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Polyphosphonium Polymers for siRNA Delivery: An Efficient and Nontoxic Alternative to Polyammonium Carriers

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Supporting Information

ABSTRACT: A water-soluble polyphosphonium polymer was synthesized and directly compared with its ammonium analog in terms of siRNA delivery. The triethylphosphonium polymer shows transfection efficiency up to 65% with 100% cell viability, whereas the best result obtained for the ammonium analog reaches only 25% transfection with 85% cell viability. Moreover, the nature of the alkyl substituents on the phosphonium cations is shown to have an important influence on the transfection efficiency and toxicity of the polyplexes. The present results show that the use of positively charged phosphonium groups is a worthy choice to achieve a good balance between toxicity and transfection efficiency in gene delivery systems.

Gene therapy brought a new hope in the fight against a variety of genetic-based diseases.[1−5] The concept includes systemic or local delivery of therapeutic nucleic acids, such as DNA, which acts on the nucleus and can induce gene expression, and short interfering RNA (siRNA), which acts on the cytosol and mediates gene silencing.[1,6−9]

siRNA has the potential to treat diseases such as nonlocalized cancers, ovarian cancer, HIV infections, neurodegenerative diseases, respiratory viruses, hepatitis B, and vascular diseases, which can be corrected by decreased expression of specific proteins.[5,10,11] The growing interest in siRNA has been catalyzed by its therapeutic properties and the possibility of its synthetic production.[1,5,6,10,11] However, siRNA alone does not cross cell membranes easily because of its relatively large size, negative charge, and hydrophilicity.[1] Essential to the success of siRNA delivery is the development of delivery systems that promote the cellular membrane crossing and that are able to protect siRNA from its degradation in the extracellular environment.[4,5,11,12]

Both viral and nonviral carriers have been developed for siRNA delivery.[1,2,5,13−22] While viral vectors may raise many safety concerns, nonviral systems can be tailored to present better biocompatibility, flexibility, and biodegradability. A common approach used for siRNA delivery involves the formation of ionic complexes (polyplexes) through noncovalent interaction between the negatively charged phosphate groups in siRNA and the cationic charges in the macromolecular vectors such as polymers, dendrimers, and lipids (Figure 1).[1,23−27] Although a wide variety of cationic systems have been developed for siRNA delivery, the correlation between transfection efficiency and cytotoxicity has been unsatisfactory because agents that are capable of high transfection efficiency often also present undesired cytotoxic effects.[1]

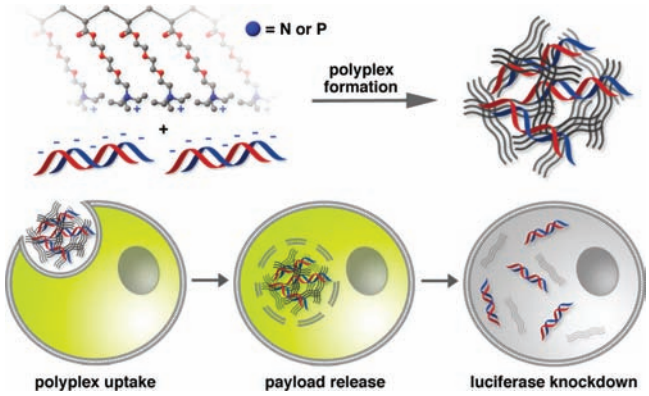

Figure 1. Representation of polyplex formation, polyplex uptake by the cell, siRNA release, and transfection causing luciferase knockdown.

Most current macromolecular siRNA delivery systems bear amine-based positively charged groups. A significant number of studies have reported that polyamines display significant cell toxicity, and efforts to minimize this problem generally focus on varying the degree of substitution of the amines or their pK_a or introducing poly(ethylene glycol) masking chains.[1,25−27] The use of other positively charged groups, such as phosphonium cations, has not yet been significantly explored for gene delivery applications.

