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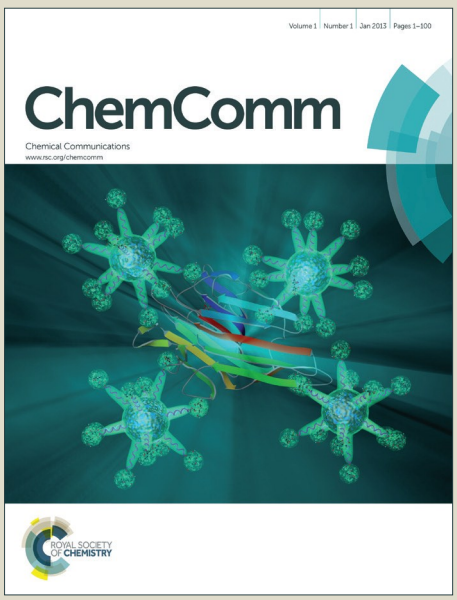

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PEG stabilized DNA - Poly(ferrocenylsilane) Polyplexes for Gene Delivery

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Polycationic poly(ferrocenylsilane)s (PFS) with tunable amounts of PEG side chains were used for the condensation of DNA into polyplexes of 110 nm in 5.0 mM HEPES. The PFS-PEG/DNA polyplexes showed negligible aggregation, a strongly reduced protein adsorption, transfection activities comparable with linear polyethyleneimine and an excellent cytocompatibility.

Gene therapy is a promising therapeutic modality for the treatment of many life-threatening and chronic diseases.[1, 2] In particular, non-viral gene delivery is attracting much attention due to its ability to overcome the immune and toxic reactions intrinsic to viral vectors, the straightforward low-cost synthesis involved and its versatility.[3-5]

Non-viral gene delivery includes approaches that rely on the use of positively charged lipids and polymers to bind and condense DNA. These cationic species spontaneously form complexes (referred to as lipoplexes and polyplexes, respectively[6]) when mixed with DNA, due to electrostatic interactions.[3] The complexation into nanoparticles is important to circumvent renal clearance, protect DNA against enzymatic degradation by nucleases and at the same time allow the DNA to pass through the endothelial layers of pathological tissues (the enhanced permeability and retention effect[7]) where they can enter the targeted cells by endocytosis.[8]

Although small size is in general considered favorable for delivery, interestingly, large, aggregated particles show in some cases a higher gene expression efficiency *in vitro* which was ascribed to the sedimentation of the particles onto the cells on the bottom of the well plates.[9] On the other hand, small singular polyplexes exhibited improved gene expression *in vivo*.[10] Comparing the toxicity of polyplexes and lipoplexes is non-trivial and it is dependent on the individual parameter sets of the specific systems. In a study of Kabanov *et al.* in which several lipid and polymeric gene delivery systems were evaluated with respect to their effectiveness and toxicity, polymeric systems had an efficiency higher than, or comparable to, lipid systems. These ensembles did not show serum dependence and were less toxic than the lipid based systems.[3]

An efficient way of reducing the cytotoxicity of polyplexes and increase their colloidal stability is PEGylation, which involves the attachment of poly(ethylene glycol) (PEG) chains through both covalent[11-15] or non-covalent[11] bonds. Pre-PEGylation, where preformed block or comb copolymers are employed for the complexation of DNA, is the most commonly used technique.[13, 14, 16] When a bifunctional PEG is used, the extremities of the PEG shell can be functionalized with targeting molecules.[12, 13, 16]

An interesting case of pre-PEGylation was shown by Zhong *et al.*[17] A low molecular weight triblock copolymer possessing a PEG middle block, PEI-PEG-PEI (4000-3000-4000 Da) was prepared, having a 3-fold higher transfection activity than linear polyethyleneimine (PEI) 25000. The polyplex conformation was proposed to have a flower-like structure, with PEI in the core condensing the DNA and PEG loops on the outside, shielding the surface positive surface charge of the polyplexes.

