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Phosphorus dendrimers as powerful nanoplatforms for drug delivery, as fluorescent probes and for liposome interaction studies: A concise overview

Dzmitry Shcharbin^a, Maria Bryszewska^b, Serge Mignani^{c,d}, Xiangyang Shi^{d,e}, Jean-Pierre Majoral^{f,g}

^a Institute of Biophysics and Cell Engineering of NASB, Minsk, Belarus

^b Department of General Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

^c Université Paris Descartes, PRES Sorbonne Paris Cité, CNRS UMR 860, Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologique, 45, rue des Saints Peres, 75006 Paris, France

^d CQM - Centro de Química da Madeira, MMRG, Universidade da Madeira, Campus da Penteada, 9020-105 Funchal, Portugal

^e State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, PR China

^f Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077, Toulouse Cedex 4, France

^g Université Toulouse 118 route de Narbonne, 31077 Toulouse Cedex 4, France

Abstract

Gene therapy is a new and promising tool to treat many severe diseases and the silencing of proteins is the safest and the most efficient tool to treat diseases because it does not induce changes in human genome and avoids a huge problem encompassing insertional mutagenesis. Using small RNAs to switch on/off target proteins is limited due to existence of some barriers for them in the human body (blood RNases, serum albumins, cell walls, etc). For therapeutic applications they need the efficient and non-toxic carrier which will deliver them into cell cytoplasm. Within the huge range of carriers available, dendrimers can be underlined as new promising efficient carriers. This review summarizes several findings in phosphorus dendrimers based on *in vitro* and *in vivo* studies. As a result, we can conclude that advantages of phosphorus dendrimers are strong interaction with siRNA/DNA and formation of small and compact positively charged complexes of high and fast penetration into cells; efficient release of siRNA/pDNA in endosomes due to "proton sponge" effect; possibility of their modification including addition of fluorescent probes - in this case fluorescent dendrimers can be used both as a gene carrier and a tracer of delivery into cells. Additional benefit of using fluorescent

phosphorus dendrimers is their ability to monitor the macrophage physiological status *in vitro* and *in vivo*.

Introduction

Gene therapy is used to treat diseases caused by missing, defective or over-expressing genes. Developing a method for introducing a therapeutic gene into target cells is the key issue to treat diseases by gene therapy. Currently two types of therapeutic approaches are carried out. One is called gene therapy *in vivo*, wherein the gene of interest is inserted into a vector and then the vector is transferred directly to the patient. The other approach is called *ex vivo*, in which the vector is introduced into cultured cells (which can be taken from the patient himself), and subsequently, these genetically engineered cells are transplanted into the patient. In any case, viral or non-viral vectors (such as liposomes, cationic polymers or dendrimers) are necessary for introducing the gene of interest into cells [1,2] Usually, nucleic acid-based therapies using plasmid DNA (pDNA), small interfering RNA (siRNA) or microRNA (miRNA) need to be achieved with the objective of increasing efficiency, safety and therapeutic value. In addition the optimal plasmid or siRNA design is of critical importance to improved delivery strategies. Nucleic acids are highly hydrophilic, therefore are unable to penetrate lipid cell membranes and additionally, can be degraded by nucleases in the blood. The successful delivery of pDNA, siRNA or miRNA, should be achieved with the lowest toxicity without inducing an immune response. For example, pDNA can be delivered by injection into muscle tissue or via hydrodynamic delivery, but in most cases requires a vector which provides for shielding, targeting and cellular uptake.[3] siRNA-based therapeutics for disease therapy has sparked rapidly growing interest to tackle difficult diseases encompassing cancers. The most important challenges in the development of naked siRNAs is their instability in the bloodstream as well their immunogenicity and their inability to cross membrane. Consequently, chemical modifications and delivery materials were developed to develop siRNAs as drugs. Over the past decade, relevant advances in engineered nanoparticles with optimal sizes, shapes and surface properties to deliver siRNAs *in vitro* and *in vivo* to its site of action (*e.g.* cells, tissues) without side effects have started to show great promise.[4] Several nanomaterial types including lipids, polymers and inorganic nanoparticles were developed.[5] In recent years, within the polymer-based nanoparticle delivery systems for the delivery of siRNAs, dendrimers have been studied extensively as nanocarriers for nucleic acid therapeutics.[6] The vehicle can be a cationic dendrimer capable of condensing genetic material containing multiple phosphate residues forming dendriplexes. Thereby, genetic material is protected from degradation and due to an excess of positive charges, complexes are able to interact with negatively charged cell membrane. Then, the dendriplex is internalized *via* endocytosis, and once inside the acidic endosomes, the dendrimer acts as "proton sponge" by buffering the pH, leading to endosomal swelling and rupture, releasing its content to cytosol and allowing DNA or RNA to eventually enter the nucleus (Figure 1).[7]

