delivery efficiency, of ON inter-polyelectrolyte complexes [5]. However, data concerning the structure property relationship of different PEI and PEGylated PEI derivatives, as well as their ability to protect ON against enzymatic degradation are scarce. In this study, we systematically investigated the stabilizing effects of several PEI and PEGylated PEI derivatives on ON. The amount of protected ON after incubation with serum or nucleases was quantified. Furthermore the influence of the pH on the protective effect was studied.

To guarantee successful delivery in vivo efficient protection of ON against nucleases within the intracellular milieu has to be achieved. PEI homopolymers and PEGylated PEI derivatives show promising characteristics for efficient protection of both d-ON and r-ON.

2. Materials and methods

2.1. Oligonucleotides

The 20 mer DNA oligodeoxynucleotide (d-ON) with the sequence 5′-TTC CAA TAC AGA AAC TCT CT-3′ and the 37 mer RNA oligonucleotide (r-ON) with the sequence 5′-UCU CUC AAA GCA GGA UUG CCU GAG UAG UCA UAA CCU U-3′ were purchased from Biospring (Frankfurt, Germany) and dissolved in sterile water.

2.2. Polymers

PEI 800 kDa was purchased from Fluka (Neu-Ulm, Germany) as an aqueous 50% (w/v) solution. PEI 2.7 kDa was synthesized as published previously [6]. PEI 25, 2, 0.8 kDa (Polymin water free, 99%) and 5 kDa (aqueous 50% (w/v) solution) were gifts from BASF (Ludwigshafen, Germany). Copolymers were synthesized using two different synthesis routes as described in detail previously [7,8]. Block copolymers were produced as described by Petersen et al. [7] by grafting linear PEG of 550, 2 and 5 kDa, respectively, onto branched PEI 25 kDa. The second so called macrostopper synthesis route [8] led exclusively to the diblock copolymer PEG(5k)-b-PEI. Polymers were stored as sterile filtered, aqueous stock solutions (0.9 mg/ml) pH 7.4. All other materials were of analytical grade.

2.3. Preparation of polymer-ON complexes

Complexes were usually prepared in 150 mM NaCl, pH 7.4 (unless otherwise mentioned) according a protocol by Boussif et al. [4]. The polymer/DNA ratio was expressed as the nitrogen/phosphate (N/P) ratio and calculated on the basis that 1 μg of DNA corresponds to 3 nmol of phosphate, while 1 μl of PEI stock solution contains 10 nmol of nitrogen [4].

2.4. ON stability investigations

Freshly prepared complexes were incubated with 5 units DNase 1 (pH optimum of 7.8) or 0.4 mU RNase A (pH optimum of 7.0–7.5) (Roche Diagnostics GmbH, Mannheim, Germany) per μg ON for 2 h, as well as for different periods of time with the indicated amount of fetal calf serum (FCS), at 37 °C. The samples were then incubated at 70 °C for 30 min to inactivate DNase 1. The ON was displaced from the polymer by adding a sodium dodecyl sulfate (SDS) solution 10% (w/v) up to a final ON concentration of 20 μg/ml. The samples were incubated 10 more minutes prior to loading 10 μl of each sample, corresponding to total amount of 0.2 μg ON, onto a 15% polyacrylamide gel containing 7M urea. Electrophoresis was carried out at 200 V for 50 min (Consort E332 power supply, Roth, Karlsruhe, Germany). Gels were stained for 5 min in a 1:1000 dilution of SYBR® Gold (MoBiTech GmbH, Göttingen, Germany) in water and imaged using the BioDocAnalyze® gel documenting system (Biometra, Göttingen, Germany) with an excitation wavelength of 312 nm.

To assess the protective effects at different pH values, r-ON-PEI 25 kDa complexes were freshly prepared in phosphate buffer saline (PBS) solution (10 mM) of different pH values and were incubated with 0.8 mU RNase A per μg r-ON for 10 min at room temperature. Afterwards, samples were incubated for a further 30 min at 70 °C and divided into two equal fractions. Both fractions were diluted to a final ON concentration of 10 μg/ml; the first fraction with pure water for determination of complex integrity and the second fraction with 10% SDS to access the stability of the liberated ON. Electrophoresis was carried out as described above.

2.5. Densitometric analysis

Electrophoretic gels were analyzed using the software program Scion Image 4.0.2. beta (Scion Corporation, Frederick, Maryland, USA). The amount of intact ON was calculated via a four point standard curve on each individual gel using three ON dilutions of 0.2, 0.1 and 0.05 μg, respectively plus the point of origin. The linear relationship between the amount of loaded ON and the optical density of the bands was determined using the mean of 5 independent 10-point calibration curves. The linear regression exhibited a correlation coefficient of 0.99469. Values were background corrected and specify either the total amount of intact ON, expressed as percentage recovery, and the difference between intact amount of ON and intact amount of the non-complexed ON control, expressed as percentage protected.

