molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

Biological Properties of New Viologen-Phosphorus Dendrimers

Karol Ciepluch,[†] Nadia Katir,[‡,§] Abdelkrim El Kadib,*[,§] Aleksandra Felczak,[∥] Katarzyna Zawadzka,[∥] Monika Weber,[†] Barbara Klajnert,[†] Katarzyna Lisowska,[∥] Anne-Marie Caminade,[‡] Mostapha Bousmina,[⊥] Maria Bryszewska,*[,†] and Jean Pierre Majoral*[,‡]

[†]Department of General Biophysics, University of Lodz, 141/143 Pomorska Street, 90-236 Lodz, Poland

[‡]Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, F-31077 Toulouse cedex 4, France

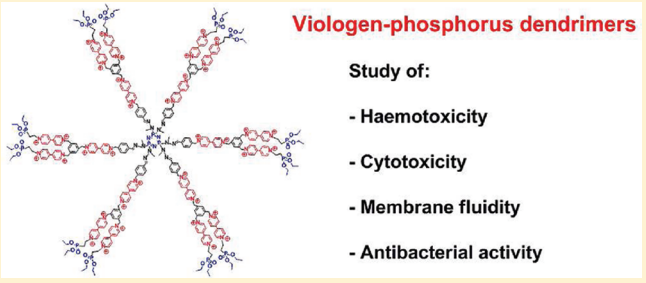
[§]Institute of Nanomaterials and Nanotechnology—MAScIR (Moroccan Foundation for Advanced Science, Innovation and Research), ENSET, Avenue de l'Armée Royale, Madinat El Irfane, 10100 Rabat, Morocco

[∥]Department of Industrial Microbiology and Biotechnology, University of Lodz, 12/16 Banacha Street, 90-237 Lodz, Poland

[⊥]Hassan II Academy of Science and Technology, Rabat, Morocco

Supporting Information

ABSTRACT: Some biological properties of eight dendrimers incorporating both phosphorus linkages and viologen units within their cascade structure or at the periphery were investigated for the first time. In particular cytotoxicity, hemotoxicity, and antimicrobial and antifungal activity of these new macromolecules were examined. Even if for example all these species exhibited good antimicrobial properties, it was demonstrated that their behavior strongly depends on several parameters as their size and molecular weight, the number of viologen units and the nature of the terminal groups.



KEYWORDS: *viologen dendrimers, hemolysis, cytotoxicity, antibacterial activity, membrane fluidity*

INTRODUCTION

4,4′-Bipyridinium salts better known under the name of viologen derivatives are showing an increasing number of applications in addition to their former use as herbicides.[1] This is mainly due to their properties as photoactive and electroactive compounds, and to their ability to give strong donor−acceptor complexes with electron donating species. However viologens by themselves can present risks for human health. As an example 1,1′-4,4′-bipyridinium dichloride is known to induce formation of superoxide (O_2^-) and to cause damage to multiple organs.[2−4] A therapeutic protocol for the treatment of viologen poisoning based on host−guest chemistry and involving the effective inhibition of viologen toxicity by complexation of *p*-sulfonatocalix[*n*]arenes was reported.[5] In marked contrast to this isolated "dark image" of viologen monomer behavior, their introduction as building blocks for the design of polycationic dendrimers allowed De Clercq et al.[6] to point out the activity of various viologen dendrimers against human immunodeficiency virus (HIV-1, strain III_b replication in MT-4 cells), as well as to a lesser extent against herpes simplex virus (HSV), vesicular stomatitis, Punta Toro virus, Sindbis virus, Reovirus and respiratory syncytial viruses. Indeed it was demonstrated that their behavior strongly depends on the number and distance of the positive charges.

Surprisingly and to the best of our knowledge no study on other biological properties of viologen dendrimers was reported. Having these observations in mind we decided to open the field of such investigations, our first goal being to design other types of dendrimers in order to have a more precise idea on their biological activities. For such a purpose we designed new viologen monomers, dendrons, and dendrimers bearing phosphorus groups as additional units incorporated either at the focal point or at the periphery or both of these key structural positions of the dendritic backbone. This choice of strategy was aimed by the fact that we already demonstrated the key role played by phosphorus dendrimers in biology and for biomedical applications due to several specificities.[7] Briefly they have a remarkable influence on cell growth, in particular for neuronal cells,[8] and for human immune blood cells such as monocytes and Natural Killer cells,[9−12] the latter playing a key role for fighting against viral infections and cancers. The uselfulness of phosphorus dendrimers was also pointed out for elaboration of highly sensitive biosensors[13−16] and for *in vitro* drug delivery, for instance as transfecting agents[17−19] or against HIV-1[20] and the scrapie form of prions.[21] *In vivo* biological properties of phosphorus dendrimers as anti- prion agents, for ocular drug delivery,[22] and for imaging rat brain blood vessels[23,24] were

Special Issue: Biological Applications of Dendrimers

Received: October 28, 2011
Revised: January 3, 2012
Accepted: January 3, 2012
Published: January 3, 2012

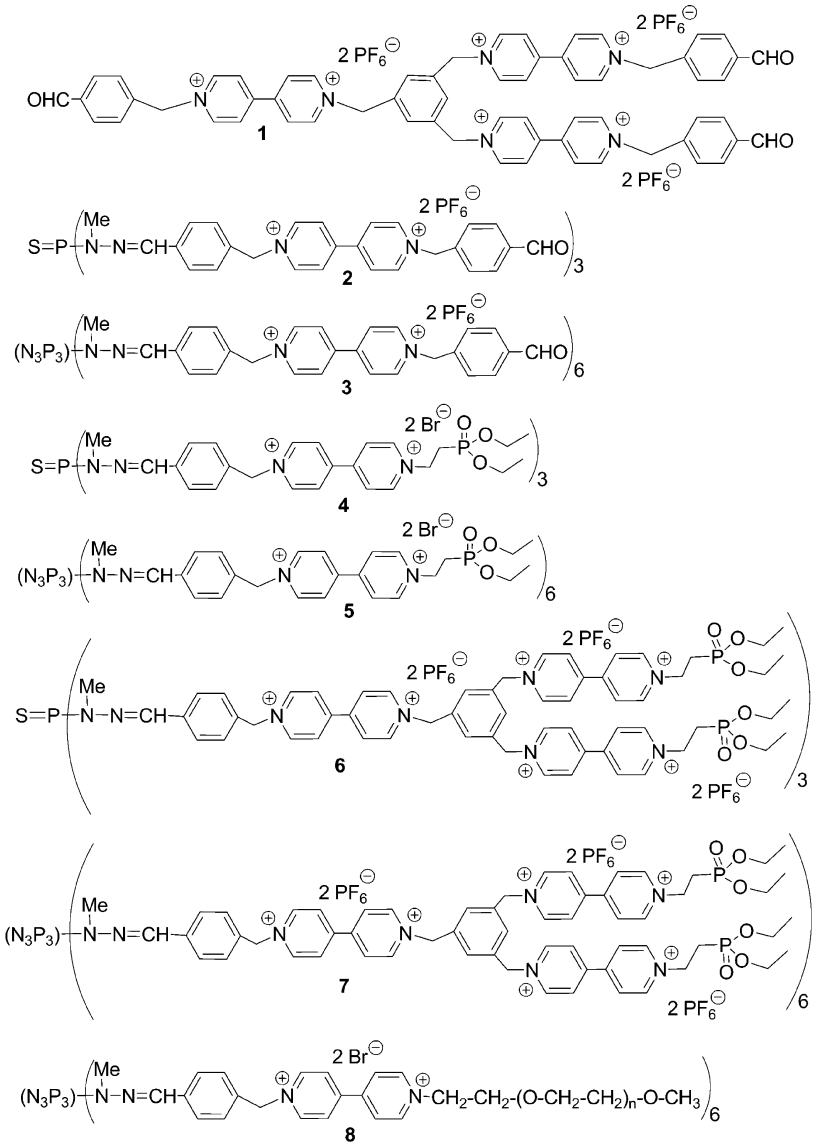


Figure 1. Chemical structures of dendrimers **1**−**8**.

reported. Some of these properties can be found also with other types of dendrimers, but others, in particular the interaction with human immune blood cells, occur only and specifically with phosphorus dendrimers. Therefore introducing phosphorus linkages within the cascade structure or at the periphery of given dendrimers may offer new properties and perspectives.