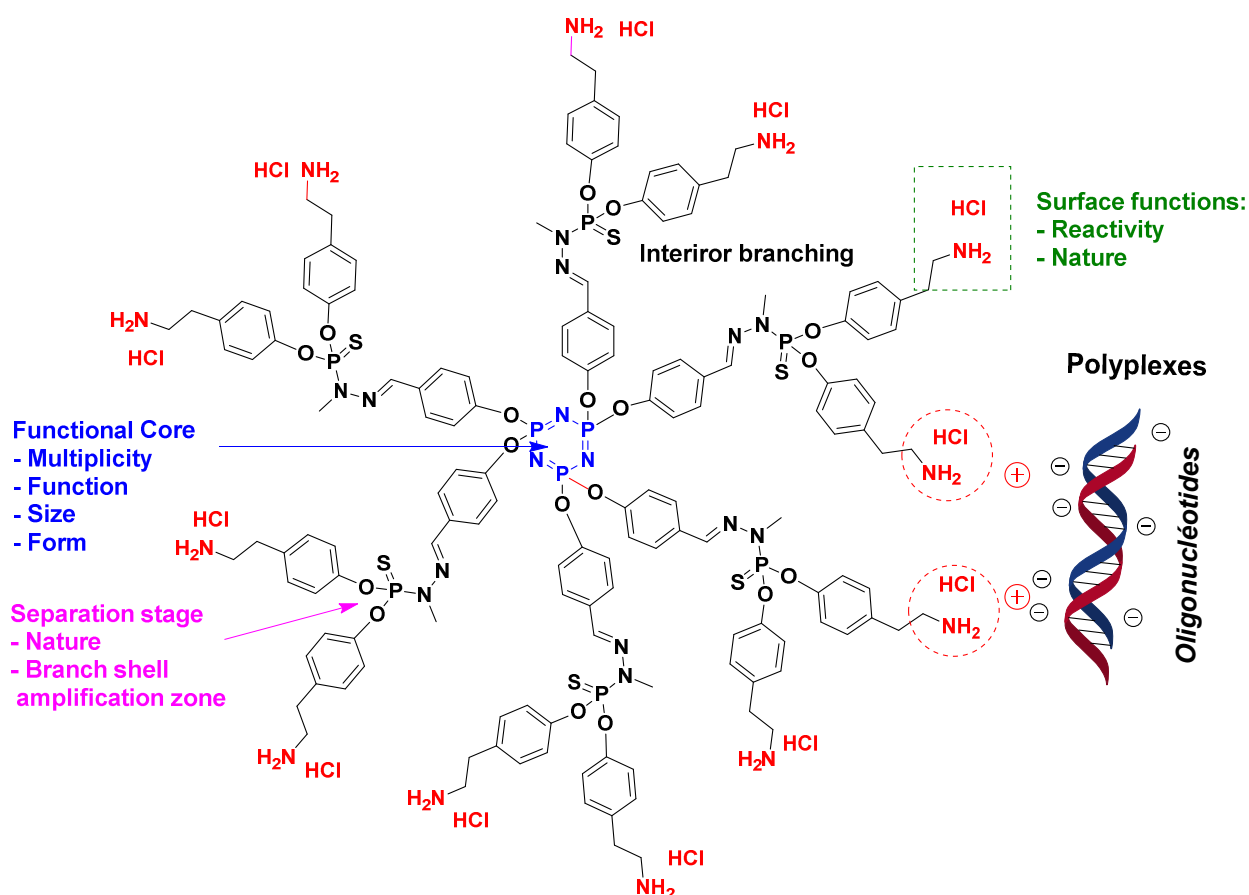


Figure 1. 2D representation of phosphorus dendrimers with main architectural components and formation of polyplexes with oligonucleotides for the delivery of siRNAs, mRNAs etc.

Phosphorus dendrimers as nanoplatforms for siRNA delivery

The first attempt to use phosphorus dendrimers for DNA separation in microarrays was made by Le Berre *at al.* in 2003.[8] It is known that successful use and reliability of microarray technology is highly dependent on several factors, including surface chemistry parameters and accessibility of cDNA targets to the DNA probes fixed onto the surface. The authors proposed to use cationic phosphorus dendrimers for functionalization of glass slides for more sensitive and reliable DNA microarrays. Covalent attachment of these dendrimers on amino-silanised glass slides generates a reactive approximately 50 Angstroms.layer onto which amino-modified DNA probes are covalently bound. Authors concluded that these features make dendrimer-activated slides ideal for manufacturing cost-effective DNA arrays applicable for gene expression and detection of mutations. Such a methodology is presently developed by the company Dendris in the field of respiratory diseases, for the identification of the 10 pathogens commonly implicated in sexual diseases or in the realm of food safety with the multiplex mode of several bacteria detection.[9] In the same year, Maksimenko and colleagues.[10] (Figure 2) proposed

to use for gene therapy the cationic phosphorus dendrimers for optimisation of dendrimer-mediated gene transfer. The authors found that anionic oligomers (oligonucleotides or destran sulfate) increased significantly the capacity of the poly(amidoamine) (PAMAM) and P-dendrimers for DNA delivery into cells when they were mixed with plasmid DNA before addition of dendrimers. The efficiency of the DNA/dendrimer penetration depends on the size, structure and charge of anionic oligomers.

