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Alkylene-bridged Viologen Dendrimers: A Versatile Cell Delivery Tool With Biosensing Properties

by

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Abstract

The synthesis of two types of viologen dendrimers with peripheral carboxy groups is described. The interaction with plasmid DNA and CT-DNA, time evolution and electrolyte influence of dendriplex formation have been electrochemically investigated. A negative potential shift appearing in cyclic voltammogramms indicates the dendriplex formation revealing a similar time scale of 15 to 19 minutes for dendriplex formation that has been determined empirically for other dendrimer types. The appearance or absence of this negative potential shift can be used to check the sodium chloride stability and different cell growth media directing to sucrose for cell incubation experiments. The electrolyte content of the commercial available cell growth media inhibits the dendriplex formation in solution prior to plasmid addition.

Furthermore, the low salt stability of 20 mmol sodium chloride for viologen dendriplexes has been confirmed also recommending the lysosomotropic sucrose. The two types of viologen dendrimers have been combined with two plasmids differing in the number of basepairs. Four immortal cell lines have been tested to check the ability of viologen dendriplexes as gene delivery system. But due to the absence of terminal amino groups and endosomolytic substances only a small cell incubation of dendriplexes at low pH has been achieved generally excluding in vivo applications. Interestingly, with the larger pHSV-eGFP plasmid (5743 bp) no transfected cells were observed indicating preference for shorter plasmids.

Keywords

Cell incubation, Cyclic voltammetry, Dendrimers, Electrochemistry, Sucrose
Introduction

The overwhelming biological application of dendrimers and dendritic substances are drug delivery systems and gene therapies. Many substances can be used for non-viral transfection of mammalian cells. Beside cationic lipids, polymers and dendrimers aminofullerenes and polycationic-modified cyclodextrins are latest examples for new transfection agents[1-4]. In the past years, the efforts to elucidate the mechanism of DNA release from dendriplexes after cell uptake has been raised. For polyethylenimine (PEI) dendrimers the proton sponge mechanism is established explaining the high transfection efficiency and for polyamidoamin dendrimers (PAMAM) the same mechanism is suspected[5]. It is known that endocytosis and endosomal inclusion of the dendrimers and dendriplexes is the step of the cellular uptake, but there is a lack of knowledge concerning the DNA release in the cell. Considering the low intracellular diffusion rate of plasmid DNA caused by the molecular crowding within mammalian cells the viologen dendrimers can help to clarify where the endosomal release occurs, in the cytoplasm or in the perinuclear zone[6,7]. Relative to the size of the polymeric and dendritic structure the toxicity of dendrimers is decreased at the step from monomeric to polymeric compounds and an application as gene and cell delivery system becomes possible, perhaps also for viologen dendrimers. Viologens are a class of organic salts containing double substituted nitrogen atoms with two persistent cationic charges per viologen (4,4'-bipyridinium) unit. The most important application is the employment for electrochromic displays[8]. Here not only the monomeric N,N'-alkyl and -aryl substituted bipyridinium...
salts exhibit electrochromic properties, but also supramolecular viologen stars showing exceptionally intramolecular color addition in their framework\cite{9,10}. The strong toxicity of monomeric viologens is well-known (Paraquat\textsuperscript{TM}) and there are investigations concerning the cytotoxicity, hematotoxicity of viologen-phosphorus dendrimers revealing for the first generation dendrimers an according toxicity compared to monomeric viologens. Surprisingly, the dendrimers zeroth generation seem to be for some cell lines non toxic but specific toxic against cancer cells\cite{11,12}.

However, viologen dendrimers offers the possibility to investigate the dendriplex formation before incubation with electrochemical methods. Cyclic voltammetry requires a redox active species such as viologens or ferrocens. Hvastkovs and Buttry observed a negative potential shift detecting the interaction of dsDNA with tetracationic diviologen compounds\cite{11}. The negative potential shift indicates a stabilization of the Vio\textsuperscript{2+} redox state in the viologens caused from the interaction with the DNA. It is assumed that such small viologens are inserted in the minor groove of the DNA. Here we describe the synthesis of two types of viologen dendrimers and the interaction of them with two expression vectors, pEGFP-C1 with 4731 bp and the larger one, pHSV-eGFP with 5734 bp. The redox-active 4,4'-bipyridinium-(viologen)-units within the dendrimers allow an unique electrochemical detection of the dendriplex formation and the feasibility to check the suitability of different cell growth media.

Furthermore, the proceeding development of ultramicroelectrodes (nanodes) offers the possibility to explore the fate of dendrimers within a single eukaryotic cell\cite{12,13}. There are some examples for the versatile utilization of micrometer- and ultra-
micrometer electrodes. The successfully performed amperiometric detection of dopamine and other catecholamines in chromaffin cells like it is reported by Mosharov et. al.\textsuperscript{[14]}. By the help of micrometer- and submicrometer-sized Pt electrodes the production and release of reactive oxidation and nitrogen species (ROS, RNS) can be amperometrically detected\textsuperscript{[15]}. For small viologens it has been shown that there is no interaction with the predominantly existing redox system NADH / NAD\textsuperscript{+} in the cytosol. Viologen dendrimers reveal the possibility to elucidate electrochemically on one hand the DNA release of dendriplexes within a mammalian cell, but also the mechanism and location of drug release whether the release is occurred in the cytosol or near the cell nucleus (Figure 1). In the recent study we found that the more flexible dendrimer type with hexamethylene groups between the viologen units leads to a better salt stability of dendriplexes compared to the methylene bridged dendrimers, and a small cell incubation (transfection) of CHO and PC12 cells were achieved with them.