A few reports demonstrated that compounds containing phosphonium groups are generally less toxic than their ammonium analogs.[28,29] Moreover, it was shown that small molecules that contain phosphonium cations can bind to DNA,[30−33] and that phospholipids bearing phosphonium groups are capable to transfect DNA.[34,35] Phosphorus-containing polymers based on phosphate, phosphonate, and phosphinate groups have found applications in drug delivery, tissue engineering, and dentistry; however, phosphonium polymers have not yet been explored in the biomedical field.[36]

Herein we report a novel and promising strategy for siRNA delivery based on phosphonium polymers. It combines the ability of phosphonium groups to bind nucleic acids and their low toxicity levels, with a polyacrylate polymer backbone that has frequently been tested in biological applications.[37]

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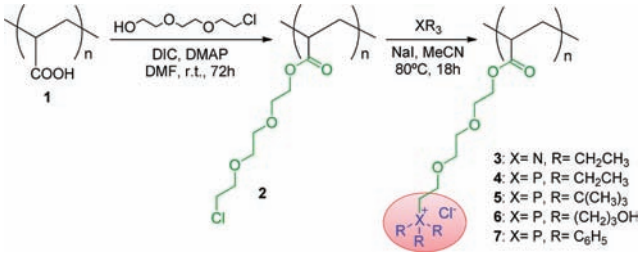
Our synthetic approach starts with the attachment of triethylene glycol monochlorohydrin to the commercially available 5 kDa poly(acrylic acid) **1**, through a hydrolyzable ester linkage (Scheme 1). The attachment of the side chains

Scheme 1. Synthesis of Ammonium and Phosphonium Polymers



resulted in polymer **2** that has a MW of about 14 kDa (PDI: 1.39, by SEC) and is soluble in organic solvents such as dichloromethane. Polymer **2** is then submitted to the nucleophilic substitution of the chlorides by phosphines, generating phosphonium terminal groups (Scheme 1). This reaction was carried out for four different phosphines: triethylphosphine, tri*tert*-butylphosphine, tris(3-hydroxypropyl)phosphine, and triphenylphosphine, yielding polymers **4**, **5**, **6** and **7** respectively. For comparison purposes, the analogous triethylammonium polymer **3** was synthesized using triethylamine.

The functionalization of polymer **2** with the phosphonium groups was verified by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. Completion of the reaction was confirmed by the disappearance of the CH_2Cl signal at 43.1 ppm on the ^{13}C NMR spectra, and the appearance of a new signal on the ^{31}P NMR spectra that corresponds to the phosphonium groups (for NMR data and ^{31}P NMR spectra, see S.I.). The resulting phosphonium polymers were easily purified by dialysis. Polymers **3**, **4**, **5** and **6** are water-soluble, whereas polymer **7** is only soluble in organic solvents and thus it was excluded from further studies. SEC analysis of polymers **3**–**6** show an average MW in the 21–26 kDa range, and as expected, their PDI is similar to the one obtained for polymer 2.

The cytotoxicity of polymers **3**–**6** was evaluated with human cervical cancer cells (HeLa cells) upon incubation in serum-containing DMEM solutions of polymers for 48 h (polymer concentrations: 50 - 500 μg/mL). The results show that the phosphonium polymer **4** present cell viability >90% up to 250 μg/mL, whereas the ammonium polymer **3** starts showing cell viability <90% at concentrations higher than 200 μg/mL (Figure 2). Therefore, the present results suggest that the

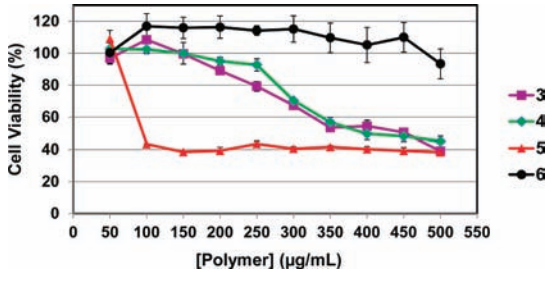


Figure 2. Cell viability obtained for polymers **3** (poly-($^{+}NEt_3$)$_n$), **4** (poly-($^{+}PEt_3$)$_n$), **5** (poly-($^{+}PtBu_3$)$_n$), and **6** (poly-($^{+}P((CH_2)_3OH)_3$)$_n$).