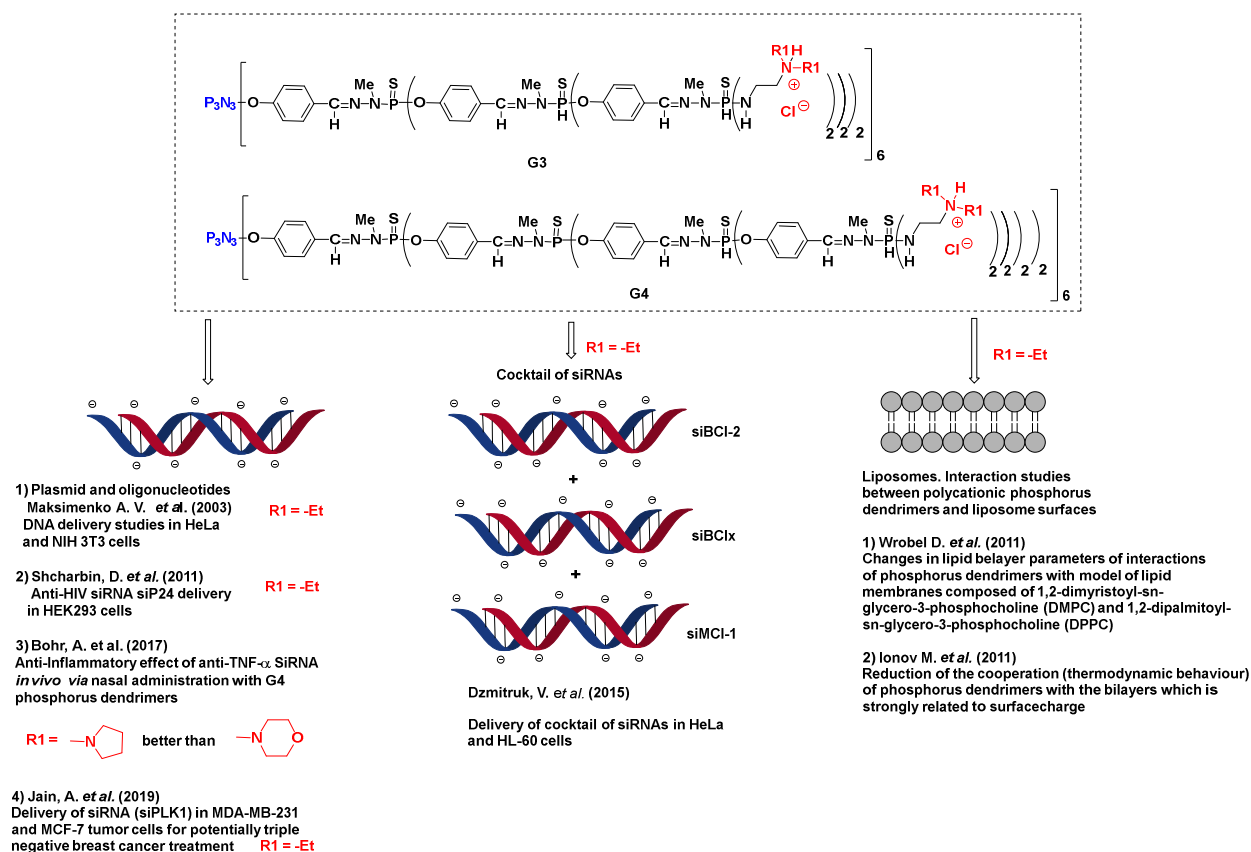


Figure 2. Formation of polyplexes (Maksimenko A. V. *et al.* (2003)[10], Ionov *et al.* (2011)[11], Wrobel *et al.* (2011)[12], Shcharbin *et al.* (2011)[13], Dzmitruk *et al.* (2015)[14], Bohr *et al.* (2017)[15] and Jain *et al.* (2019)[16])

Shcharbin and colleagues.[13] studied 4th generation cationic phosphorus dendrimer (Figure 2) as a prospective candidate for gene transfer. The binding constant and the number of binding centers per one molecule of this dendrimer were determined. The dendriplex between it and anti-HIV siRNA, was characterized using circular dichroism, fluorescence polarization and zeta-potential methods; the average hydrodynamic diameter of the dendriplex was calculated using dynamic light scattering measurements.

