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Multifunctional poly(methacrylate) polyplex libraries: A platform for gene delivery inspired by nature

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ABSTRACT

Polymer-based gene delivery systems have enormous potential in biomedicine, but their efficiency is often limited by poor biocompatibility. Poly(methacrylate)s (PMAs) are an interesting class of polymers which allow to explore structure–activity relationships of polymer functionalities for polyplex formation in oligonucleotide delivery. Here, we synthesized and tested a library of PMA polymers, containing functional groups contributing to the different steps of gene delivery, from oligonucleotide complexation to cellular internalization and endosomal escape. By variation of the molar ratios of the individual building blocks, the physicochemical properties of the polymers and polyplexes were fine-tuned to reduce toxicity as well as to increase activity of the polyplexes. To further enhance transfection efficiency, a cell-penetrating peptide (CPP)-like functionality was introduced on the polymeric backbone. With the ability to synthesize large libraries of polymers in parallel we also developed a workflow for a mid-to-high throughput screening, focusing first on safety parameters that are accessible by high-throughput approaches such as blood compatibility and toxicity towards host cells and only at a later stage on more laborious tests for the ability to deliver oligonucleotides. To arrive at a better understanding of the molecular basis of activity, furthermore, the effect of the presence of heparan sulfates on the surface of host cells was assessed and the mechanism of cell entry and intracellular trafficking investigated for those polymers that showed a suitable pharmacological profile. Following endocytic uptake, rapid endosomal release occurred. Interestingly, the presence of heparan sulfates on the cell surface had a negative impact on the activity of those polyplexes that were sensitive to decomplexation by heparin in solution. In summary, the screening approach identified two polymers, which form polyplexes with high stability and transfection capacity exceeding the one of poly(ethylene imine) also in the presence of serum.

1. Introduction

Polycationic polymers, either from natural or from synthetic origin, have been widely investigated as vectors for nucleic acid therapeutics, like DNA, siRNA or antisense oligonucleotides [1]. Prominent examples are the poly(ethylene imines) (PEI) [2] and polyamidoamides (PAA) [3] that have shown effectivity in vitro and in vivo. Complexation into so-called polyplexes occurs through electrostatic interactions with the negatively charged nucleic acids. In these complexes, oligonucleotides are protected from degradation. Furthermore, an excess of positive charge on the surface of the polyplexes is supposed to facilitate cellular uptake [4,5].

To guarantee an efficient delivery, polyplexes have to meet several requirements: Outside the cells effective condensation of oligonucleotides into monodisperse nanoparticles needs to take place. For in vivo applications, these polyplexes need to be stable in serum and free of hemolytic activity. In contact with cells, efficient induction of uptake has to occur.

According to the present model, cellular uptake involves the interaction of the positively charged polyplexes with the negatively charged glycocalyx. Along with charge, particle size is an important determinant for endocytic uptake. Ideally, polyplexes should have a size of 200 to 600 nm in order to show effective delivery [6–8]. Engagement of the glycocalyx constituent heparan sulfate (HS) has also directly been associated with the induction of endocytic cellular uptake [9,10]. However, given the capacity of negatively charged oligosaccharides to disassemble polyplexes [11], HS may also sequester the polyplexes on the cell membrane and displace the DNA from the complexes thereby compromising the uptake of oligonucleotides.

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Following endocytosis, endosomal escape is required to guarantee an appropriate delivery of the cargo into the cytoplasm and nucleus. Endosomal escape can occur through two main mechanisms. For membrane viruses and liposomes, release occurs by membrane fusion [12,13]. For cationic polymers, the "proton sponge effect" has been discussed as the main mode-of-action [14]. Briefly, protonable groups of tertiary amines with a mildly acidic pKa value such as imizadoles sequester protons, which counteract the endosomal acidification leading to further import of protons and chloride counterions that will cause an osmotic swelling and the subsequent rupture of the endo(lyso)somes [4]. Also decomposition of polyplexes leads to an increase in osmolarity [15]. Finally, in the cytoplasm release of the oligonucleotides has to occur.

To further improve internalization efficiency the functionalization of polyplexes with cell-penetrating peptides (CPPs) has been explored [16]. These peptides have been shown to facilitate cellular uptake of membrane impermeable macromolecules. Several classes of CPPs can be distinguished [17]. The well-studied CPPs Tat and nonaarginine belong to the class of arginine-rich CPP. For induction of cellular import arginine is a privileged structure as it can effectively interact with glycosaminoglycans on the cell surface and form bidentate hydrogen bonds with negatively charged molecules to partition into lipid bilayers [18].

To improve the transfection efficiency of polyplexes, significant efforts have been invested into the synthesis and screening of polymers that possess a better pharmacological profile. However, only a limited set of functionalities was tested so far. In contrast, for polypeptides, the role of a variety of amino acid side chains with respect to oligonucleotide complexation, membrane binding and intracellular trafficking has been explored. For example, complexation of negatively charged nucleic acids can be achieved by incorporation of lysines [19], while histidines can induce endosomal release [20]. Tryptophans can be introduced to promote interaction with the plasma membrane [21]. The solubility can further be modulated by tuning the ratio between hydrophilic and hydrophobic residues.

