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Combining reactive triblock copolymers with functional cross-linkers: A versatile pathway to disulfide stabilized-polyplex libraries and their application as pDNA vaccines

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ABSTRACT

Therapeutic nucleic acids such as pDNA hold great promise for the treatment of multiple diseases. These therapeutic interventions are, however, compromised by the lack of efficient and safe non-viral delivery systems, which guarantee stability during blood circulation together with high transfection efficiency. To provide these desired properties within one system, we propose the use of reactive triblock copolypept(o)ides, which include a stealth-like block for efficient shielding, a hydrophobic block based on reactive disulfides for cross-linking and a cationic block for complexation of pDNA. After the complexation step, bifunctional cross-linkers can be employed to bio-reversibly stabilize derived polyplexes by disulfide bond formation and to introduce endosomolytic moieties at the same time. Cross-linked polyplexes show no aggregation in human blood serum. Upon cellular uptake and cleavage of disulfide bonds, the cross-linkers can interact with the endosomal membrane, leading to lysis and efficient endosomal translocation. In principal, the approach allows for the combination of one polymer with various different cross-linkers and thus enables the fast forward creation of a polyplex library.

Here, we provide a first insight into the potential of this concept and use a screening strategy to identify a lead candidate, which is able to transfect dendritic cells with a model DNA vaccine.

1. Introduction

The implementation of nucleic acids like plasmid DNA (pDNA), mRNA, siRNA and others as a new class of drugs promises to broaden the pharmaceutical repertoire from conventional direct interaction with accessible targets such as proteins and receptors to more sophisticated cellular manipulations *via* the transcriptional and translational machinery [1]. This strategy provides opportunity to make formerly "undruggable" therapeutic targets accessible. DNA vaccines in particular provide enormous potential, because in contrast to conventional vaccines, antigen and immune activator can be encoded on one plasmid and thus be combined on DNA level. In addition, the plasmid can contain a cell specific promotor, which prevents transcription in non-target cells [2].

The transfection of dendritic cells with DNA vaccines, however, is challenging and thus complex demands arise for non-viral vectors [3]. To find and optimize suitable systems, one needs to establish synthetic pathways allowing rapid synthesis and screening of vector compositions. While screening approaches are commonly used for low molecular weight lipid and lipoid formulations [4,5], these approaches are more challenging for block ionomer-based complexes (polyplexes) for systemic gene delivery. Various polymeric materials have been examined intensively for gene delivery application [1]. Yet, in most cases, optimization steps require the complete synthesis of new polymers [6–8], which might complicate efficient screening approaches.

Over the last decades, a series of researchers rationalized a chemical design principle for systemic pDNA delivery systems based on polyplexes, which can ensure efficient transfection *in vitro* and even in the more challenging *in vivo* environment [9–12]. To achieve these properties, core shell structures turned out to be beneficial and thus need to be

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generated during polyplex formulation [13]. The most common way to gain such structures is the self-assembly of pDNA and block ionomers. Block ionomers are block copolymers combining a neutral, hydrophilic and protein resistant block, *e.g.* polyethylene glycol (PEG) [14], polyhydroxypropyl methacrylamide (PHPMA) [15,16] or more recently, polysarcosine (PSar) [17–19] with a cationic block, which provides two functionalities. First, it interacts with the anionic nucleotides, forming a stable complex, thereby condensing the pDNA to a more compact structure, often with a diameter of half or a third of its original size. Second, the cationic block commonly provides endosomolytic properties or is functionalized with endosomolytic groups to further enhance endosomal escape, which is a main hurdle for an efficient transfection of cells [20]. Prominent examples for endosomolytic groups on the one hand include pH buffering secondary and tertiary amines – as found in PEI [21], histidine [22] and PAMAM [23,24] – acting through the hypothesized proton sponge effect triggered by the endosomal protonation and through cationic membrane interaction [20]. On the other hand, amphipathic motives, being the key component in many artificial or virus-derived fusogenic peptides, are well known to foster endosomal escape through hydrophobic membrane interaction [25,26].

Complementary to these basic prerequisites for transfection, several groups have demonstrated that *in vivo* performance of polyplexes can be improved significantly, if they are additionally stabilized by covalent cross-linking [11,27–30]. In this respect, cross-linking *via* disulfide bond formation seems particularly attractive, since it leads to redox-responsive stabilization, as disulfides can be cleaved by elevated concentrations of the natural reducing agent glutathione [31]. As the glutathione concentration differs by a factor of 50–1000 [31] between extra- and intracellular environments, Kataoka and *co*-workers could show that disulfide cross-linking of polyplexes provides stability in extracellular milieu and during blood circulation, while allowing efficient disintegration of polyplexes after cellular uptake [32–34].