Post-PEGylation is used to ensure unhindered DNA complexation. The polyplexes are preformed, after which the PEG chains are reacted with the particle surface to create a protective shell.[10, 11, 15] In this way, it can be assured that targeting moieties are attached onto the complexes at the distal ends of the PEG shell, making them more accessible for their receptors.[12, 13] However, clinical application of post-PEGylation procedures may be hindered by the elaborate synthetic work needed to prepare the polyplexes.[10, 18]

In view of these drawbacks of post-PEGylation, efforts have been made to develop a pre-made mixture of condensing,

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shielding and targeting agents for the direct preparation of polyplexes. These polyplexes were also used in transfection experiments after freezing and thawing, to test storage possibilities. Freeze-thawed and pre-made mixtures were both successfully applied for transfection, yielding efficiencies higher than their linear PEI (22 kDa) control polyplexes.[18]

To improve biodegradability, disulfide bonds, which are cleavable by intracellular reduction, can be used for the attachment of PEG.[18, 19] Alternatively, pH-sensitive hydrazone linkages can be used, which can be broken in endosomal environment, pH 5.[20]

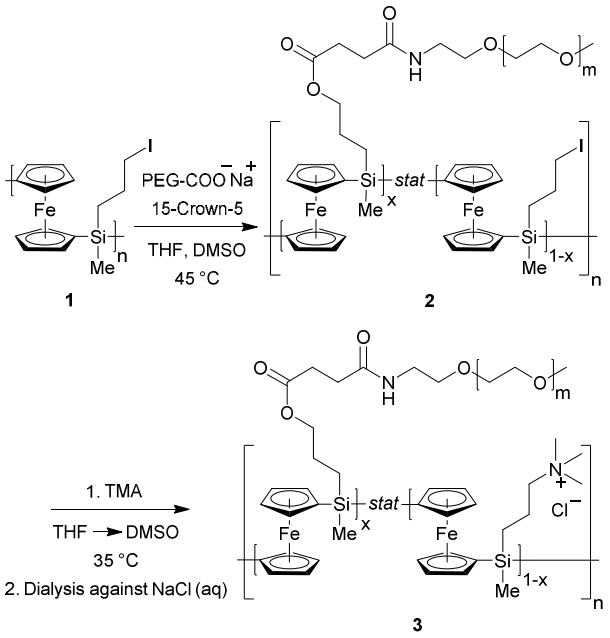
In addition to reducing cytotoxicity, PEGylation can increase colloidal stability and prevent aggregation under physiological conditions.[10, 11, 17, 21, 22] The hydrophilic polymer shell reduces protein interaction and activation of the complement system (a part of the immune system that helps to clear pathogens from an organism), thereby increasing the circulation time of the polyplexes.[15, 22] Furthermore, the transfection efficiency of the polyplexes was reported to remain comparable or even to improve.[17, 21, 23]

Recently, redox-active alkyl-ferrocene modified branched PEI was investigated as transfection agent. Polyplexes based on this polymer showed improved transfection efficiencies when compared to branched PEI (25 kDa) and Lipofectamine 2000. Oxidation of the ferrocene moieties increased the size and the zeta potential of the polyplexes and drastically reduced the gene transfection capabilities of the polyplexes.[24]

Organometallic polymers have drawn interest by their interactions with DNA.[25] Recently it was shown that the positively charged oxidized metal center of this kind of polymers could interact with the negatively charged phosphate groups of DNA.[26] In this case, efforts were focused on the structural replication of the anionic chiral template by poly(cobaltoceniumethylene).

An interesting class of versatile and inherently redox-active organometallic polymers encompasses poly(ferrocenylsilane)s (PFS).[27-29] Water-soluble cationic poly(ferrocenylsilane)s were shown to be effective DNA condensation and transfection agents by Zhong *et al.*, who showed a higher transfection efficiency compared to pDMAEMA polyplexes.[30] However, significant agglomeration of the polyplexes was observed under physiological conditions, which limits their applicability *in vivo*. Similar polymers where used for the redox triggered release of Nile Red and paclitaxel.[31] By introducing about 25 % of decyl hydrocarbon chains to the cationic polymer, it formed micellar assemblies of 100 nm which could be loaded with the aforementioned payloads. By varying the concentration of redox agents, control of the payload release profile was demonstrated. In addition, a significantly higher transfection efficiency compared to the non-micellar polymer of Zhong *et al.* was observed. In this study we address the transfection efficiency, stability and protein adsorption of polyplexes formed from PEGylated cationic PFSs and DNA.

A polycationic poly(ferrocenylsilane) with tunable amounts of PEG side chains was synthesized by side group modification of PFS **1** (Scheme 1). To introduce PEG side chains, PFS **1** was reacted with the sodium salt of monocarboxylic acid functional



Scheme 1. Synthesis of poly(ferrocenylsilane) polycations with PEG side chains.