In this paper we are considering viologen-phosphorus dendrimers built from a trifunctionalized core $P(S)(NCH_3NH_2)_3$ or from a hexafunctionalized one $(P_3N_3)(NCH_3NH_2)_6$ and decorated on their surface either with aldehyde groups or with phosphonate groups, the latter being well-known for their biological properties. Other structural parameters were also considered: (i) the number of charges from 6 to 36, i.e. the number of incorporated viologen units from 3 to 18, (ii) the nature of the counteranions: Br or PF_6, (iii) the size and generation of dendrons and dendrimers, the nature of terminal groups, aldehyde, phosphonate or PEG, (iv) the presence or not of phosphorus groups, (v) the solubility in water or in organic solvents.

The biological properties of eight of these dendritic structures, hemolysis, cytotoxicity, antimicrobial and antifungal activity, morphology and membrane changes of red blood cells were examined in this work.

EXPERIMENTAL SECTION

Synthesis of Viologen Dendrimers. The structure of the considered viologen−phosphorus dendrimers is shown in Figure 1 and Table 1, and their synthesis is detailed in "Materials and methods" in the Supporting Information.

Hemotoxicity. Blood from healthy donors was obtained from Central Blood Bank in Lodz. Blood was anticoagulated with 3% sodium citrate. Erythrocytes were separated from blood plasma and leukocytes by centrifugation (4000*g*, 10 min) at 4 °C and washed three times with PBS (phosphate buffered saline) (13.7 mmol/L NaCl, 0.27 mmol/L KCl, 0.43 mM Na_2HPO_4, 0.147 mmol/L KH_2PO_4). Erythrocytes were used immediately after isolation. To study the effect of viologen-phosphorus dendrimers on erythrocyte hemolysis, dendrimers were added to erythrocytes of 1% hematocrit and incubated at temperature of 37 °C. After 2, 8, 13, and 24 h suspensions were centrifuged (1000*g*, 5 min) and a supernatant taken for analysis. For reference, red blood cells were treated with double-distilled

dx.doi.org/10.1021/mp200549c | *Mol. Pharmaceutics* 2012, 9, 448−457

Table 1. Key Features of Dendritic Structures 1−8

compd	core	generation	charges (anion)	surf. groups	no. of surf. groups
1	$C_6H_3(CH_2)_3$	0	6 (PF_6^-)	CHO	3
2	S=P(NMe−N=)$_3$	0	6 (PF_6^-)	CHO	3
3	(N_3P_3)(NMe−N=)$_6$	0	12 (PF_6^-)	CHO	6
4	S=P(NMe−N=)$_3$	0	6 (Br^-)	P(O)(OEt)$_2$	3
5	(N_3P_3)(NMe−N=)$_6$	0	12 (Br^-)	P(O)(OEt)$_2$	6
6	S=P(NMe−N=)$_3$	1	18 (PF_6^-)	P(O)(OEt)$_2$	6
7	(N_3P_3)(NMe−N=)$_6$	1	36 (PF_6^-)	P(O)(OEt)$_2$	12
8	(N_3P_3)(NMe−N=)$_6$	0	12 (Br^-)	PEG	6

water, which corresponds to 100% hemolysis. To study the effect of HSA complexation with dendrimers on hemolysis of red blood cells, 2 mg/mL of HSA was added to 20 μmol/L viologen dendrimer solution, and after 5 min, red blood cells were added to 1% hematocrit. The samples were then incubated for 2, 8, and 24 h, and the percentage of hemolysis was determined on the basis of released hemoglobin in supernatants and measured spectrophotometrically from the absorbance at 540 nm.

Morphology of Erythrocytes. For microscopy, erythrocytes at a hematocrit of 1% were suspended in solution containing 20 μmol/L viologen-phosphorus dendrimers and incubated at 37 °C for 24 h. 10-fold diluted samples were then viewed under an optical microscope at a magnification of 400×.

Measurements of Membrane Fluidity. Erythrocytes at a hematocrit of 1% were suspended in solution containing 1, 2.5, 5 and 10 μmol/L viologen-phosphorus dendrimers and incubated at room temperature for 0.5 h. Next, erythrocytes were centrifuged (10 min, 2000g) and PBS was added. To evaluate membrane fluidity by fluorescence spectroscopy, erythrocytes (H = 0.04%), were incubated with the fluorescence probe TMA DPH (4-trimethylammonio-1,6-diphenyl-1,3,5-hexatriene) of 10^{-6} mol/L concentration at room temperature for 10 min. The polarization values (r) of the samples were calculated by the fluorescence data manager program using the following Jablonski's equation:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

where I_{VV} and I_{VH} are the vertical and horizontal fluorescence intensities, respectively, to the vertical polarization of the excitation light beam. The factor $G = I_{HV}/I_{HH}$ (grating correction factor) corrects the polarizing effects of the monochromator.

Measurements were made at room temperature using an LS-50B Perkin-Elmer spectrofluorimeter. The excitation and emission wavelengths for TMA-DPH were λ_{ex} = 358 and λ_{em} = 428 nm, respectively.

***In Vitro* Cytotoxicity.** Dendrimers and the reference samples containing either 1% acetonitrile or PBS respectively (when acetonitrile was used, the same amount of acetonitrile without dendrimer was added to a control sample in PBS) were incubated with B14 Chinese hamster peritoneal fibroblasts and N2a mouse neuroblastoma cell lines. Cells were seeded at a density of 1×10^5 cells per mL into 96-well microtitrate plates using DMEM medium. To recover, cells were left for 24 h before the addition of dendrimers. After this time dendrimers were added and cells were incubated for 24 h. After 24 h MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) concentration of 5 mg/mL (20 μL) (final concentration was 0.5 mg/mL) was added, and cells were left for final 4 h. After 4 h medium was removed, DMSO was added to dissolve the MTT crystals, and the absorption of samples was measured at 570 nm with background correction at 630 nm using a microplate reader. Results were expressed as a percent of absorbance relative to untreated control cells. The results of the cytotoxicity assay were used for the calculation of cell viability after incubation with dendrimers: viability [%] = $X/X_c \times 100\%$ (2) where X is the absorbance in a well containing a particular dendrimer concentration and X_c is the absorbance for untreated control cells.