The efficiency of transfection of pGFP using P4 was determined in HEK293 cells and human mesenchymal stem cells, and the cytotoxicity of the P4-pGFP dendriplex was studied. Based on these results, the fourth generation cationic phosphorus-containing dendrimers seem to be a

good drug and gene delivery carrier candidate. Shakhbazau *et al.*[17,18,19] used poly(amidoamine) (PAMAM) and phosphorus dendrimers for *ex vivo* modification of human and rodent stem cells to over-express the neurotrophic factors in them (Figure 3).

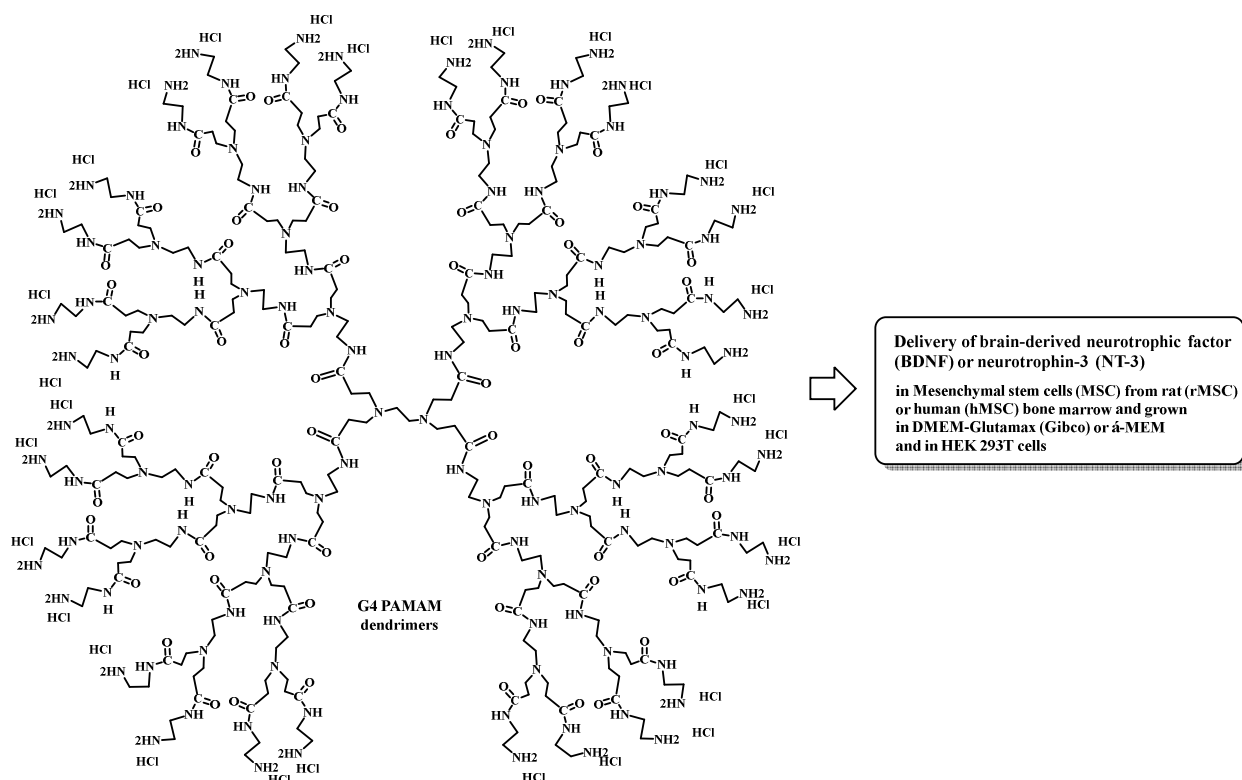


Figure 3. Delivery of neurotrophic factors (NTFs) by protonated G4 PAMAM dendrimers

Dendrimer-based transfection resulted in a time-restricted overproduction of factors, such as BDNF, NT-3, and CNTF, representing different families of proteins essential for the survival, maintenance and regeneration of neurons. In the 'proof-of-concept' study Shakhbazau and colleagues engineered Schwann cells with GDNF expression using dendrimers or lentiviral transduction. Injection of GDNF-modified cells into the rodent injured peripheral nerve resulted in the increase of the extent of axonal regeneration and was associated with improved functional recovery. This approach can improve the therapeutic outcome in patients with nerve trauma and neurodegenerative disorders. Macrophages play an important role in the course of CNS injuries and diseases, and the role and potential of macrophages, alone or with modifying agents, in CNS recovery is currently of significant academic and clinical interest[.20,21]

Phosphorus dendrimers successfully used as nano-sized gene delivery vehicles to interfere with gene expression, represent a promising approach for preventive therapy against HIV/AIDS pandemic as reported by Briz *et al.*[22] Authors evaluated the phosphorus-containing dendrimer G4(NH+Et₂Cl)-96 (generation 4, Figure 4) as a delivery agent of ODNs and siRNAs in HIV-1 infected cells. It was shown that dendrimer formed stable complexes with ODNs or siRNAs and exhibited very low cytotoxicity in Sup T1 cells or PBMC. Functional validation was performed by using specific siRNA against HIV-1 Nef, i.e. siNEF to interfere in HIV-1 replication.

