CHARACTERIZATION OF POLYMER VECTOR SYSTEMS BASED ON PARTIALLY HYDROLYZED POLYOXAZOLINE FOR GENE TRANSFECTION

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Abstract: In this work, partially hydrolyzed thermoresponsive polyoxazoline was used for complexation with DNA. The resulting polyelectrolyte complex particles were characterized by dynamic and electrophoretic light scattering. They showed narrow size distribution and hydrodynamic diameter of 183 ±5 nm at 65 °C. To improve stability of the system at physiological temperature, the particles were coated with cross-linked polymer shell on their surface. A cytotoxicity study indicated lower toxicity of the investigated systems compared to the referent polyethylenimine. In addition, the transfection ability of the resulting vector systems was evaluated by flow cytometry. Transfection efficiencies up to 65 % that of the referent polyethylenimine indicated the potential of the vectors for DNA delivery.

Key words: gene therapy; gene delivery; polymer vectors; polyethylenimine; polyoxazolines

Introduction

Gene therapy is an approach for treating of patients with inherited or acquired diseases through the introduction of therapeutic genes in the nuclei of pathologically altered cells [1,2]. A critical parameter for effective gene therapy is the choice of an appropriate vector for transfection. In the recent years polymer vectors are of great interest because they are less hazardous, less pathogenic, and less immunogenic than the viral ones [3-6]. Typically, the negatively charged DNA interacts electrostatically with positively charged polymers thus forming nano- or microsized particles called *polyplexes* [7,8]. A wide variety of cationic polymers have been investigated to compact and deliver DNA molecules [8,9]. Among them polyethylenimine (PEI) has been found to exhibit the highest transfection efficiency and, accordingly, has been referred to as the "gold standard" [10,11]. The high efficiency of PEI, however, is compromised by its cytotoxicity [12,13]. Therefore, the search and investigations of polymer vectors that combine high efficiency and low toxicity is an active research area.

Poly(2-oxazoline)s (POx) are biocompatible pseudo-polypeptides that have received significant attention for biomedical applications in recent years [14,15]. They are known as thermoresponsive polymers that are able to undergo a reversible soluble-to-insoluble state transition in response to small changes in temperature [14,15]. Furthermore, POx are widely used as precursors for the synthesis of PEI through its hydrolysis [16]. Combining the properties of PEI and POx, a robust vector system for gene delivery can be designed. In this work, we used partially hydrolyzed POx for complexation with DNA. The resulting nanosized complexes were characterized by dynamic and electrophoretic light scattering. They were stabilized by creating a cross-linked polymer shell on their surface. A cytotoxicity investigation of the systems was carried out on a spectrum of human cell lines. The transfection ability of the resulting vector systems has been evaluated by flow cytometry.

Materials and Methods

Materials

All reagents and solvents were purchased from Sigma-Aldrich. The solvents were purified by standard procedures. Plasmid DNA containing the gene encoding for the enhanced green fluorescent reporter protein pEGFP-N1 (4730 bp) was isolated from glycerin culture E. coli DH5 alpha. For the preparation of the polyplexes a stock solution of 50 mg

mL^{-1} in ultra-pure water (>18 MU) was used. The poly(2-propyl-2-oxazoline)-poly(ethylenimine) (POx-PEI) copolymer was prepared by controlled partial hydrolysis of POx. The hydrolysis was performed in 6M HCl at 100° C and was determined via ^1H NMR spectroscopy. The molecular characteristics of polymer are as follows: M_n = 34000 $g.mol^{-1}$, Đ=1.2, PEI content =9 %.

Cell lines and culture conditions

The cell lines HL-60 (acute promyelocyte leukemia), RPMI-8226 (multiple myeloma), EJ (bladder cancer), REH (acute lymphocytic leukemia) and HEK-293 (human embryonic kidney cells) were cultured routinely in a controlled environment: 37°C in 5% CO_2 humidified atmosphere and were maintained in RPMI-1640 medium, supplemented with 2 mM L-glutamine and 10% fetal calf serum.

