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Efficient delivery of intact phosphodiester oligonucleotides by poly-β-amino esters

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ABSTRACT

Due to their great instability, phosphodiester antisense oligonucleotides (PO-ODNs) are rapidly degraded in the intracellular environment, which limits their biological activity. The release of PO-ODNs during a prolonged period of time could however greatly enhance their antisense effect by creating a pool of intact PO-ODNs at any time point. Poly-β-aminoesters are biodegradable cationic polymers which show potential for the controlled release of short DNA fragments like ODNs and small interfering RNA (siRNA). In this research we evaluated biodegradable poly-β-aminoesters as carriers for PO-ODNs and compared the antisense activity with nuclease stable phosphothioate (PS) ODNs. PBAE1 polymers were not able to generate an antisense effect with PO- or PS-ODNs, most likely due to their poor cellular uptake. When complexed to PBAE2 polymers at N/P ratio 10, both PO- and PS-ODNs downregulated the targeted protein expression with 70%. By confocal imaging we observed a high concentration of released PO-ODNs that formed nuclear bodies in the nucleoplasm. The ODNs in these nuclear bodies were still intact as could be demonstrated by Fluorescence Resonance Energy Transfer (FRET) and acceptor photobleaching. This was in clear contrast to PO-ODNs delivery by cationic liposomes where the ODNs that accumulated in the nucleus were degraded and nuclear bodies were not observed. We conclude that PBAE2 shows potential for the delivery of nuclease sensitive PO-ODNs. This occurs however not through a time controlled release profile, but rather due to the rapid delivery of a high concentration of intact PO-ODNs that form nuclear bodies in the nuclei of the cells. These nuclear bodies can most likely act as a depot of intact PO-ODNs, resulting in efficient antisense activity.

1. Introduction

Delivery of small DNA fragments such as antisense oligonucleotides (ODNs) and small interfering RNA (siRNA) remains an attractive research domain to achieve the therapeutic regulation of gene expression. Antisense oligonucleotides are short single stranded DNA strands which naturally consist of a phosphodiester (PO) backbone. Due to the high instability of this PO backbone, however, the routinely used ODNs consist of the more stable phosphothioate (PS) backbone [1,2]. ODNs can act both in the cytoplasm and the nucleus by a sequence-specific recognition of the complementary mRNA. Through a variety of mechanisms, the complementary mRNA is then destroyed which results in the downregulation of the corresponding protein [3,4].

siRNAs are short double-stranded RNA fragments with a 2 nucleotides overhang at the 3′ end. They are very potent molecules since they are recognised by intracellular RNA-induced silencing complexes (RISCs) which specifically and repeatedly destroy the targeted mRNA molecules. SiRNA can be used in much lower concentrations and generally achieves a higher knockdown efficiency when compared to ODNs [5,6].

Both ODNs and siRNA need to reach the cytoplasm or nucleus of the cells before a biological effect can be obtained. As naked ODNs and siRNA are not efficient in crossing the cellular membrane, they are mostly complexed to carriers such as cationic liposomes or polymers [1]. The positively charged complexes can relatively easy enter the cells by endocytosis but then still need to escape from the endosomal compartment and release the ODNs or siRNA in the intracellular environment. To allow us to explore the therapeutic potential of the simple mechanism of antisense therapy, the search for suited gene delivery carriers continues.

Previously we demonstrated that cationic liposomes are suitable for delivery of PS- but not PO-ODNs [7]. We found that cationic liposomes deliver the ODNs they were carrying upon endosomal escape and that the released ODNs subsequently accumulated rapidly in the nuclei of the transfected cells. We showed experimentally that in the case of a PS backbone the delivered ODNs remained intact while PO-ODN became degraded by intracellular nucleases before a biological effect could be obtained. As the delivery of PO-ODNs remains attractive, we reasoned that due to their rapid degradation, successful delivery would only be possible with carriers that slowly release the ODNs during a prolonged period of time, thus creating a pool of intact PO-ODNs at any time during the delivery process.