Two different methodologies for the incorporation of disulfide bonds into polyplexes are reported in literature. The first strategy involves reaction of preformed polyplexes with amine-to-amine cross-linking reagents such as dimethyl 3,3′-dithiobispropionimidate (DTBP) [27,35] or dithiobis(succinimidyl propionate) (DSP) [36], which already contain a disulfide bond. The second strategy is based on the functionalization of polymers with disulfide or sulfhydryl groups before polyplex formation either by polymerizing thiol-containing monomers [29,37] or by polymer-analogous reaction [32], respectively. The latter procedure was performed by Kataoka and co-workers using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or 2-iminothiolane (Traut's reagent) to introduce thiols to a poly-L-lysine (PLys) segment of PEG-*b*-PLys block copolymers.

In conclusion, an ideal polymer for the formation of polyplexes should combine a shielding block based on a stealth-like material, a cationic segment for pDNA complexation, endosomolytic moieties for efficient endosomal translocation and thiols for bioreversible cross-linking. While these requirements seem to create a need for highly complex materials, we herein would like to introduce a design concept, which meets the aforementioned requirements by robust and scalable chemistry. Our concept is based on the use of reactive triblock copolypept(o)ides [19,38,39] which comprise a hydrophilic polysarcosine block for shielding of the cationic core, an activated thiol containing segment for asymmetric disulfide formation and a cationic polylysine block for complexation. After polyplex formation between triblock copolypept(o)ides and pDNA, bifunctional cross-linkers, containing endosomolytic moieties and two or more thiols, can simply be added for the disulfide formation. Thereafter, these endosomolytic cross-linkers provide polyplex stability in extracellular space, while fostering endosomal escape by proton sponge effect or membrane interaction after cellular uptake and being released from the polyplexes upon disulfide cleavage [40–45] (see Scheme 1).

The versatility of this approach allows control over charge density and degree of cross-linking, but most importantly, it enables the straightforward synthesis of polyplex libraries based on just one single triblock copolymer, which can be combined with an array of different cross-linkers. To validate this novel synthetic concept for cross-linked polyplexes, we report the generation, characterization and evaluation of a polyplex library in search for an efficient non-viral vector for pDNA vaccination.

2. Material and methods

2.1. Materials

DMF was purchased from Acros (99.8%, Extra Dry over Molecular Sieve) dried over BaO and distilled *in vacuo* followed by excessive stirring *in vacuo* to remove traces of dimethylamine. THF and hexane were purchased from Sigma-Aldrich and distilled from sodium. Triethylamine was purchased from Carl Roth and distilled from potassium hydroxide Other solvents were used as received. Diphosgene and chlorotrimethylsilane were purchased from Alfa Aesar and used as provided. Neopentylamine was purchased from TCI Europe, dried over NaOH and fractionally distilled. Sarcosine was purchased from Sigma-Aldrich, all other amino acids were purchased from ORPEGEN. Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Thermo Fisher Scientific.

2.2. Polymer characterization

400 MHz ^{1}H NMR spectra and ^{19}F NMR spectra were recorded on a Bruker Avance II 400. DOSY NMR measurements were performed on a Bruker Avance III HD 400. All spectra were recorded at room temperature and analyzed with MestReNova software. Degrees of polymerization as well as molecular weights were calculated by comparing the integral of the neopentylamine initiator peak either with the integrals of the side chain amide protons (for PLys(Boc) and PLys(TFA)) or with the average of integrals of methyl group and α-protons (for PSar) in the 400 MHz ^{1}H NMR spectra.

Attenuated total reflectance Fourier transformed infrared (ATR-FTIR) spectroscopy was performed on a FT/IR-4100 (JASCO) with an ATR sampling accessory (MIRacleTM, Pike Technologies). IR Spectra were analyzed using Spectra Manager 2.0 (JASCO).

Melting points were determined using a METTLER FP62 (METTLER WAAGEN GMBH).

Hexafluoroisopropanol (HFIP) gel permeation chromatography (GPC) was performed with HFIP containing 3 g/L potassium trifluoroacetate as eluent at 40 °C and a flow rate of 0.8 mL min^{-1}. The columns were packed with modified silica (PFG columns; particle size: 7 μm, porosity: 100 & 1000 Å). Polymers were detected with a refractive index detector (G 1362A RID, JASCO) and a UV/Vis Detector (UV-2075 Plus, JASCO). Molecular weights were calculated using a calibration performed with PMMA standards (Polymer Standards Services GmbH) and toluene as internal standard. Elution diagrams were analyzed using WinGPC UniChrome 8.00 (Build 994) software from Polymer Standards Services.

2.3. Synthesis of N-ε-tert-butyloxycarbonyl-L-lysine N-carboxyanhydride

9.8 g (39.8 mmol) of *N*-ε-Boc-protected lysine were weighed into a three-neck flask and dried under high vacuum overnight. The solid was suspended in 300 mL of absolute THF under a steady flow of dry nitrogen. Afterwards, 9.9 ml (79.6 mmol) of absolute trimethylsilylchloride and 11.0 ml (79.6 mmol) of absolute triethylamine were added slowly *via* syringe and the nitrogen stream was reduced. The suspension was stirred for two hours followed by addition of 4.8 ml (39.8 mmol) of diphosgene, again *via* syringe. The suspension was mildly refluxed for another 2 h. Overnight, a steady flow of dry nitrogen was lead through the solution into two gas washing bottles filled with