Dendrimer/siNEF dendriplex showed a high efficiency in Nef silencing. Furthermore, *in vitro* treatment of HIV-infected PBMC with G4(NH+Et₂Cl)-96 dendrimer/siNEF dendriplex significantly reduced the viral replication. Thus, the authors proved the usefulness of this phosphorus-containing dendrimer to deliver and transfect siRNA into CD4-T cells as a potential alternative therapy in the HIV-1 infection. Dzmitruk and colleagues.[14] investigated the possibility to use phosphorus dendrimers to deliver anticancer siRNA cocktails to treat cancer cells (Figure 2). For such a purpose the potential of various dendrimers as nanocarriers for anticancer Bcl-xl, Bcl-2, Mcl-1 siRNAs and additionally a scrambled sequence siRNA has been explored. The transfection of complexes in HeLa and HL-60 cells was analyzed using both single apoptotic siRNAs and a mixture (cocktail) of them. Cocktails were more effective than single siRNAs, allowing to decrease siRNAs concentration in treated cells. The PAMAM, carbosilane and phosphorus dendrimers were compared as siRNA carriers, the most effective being the phosphorus-based ones. However, they were also the most cytotoxic on their own.

Inflammation is an essential component of many lung diseases, including asthma, chronic obstructive pulmonary disease, or acute lung injury. Bohr *et al.*[15] designed cationic phosphorus dendrimers for lung delivery of siRNA targeting tumor necrosis factor (TNF- α) in an acute lung injury model. They synthesized two different types of phosphorus-based dendrimers with either pyrrolidinium or morpholinium as terminal protonated amino groups which were selected for their better biocompatibility compared to other dendrimers. Dendriplexes containing pyrrolidinium surface groups demonstrated a stronger siRNA complexation, a higher cellular uptake, and enhanced *in vitro* silencing efficiency of TNF- α in the lipopolysaccharide (LPS)-activated mouse macrophage cell line RAW264.7, compared to morpholinium-containing dendriplexes. The better performance of the pyrrolidinium dendriplexes was attributed to their higher pKa value leading to a stronger siRNA complexation and improved protection against enzymatic degradation thus resulting in a higher cellular uptake. The superior silencing effect of the pyrrolidinium dendriplexes, compared to noncomplexed siRNA, was confirmed *in vivo* in an LPS-induced murine model of short-term acute lung injury upon lung delivery via nasal administration. These data suggest that phosphorus dendriplexes have a strong potential in lung delivery of siRNA for treating inflammatory lung diseases. Deriu and colleagues.²³ analyzed cationic phosphorus dendrimers functionalized with either pyrrolidinium or morpholinium terminal groups to estimate their complexation with siRNAs using experimental and computational approaches and found that chemical surface modifications may drive the overall dendrimer-siRNA affinity by influencing enthalpic and entropic contributions of binding free energy. Moreover, this study elucidates complexation stoichiometry that may be crucial in determining the dendrimer complexation efficiency.

Due to the irresponsiveness of triple negative breast cancer toward conventional therapies efforts were focused on the formation and properties of siRNA therapeutics using phosphorus dendrimers and polo-like kinase (PLK1) which is reported as a potential target for triple

negative breast cancer¹⁶. Dendriplexes were found to be stable in presence of ribonuclease and serum. Dendriplexes resulted in enhanced cell uptake of siPLK1 compared to siPLK1 solution in MDA-MB-231 and MCF-7 cells (Figure 2). Dendriplexes caused increased cell arrest in sub-G1 phase compared to solution thus demonstrating again the interest to use phosphorus dendrimers as nanocarriers.

In a recent study Chen *et al.* [24] synthesized a series of cationic phosphorus dendrimers of different generations (G1-3) and surface ligands (1-(2-aminoethyl) pyrrolidine, 1-(3-aminopropyl) piperidine, or 1-(2-aminoethyl) piperidine) for optimized gene delivery toward cancer gene therapy applications (Figure 4). First, the synthesized dendrimers were evaluated to condense plasmid DNA (pDNA) encoding enhanced green fluorescent protein (EGFP) to optimize their gene delivery efficiency by varying the dendrimer generations and the nature of the polycationic ligand end groups. They showed that all dendrimer/pDNA polyplexes displayed noteworthy cytocompatibility, and the 1-(2-aminoethyl) pyrrolidine-modified protonated G1 dendrimers (1-G1) displayed the best gene delivery efficiency to HeLa cells. Hence, 1-G1 dendrimers were then used as a vector to transfect pDNA encoding both EGFP and p53 protein into cancer cells. Under the optimized conditions, the transfection of pDNA induces the significant p53 protein expression. The cancer gene therapy potential of the polyplexes was finally validated through therapy of a xenografted tumor model after intratumoral injection without systemic toxicity. Thus, the developed cationic 1-G1 dendrimers may be adopted as a powerful vector system for gene therapy of cancer, as well as for highly effective gene therapy of other diseases.