Methods

Preparation of polyplexes

The polyplex was formed by drop-wise addition of DNA aqueous solution (50 $\mu g.mL^{-1}$) into copolymer micellar solution (0.5 $mg.mL^{-1}$) at 65 °C under vigorous stirring. The polyplex was prepared in amine to phosphate groups ratios (N/P) of 4.

Coating procedure

To form a cross-linked polymer shell around the polyplex particles, seeded radical polymerization of N-isopropylacrylamide (NIPAM) was used. The polyplex solution was heated to 65 °C under nitrogen atmosphere and vigorous stirring. NIPAM (5 $mmol.L^{-1}$) and N,N-methylenbis(acrylamide) (BIS) as a cross-linker (0.26 $mmol.L^{-1}$) were dissolved in water and the mixture was added into the polyplex solution. After 30 min, α,α-azodiisobutyramidine (1 $mmol.L^{-1}$) was introduced to initiate the copolymerization of NIPAM and BIS. The cross-linking reaction was allowed to proceed for 5 h at 70 °C.

Cytotoxicity assay

The cellular viability was assessed using the MTT-dye reduction assay as described by Mosmann [17] with slight modifications [18]. The assay is based on the metabolic transformation of the yellow tetrazolium dye MTT to a violet formazan product via the mitochondrial succinate dehydrogenase in viable cells. In brief, exponentially proliferating cells were seeded in 96-well flat-bottomed microplates (100 µl/well) at a density of 1×10^5 cells per ml and after 24h incubation at 37°C they were exposed to various concentrations of the tested compounds for 72h. For each concentration a set of at least 8 wells were used. After the treatment period 10µl MTT solution (10mg/ml in PBS) aliquots were added to each well. Thereafter the microplates were incubated for 4h at 37°C and the MTT-formazan crystals formed were dissolved through addition of 100 µl/well 5% formic acid solution in 2-propanol. The MTT-formazan absorption was measured using Beckman-Coulter DTX800 multimode microplate reader at 580 nm. Cell survival fractions were calculated as percentage of the untreated control. In addition, IC_{50} values were derived from the concentration-response curves.

Transfection experiments

The transfection efficacy of the tested POx based polyplexes with a plasmid DNA encoding the synthesis of fluorescent protein, was monitored by flow cytometry. For this purpose, human cell cultures with different type and origin namely, REH (acute myeloid leukemia) and non-malignant HEK-293 (human embrional kidney cells) were treated with the tested polyplexes at plasmid concentration of 100 ng/ml for 24 h at 37°C. As a positive control PEI based polyplexes were used. At the end of the incubation period the cells were pelleted after centrifugation, washed twice with ice cold phosphate buffer and resuspended in 500 µl phosphate buffer. So prepared cell suspensions were analyzed by flow cytometer FACSCantoII in order to demonstrate the synthesis of fluorescent protein and the respective transfection. For each sample at least 20 000 cells were analyzed. The fluorescence of the cells treated with the tested POx polyplexes is expressed as % relative fluorescence compered to the fluorescence of cell treated with referent PEI polyplexes set as 100 %.

Dynamic and electrophoretic light scattering

The measurements were carried out at 65, 37 and 25ºC on a Zetasizer Nano-ZS instrument (Malvern Instruments), equipped with a He-Ne laser (λ = 633 nm) and a non-invasive back scatter system, allowing measurements of the scattered light at a scattering angle of 173º.