triethylphosphonium polymer **4** is slightly less toxic than its ammonium analog **3**. It was also found that the nature of the alkyl substituents (R in Scheme 1) on the phosphonium cations has an important influence on cytotoxicity, since polymer **5** (*R* = *t*Bu) is highly toxic at concentrations higher than 50 μg/mL, whereas polymer **6** (R = $(CH_2)_3OH$) does not present any significant toxicity up to 500 μg/mL (Figure 2).

The ability of the new cationic polymers to bind siRNA was evaluated by gel electrophoresis. It was found that all the phosphonium polymers completely bind siRNA at 1:1 P^+/P^- ratio while the analogous ammonium polymer **3** needs a N^+/P^- ratio higher than 5:1 to completely bind the siRNA (Figure 3);

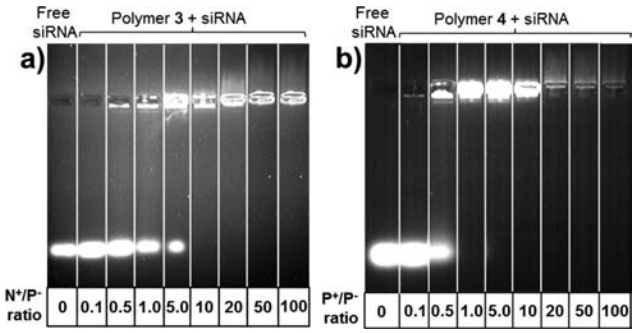


Figure 3. Gel electrophoresis of the siRNA polyplexes formed with polymers (a) **3** and (b) **4** (for polyplexes formed with polymers **5** and **6**, see S.I). X^+ (X = N or P) refers to the ammonium or phosphonium groups present on the polymers, and P^- refers to the phosphate groups present on the siRNA structure.

X^+ (X = N or P) refers to the ammonium or phosphonium groups present on the polymers, and P^- refers to the phosphate groups present in the siRNA structure. These experiments suggest that the phosphonium groups present higher binding affinity to siRNA than the ammonium analogs. These findings are in agreement with theoretical calculations on the charge distribution of different cations.[27] For the phosphonium groups, the positive charge is centered at the P atom, whereas the positive charge of the ammonium moiety is distributed through the adjacent carbons, resulting in a weaker cationic charge in the ammonium groups.[38]

The sizes of the polyplexes formed with polymer **4** were analyzed by DLS (Figure 4 and S.I.).[39–42] As expected, the size of the polyplexes decreases with decreasing their concentration. Thus, all the values reported here were measured with freshly prepared polyplexes after 30 min of incubation, using the same concentrations used in the transfection experiments. The polyplexes were first formed by mixing the siRNA with polymer **4** in PBS, and then diluted 10× with DMEM (for experimental details, see S.I.). At 5:1, 10:1, 20:1 P^+/P^- ratios in PBS all polyplexes present around 300 ± 100 nm, being significantly bigger at 1:1 P^+/P^- ratio (450 ± 100 nm). When diluted with DMEM to achieve the siRNA concentration (5 μg/mL) used in the transfection experiments, all polyplexes have a hydrodynamic diameter of about 120 ± 50 nm (Figure 4 and S.I.).