PEG (750 Da). Successful substitution was confirmed by NMR and FTIR analysis (Figure S1, S2 and S3, Supplementary Information). Next to the characteristic peaks for PFS, the methoxy end group signal of the grafted PEG sidechains was present in the ^{1}H NMR spectra at δ = 3.24 ppm and in the ^{13}C NMR spectra at δ = 58.04 ppm. Furthermore, the signal associated with the oxyethylene groups of PEG is present in the ^{1}H NMR spectra at δ = 3.51 ppm and in the ^{13}C NMR spectra at δ = 69.76 ppm. Also, the peak of the methyl groups present in the cationic NMe_3Cl units of polymer is clearly visible in the ^{1}H NMR spectra at δ = 3.11 ppm and in the ^{13}C NMR spectra at δ = 52.00 ppm.

The targeted degrees of substitution (namely 10 and 25 mol%) were reached, as was confirmed by comparing the CH_3-Si signals of the PFS main chain with the CH_3-O signal of PEG in the ^{1}H NMR spectrum.

Subsequently, positively charged moieties were introduced by amination of the iodopropyl side groups of PFS **2** using trimethylamine. Exchange of the iodide to chloride counterions further improved the water-solubility of the polycations. FTIR confirmed the expected functional groups; 1700 cm^{-1} (carbonyl, PEG), 1632 and 1479 cm^{-1} (–NMe_3Cl), 1111 cm^{-1} (PEG), 1035 and 774 cm^{-1} (PFS) (Figure S3, Supplementary Information). GPC data showed that the organometallic main chains remained intact when the PEG side chains were attached (Table S4, Supplementary Information). Introduction of ammonium groups to PFS main chains was previously demonstrated to proceed without molar mass decline.[32]

Polymer/DNA complexes were prepared by mixing PFS **3** with pCMV-GFP reporter plasmid with amine to phosphate (N/P) ratios ranging from 1–8 (mol/mol ratio) in a 5.0 mM HEPES

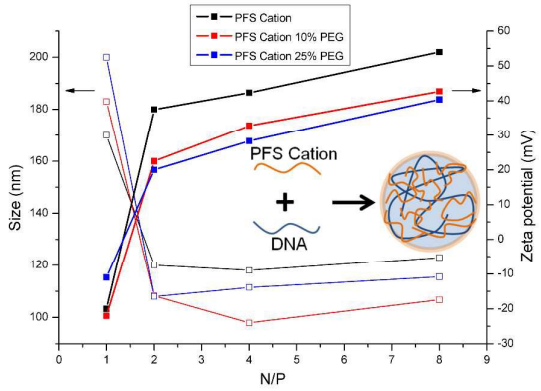


Figure 1. Size and zeta potential of PFS/DNA polyplexes as a function of the N/P (mol/mol ratio) as measured by DLS in 5.0 mM HEPES buffer. The concentration was fixed at 100 µg/ml and the incubation time was 30 minutes.

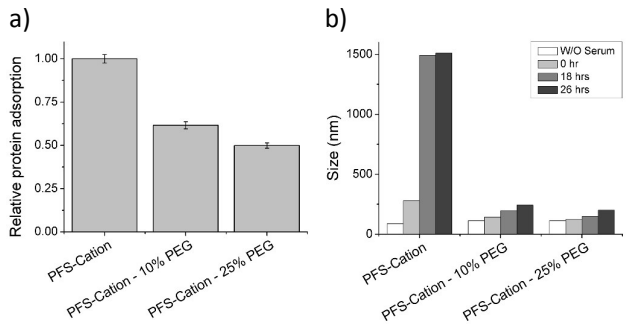


Figure 2. a) Relative protein adsorption (the protein adsorption for non-PEGylated PFS polyplexes was set to 1), determined by Surface Plasmon Resonance (SPR), and b) colloidal stability of polyplexes with different degrees of PEGylation (N/P = 4).

buffer solution at pH 7.4. Increasing the ratio of amine (PFS cation) to phosphate (DNA) to 2 or higher resulted in condensation of PFS **3** and DNA into polyplex particles as shown by an agarose gel retardation assay (Figure S5, Supplementary Information) and confirmed by DLS, displaying diameters of about 110 nm (Figure 1). AFM measurements on polyplexes with N/P ratio 4 deposited on silicon substrates showed similar sizes (Figure S6, Supplementary Information) although the measured height was significantly lower with respect to the diameter, suggesting a collapse of the particles upon drying. Dynamic light scattering (DLS) measurements in 5.0 mM HEPES buffer at pH 7.4 gave PFS/DNA polyplex diameters of about 110 nm for the PEGylated polyplexes and 120 nm for the non-PEGylated polyplexes. Upon condensation of DNA at an N/P ratio of 2, the zeta potential switched from negative (-10 to -20 mV) to positive (+40 to +55 mV), indicating the inclusion of DNA inside the PFS polycations (Figure 1).