Results and Discussion

Preparation of polyplexes

Poly(2-alkyl-2-oxazoline)s are thermoresponsive polymers that exhibit a phase transition temperature in the 24-70 °C range, depending on their molar mass as well as the length of the alkyl side chain [14,15,19]. The POx-PEI copolymer used in

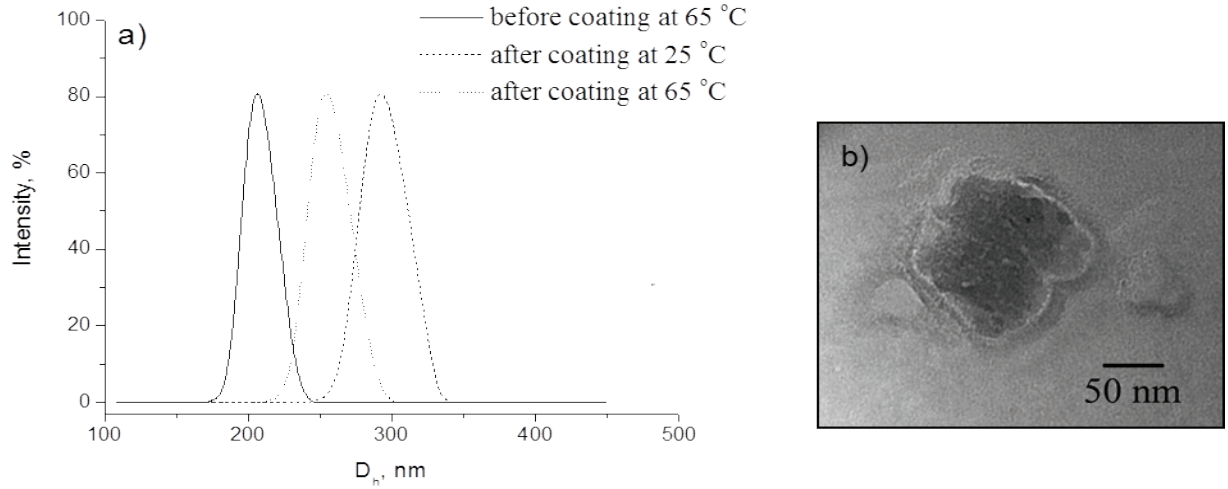

Figure 1. Size distributions (a) and TEM micrograph (b) of polyplex formed from POx-PEI polymer and DNA at N/P ratio of 4 coated with crosslinked PNIPAM shell.

this study displays a phase transition temperature around 30 °C. The complexation with DNA at N/P = 4 was carried out at 65 °C, that is, well above the transition temperature. As seen from Figure 1a, the polyplex particles are characterized with narrow size distribution and hydrodynamic diameter of 183 ± 5 nm at 65 °C (solid line). As expected, the ζ potential of the polyplex was positive due to the excess of copolymer. However, upon decreasing temperature to below 30 °C a large instability in the system was introduced. It was observed as widening of particle size distribution, substantial increase in dimensions (D_h>800 nm), and dramatic altering of the ζ potential to a negative value (-1.9 mV).

To stabilize the particles, a cross-linked polymeric shell was constructed on the polyplex surface. The shell was formed by seeded radical copolymerization of NIPAM and N,N-methylenbisacrylamide-used as a cross-linking agent initiated by α,α-azodiisobutyroamidine. Based on previous experience [20-22], optimal reaction conditions were selected with regard to the reaction efficiency and reproducibility of results. Shell thickness of about 30 nm was obtained by controlling the initiator/monomer molar ratio. An indication for successful construction of PNIPAM shell was the slight but noticeable increase in particle size. As evident from Figure 1a (dash line), the hydrodynamic diameter of polyplexes at 65 °C after coating was 250 nm. The resulting thickness of the polymer shell was 33.5 nm, calculated from the difference in hydrodynamic dimensions of the particles before and after coating, was in good agreement with the theoretical value. The stabilization effect of the cross-linked polymer shell was immediately seen upon decreasing temperature (Figure 1a, dot line): the size dispersity was not affected and only slight increase in particle dimensions due to swelling of the shell was detected. The morphology of the resulting vector system was visualized by TEM. A representative micrograph of polyplex coated with cross-linked PNIPAM shell is shown in Figure 1b: a feature of the particles is their irregular spherical shape with a distinctive well-defined shell.