Determination of Antibacterial Activity. Screening for antibacterial activities was carried out using the broth microdilution method following the standards defined by the National Committee for Clinical Laboratory Standards with modifications. The methods involved incubation of tested microorganisms with dendrimers on microtiter plates and spectrometric measurement of cell density at 630 nm (the initial cell density, in all experiments, was approximately 0.1 (OD_{630})). Antimicrobial properties of dendrimers were expressed as the percentage of growth compared to control samples of the bacteria, incubated in medium without macromolecules.

RESULTS

Hemolysis Assay. The results presented as the percentage of released hemoglobin are shown in Figure 2. The red blood cell hemolysis caused by dendrimers was concentration- and time-dependent. The first generation dendrimers **6** and **7** were the highest hemolytic, whereas the dendrimers **1** and **8** were the least. Adding HSA to the system slightly reduced the amount of hemolysis especially for those dendrimers which were weakly hemolytic (Figure 3). The concentration of albumin (2 mg/mL) was chosen to achieve the same ratio of hematocrit to albumin concentration as under physiological conditions.

Cell Morphology. The influence of viologen-phosphorus dendrimers on red blood cell morphology was checked by optical microscopy (Figure 4). The control cells were mostly discocytes (Figure 4A). For some dendrimers **2**, **3**, **6**, and **7** most of the erythrocytes had been destroyed and only few intact cells were visible. Dendrimer **1** induced the echinocytic transformation that led to cell disruption (Figure 4D).

Membrane Fluidity. Membrane fluidity of red blood cells was measured as anisotropy of fluorescence (r) of the fluorescent label TMA-DPH incorporated into the lipid phase of the membrane. Results are presented in Figure 5. Slight increase in fluorescence anisotropy was observed for red blood cells incubated with viologen-phosphorus dendrimers in comparison to untreated control: that means the increased rigidity of the membrane. This increase was statistically significant only for dendrimers **4**, **6**, **7** and **8** for their highest concentrations.

***In Vitro* Cytotoxicity.** The effect of viologen-phosphorus dendrimers on cell viability was determined using the MTT assay.[25] Reduction of MTT by cells indicates mitochondrial activity, which may be interpreted as a proof of cell viability. B14 was a control cell line used in this experiment. The results show that the strongest influence on cell viability was observed for dendrimers **3**, **5**, **6** while dendrimers **1** and **8** were the least cytotoxic in the whole range of concentrations (Figure 6).

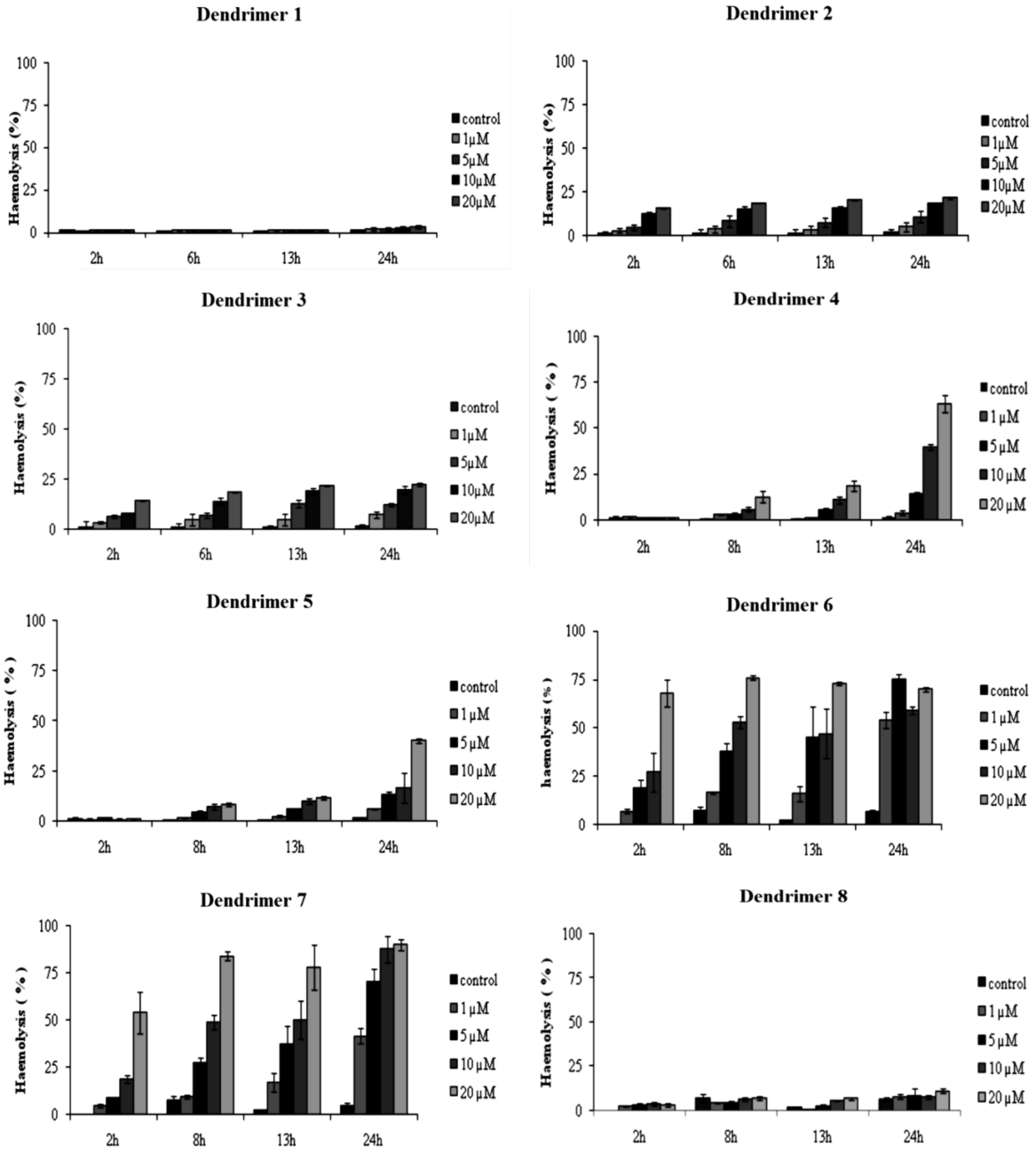


Figure 2. Red blood cell hemolysis caused by viologen-phosphorus dendrimers.

As to the N2a cell line, the strongest influence on cell viability was observed for dendrimers **4**, **5**, **6**, and **8**. All water-soluble dendrimers exerted cytotoxicity against the N2a cell line. Among acetonitrile-soluble dendrimers only dendrimer **6** was toxic. The generation-dependent effect was not observed (Figure 7).

Antimicrobial Activity. Viologen-phosphorus dendrimers were evaluated for an antibacterial activity against a Gram-positive bacterium (*Staphylococcus aureus* ATCC 6538), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315 and *Pseudomonas aeruginosa* ATCC 15442) and yeast (*Candida albicans* ATCC 10231). Antimicrobial activity of all dendrimers was evaluated at concentrations ranging from 0.1 μmol/L to 20 μmol/L and expressed as the percentage of growth compared to the control samples of bacteria/fungi incubated in medium without dendrimers.

Among the studied molecules, dendrimer **1** exhibited the greatest antimicrobial activity against *S. aureus* and *E. coli.* In the highest tested concentration (20 μmol/L) dendrimer **1** reduced the growth of *S. aureus* by 80% and limited the growth of *E. coli* by 50%. It is worth mentioning that dendrimer **1** had also good antifungal properties. The growth inhibition of *C. albicans*, in culture containing 20 μmol/L of dendrimer **1**, reached the value of 70% (Figure 8).