The efficiency of the new polymers as siRNA carriers was initially tested in HeLa Luc cells (genetically engineered HeLa cells that express luciferase). The polyplexes were formed with a custom siRNA sequence which, if properly delivered, has the ability to knockdown luciferase expression. The HeLa Luc cells were incubated with the polyplexes for 5 h in serum-free media

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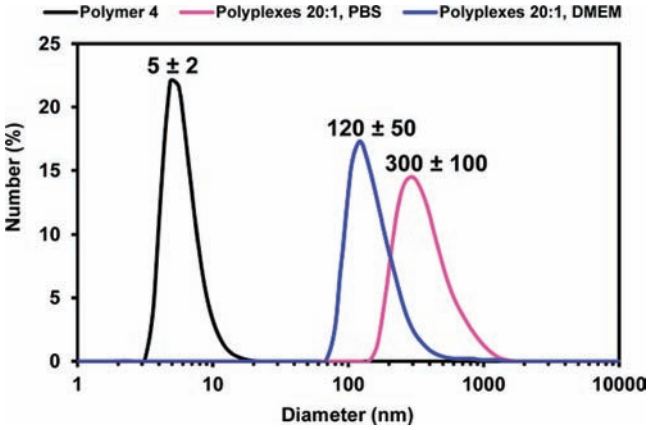

Figure 4. DLS data obtained for black line, polymer **4** at 2 mg/mL in PBS, 5 ± 2 nm; pink line, polyplexes formed with polymer **4** at 20:1 P^+/P^- ratio in PBS, using 1000 μg/mL of polymer and 50 μg/mL of siRNA, 300 ± 100 nm; blue line, polyplexes formed with polymer **4** at 20:1 P^+/P^- ratio in DMEM, using 100 μg/mL of polymer and 5 μg/mL of siRNA, 120 ± 50 nm.

(DMEM), followed by 43 h of incubation in serum containing media. All experiments were carried out at a constant concentration of siRNA (5 μg/mL), and the concentration of the polymers were varied according to the X^+/P^- ratios (for example, 20:1 ratio corresponds to 100 μg/mL of polymer, for details see S.I.). For comparison, parallel experiments were carried out using polyplexes formed with inactive siRNA as a negative control. As positive controls, the amine-based polymer PEI (25 kDa) and the transfection reagent Lipofectamine 2000 were used. The transfection efficiency is expressed in % knockdown of luciferase production, and is normalized to the total amount of protein present in the cells. Along with the transfection experiments, the toxicity of the polyplexes was evaluated (see S.I. for experimental details).

The toxicity of the polyplexes follows the same trend as the toxicity measured for the polymers alone. The polyplexes formed with the phosphonium polymers **4** and **6** are less toxic than the polyplexes formed with the ammonium analog **3** (Figure 5). Polyplexes based in the tri*tert*-butylphosphonium polymer **5** are highly toxic precluding its use as a siRNA carrier.

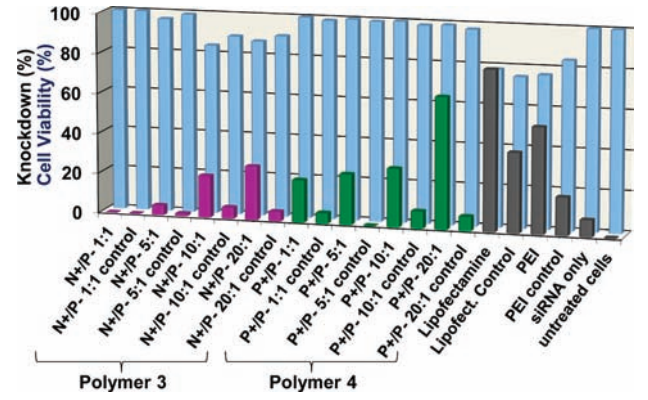

Figure 5. Cell viability (back columns in blue) and transfection efficiency (front columns) obtained for polyplexes formed with polymers **3** (purple) and **4** (green), and with lipofectamine and PEI (gray), at different X^+/P^- ratios (X = N or P). For data related to polymer **5** and **6**, and all standard deviations (SD), see S.I. Control refers to data obtained with polyplexes formed with inactive siRNA.

