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# Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: In vitro characterization and in vivo studies in mice

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**Abstract**

Crosslinked poly(ethylene imine) (PEI) polyplexes for intracellular DNA release were generated using a low molecular weight crosslinking reagent, Dithiobis(succinimidyl propionate) (DSP). Disulfide bonds of the crosslinked polyplexes were susceptible to intracellular redox conditions and DNA release was observed using an ethidium bromide exclusion assay and dynamic light scattering. Transfection experiments were performed to elucidate the effect of extra- and intracellular redox conditions. Pharmacokinetics and organ accumulation of uncrosslinked and crosslinked polyplexes were compared and gene expression patterns were measured in mice 24 h after intravenous injection.

Crosslinked PEI and plasmid DNA formed stable polyplexes in a size range of 100–300 nm, with zeta potentials between +16.4 and +26.1 mV. DNA release occurred after cleavage of the disulfide bonds. Cell culture experiments under reducing conditions as well as with glutathione loaded cells confirmed the proposed intracellular activation. A significant influence of the intracellular glutathione status on the transfection efficiency was observed.

Pharmacokinetic profiles of crosslinked PEI/DNA polyplexes in mice after intravenous administration showed higher blood levels for crosslinked polyplexes. These polyplexes accumulated mainly in the liver and the lungs. In vivo transfection data revealed significantly reduced (unwanted) lung transfection while liver transfection predominated. These studies suggest that crosslinked polyplexes are more stable in circulation and retain their transfection efficiency after intravenous administration.


*Keywords:* Poly(ethylene imine); Polyplex; Pharmacokinetics; Biodistribution; In vivo transfection

## 1. Introduction

Gene therapy using non-viral vectors has received significant interest over the last decade. Despite the current interest in cationic polymer based gene delivery systems, stability of these vectors in the biological milieu as well as their circulation times after intravenous administration need to be improved significantly to allow therapeutic application [1,2]. While local administration of nucleic acids has been successfully applied in the treatment of bladder cancer [3], systemic application of positively charged polyplexes is limited by their rapid clearance from the bloodstream following intravenous injection [4–7]. In fact, most of these vectors are cleared in less than 10 min by first pass organs such as the liver or the spleen [1].

Enhanced stability of polyplexes in blood circulation is thought to be an important prerequisite for successful systemic gene delivery. Also, gene delivery systems sensitive to intracellular trigger mechanisms have been subject of intensive scrutiny as they would allow formation of polyplexes stable in circulation that dissociate after intracellular uptake [8]. Various trigger mechanisms, such as sensitivity to pH changes [9,10], temperature [11], enzymatic lability to lysosomal enzymes [12] or redox conditions [13,14] have been put forward in this context [15].

Redox sensitive vector systems rely on the higher intracellular reduction capacity compared to the extracellular milieu

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[16]. Natural cationic polymers playing a role in DNA compaction, such as protamines, were reported to be stable at high ionic strengths due to the presence of disulfide linkages [17]. Thus, a redox-triggered destabilization mechanism could be a promising strategy to design synthetic non-viral vectors with engineered intracellular release characteristic. Polyplexes, that are known to be taken up by endocytic pathways [18], may undergo disulfide cleavage in the lysosomal compartments [19]. It is reasonable to assume that the glutathione pathway, which controls the intracellular redox environment [20], is significantly involved in this trigger mechanism. Large differences in the glutathione (GSH) concentration can be found in different tissues (ranging from 0.5 to 11 mM in liver cells [20]) as well as between the intracellular and extracellular environment (in the cytosol about 1000 fold higher and in the nucleus approximately the same or greater than in the cytosol [16]). This difference in glutathione concentrations could potentially be exploited as a trigger mechanism. Moreover, cancer cells resistant to apoptosis or to cisplatin seem to show a higher activity of glutathione-*S*-transferase (GST) and hence higher glutathione levels, thus displaying increased reduction capacity [21,22].

Polylysine (PLL) crosslinked by disulfide bonds was tested in the context of redox-triggered intracellular DNA release and showed reduced transfection efficiency probably due to a failure of endosomal escape [23]. Therefore, we employed PEI as polycation since it possesses an intrinsic endosomal release capacity, known as the "proton sponge effect" [24,25]. Additionally, polyplex stability, protection of the DNA against DNase I, and in vivo transfection efficiency of branched PEI have been reported to exceed those of PLL [26,27].