The growth of two tested bacteria (*S. aureus* and *E. coli)* was effectively limited by dendrimers **4**, **6**, and **7**. In case of *S. aureus* growth inhibition reached the value of 50% in samples containing 1 μmol/L of dendrimers **6** and **7**. For *E. coli* addition of 5 μmol/L dendrimer **7** caused 45% reduction of growth. In the case of dendrimer **6**, 50% limitation of growth was noted in samples with 10 μmol/L (Figure 8).

dx.doi.org/10.1021/mp200549c | *Mol. Pharmaceutics* 2012, 9, 448–457

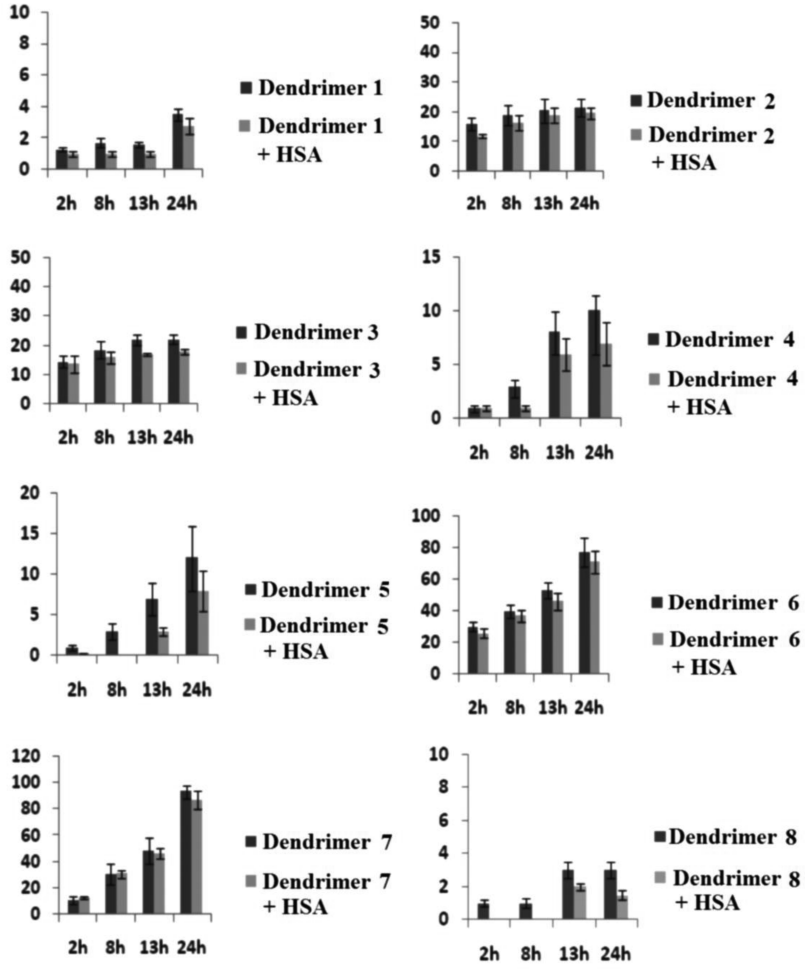


Figure 3. Red blood cell hemolysis caused by viologen-phosphorus dendrimers (20 μmol/L) in the presence or absence of human serum albumin (2 mg/mL).

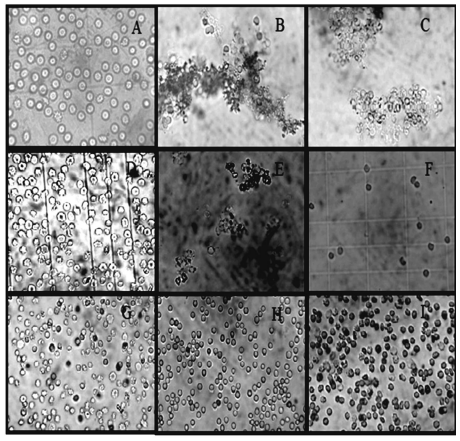


Figure 4. Morphology of erythrocytes affected by viologen-phosphorus dendrimers (concentration of dendrimers, 20 μM; dendrimer expositions, 24 h): (A) control, (B) dendrimer 3, (C) dendrimer 2, (D) dendrimer 1, (E) dendrimer 7, (F) dendrimer 6, (G) dendrimer 5, (H) dendrimer 4, (I) dendrimer 8.

Also addition of dendrimer **4**, in concentration of 20 μmol/L, reduced the growth of *S. aureus* and *E. coli* almost by a half (Figure 8).

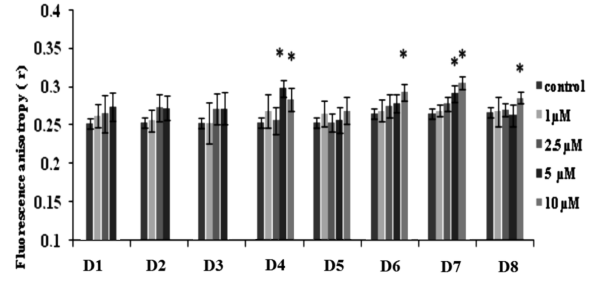


Figure 5. Fluidity of red blood cell membranes treated with viologen-phosphorus dendrimers (D).

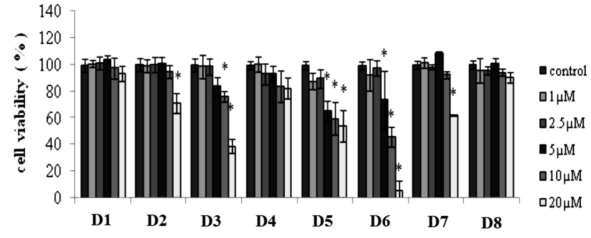


Figure 6. Viability of B14 fibroblasts treated with viologen-phosphorus dendrimers (D).

Among studied dendrimers, dendrimers **5** and **8** displayed the lowest antimicrobial activity against tested microorganisms (Figure 8).

dx.doi.org/10.1021/mp200549c | *Mol. Pharmaceutics* 2012, 9, 448–457

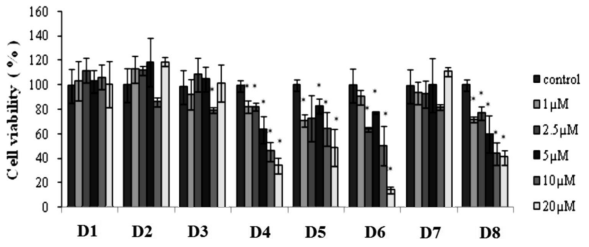


Figure 7. Viability of N2a cells treated with viologen-phosphorus dendrimers (D).

DISCUSSION

It has been shown that positively charged dendrimers can be used in many medical applications, e.g. gene therapy or drug delivery.[26] The problem is that polycationic dendrimers in most cases are very toxic.[27] If clinical applications of dendrimers are considered, knowledge about their biological activity is very important. It was demonstrated that low molecular mass dendrimers are less cytotoxic and hematotoxic than higher mass dendrimers.[28] That is the reason why we investigated the biological properties of viologen-phosphorus dendrons and dendrimers of generation 0 and 1.