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In this research we evaluated the use of biodegradable poly-β-aminoesters as carriers for PO-ODNs. Poly-β-aminoesters are cationic polymers that spontaneously form nanocomplexes with the negatively charged DNA molecules and show broad potential for DNA delivery both for *in vitro* and *in vivo* applications [8,9]. Apart from a positive charge, the presence of tertiary amines gives the polymers a certain buffering capacity, which is favourable to enhance endosomal escape by the so-called "proton sponge" effect [10]. Also, the hydrolysable ester bonds make them interesting candidates for the controlled release of DNA due to polymer backbone degradation. Furthermore, biodegradable polymers are potentially more biocompatible for long-term or repeated applications. We previously demonstrated that poly-β-aminoesters are suitable for the long-term delivery of siRNA to hepatoma cells, probably because they may create a depot of complexed siRNA that is slowly being released in the cytoplasm of the cells [11]. Therefore we wanted to evaluate if these carriers would be suitable to deliver intact PO-ODNs during a prolonged period of time, resulting in a certain extent of protein downregulation.

2. Materials and methods

2.1. Materials

For cytotoxicity and antisense activity measurements, anti-ICAM-1 20mer ODNs with a phosphodiester backbone (PO-ODN) or a phosphothioate backbone (PS-ODN) were used (Isis1939: 5′ CCC-CCA-CCA-CTT-CCC-CTC-TC 3′). For the Fluorescence Correlation Spectroscopy (FCS) experiments and confocal imaging, the Isis1939 PS-ODNs were double-labeled with a rhodamine green fluorophore at the 3′ end ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 532$ nm) and a Cy5 fluorophore at the 5′ end ($\lambda_{ex} = 647$ nm, $\lambda_{em} = 670$ nm). As a PO-ODN, a double-labeled 40 mer phosphodiester ODN (5′ GCC-GTC-TCT-GAC-TGC-TGA-TGA-CTA-CTA-TCG-TAT-AGT-GCG-G 3′; 13,388, 1 g/mol) was used, since we demonstrated in previous research that this 40 mer PO-ODN has a higher FRET efficiency when compared to the double-labeled Isis1939 PO-ODNs [12]. It should be noted that the difference in length does not interfere with the complexation and dissociation profile of the PO-ODNs, nor does it influence the intracellular distribution upon microinjection or transfection experiments. All ODNs (labeled and non-labeled) were purchased from Eurogentec (Seraing, Belgium) and were purified by polyacrylamide gel electrophoresis (PAGE) by the supplier. In the intact double-labeled ODNs used in this study Fluorescence Resonance Energy Transfer (FRET) occurs between the rhodamine green fluorophore on the 3′ end and the Cy5 fluorophore on the 5′ end [12].

The polymers used in the experiments are biodegradable poly(β-amino esters), namely PBAE1 and PBAE2 (the molecular weight of the monomers is respectively 408 g/mol and 436 g/mol) (Fig. 1). PBAE1 and PBAE2 had an average molecular weight of respectively 18 kDa and 22 kDa and were synthesized as described elsewhere [11]. As these PBAEs were not soluble at a neutral or basic pH, they were dissolved in 0.1 M acetate buffer (pH 5.4) and were kept at -20 °C to avoid hydrolysis of the ester functions of the polymers after production.

DNase I (Pulmozyme®, 1 unit/μl) was kindly provided by NV Roche, Brussels, Belgium. Dextran sulfate (DS) was purchased from Sigma (St Louis, USA). The molar mass and sulfate content, as provided by the supplier, equaled respectively 500 kDa and 2.3 sulfate groups per glucosyl residue. A stock solution of 10 μg/μl was prepared in 20 mM Hepes buffer. Lipofectamine™ 2000 was purchased from Invitrogen (Merelbeke, Belgium).