**Results and Discussion**

1. Syntheses

The synthetic route to obtain the two types of viologen dendrimers is shown in scheme 1 and 2. Periphery unit 2 and the adequate mono-quarternary- 4,4\textsuperscript{-}bipyridinium salts 3, 4 and 5 have been achieved via Metschukin reaction of 4,4\textsuperscript{-}bipyridine in acetonitrile in good yields. After a subsequently performed anion exchange with 3 M aqueous ammonium hexafluorophosphate these compounds were ready for closed reaction of dendrimer build up (scheme 1). Starting from 1,3,5-tris-bromomethyl-benzene both types of viologen dendrimers have been obtained in
Metschukin reactions to form in acetonitrile first the hydroxymethyl-(p$_1$OH) and hydroxyhexylene precursor (p$_2$OH). The direct conversion of 1,3,5-tris-bromo-methylbenzene with 1-(2-hydroxy-ethyl)-4,4'-bipyridinium-hexafluorophosphate (5) (prepared according to literature[18]) in acetonitrile resulted in dendrimer 0th generation G$_0$-OH in a yield of 26%. In case of methylene bridged viologen dendrimers the build up proceeded after activation of precursor p$_1$OH with 5.7 M acetic hydrobromic acid to the p$_1$Br bromide salt. The following conversion to the hexafluorophosphate salts prepared precursor p$_1$Br for the last reaction step with 1-(2-carboxyethyl)-4,4'-bipyridinium-hexafluorophosphate (2) to afford the according methylene-bridged dendrimer 1th generation G$_1$-COOH in a yield of 39.8%. In case of the hexylene-bridged dendrimer the spacer hexylene chain has been inserted via Metschukin reaction of 1,3,5-tris-bromomethylbenzene with 1-(6-hydroxyhexyl)-4,4'-bipyridinium salt (6) to form p$_2$OH. The following activation with hydrobromic acid resulted in precursor p$_2$Br and facilitates the attachment of branching unit (1) to yield p$_3$OH. The last activation step with hydrobromic acid prepared precursor p$_3$Br for the closing with 1-(5-carboxypentyl-4,4'-bipyridinium-hexafluorophosphate (5) to achieve dendrimer G$_1$-Hexyl-COOH in a yield of 41 %.

2. Cyclic voltammetry

In Figure 2, the time evolution for dendriplex formation of G$_1$-COOH and plasmid pC1-eGFP is shown. 20.4 µM G$_1$-COOH yielded an about similar negative potential shifting and current like it has been described by Hvastkovs and Buttry for tetracationic diviologens$^{[11]}$. The decrease in current from scan 1 to 5 indicates a loss
of dendrimer molecules in solution bounded by the DNA. The complex formation has been extended about 19 minutes, then no further potential shifting was observed. The formal negative potential shift was -0.105 V and is comparable to the -0.123 V for the dsDNA complexed diviologen compound by Hvastkovs and Buttry. A similar potential shift and time dependence has been observed for both types of viologen dendrimers.

Changing the surface group leading to no significant differences in the electrochemical properties of the dendrimers. Synthesis of the more flexible dendrimer had the aim to obtain dendriplexes with better electrolyte stability. In Figure 3, the electrochemical features of \( G_1\)-Hexyl-COOH / pC1-eGFP under the influence of sodium chloride is shown. We observed a same decrease in current and the potential shifting was preserved up to 20 mM sodium chloride (scan 1 and 2, Figure 3). After that, the current increased and a potential shifting to the left side has been observed, which is in contrast to the behaviour of DNA-containing methylene-bridged viologen dendrimers and is probably caused by the sodium chloride (scan 3 and 4, Figure 3).

The same experiment for \( G_1\)-COOH resulted in a lower salt stability. We confirmed for methylene-bridged viologen dendrimers a value of 10 mM showing the influence of both ions. A low salt stability that was previously found by Marchioni et. al. in methylene-bridged viologen dendrimer complexes with eosin\(^{[19]}\). That means the substitution of methylene groups by hexyl spacers resulted in a 100% better electrolyt stability.