Hematotoxicity is the first step to check the impact of dendrimers on human cells. Erythrocytes are a good model for studying alterations in membrane conditions. The loss of integrity of erythrocyte membrane is accompanied by a leakage of hemoglobin. The strongest hemolytical effect was observed for the water-soluble dendrimers **6** and **7** (Figure 2). These dendrimers are of generation 1 and possess phosphonate end groups, 9 or 18 viologen units and 18 or 36 positive charges, respectively, that is to say the highest number of viologen groups and consequently the highest number of positive charges. Other dendrimers with phosphonate end groups caused moderate hemolysis; however this was observed only after 24 h of administration. It seems that viologen dendrimers with phosphonate end groups are more hematotoxic than dendrimers having aldehyde end groups. Probably not only end groups are important, but also the number of viologen units and positive charge play a crucial role. It was proven that viologens are toxic.[29] The more viologen units and positive charges were present, the more hemolysis was observed. Dendrimers **1** and **8** caused the least hemolysis, and among these two dendrimers of generation 0, dendrimer **8** is the least toxic. The first one has aldehyde end groups, whereas dendrimer **8** is coated with polyethylene glycol (PEG) on the surface. This corroborated the observation that PEGylation significantly decreased the *in vitro* and *in vivo* cytotoxicities and hemolysis of G5 and G6 PAMAM dendrimers, especially at higher PEG molar ratios.[30]

The surface of the red blood cells is negatively charged under physiological conditions. Therefore, some viologen dendrimers may exert a hemotoxic effect due to disruption of the cell membrane through initial adhesion to the cell surface by electrostatic attraction.[31] It explains that more positive charge causes more hemolysis. Previous studies have shown that hemolysis is usually preceded by echinocytic transformation.[32,33] This situation was observed for dendrimers **1** and **5**. The presence of erythrocyte ghosts was observed for dendrimers **2**, **3**, **6** and **7**. For other dendrimers normal morphology of cells was observed (Figure 4).

Under physiological conditions red blood cells are suspended in the plasma which contains proteins, among which serum albumin is the most abundant. There are many proofs that serum albumin in the incubation medium significantly diminished the membrane perturbations caused by dendrimers.[34] For viologen-phosphorus dendrimers no significant effect of albumin on red blood cells hemolysis was observed (Figure 3); this would suggest the lack of protective effect of albumin due to weak interactions between dendrimers and albumin.

The erythrocyte membrane is a first barrier to cross for dendrimers. It is very important how they behave in contact with membrane. TMA-DPH is a hydrophobic molecule, which is anchored at the water/lipid interface due to its charged trimethylammonium group. This fluorophore reports then on the mobility of the lipid headgroup region of membrane.[35] The highest dendrimer concentrations induced statistically significant decrease in membrane fluidity for dendrimers **4**, **6**, **7** and **8** (Figure 5). These dendrimers, except dendrimer **4**, possess the highest molecular weights. The results suggest that dendrimers can interact with the polar headgroup region of the phospholipid bilayer. Higher generation dendrimers interact more strongly with membranes, and the concentration, as well as the generation, is of similar importance.[36]

Viability of B14 cells (control cell line) and N2a cells (cancer cell line) treated with dendrimers was determined by MTT assay. For B14 cells five, out of eight, dendrimers were cytotoxic: dendrimers **2**, **3**, **5**, **6**, and **7** (Figure 6). However, they were cytotoxic only when the concentration exceeded 5 μmol/L. Three of them, **5**, **6**, and **7**, have many viologen units and high positive charge (12, 18 and 36, respectively), and they have phosphonate end groups while dendrimers **2** and **3** have aldehyde terminal groups. A similar situation was observed for the hemolytical assay. Modification of the dendrimer surface plays a crucial role in their biological activity. Some modifications of dendrimers, e.g. PEGylation, cause lower cytotoxicity to normal cell line than unmodified polymers.[37] Also in our studies PEGylated dendrimer **8** was not toxic to B14 cells. Cell viability of cancer cell line N2a decreased after administration of dendrimers **4**, **5**, **6**, and **8** (Figure 7). Three of them are ended with phosphonate groups, and dendrimer **8** is coated with PEG. The behavior of dendrimer **4** and PEGylated dendrimer **8** is fascinating: contrary to the control of cell line B14, dendrimer **4** and PEGylated dendrimer **8** were very cytotoxic toward cancerous cell line N2a. The altered structure of cancer cell membranes may be one of the reasons for that. Having a compound which is harmless to normal cells being at the same time highly toxic to cancer cells may open very interesting perspectives for biomedical applications.

The next step was to check antimicrobial activity of viologen dendrimers. The chemicals which contain viologen units present excellent antibacterial activity against the bacteria pathogens, e.g. *Staphylococcus aureus* and *Escherichia coli* .[38] Many studies have shown that dendrimers are good carriers of biologically active agents, but only few describe their antimicrobial activity. Lopez et al.[37] reported that amino-terminated PAMAM dendrimers unmodified and modified with poly(ethylene glycol) groups exhibited antimicrobial activity against *S. aureus* and *P. aeruginosa*. The amine- and ammonium-terminated carbosilane dendrimers have been described as potent antimicrobial agents.[39−42] Antimicrobial and antifungal activity was also demonstrated for amine-terminated dendrimers with poly(propylene oxide) amine core and methylacrylate and ethylenediamine core[43] and for functionalized PPI dendrimers.[41] To the best of our knowledge there is no report of antimicrobial activity of viologen containing dendrimers. The possible role of viologen monomers was mentioned in two