The PEG side chains were used to reduce protein adsorption on the polyplexes. Next to shielding the positive core from the negatively charged proteins, these PEG side chains also introduce steric hindrance, stabilizing the polyplexes. Even though protein adsorption, measured by SPR, was still apparent after PEGylation of the PFS for polyplexes, it significantly decreased compared with the non-PEGylated PFS polyplexes (Figure 2A, the protein adsorption for non-PEGylated PFS polyplexes was set to 1). The same trend in BSA protein adsorption was found by Crielaard *et al.* for non-PEGylated versus PEGylated liposomes.[33] However, upon PEGylation, the colloidal stability increased significantly (Figure 2B).

Transfection activity and cytocompatibility of the PFS/DNA polyplexes were tested and compared with polyplexes based on linear PEI (25 kDa). The N/P ratio was set to the optimum values obtained from previous experiments (results not reported); for PFS 6/1 and for linear PEI 8/1 was used with 150 ng/mL pCMV-GFP reporter plasmid. The experiments were performed in medium with 0 %, 5 % and 10 % of serum. The results of transfection and cell viability assays are summarized in Figure 3 (Fluorescence microscopy images are provided in Figure S7, Supplementary Information).

Among the tested polymers, unmodified PFS cation has the highest efficiency regardless of the serum content. Interestingly, even in 10 % serum, the unmodified PFS cation has high transfection efficiency which shows the superior efficiency of PFS in cell transfection *in vitro*, regardless of the protein adsorption and agglomeration of the particles. These relatively high transfection efficiencies can be explained by sedimentation of the particles onto the cells on the bottom of the well plates.[9, 34] This is supported by the lower transfection efficiency of the PEGylated cationic PFS polyplexes which have an only slightly lower zeta potential, important for polyplex-cell membrane interactions,[35, 36] but a significantly reduced agglomeration.

Although the transfection efficiency decreased by PEGylation, it increased the cell viability during transfection in serum. By increasing the serum concentration, the cell viability decreased for the unmodified PFS cation, while for the PEGylated cations, the cell viability increased. This can be explained by the aggregation of the unmodified cationic PFS polyplexes (Figure 2). These charged aggregates interact with negatively charged cell membranes resulting in cell permeabilization and finally in cell death.

When comparing PFS at a higher degree of PEG grafting, with linear PEI, the cell viability and transfection efficiency for PFS polyplexes is comparable or higher (Figure 3). In combination with the facile side group modification of PFS, desired membrane disruptive peptides or targeting units could be incorporated during the synthesis, which is a promising future strategy to increase the transfection activity of the polyplexes and to render them cell specific.[37, 38]

In this study, it was shown that cationic PFS is an interesting polymer for transfection studies. The cationic PFS polyplexes were stabilized by short PEG side chains increasing their colloidal stability and reducing protein adsorption onto their surface. These PEGylated polyplexes did not compromise, in contrast to e.g. PEI-bases formulations, the cell vialibility. Even though the transfection efficiency of the PFS-polyplexes was

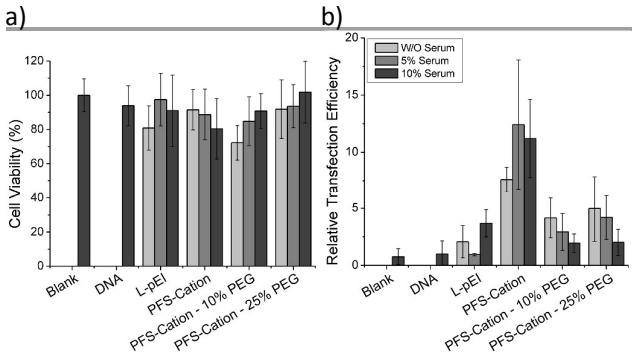


Figure 3. a) Cell viability and b) relative transfection efficiency (the transfection efficiency of bare DNA was set to 1) of polyplexes with different degrees of PEGylation, N/P (mol/mol) ratios were for PFS 6/1 and for linear PEI 8/1.

reduced by PEGylation, the efficiency of the PEGylated PFS polyplexes remained comparable to non-PEGylated linear PEI polyplexes. These results show the opportunity to prepare tailor-made cationic polyplexes based on the versatility of cationic PFS.

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