3. Results and discussion

The protection efficiency of different PEI and PEGylated PEI derivatives was determined by incubating freshly prepared complexes at a N/P ratio of 20 over 2 h with different nucleases. Complexes containing d-ON were incubated with DNase 1 and r-ON containing complexes with RNAse A. The quantification of the ON displaced from the complexes is shown in Fig. 1. On the left *y*-axis the total amount of intact ON is shown. About 50% of the non-complexed d-ON control was degraded by the double-strand specific endonuclease DNase 1 after 2 h. In contrast, the r-ON was totally degraded. When complexed with PEI homopolymers of molecular weight ranging from 800 to 2 kDa, more than 90% of the d-ON remained intact. In this case, the recovery was about 40% for the r-ON. The right *y*-axis shows the difference between the total recovery and the intact non-complexed control, expressed as percentage protected. This demonstrated that the efficiencies of PEI homopolymers to protect the two different types of ON were similar. PEI 0.8 kDa showed a slightly decreased protection efficiency compared to the other PEI homopolymer derivatives. This effect could be reproduced for both types of ON. A possible explanation for this observation might be the low molecular weight of the PEI, which is approximately 10 times smaller than that of the ON. It is assumed that soluble interpolyelectrolyte complexes can be produced only with sufficiently long polycations. Therefore, full compaction of ON within a polycationic core restricting access of nucleases is unlikely [5]. Similar observations have been made by Godbey et al. for plasmid DNA. They concluded that branched and higher molecular weight PEI provide more effective barriers for DNases [9]. A trend with regard to the efficiency of PEI-g-PEG block copolymers to protect ON could be observed. The derivative with 100 short PEG chains (PEI-g-PEG(550)$_{100}$) protected more efficiently with nearly 100 and 70% intact d-ON and r-ON, respectively, compared to derivatives containing fewer PEG chains of a higher molecular weight. This effect was more prominent for the r-ON than for d-ON. Due to the fact that the total PEI content of PEI-g-PEG(550)$_{100}$ and PEI-g-PEG(5k)$_{15}$ is approximately the same, it is unlikely that the increased protection can be explained by a higher degree of hydrophilic PEG. We assume that a brush-like hydrophilic shell, with a high number of short PEG chains, can more effectively protect against nucleolytic attacks. Longer PEG chains with lower degrees of PEG substitution might allow access of nucleases to the cationic core. Also, PEI/ON complexes exist in a state of a dynamic equilibrium requiring an excess of polycation to fully mask the ON [10]. PEG(5k)-b-PEI, represents an A–B-type block copolymer, which may offer a more effective orientation of the PEG shell. Although excellent condensation properties were described for this type of block copolymer [8], the protection of ON against enzymatic degradation seems to be more favorable with higher grafted PEG-PEI block copolymers, suggesting again that the degree of PEG substitution is a critical factor for protection.

During delivery, non-viral vector systems are exposed to different pH environments, e.g. a neutral pH within the blood stream or an acidic pH within the lysosomal compartment. Therefore, variations in the ability of a carrier to protect ON caused by a changing pH are of importance. As shown in Fig. 2, PEI 25 kDa expressed the highest protection efficiency at pH 6.7. At an acidic pH of 3, the protection efficiency was slightly decreased. A possible explanation might be the swelling of the polymer during protonation as described by the proton sponge theory [4]. Hence, in the swollen state the polymer coverage of the ON might be decreased. This effect seems to be even more pronounced at pH 1.5. At a N/P ratio of 3, the protective

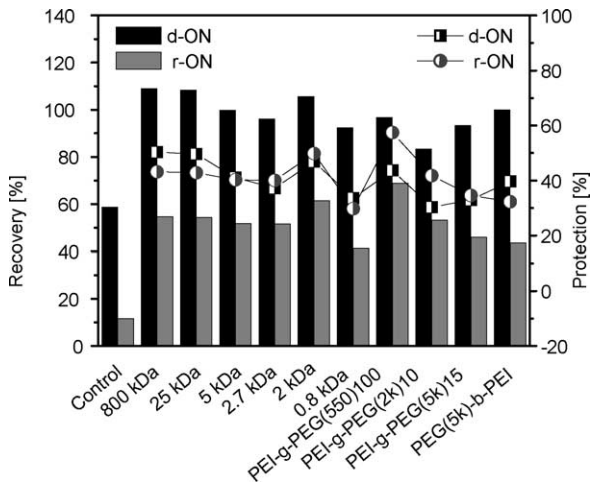

Fig. 1. Incubation of d-ON and r-ON complexes of different PEI and PEGylated PEI derivatives at an N/P ratio 20 over 2 h with DNase 1 and RNAse A, respectively. It is shown the percentage recovery of intact ON and the percentage of protected ON in reference to the intact amount of non-complexed control ON.