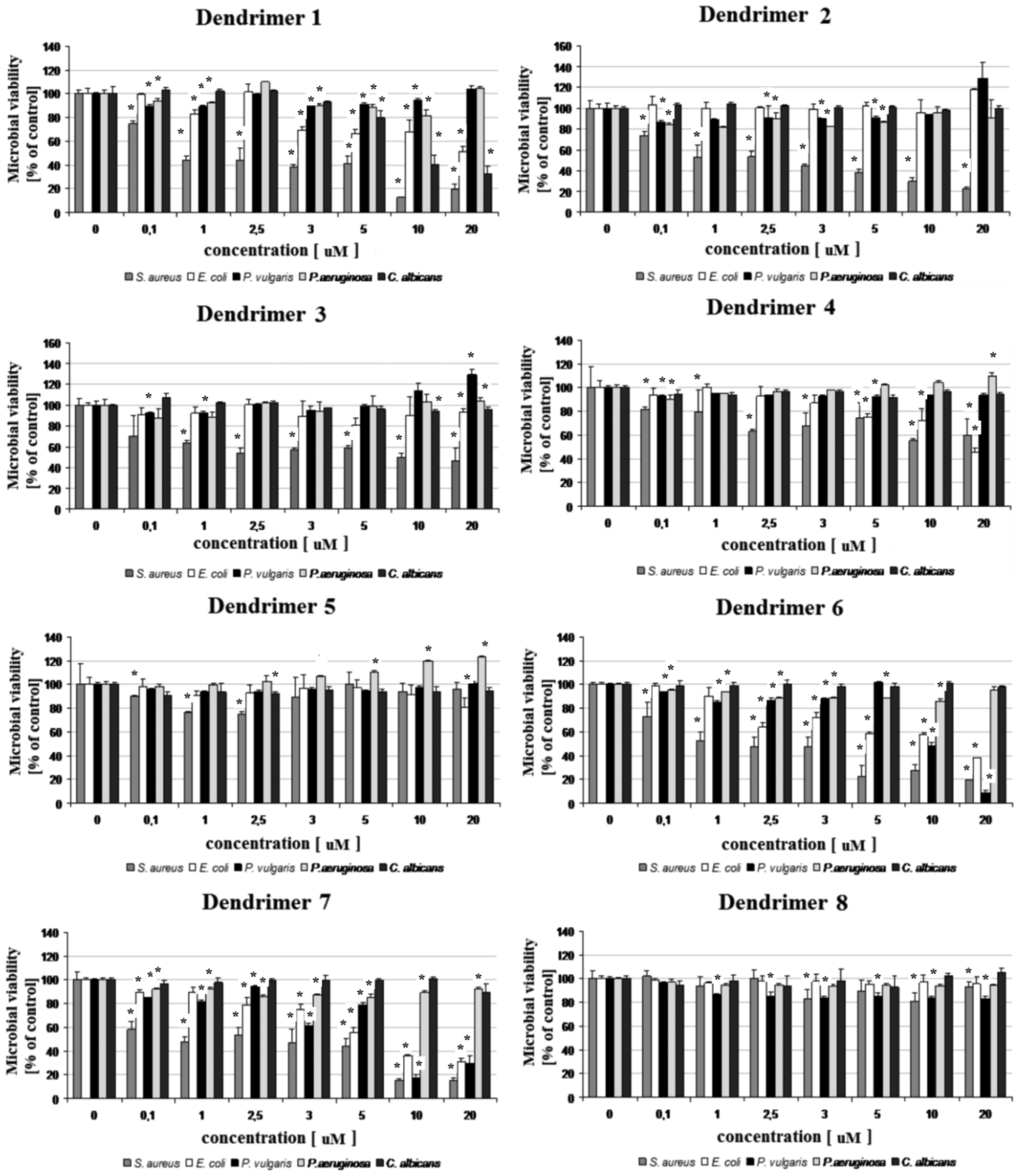


Figure 8. Antimicrobial activity of viologen-phosphorus dendrimers against tested microorganisms after 24 h incubation.

reports: in each of them viologen units are incorporated into the backbone of polymers. In a previous report a viologen, namely, the *N*-hexyl-*N*-(4-vinylbenzyl)-4,4′-bipyridinium dinitrate (HVVN), was prepared and subsequently graft-copolymerized on poly(ethylene terephthalate) (PET) films on which silver nanoparticles were deposited (silver as a powerful antibacterial agent has been used for many years). The HVVN chains stabilized silver nanoparticles and were claimed to confer an antibacterial property to PET; this antibacterial effect is very significantly enhanced by the presence of silver nanoparticles.[44] Antibacterial activity of viologen pendant indole stabilized silver nanoparticles was also reported: nevertheless, apart from the stabilization of silver nanoparticles by the polymer, no real evidence was given for a direct effect of viologen monomers on the antibacterial properties of the resulting macromolecules.[38]

dx.doi.org/10.1021/mp200549c | *Mol. Pharmaceutics* 2012, 9, 448−457

Antimicrobial activity of viologen-phosphorus dendrimers of generation 0 and generation 1 toward Gram-positive bacterium *Staphylococcus aureus* ATCC 6538, Gram-negative bacteria *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315 and *Pseudomonas aeruginosa* ATCC 15442 and yeast *Candida albicans* ATCC 10231 was determined. First of all the influence of viologen dendrimers with peripheral aldehyde groups on microbial growth was evaluated. All these dendritic structures were of generation 0 (dendrimers **1**, **2** and **3**). Dendrimer **2** and dendron **1** possess on their periphery 3 aldehyde groups while dendrimer **3** has 6 aldehyde end groups. The obtained results indicate that all these dendritic structures limited the growth of *S. aureus*, but the dendron and the dendrimer with 3 end groups were more effective and caused 80% growth reduction in samples containing 20 μmol/L of compounds. In the same concentration dendrimer **3** limited the growth of *S. aureus* only by 60%. The reason for the lower toxicity of **3** may be connected with the number of end groups and the high mass of tested macromolecules. It is worth mentioning that, among aldehyde-terminated dendrimers, only dendron **1** exhibited good antimicrobial properties toward Gram-negative bacterium *E. coli* and yeast *C. albicans*. Addition of **1** in the highest tested concentration caused 50% and 70% growth inhibition of *E. coli* and *C. albicans*, respectively. It can be concluded that not only the number of end groups determines the antimicrobial activity of macromolecules but also the chemical structure of core region.

Next the microbial susceptibility to viologen-phosphorus dendrimers of generation 0 terminated with phosphonate groups was carried out. Studies were performed with dendrimers **4** and **5**, the chemical structure (core and interior) of which corresponds with the structure of dendrimers **2** and **3**, respectively. The obtained results revealed that replacement of aldehyde groups by phosphonate groups caused reduction of antimicrobial activity of compounds against *S. aureus*. The addition of dendrimer **5** to *S. aureus* culture limited the growth of bacterium by 10−15%, whereas in samples containing dendrimer **3** the growth was reduced by 40−50%. A similar situation was observed for **4**, which in the highest tested concentration limited the growth of *S. aureus* only by 40%, while in samples containing dendrimer **2** inhibition of growth reached the value of 80%. These results indicate that among dendrimers of generation 0 the macromolecules with aldehyde groups are more potent antimicrobial agents.

Among dendrimers of generation 1, we tested only dendrimers terminated with phosphonate groups (dendrimers **6** and **7**). These dendrimers possess the highest numbers of viologen units and positive charges. They were able to reduce the growth of *S. aureus*, *E. coli* and additionally *P. vulgaris*. They showed the strongest antimicrobial activity against tested Gram-positive bacterium compared to Gram-negative bacteria. The addition of 20 μmol/L of dendrimers **6** or **7** caused 80% growth reduction of *S. aureus*, in comparison to control samples without dendritic structures. In case of *E. coli* the limitation of growth reached values of 70% and 60% for dendrimers **7** and **6**, respectively. Tested viologen dendrimers exhibited satisfactory antimicrobial properties also against *P. vulgaris*. In samples containing the highest tested concentration of dendrimers, inhibition of growth ranged from 70% to 90%. During incubation of *P. vulgaris* bacteria with dendrimers a change of color (violet) was observed for all the dendrimer derivatives. This phenomenon was not observed in abiotic control, containing only medium and dendrimers, which indicates that *P. vulgaris* is able to convert the dendrimers to unknown derivatives. It must be mentioned that in this described situation we cannot clearly state which compound limited the growth of *P. vulgaris*, and the ability of *P. vulgaris* in bioconversion of dendrimers needs future research.

■ CONCLUSION

A series of viologen-phosphorus dendrimers of generation 0 and 1 and a dendron of generation 0 were prepared and their biological properties investigated. Attention was focused on hemotoxicity, erythrocyte morphology, membrane fluidity, *in vitro* cytotoxicity and antibacterial activity of these new dendritic structures. Even if their versatile behavior was observed, several features of these dendrimers can be emphasized. Dendrimers bearing the highest number of charges, i.e. the highest number of viologen units, cause more hemolysis. Higher generations also interact more strongly with membranes. Remarkably two of these dendrimers, dendrimer **4** and the PEGylated dendrimer **8**, are not toxic to B14 cells but are very cytoxic toward cancerous cells N2a. It is known that the chemical structure, surface charge, 3D structure and size play a crucial role in determining antimicrobial activity. All tested dendrimers exhibited good antimicrobial properties toward the Gram-positive strain *S. aureus*. Some of them—dendrimers **6** and **7**, bearing the highest numbers of viologen units and positive charges—also limited the growth of Gram-negative strains *E. coli* and *P. vulgaris*. It may depend on both structure of macromolecules and structure of the bacterial cell wall, which is different for Gram-positive and Gram-negative bacteria. Additional studies are underway aiming to get a better understanding on the behavior of these bioreactive dendritic structures as well as those of their higher generation congeners.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of synthesis and full characterization of building blocks necessary for the preparation of dendrimers **1**−**8**, and of dendrimers **1**−**8** (17 compounds). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*J.P.M.: CNRS, Laboratoire de Chimie de Coordination, 205 route de Narbonne, F-31077 Toulouse cedex 4, France; e-mail, majoral@lcc-toulouse.fr; tel, 00 33 5 61 33 31 23; fax, 00 33 5 61 55 30 03. M.B.: e-mail, marbrys@biol.uni.lodz.pl. A.E.K.: e-mail, a.elkadib@mascir.com.