Cytotoxicity study

The cytotoxicity of partially hydrolyzed thermoresponsive polyoxaziline and its non-coated or coated polyplexes with plasmid DNA was evaluated in a panel of human tumor cell lines with distinct cell types and origin, namely HL-60 (acute promyelocyte leukemia), RPMI-8226 (multiple myeloma) and EJ (bladder cancer) and compared with cytotoxicity of the referent PEI. Following 72 h exposure, the POx-PEI copolymer exerted prominent concentration-dependent decrease of the cellular viability in the tested cell lines, causing considerable eradication of viable cells at concentrations exceeding 40 μM (Figure 2a). The equieffective IC_{50} values were derived using non-linear regression (curve-fit) analysis and are shown in Figure 2b. It is noteworthy that in comparison with the referent PEI, POx is characterized with lower cytotoxic potential. Thus, the POx-PEI copolymer had ca 20 % higher IC_{50} values in HL-60 and RPMI-8226 cells, as compared to the PEI.

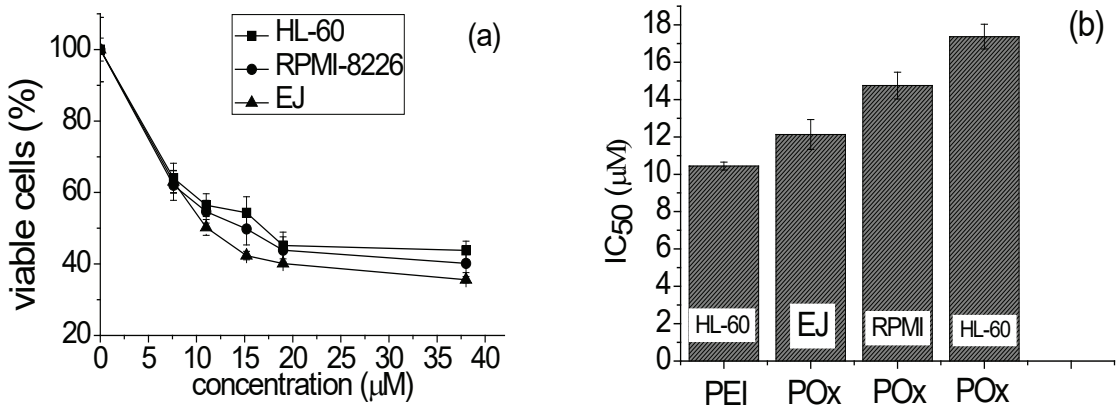

Figure 2. Cytotoxic activity (a) and IC50 values (b) of POx and PEI polymers against a panel of human cell lines: HL-60; RPMI-8226; EJ after 72 h exposure. Data represent the arithmetic mean ± sd from 8 independent experiments.

The study of cytotoxicity of free copolymers was followed by monitoring the cytotoxic potential of the derived non-coated and coated polyplexes with DNA. Concentration-effect curves of are shown on Figure 3.

As evident from the results, the formation of complexes with the DNA is followed by a considerable reduction of cytotoxicity, thus, even at the highest tested concentrations, the inhibition of cell proliferation was less than 30% as compared to the untreated control. The coating of polyplexes with PNIPAM is accompanied by additional reduction in their cytotoxicity potential. After 72 hours of treatment with the coated polyplexes the inhibition of cell viability was less than 20% compared to cells of the control group.

Transfection study

The transfection efficacy of the prepared vector systems was evaluated on two human cell lines non-malignant HEK-293 (human kidney embryonal cells) and malignant REH (acute leukemia) by flow cytometry. Branched PEI was used as a standard transfection agent. Figure 4 shows the percentages of the transfected cells after treatment with the test polyplexes either non-coated or coated with PNIPAM shell. Evident from the presented results, the non-coated polyplexes showed app. 65% transfection efficiency as compared with referent PEI vector. In contrast, the coated counterparts are characterized with lower transfection efficacy - about 45% as compared with PEI vector. Probably the lower transfection efficiency is due to the polymer shell, requiring a longer time for degradation and internalization into the cells. However, the results clearly show that despite their lower transfection the coated polyplexes are potential vectors for successful transfer of pEGFP-N1 into HEK 293 and REH cells.