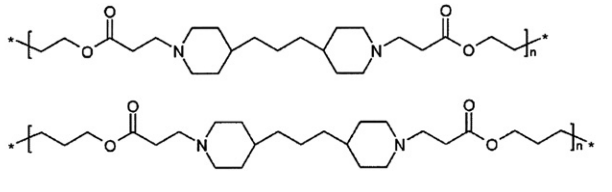

Fig. 1. Schematic representation of PBAE1 (A) and PBAE2 (B) as used in this study. The molecular weight of PBAE1 and PBAE2 monomers is respectively 408 g/mol and 436 g/mol. The molecular weight of one nitrogen containing monomer was calculated as half of the molecular weight due to symmetrical nature of polymers (A) and (B).

Human lung carcinoma cells (A549 cells, ATCC number: CCL-185) (DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Gibco, Merelbeke, Belgium) containing 2 mM glutamine, 10% heat deactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Preparation of PBAE1/ODN and PBAE2/ODN polyplexes

The N/P ratio of the polyplexes is defined as the molar ratio of the total number of nitrogen atoms of the PBAE polymers to the number of DNA phosphates. Polyplexes with different N/P ratios were prepared by adding a cationic polymer solution to an equal volume of an ODN solution, followed by vortexing the dispersion for 10 s. Every polymer and ODN solution was diluted in 0.1 M acetate buffer (pH 5.4) prior to mixing, to assure that the polymer was soluble and able to interact with the ODNs. The polyplexes were allowed to equilibrate at room temperature for 30 min prior to use. Then, for gel electrophoresis, FCS measurements and ODNs degradation experiments, the complexes were further diluted in the so-called "degradation buffer" (2 mM magnesium acetate, 110 mM potassium acetate and 20 mM Hepes, pH 7.4). In this buffer it is sure that the enzyme DNase I is active. For cytotoxicity, uptake, antisense activity and acceptor photobleaching experiments, the complexes were diluted in Opti-MEM® instead of degradation buffer before adding the complexes to the cells. The hydrodynamic size and zeta potential of the polyplexes was checked by respectively dynamic light scattering and surface potential measurements (NanoZS, Malvern, Worcestershire, UK), as previously described [13].

2.3. Gel electrophoresis measurements on the polyplex dispersions and ODNs degradation

To study the complexation between ODNs and respectively PBAE1 and PBAE2 by agarose gel electrophoresis, 10 μl of the ODN solution (60 μg/ml) was mixed with 10 μl of a PBAE1 respectively PBAE2 solution (the concentration being dependent on the desired N/P ratio). The polyplexes were allowed to equilibrate for 30 min before further use. To study the displacement of the ODNs from the polyplexes an increasing amount of dextran sulfate was added to the dispersions. All samples were diluted with degradation buffer to a total volume of 30 μl. Also, 5 μl was taken from each sample and diluted with degradation buffer to a total volume of 50 μl to perform FCS measurements. Before loading the remaining samples on the 1% agarose gel, 5 μl of 50% sucrose was added.

The degradation of the 40 mer ODNs, upon exposure of the polyplexes to DNase I, was studied by polyacrylamide gel electrophoresis (PAGE) as follows. 2 μl of the ODN solution (100 μg/ml) was mixed with 2 μl of the PBAE1 (1219 μg/ml) respectively PBAE2 (1302 μg/ml) solution, resulting in polyplexes with N/P ratio 20. After equilibration, the samples were diluted with degradation buffer to a total volume of 12 μl. Then, the samples were incubated, respectively with or without 1 unit of DNase I, during 24 h. After the desired incubation time, 5 μl 10× EDTA-enriched TBE buffer was added (10.8 g/l tris base, 5.5 g/l boric acid and 3.7 g/l EDTA) to inhibit DNase I. Subsequently, 5 μl of dextran sulfate (10 μg/μl) was added to release the ODNs from the polyplexes before loading the samples on the polyacrylamide gel. Also, from these dispersions 5 μl was removed and diluted with degradation buffer to a total volume of 50 μl to perform FCS measurements. Before loading the remaining samples on the polyacrylamide gel, 5 μl of 50% sucrose was added.