■ ACKNOWLEDGMENTS

The authors thank COST—European Cooperation in Science and Technology TD0802 for financial support. Studies were also funded by project "Biological Properties and Biomedical Applications of Dendrimers" operated within Foundation for Polish Science TEAM/2008-1/5 programme cofinanced by the European Regional Development Fund and by the CNRS (France) and MAScIR (Moroccan Foundation for Advanced Science, Innovation and Research).

■ REFERENCES

(1) Ross, J. H.; Krieger, R. I. Synthesis and properties of paraquat (methyl viologen) and other herbicidal alkyl homologs. *J. Agric. Food Chem.* **1980**, *28*, 1026−1031.

(2) Fukushima, T.; Tanaka, K.; Lim, H.; Moriyama, M. Mechanism of cytoxicity of paraquat. *Environ. Health Prev. Med.* **2002**, *7*, 89−94.

(3) Moran, J. M.; Ortiz-Ortiz, M. A.; Ruiz-Mesa, L. M.; Fuentes, J. M. Nitric Oxide in Paraquat-Mediated Toxicity: A Review. *J. Biochem. Mol. Toxicol.* **2010**, *24*, 402−409.
(4) Castonguay, A.; Wilson, E.; Al-Hajaj, N.; Petitjean, L.; Paoletti, J.; Maysinger, D.; Kakkar, A. Thermosensitive dendrimer formulation for drug delivery at physiologically relevant temperatures. *Chem. Commun.* **2011**, *47*, 12146−12148, DOI: 10.1039/C1CC15354D.
(5) Wang, K.; Guo, D.-S.; Zhang, H.-Q.; Li, D.; Zheng, X.-L.; Liu, Y. Highly effective binding of viologens by *p*-sulfonatocalixarenes for the treatment of viologen poisoning. *J. Med. Chem.* **2009**, *52*, 6402−6412.
(6) Asaftei, S.; De Clercq, E. "Viologen" Dendrimers as Antiviral Agents: The Effect of Charge Number and Distance. *J. Med. Chem.* **2010**, *53*, 3480−3488.
(7) Caminade, A. M.; Turrin, C. O.; Majoral, J. P. Biological properties of phosphorus dendrimers. *New J. Chem.* **2010**, *34*, 1512−1524.
(8) Hernandez-Lopez, J. L.; Khor, H. L.; Caminade, A. M.; Majoral, J. P.; Mittler, S.; Knoll, W.; Kim, D. H. Bioactive multilayer thin films of charged N,N-disubstituted hydrazine phosphorus dendrimers fabricated by layer-by-layer self-assembly. *Thin Solid Films* **2008**, *516*, 1256−1264.
(9) Poupot, M.; Griffe, L.; Marchand, P.; Maraval, A.; Rolland, O.; Martinet, L.; L'Faqihi-Olive, F. E.; Turrin, C. O.; Caminade, A. M.; Fournié, J. J.; Majoral, J. P.; Poupot, R. Design of phosphorylated dendritic architectures to promote human monocyte activation. *FASEB J.* **2006**, *20*, 2339−2351.
(10) Griffe, L.; Poupot, M.; Marchand, P.; Maraval, A.; Turrin, C. O.; Rolland, O.; Metivier, P.; Bacquet, G.; Fournie, J. J.; Caminade, A. M.; Poupot, R.; Majoral, J. P. Multiplication of human natural killer cells by nanosized phosphonate-capped dendrimers. *Angew. Chem., Int. Ed.* **2007**, *46*, 2523−2526.
(11) Rolland, O.; Griffe, L.; Poupot, M.; Maraval, A.; Ouali, A.; Coppel, Y.; Fournie, J. J.; Bacquet, G.; Turrin, C. O.; Caminade, A. M.; Majoral, J. P.; Poupot, R. Tailored control and optimization of the number of phosphonic acid termini on phosphorus-containing dendrimers for the ex-vivo activation of human monocytes. *Chem.—Eur. J.* **2008**, *14*, 4836−4850.
(12) Hayder, M.; Poupot, M.; Baron, M.; Nigon, D.; Turrin, C. O.; Caminade, A. M.; Majoral, J. P.; Eisenberg, R. A.; Fournie, J. J.; Cantagrel, A.; Poupot, R.; Davignon, J. L. A Phosphorus-Based Dendrimer Targets Inflammation and Osteoclastogenesis in Experimental Arthritis. *Sci. Transl. Med.* **2011**, *3*, 81ra35.
(13) Caminade, A. M.; Padie, C.; Laurent, R.; Maraval, A.; Majoral, J. P. Uses of dendrimers for DNA microarrays. *Sensors* **2006**, *6*, 901−914.
(14) Slomkowski, S.; Miksa, B.; Chehimi, M. M.; Delamar, M.; Cabet-Deliry, E.; Majoral, J. P.; Caminade, A. M. Inorganic-organic systems with tailored properties controlled on molecular, macromolecular and microscopic level. *React. Funct. Polym.* **1999**, *41*, 45−57.
(15) Le Berre, V.; Trevisiol, E.; Dagkessamanskaia, A.; Sokol, S.; Caminade, A. M.; Majoral, J. P.; Meunier, B.; Francois, J. Dendrimeric coating of glass slides for sensitive DNA microarrays analysis. *Nucleic Acids Res.* **2003**, *31*, e88.
(16) Trevisiol, E.; Le Berre-Anton, V.; Leclaire, J.; Pratviel, G.; Caminade, A. M.; Majoral, J. P.; Francois, J. M.; Meunier, B. Dendrislides, dendrichips: a simple chemical functionalization of glass slides with phosphorus dendrimers as an effective means for the preparation of biochips. *New J. Chem.* **2003**, *27*, 1713−1719.
(17) Loup, C.; Zanta, M. A.; Caminade, A. M.; Majoral, J. P.; Meunier, B. Preparation of water-soluble cationic phosphorus-containing dendrimers as DNA transfecting agents. *Chem.—Eur. J.* **1999**, *5*, 3644−3650.
(18) Maszewska, M.; Leclaire, J.; Cieslak, M.; Nawrot, B.; Okruszek, A.; Caminade, A. M.; Majoral, J. P. Water-soluble polycationic dendrimers with a phosphoramidothioate backbone: Preliminary studies of cytotoxicity and oligonucleotide/plasmid delivery in human cell culture. *Oligonucleotides* **2003**, *13*, 193−205.
(19) Maksimenko, A. V.; Mandrouguine, V.; Gottikh, M. B.; Bertrand, J. R.; Majoral, J. P.; Malvy, C. Optimisation of dendrimer-mediated gene transfer by anionic oligomers. *J. Gene Med.* **2003**, *5*, 61−71.
(20) Blanzat, M.; Turrin, C. O.; Aubertin, A. M.; Couturier-Vidal, C.; Caminade, A. M.; Majoral, J. P.; Rico-Lattes, I.; Lattes, A. Dendritic catanionic assemblies: In vitro anti-HIV activity of phosphorus-containing dendrimers bearing Gal beta(1)cer analogues. *ChemBioChem* **2005**, *6*, 2207−2213.
(21) Solassol, J.; Crozet, C.; Perrier, V.; Leclaire, J.; Beranger, F.; Caminade, A. M.; Meunier, B.; Dormont, D.; Majoral, J. P.; Lehmann, S. Cationic phosphorus-containing dendrimers reduce prion replication both in cell culture and in mice infected with scrapie. *J. Gen. Virol.* **2004**, *85*, 1791−1799.
(22) Spataro, G.; Malecaze, F.; Turrin, C. O.; Soler, V.; Duhayon, C.; Elena, P. P.; Majoral, J. P.; Caminade, A. M. Designing dendrimers for ocular drug delivery. *Eur. J. Med. Chem.* **2010**, *45*, 326−334.
(23) Mongin, O.; Krishna, T. R.; Werts, M. H. V.; Caminade, A. M.; Majoral, J. P.; Blanchard-Desce, M. A modular approach to two-photon absorbing organic nanodots: brilliant dendrimers as an alternative to semiconductor quantum dots? *Chem.Commun.* **2006**, 915−917.
(24) Terenziani, F.; Parthasarathy, V.; Pla-Quintana, A.; Maishal, T.; Caminade, A. M.; Majoral, J. P.; Blanchard-Desce, M. Cooperative Two-Photon Absorption Enhancement by Through-Space Interactions in Multichromophoric Compounds. *Angew. Chem., Int. Ed.* **2009**, *48*, 8691−8694.
(25) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(26) Shcharbin, D.; Pedziwiatr, E.; Nowacka, O.; Kumar, M.; Zaborski, M.; Ortega, P.; de la Mata, F. J.; Gómez, R.; Muñoz-Fernandez, M. A.; Bryszewska, M. Carbosilane dendrimers NN8 and NN16 form a stable complex with siGAG1. *Colloids Surf., B* **2011**, *83*, 388−391.
(27) Padié, C.; Maszewska, M.; Majchrzak, K.; Nawrot, B.; Caminade, A. M.; Majoral, J. P. Polycationic phosphorus dendrimers: synthesis, characterization, study of cytotoxicity, complexation of DNA, and transfection experiments. *New J. Chem.* **2009**, *33*, 318−326.
(28) Klajnert, B.; Janiszewska, J.; Urbanczyk-Lipkowska, Z.; Bryszewska, M.; Shcharbin, D.; Labieniec, M. Biological properties of low molecular mass peptide dendrimers. *Int. J. Pharm.* **2006**, *309*, 208−217.
(29) Summers, L. A. *The bipyridinum herbicides*; Academic Press: New York, 1980.
(30) Qi, R.; Gao, Y.; Tang, Y.; He, R. R.; Liu, T. L.; He, Y.; Sun, S.; Li, B. Y.; Li, Y. B.; Liu, G. PEG- conjugated PAMAM dendrimers mediate efficient intramuscular gene expression. *AAPS J.* **2009**, *11*, 395−405.
(31) Hong, S.; Bielinska, A. U.; Mecke, A.; Keszler, B.; Beals, J. L.; Shi, X.; Balogh, L.; Orr, B. G.; Baker, J. R. Jr.; Banaszak Holl, M. M. Interaction of poly(amidoamine) dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport. *Bioconjugate Chem.* **2004**, *15*, 774−782.
(32) Malik, N.; Wiwattanapatapee, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J. W.; Meijer, E. W.; Paulus, W.; Duncan, R. Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. *J. Controlled Release* **2000**, *65*, 133−48.
(33) Domański, D. M.; Klajnert, B.; Bryszewska, M. Influence of PAMAM dendrimers on human red blood cells. *Bioelectrochemistry* **2004**, *63*, 189−191.
(34) Klajnert, B.; Pikala, S.; Bryszewska, M. Haemolytic activity of polyamidoamine dendrimers and the protective role of human serum albumin. *Proc. R. Soc. A* **2010**, *466*, 1527−1534.
(35) Prendergast, F. G.; Haugland, R. P.; Callahan, P. J. 1-[4-(Trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties, and use as a fluorescence probe of lipid bilayers. *Biochemistry* **1981**, *20*, 7333−7338.
(36) Wrobel, D.; Ionov, M.; Gardikis, K.; Demetzos, C.; Majoral, J. P.; Palecz, B.; Klajnert, B.; Bryszewska, M. Interactions of phosphorus-containing dendrimers with liposomes. *Biochim. Biophys. Acta* **2011**, *11*, 221−226.