Recently, poly(methacrylate)s (PMAs) have been emerging as an alternative vector class in gene delivery, either alone [22,23] or as copolymers with PEI [24,25]. Their applications range from brain-targeted gene delivery [26] to intradermal administration [27]. Their accessible chemistry allows the synthesis of a variety of monomers which mimic the physicochemistry and functionality of amino acid side chains. Utilizing controlled radical polymerization methods (atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain-transfer (RAFT) polymerization [28]), polymers with tunable solubility, complexation capacity and transfection efficiency can be formed. Frequently, complexation with nucleic acids is granted by the presence of dimethylaminoethyl methacrylate (DMAEMA) or diethylaminoethyl methacrylate (DEAEMA) [22], which also act as a proton sponge and facilitate cytoplasmic delivery; in addition, endosomal escape is often achieved by the hydrophobic butyl methacrylate (BMA) [22,29], which is known to destabilize cellular membranes. In order to prolong the circulation time and to reduce cell toxicity, PEG monomers (PEG-methylether methacrylate, ethylene glycol dimethacrylate and PEG-DMAEMA) are inserted into the polymer backbone and act as a protective shell [26,30].

Thus, given the well-established polymerization chemistry and the capacity to vary the side chain, PMAs represent a highly interesting class of polymers to explore the structure space for oligonucleotide delivery.

The aim of this study was two-fold. First, we aimed at identifying the structure–activity relationship between the polymer structure and the physicochemical and biological properties for PMA copolymers with functionalities intended to cover major structural characteristics relevant to oligonucleotide complexation, cellular uptake and endosomal release. Second, we aimed to do so by establishing a screening strategy that would allow for a rapid and reliable selection of the most promising candidates for pre-clinical trials. Therefore, toxicity tests that can be performed in a high-throughput approach and that conventionally are only conducted at a later stage in testing for those polymers that show transfection activity were introduced early in the test protocol. Following an assessment of the capacity to form polyplexes with oligonucleotides, the resulting polyplexes were then tested for their stability in the presence of serum protein and polyanions, their hemolytic activity and their acute toxicity towards host cells. Finally, the activity in oligonucleotide delivery was determined. For this purpose we made use of a 2′-O-methylated (2-OMe)-oligonucleotide that induces splice correction of an aberrant primary luciferase gene transcript [31]. Moreover, the effect of the presence of HS on host cells was investigated. Using time-lapse confocal microscopy, we furthermore demonstrate that the most active polymer induces a wave of massive endosomal uptake followed by a dispersion of endosomal structures and endosomal release.

2. Materials and methods

2.1. Materials

N,*N*′-(dimethylamino)ethyl methacrylate (DMAEMA), oligo (ethyleneglycole) methacrylate (OEGMA), 2-cyano-2-propyl benzodithioate (CPDB), and 2,2′-azobis(2-isobutyronitrile) (AIBN) were purchased from Sigma-Aldrich. The monomers were passed over a column of inhibitor remover (Sigma-Aldrich) preliminary to the reaction. AIBN was recrystallized from methanol whereas CPDB was used without further purification. 2-(*N*-imidazol)ethyl methacrylate (ImEtMA) and but-3-ene-1-yl methacrylate (BEMA) were synthesized by conversion of the corresponding alcohol with methacrylic acid chloride in chloroform/triethylamine at 25 °C. An acetylated cysteinyl tetraarginyl amide CPP (Ac-CRRRR-NH_2) was purchased from EMC microcollections, Tuebingen, Germany.

Resazurin, heparin from porcine intestinal mucosa (average MW 5 kDa) and 25,000 g/mol branched PEI were obtained from Sigma-Aldrich, Zwijndrecht, NL. Peri-phosphorothioate Cy5-labeled 2′-OMe-ON-705 was purchased from Biolegio, Nijmegen, NL. A luciferase assay system was purchased from Promega, Leiden, NL. Rhodamine-labeled dextran (neutral, 10,000 Da) was obtained from Invitrogen, Bleiswijk, NL. Heparinase III was purchased from Ibex, Montreal, Canada. cDNA encoding for a Rab5-GFP protein was kindly donated by Dr. Sandra de Keijzer, Dept. of Tumour Immunology, Radboud University Medical Center, Nijmegen.

2.2. Methods

2.2.1. Polymer synthesis

The polymerization conditions are described for the typical example IM-1: In a microwave vial, 505 μL (471 mg, 3×10^{-3} mol) DMAEMA, 429 μL (463 mg, 9.75×10^{-4} mol) OEGMA, and 108 mg (6×10^{-4} mol) ImEtMA were mixed with 4.4 mg (2×10^{-5} mol) CPDB and 0.8 mg (5×10^{-6} mol) AIBN. Subsequently, 1.258 mL ethanol was added, and the vial was capped. The mixture was flushed with argon for 30 min to remove the oxygen and placed in an oil bath at 60 °C for 12 h. After the reaction, the polymer was precipitated into hexane and dried under vacuum. The polymers were analyzed by size exclusion chromatography (SEC) as well as ^{1}H NMR spectroscopy. The polymerization of the other polymers was performed in a similar manner using the corresponding amounts of each monomer (Table S1).

Polymer series IM + V-1-3 was functionalized with a cell-penetrating peptide via UV induced thiol-ene coupling reaction using 2,2-dimethoxy-2-phenylacetophenone (DMAP) as photoinitiator. The representative procedure is presented for polymer IM + CPP-1.

The polymers IM + V-1 (50 mg, 2×10^{-6} mol) and Ac-CRRRR-NH_2 (3 mg, 4×10^{-6} mol) were dissolved in 3 mL ethanol. 2 mg DMAP (8×10^{-6} mol) were added, and the whole mixture was flushed with argon for 30 min. The reaction solution was irradiated with UV light for 24 h. Afterwards, the solvent was evaporated and the residues