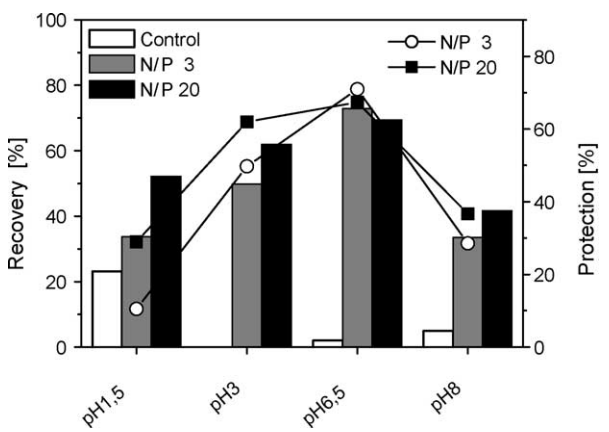

Fig. 2. Incubation of r-ON PEI 25 kDa complexes with RNAse A in PBS of different pH over 10 min. It is shown the percentage recovery of intact ON and the percentage of protected ON in reference to the intact amount of non-complexed control ON.

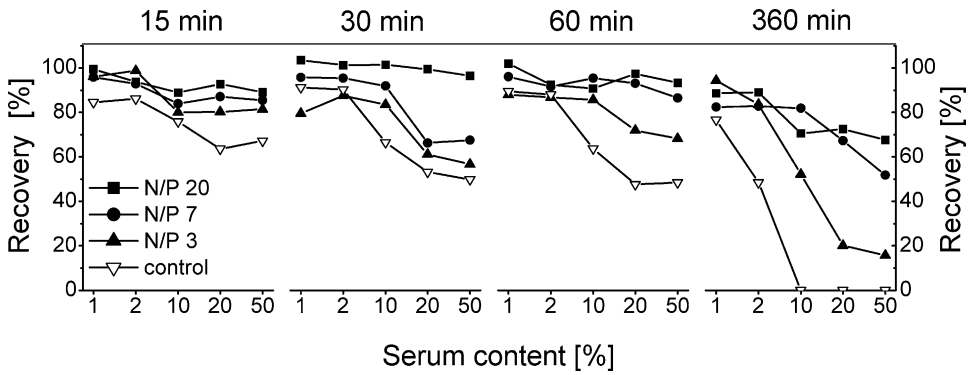

Fig. 3. Incubation of d-ON PEI 25 kDa complexes with 1, 2, 10, 20 and 50% FCS over 15, 30, 60 and 360 min at three different N/P ratios 3, 7 and 20. It is shown the percentage recovery of intact ON.

effect seemed to be reduced, whereas an excess of polymer at N/P 20 protected more efficiently [10]. However, it has to be taken into consideration that the nuclease activity in this environment is also decreased. Secondly, it has to be considered that additionally protonation of the nucleic acid at acidic pH could lead to destabilization of the complex. As expected, protection was decreased at alkaline pH values as well. This is due to the diminished protonation of the polymer, which results in a decreased number of positive charges available for cooperative binding with the ON [11]. Thus, the enzymatic access is more likely.

D-ON PEI 25 kDa complexes were incubated up to 6 h with different percentages of fetal calf serum (FCS) from 1 to 50%. The generated degradation profiles demonstrated the influence of the N/P ratio on the protection efficiency (Fig. 3). Already after 30 min, half of the non-complexed control ON was degraded in 20 and 50% FCS. After 6 h, the control was completely degraded in 10% FCS, as well. At all N/P ratios the ON could be protected against 1 or 2% FCS over the 6 h period. Using higher amounts of FCS, the ON within the complex at N/P 3 was insufficiently protected. However, after 6 h the recovery of intact ON could be increased up to 50 or 70% for N/P ratios of 6 and 20, respectively. This again could be explained by the dynamic processes of binding and dissociation within the complex, which is even more pronounced for small polynucleotides and polycations [5], allowing the enzymatic attack [10]. Using an excess of polycation the ON is efficiently masked therefore, preventing access to the ON. Additionally, other serum proteins are known to interact with interpolyelectrolyte complexes. Negatively charged proteins, such as serum albumin, potentially displace the ON from the polymer complex, exposing it to nuclease attack. An excess of polycation could reduce displacement and non-complexed surplus polymer could possibly intercept a fraction of the proteins.

In conclusion, this study demonstrated that PEI derivatives effectively protected different types of ON. This information provided regarding the time-dependency, the N/P dependency and the influence of polymer structure on the protective effects of PEI derivatives is important for the development of efficient delivery systems for ON.

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