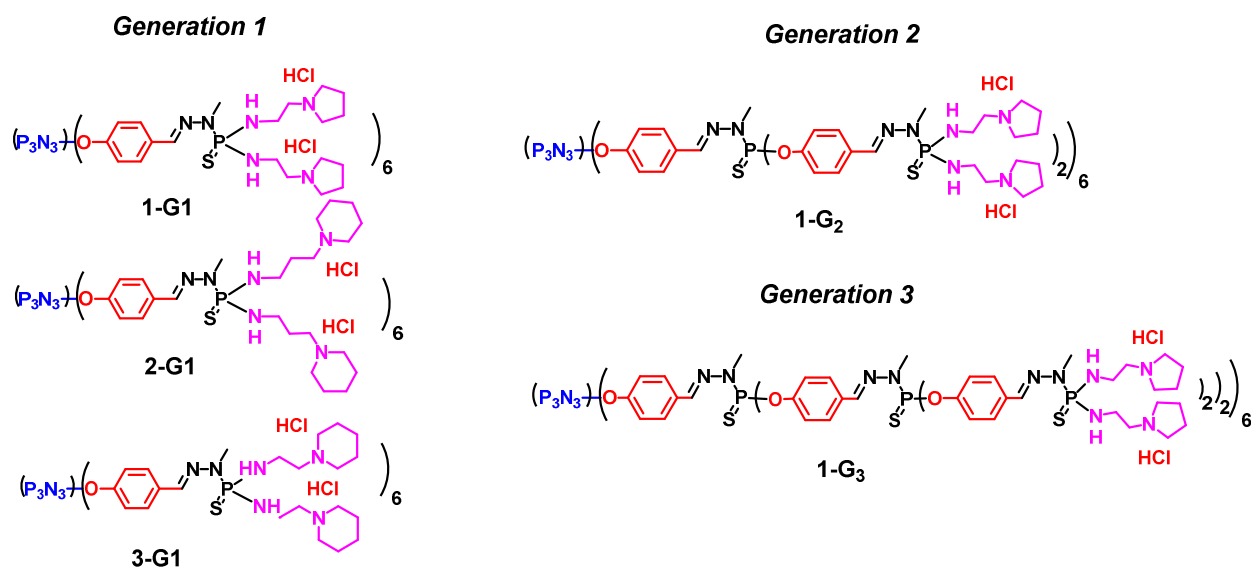


Figure 4. 2D chemical structure of G1-G3 polycationic phosphorus dendrimers

Also, the toxicity of dendrimers *in vivo* and their redistribution in the body is an important question for their biomedical applications. Shcharbin and colleagues.[25] reviewed the

biodistribution, toxicity and pharmacokinetics of pure dendrimers and their complexes with nucleic acids in animals, including mice, rats, rabbits, and guinea pigs. The data have shown that almost all dendrimers of low and middle generations were non-toxic *in vivo*, despite showing some cytotoxic effects *in vitro*. Only the high generations of unmodified cationic dendrimers in high doses had some toxicity *in vivo*. Modifications of dendrimers decreased further their toxicity with regard to unmodified dendrimers. It means that dendrimers can be successfully used for *in vivo* applications.

Phosphorus dendrimers as nanoplatforms for small molecules delivery

The first attempt to use cationic phosphorus dendrimers for *in vivo* (in rabbits) ocular delivery was made by Spataro *et al.*[26] As shown in Figure 5, new series of phosphorus-containing dendrimers, having one quaternary ammonium salt as a core and carboxylic acid terminal groups have been synthesized from generation 0 (3 carboxylic acid terminal groups, named G0) to generation 2 (12 carboxylic acid terminal groups, named G2). These dendrimers react with the neutral form of carteolol (an ocular anti-hypertensive drug used to treat glaucoma) to afford ion pair (saline) species. These dendrimers have been tested *in vivo*, as a vehicle for ocular drug delivery of carteolol to rabbits.