(37) Lopez, A. I.; Reins, R. Y.; McDermott, A. M.; Trautner, B. W.; Cai, C. Antibacterial activity and cytotoxicity of PEGylated poly-(amidoamine) dendrimers. *Mol. Biosyst.* **2009**, *5*, 1148−56.
(38) Ganesh Kumar, V.; Govindaraju, K.; Singaravelu, G.; Adhikesavalu, D. Antibacterial activity of viologen pendant indole stabilized silver nanoparticles. *J. Biopestic.* **2009**, *2*, 217−221.
(39) Ortega, P.; Copa-Patiño, J. L.; Muñoz-Fernandez, M. A.; Soliveri, J.; Gomez, R.; de la Mata, F. J. Amine and ammonium functionalization of chloromethylsilane-ended dendrimers. Antimicrobial activity studies. *Org. Biomol. Chem.* **2008**, *6*, 3264−3269.
(40) Calabretta, M. K.; Kumar, A.; McDermott, A. M.; Cai, C. Antibacterial activities of poly(amidoamine) dendrimers terminated with amino and poly(ethylene glycol) groups. *Biomacromolecules* **2007**, *6*, 1807−1811.
(41) Chen, C. Z.; Beck-Tan, N. C.; Dhurjati, P.; van Dyk, T. K.; LaRossa, R. A.; Cooper, S. L. Quaternary ammonium functionalized poly(propylene imine) dendrimers as effective antimicrobials: structure-activity studies. *Biomacromolecules* **2000**, *1*, 473−480.
(42) Rasines, B.; Hernández-Ros, J. M.; de las Cuevas, N.; Copa-Patiño, J. L.; Soliveri, J.; Muñoz-Fernández, M. A.; Gómez, R.; de la Mata, F. J. Water-stable ammonium-terminated carbosilane dendrimers as efficient antibacterial agents. *Dalton Trans.* **2009**, *40*, 8704−8713.
(43) Tulu, M.; Aghatabay, N. M.; Senel, M.; Dizman, C.; Parali, T.; Dulger, B. Synthesis, characterization and antimicrobial activity of water soluble dendritic macromolecules. *Eur. J. Med. Chem.* **2009**, *44*, 1093−1099.
(44) Shi, Z.; Neoh, K. G.; Kang, E. T. Surface-grafted viologen for precipitation of silver nanoparticles and their combined bactericidal activities. *Langmuir* **2004**, *20*, 6847−6852.