Tumor tissue is known to be characterized by enhanced vascular permeability and impaired lymphatic clearance [28]. Thus, long circulating vectors are thought to accumulate in tumors due to the EPR effect [1,29]. A combination of enhanced circulation times due to surface stabilization and redox-triggered intracellular release was believed to be a promising way for intravenous plasmid delivery. Hydrophilic copolymers are known to increase circulation times of polyplexes by steric shielding, thereby reducing unwanted interaction with endothelia or blood compounds, such as erythrocytes and proteins [30]. Redox sensitive polyplexes were obtained by coupling Poly(ethylene glycol) (PEG) or Poly[*N*-(2-hydroxypropyl) methacrylamide] (PHMPA) to polycations. Redox sensitive PEGylated PLL polyplexes showed enhanced blood levels in mice, but the effect could be predominantly attributed to PEG [23]. Similarly, no control measurements without PEG were reported for thiolated PEG–PLL showing liver expression in mice [31]. Polyplexes formed with thiolated PEI followed by PHPMA coating enhanced the stability of the polyplexes against polyanion displacement, and plasmid release could be triggered under reducing conditions. An addition of free PEI to polyplexes was necessary to obtain transfection under in vitro conditions and no in vivo data were reported [32].

PEI polyplex stabilization using disulfide bonds without additional steric stabilization with hydrophilic copolymers was investigated systematically utilizing a low molecular weight crosslinking reagent, Dithiobis(succinimidyl propionate) (DSP), as redox sensitive moiety (Scheme 1). This procedure avoids drawbacks of a two-step crosslinking procedure, i.e. initially introducing thiol groups which were subsequently oxidized or crosslinked via thiol reactive copolymers [32,33]. High molecular weight poly(ethylene imine) (PEI 25 kDa) was used to complex plasmid DNA, followed by surface stabilization with DSP by crosslinking the primary amines in PEI. Successful surface crosslinking of such polyplexes was recently described from our group [34]. At the same time, potentially cleavable disulfide bridges are incorporated by DSP to allow the release of the DNA. Polyplexes were tested for their sensitivity to reducing environment and the factors influencing DNA release. Pharmacokinetic parameters, biodistribution of the plasmid, and the transfection efficiency in mice were studied after intravenous injection and related to the crosslinking degree of the polyplexes.

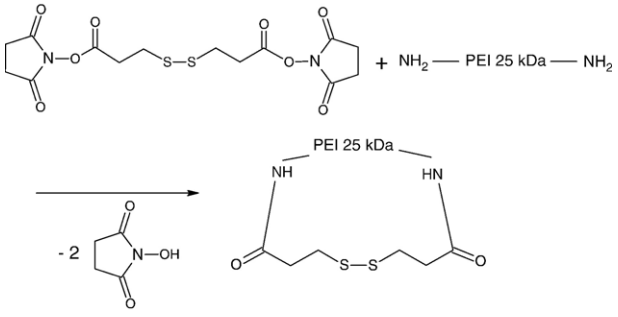
## 2. Experimental section

### 2.1. Materials

*Polymers*: poly(ethylene imine) (25 kDa, HMW PEI) was a gift from BASF, Ludwigshafen, Germany. *DNA*: the plasmid pCMV-GL3 encoding the firefly luciferase gene was amplified in JM-109 competent cells and purified using a commercial kit (Qiagen Hilden, Germany). pCMV-Luc plasmid was purchased from PlasmidFactory (Bielefeld, Germany). Salmon testes DNA (Sigma, Taufkirchen, Germany) was used for light scattering experiments. Dithiobis(succinimidyl propionate) (DSP), water free dimethylsulfoxide (DMSO) HPLC grade, glutathione-monoethyl ester (GSHMEE) and D,L-buthionine-[*S*,*R*]-sulfoximine (BSO) were purchased from Sigma, Taufkirchen, Germany. All other reagents used were of analytical grade.

### 2.2. Formation of PEI/DNA polyplexes and crosslinking

Polyplex formation was performed according to a recently reported procedure [35]. Luciferase reporter gene plasmids (pCMV-GL3 or pCMV-Luc) and the appropriate amounts of PEI 25 kDa were dissolved separately in low ionic strength buffer (5% glucose/25 mM HEPES at pH 7.5), mixed by vigorous pipetting and incubated for 10 min to allow polyplex




Scheme 1. Reaction scheme for the conversion of PEI primary amines with Dithiobis(succinimidyl propionate) (DSP). The resulting amide bonds lead to a reduction of protonable primary amines of the polymer.