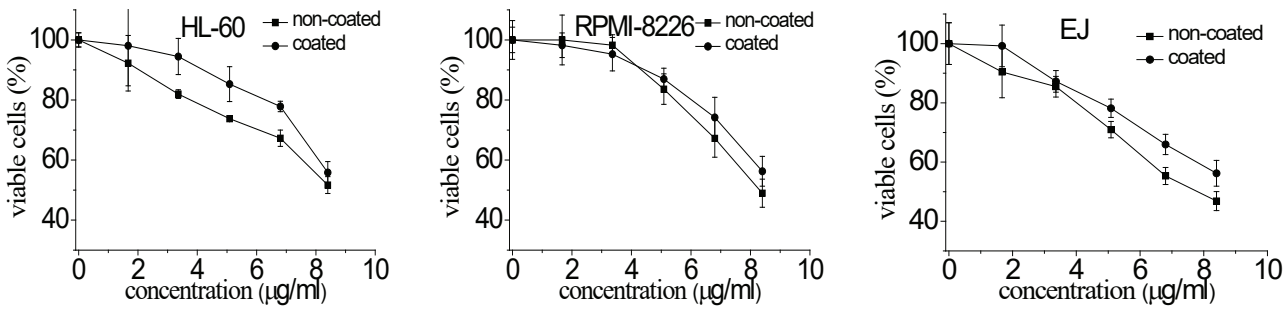

Figure 3. Cytotoxic activity of non-coated and coated POx-PEI polyplexes against a panel of human cell lines: HL-60; RPMI-8226; EJ after 72 h exposure. Data represent the arithmetic mean ± sd from 8 independent experiments.

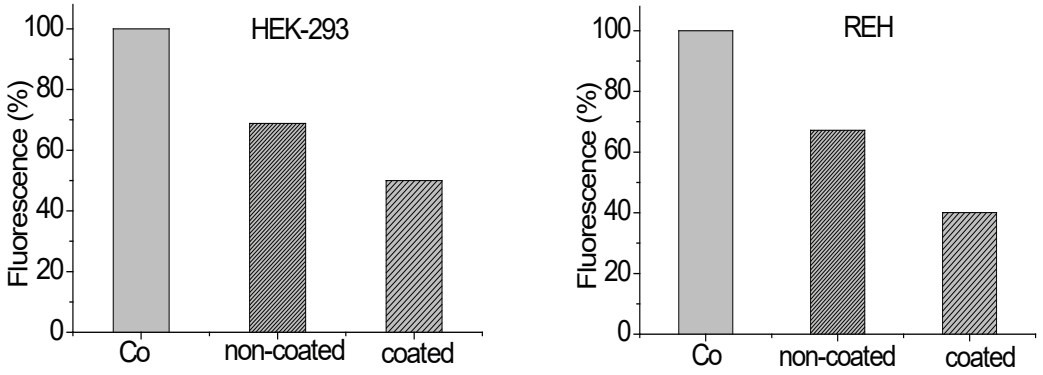

Figure 4. Transfection efficiency of non-coated and coated POx-PEI polyplexes at N/P ratio of 4 expressed as % relative to fluorescence of PEI-polyplexes taken as 100%. HEK-293 (human embryonic kidney cells) and REH (acute lymphocytic leukemia).

Conclusions

A partially hydrolyzed thermoresponsive polyoxaziline (POx-PEI) was used for elaboration of polymer vectors of plasmid DNA. The resulting polyplex particles were characterized with a small size and narrow size distribution suitable for systemic delivery. Additional stabilization was achieved by coating them with cross-linked polymer shell. The investigated polyplexes showed significantly lower cytotoxicity compared to the standard PEI vectors. Furthermore, they exhibit transfection efficacy of 65% comparable to that of the referent transfection reagent. On this ground it can be concluded that the investigated POx based polyplexes are potential platforms for successful DNA delivery.

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