Polymer **6** did not show significant transfection efficiency probably due to poor siRNA release in the cytoplasm.

The best results obtained for the ammonium polymer **3** show 25% transfection with 85% cell viability, while the analogous phosphonium polymer **4** shows up to 65% transfection efficiency with 100% cell viability (Figure 5). These results indicate that the phosphonium polymer **4** is significantly more efficient than the ammonium polymer **3**, suggesting that using phosphonium instead of ammonium groups is highly beneficial for siRNA delivery systems. The results obtained for polymer **4** reveal a good balance between toxicity and transfection efficiency, exceeding the results obtained for the commercially available transfection reagent Lipofectamine 2000 and the amine-based polymer PEI (25 kDa), which show only 75% cell viability under the same conditions (Figure 5).

Several transfection experiments using higher concentrations of polymers (X^+/P^- ratios: 30:1, 40:1 and 50:1) and higher concentration of siRNA (10 μg/mL) were carried out. However, it resulted in higher toxicity levels without increasing the transfection efficiency (see S.I).

The best performing polymer, **4**, its ammonium analog **3** and PEI were tested in media containing different amounts of serum proteins. No significant difference in transfection efficiency was detected between experiments carried in serum-free DMEM and Opti-MEM (reduced serum content). However, in the challenging environment of serum-containing DMEM, the ammonium polymer **3** and PEI do not show any transfection while the phosphonium polymer **4** shows about 40% transfection (Figure 6). These results suggest that the polyplexes formed with the phosphonium polymer **4** are more

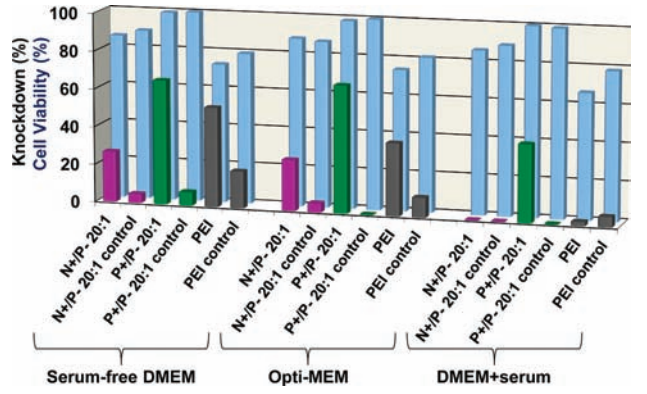

Figure 6. Cell viability (back columns in blue) and transfection efficiency (front columns) obtained for polyplexes formed with polymers **3** (purple), **4** (green) and PEI (gray) in serum-free DMEM, Opti-MEM and serum-containing DMEM. Control refers to polyplexes formed with inactive siRNA.

stable in the presence of proteins than the polyplexes formed with either the ammonium analog or the amine-based polymer PEI. Although the transfection efficiency decreases in serum-containing DMEM when compared to serum-free media, the positive transfection with 100% cell viability makes the triethylphosphonium polymer **4** a promising candidate for siRNA delivery *in vivo*.

In conclusion, we have demonstrated that polyphosphonium polymers are efficient and nontoxic alternatives to polyammonium-based siRNA delivery systems. The new triethylphosphonium polymer described here shows transfection efficiency up to 65% with 100% cell viability, while the best result obtained

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for the ammonium analog reaches only 25% transfection with 85% cell viability. We have also shown that the nature of the alkyl substituents on the phosphonium groups have a significant influence on toxicity and transfection efficiency.

Incorporation of phosphonium groups into gene delivery systems has the potential to further the field of polymeric vectors by improving their performance. Our efforts are now focused on the functionalization of other polymeric structures[43,44] with phosphonium cations for siRNA and plasmid DNA delivery, and on the evaluation of the phosphonium polymers for siRNA delivery *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, ^{31}P NMR spectra, gel electrophoresis images, toxicity and transfection efficiency data for all polymers, DLS data for polyplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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