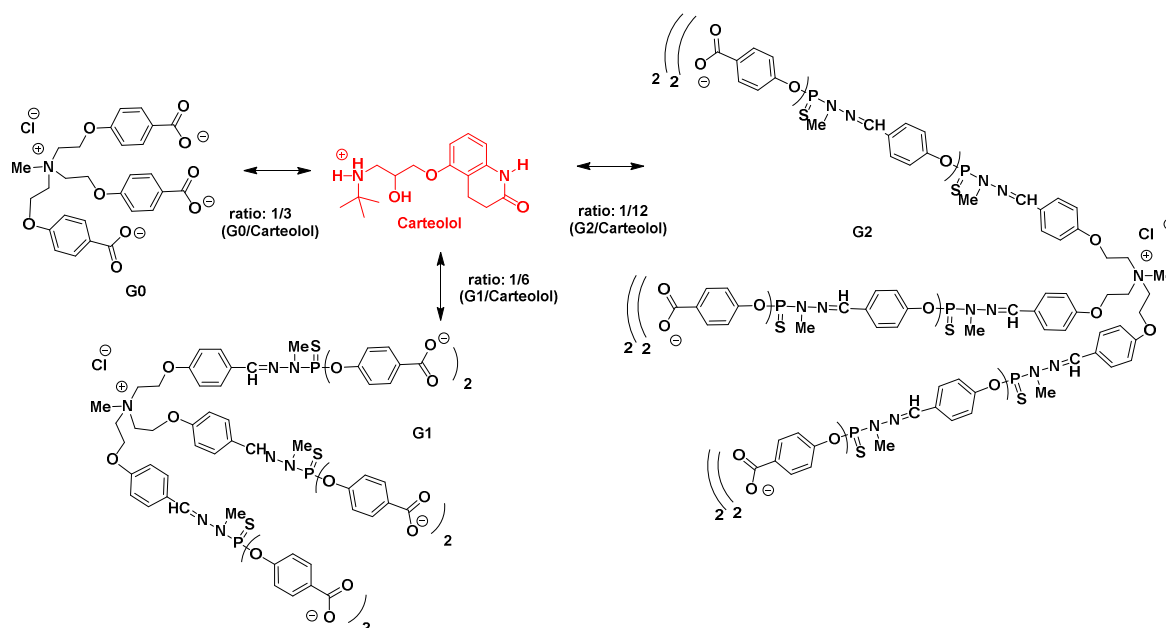


Figure 5. Chemical structure of complexes between phosphorus dendrimers (G0, G1 and G2) and carteolol

Phosphorus dendrimers as fluorescent probes

The important tool to improve (also broaden) the application of phosphorus dendrimers is construction of their fluorescent analogs for both gene/drug delivery and for monitoring.

Kazmierczak-Baranska and colleagues[27] used a water-soluble phosphorus-containing dendrimer possessing a fluorophore (maleimide-type) linked to the core (Figure 6). The cytotoxicity of this dendrimer was relatively low towards HeLa and A549 cells, and less toxic after 48 hours than after 24 hours. Association of this dendrimer with plasmid DNA (BACE-GFP) analyzed with circular dichroism (CD) indicates a possible disturbing of the helical B-type structure of DNA. The strength of this association (a "dendriplex") with BACE-GFP (also with HygEGFP) was measured by electrophoresis.

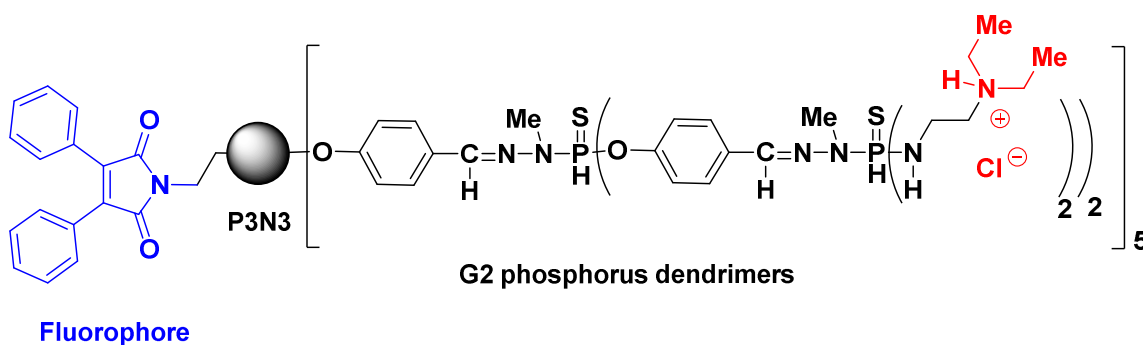


Figure 6. 2D chemical structure of G2 fluorescent phosphorus dendrimer

Shakhbazau *et al.*[28] advocated that a phosphorus dendrimer incorporating a two photon absorption group within the cascade structure of the dendrimer is readily internalized and maintained by bone marrow derived macrophages, making it a useful cytoplasmic tracer both *in vitro* and *in vivo* (Figure 7).

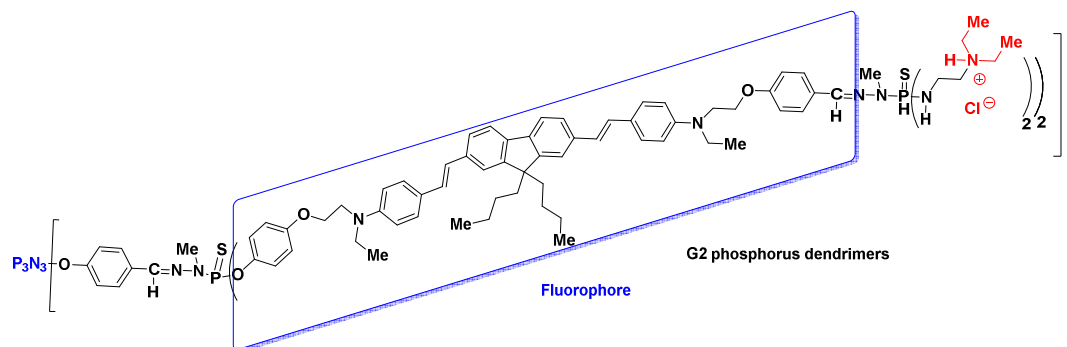


Figure 7. 2D Chemical structure of G2 fluorescent phosphorus dendrimers

The stable fluorescence of fluorescent phosphorus dendrimers allows specific tracing of transfected cells facilitating the study of their function and fate *in vivo*. The transplantation of dendrimer-labeled macrophages in spinal cord *in vivo* complements and supports the existing paradigms on retention and function of blood-borne phagocytes in spinal cord injury model. Sensitivity of dendrimer emission profile to the macrophage physiological status allows for using dendrimers to monitor the different stages of macrophages *in vitro* providing the useful tool to track macrophages physiological status *in vivo*. [28]

Phosphorus dendrimers for liposome interaction studie

To understand the mechanisms of entrance of dendrimers into cells, Wrobel and colleagues[11,12] performed the model studies of interaction between phosphorus dendrimers and liposomes (Figure 2). The results of this study demonstrated that cationic phosphorus dendrimers can alter the thermotropic behaviour of the bilayer by reducing the cooperativity of phospholipids and this effect strongly depends on membrane surface charge. Also, dendrimers of both generations influence lipid bilayers by decreasing membrane fluidity. These data are important to understand the primary interactions between lipid bilayer of cell-membranes and dendrimers.

Conclusion and perspectives

Phosphorus dendrimers incorporating phosphorus in various positions in their structures (core, internal branches and external surfaces) can be easily prepared based on the strong versatility of organophosphorus chemistry. A large number of synthetic procedures allowed to prepare diverse type of phosphorus dendrimers including: 1) phosphorhydrazone linkages in the internal branches; 2) P=N-P=S linkages in the internal branches; 3) viologen dendritic structures; 4) onion peel dendritic structures; and 5) Janus phosphorus dendrimers.[29] These synthesis were performed under mild reaction conditions and non-toxic by-products (e.g. HCl, NaCl) being removed without sophisticated techniques.

The main advantages of phosphorus dendrimers *versus* PAMAM dendrimers are worth noting: 1) several routes of administration; 2) GMP grade manufacturing for clinical use; 3) higher lot-to-lot chemical stability; 4) easily tunable as active drugs *per se* and for gene delivery strategy; and 5) low generation showing potent anti-cancer and anti-tuberculosis activities. Interestingly several biocompatible phosphorus dendrimer types displayed good *in vivo* activities as anticancer agents²⁴, anti-inflammatory agents[30], and for gene delivery [31]

Twenty years passed after the discovery of RNA interference as a gene silencing tool[32] before the first therapeutic drug ONPATRO® (patisiran) based on RNA interference and nanoparticle carrier was approved by U.S. FDA[33]. The discovery of mechanism of RNA interference and its application in medicine opened a wide way to create a completely new class of drugs, at which any protein can be targeted and switched on or off. Also, this tool allows to normalize the disturbed regulation of a cell, involving both gene downregulation and upregulation. However, this field of study is in the beginning of its development and a number of problems related to toxicity, targeting, biological barriers, and delivery remain unsolved. In particular, the siRNA delivery needs the efficient and safe carriers and multifunctional nano-conjugates combining the features of delivery, bioavailability, monitoring and tracking. In this regard phosphorus dendrimers showed themselves as a new promising tool for gene therapy and other biomedical applications. Phosphorus dendrimers were successfully evaluated for *in vivo* drug/gene delivery in several models: ocular drug delivery of carteolol to rabbits[26], for *ex vivo* modification of

human and rodent stem cells to over-express the neurotrophic factors in them and for *ex vivo* modification of macrophages[17,18,34,28] for lung delivery of small interfering RNA targeting tumor necrosis factor (TNF- α) in an acute lung injury model[15], for delivery of pDNA encoding both EGFP and p53 protein into xenografted tumor model after intratumoral injection without systemic toxicity.[16] In all cases cationic phosphorus dendrimers were effective gene carriers *in vitro or in vivo*, thus opening new perspectives as pDNA and siRNA nanocarriers for future gene therapy.

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ORCID

Dzmitry Shcharbin: 0000-0002-1414-4465

Maria Bryszewska 0000-0003-4676-3743

Serge Mignani : 0000-0002-1383-5256

Xangyang Shi : 0000-0001-6785-6645

Jean Pierre Majoral 0000-0002-0971-817X

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