The shift in potential can be used to check the different cell growth media for the electrolyte effect during the incubation of transfection experiments. In standard cell growth media such as DMEM or OptiMEM no negative potential shifting could be observed, which is most likely due to the high content of electrolytes in these media.
that prevent the dendriplex formation. For this purpose cyclic voltammetry has been used in a qualitative way, the appearance of a negative potential shifting indicates the preservation and a return the dissoziation of the DNA complexes. In Figure 4, the CV’s of $G_0$-$\text{OH}$ / calf thymus DNA complexes in DMEM and OptiMEM are compared to physiological sodium chloride and 0.25 M aqueous sucrose. Here the measurements were performed in a three electrode system, in a cell volume of 10 ml and calf thymus DNA instead of plasmids. The scan rate was 400 mV/s. The electrode was not polished after first and following voltammetric scans. In counterpart to the measurements of Figure 2 where the current decreased after plasmid addition, here the the current increased indicating an adsorption onto the electrode surface. The addition of 150 mM sodium chloride lead to a return to current values of pure dendrimer solution indicating the dissoziation of the dendrimer / DNA complex (scan 2 to 3, Figure 4) for the $G_0$-$\text{OH}$ / calf thymus DNA system. The cyclic voltammetric measurements with DMEM and OptiMEM were carried out in reversed modi operandi. The dendrimer $G_0$-$\text{OH}$ was soluted in the aqueous cell growth media by a pH 8.5 and the calf thymus DNA was added (scan 2, Figure 4 c,d). The experiment with DMEM has been performed with 9.5 $\mu$M $G_0$-$\text{OH}$ and 130 $\mu$M CT-DNA, the analog OptiMEM trial was carried out with 4 times more dendrimer and nearly the double amount of CT-DNA. Despite there was no negative potential shifting observed but a small shift of about 10 mV in positive direction, an increase in current of 1 $\mu$A was visible in both cases. This indicates a precipitation onto the electrode surface but was not interpreted as complex formation due to the four times more dendrimer concentration and double amount of DNA. Only in 0.25 M sucrose the potential shift and a stable current have been preserved and in this medium dendriplex incubations
(transfections) were successfully achieved.

3. Transfection experiments

Cell incubations (transfections) were achieved with dendrimer $G_1$-$\text{Hexyl-COOH}$ for CHO and NIH 3T3 cells, albeit with very low transfection efficiency. Two expression vectors, pC1-eGFP with 4731 basepairs (bp) and pHSV-eGFP with 5743 bp have been tested with the result that only dendriplexes of the smaller one (2 $\mu$g) combined with $G_1$-$\text{Hexyl-COOH}$ (2 $\mu$g) showing successfully transfected cells (pictures of transfected CHO cells can be seen in the supporting information).

The all-deciding experimental condition was the fully exclusion of all electrolytes during the incubation of the dendriplexes to the cells. Interestingly, with the larger pHSV-eGFP plasmid (5743 bp) no transfected cells were observed indicating preference for shorter plasmids. In Figure 4 it can be seen that only in 0.25 M aqueous sucrose the negative potential shift is obtained indicating the uphold of the dendriplexes, whereas in DMEM and OptiMEM, the common used cell growth media, and sodium chloride at physiological concentration the dendriplexes dissoziated.

Therefore, in aqueous sucrose the two cell types were transfected with an efficiency between 0.03 to 1.1 % related to the Superfect transfection agent and an overall transfection efficiency of 0.003% related to the number of all treated cells.

However, sucrose combined with the synthetic buffer MES allows for viologen dendrimers the use as gene delivery system for \textit{in vitro} applications. Ciftci and Levy have been demonstrated that sucrose shows not only a lysosomotropic effect, but also an enhancement in transfection efficiency$^{[20]}$. 

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Conclusion

In summary two types of alkylene-bridged viologen dendrimers have been synthesized with intention to elucidate an application for in vivo and in vitro gene delivery system. According to the well-known poly(N-ethyl-4-vinylpyridinium) PEVP polymers we expected a significant transfection efficiency for viologen dendrimers even without terminal amino groups\(^{[21]}\).

But due to the absence of terminal amino groups the deciding structural feature in PAMAM- and PEI dendrimers and responsible for the high transfection efficiency, the absence of endosomolytic substances we found only a very small cell incubation for viologen dendriplexes. The disadvantage for PAMAM and PEI dendrimers with a more or less cytotoxicity based on the pH depending ratio of protonated to non protonated primary amino groups preventing in vivo application. The persistent pH independent positive charges in the framework of viologen dendrimers endow them with a low cytotoxicity compared to PAMAM and PEI dendrimers but providing a sufficient drug and DNA complexation.

The cellular uptake, haemotoxicity and cell viability of structural cognated viologen-phosphorus dendrimers in erythrocytes has been confirmed\(^{[12]}\). The advantage of quarternary amino groups in bipyridinium units containing viologen dendrimers culminate for viologen dendrimer 0\(^{th}\). generation in specific toxicity against cancer cells and almost non toxic behaviour against common immortal cell lines.

Surprisingly, only the combination of the smaller plasmid together with the more flexible hexylene-bridged viologen dendrimer show positive results. Having in mind the low electrolyte stability that has been confirmed for eosin / viologen dendrimer complexes in vivo drug delivery systems may be excluded.

But the redoxible viologen units within the dendrimers together with the extraordinary
progress in the developing of ultramicroelectrodes (nanodes) viologen dendrimers are a promising tool for a wide range of bioanalytical and biomedical applications. Related to the methyl-(benzyl) viologen / hydrogenase assay\cite{22} the electrochemical detection of reduced viologen dendrimer species in single mammalian cells is currently under investigation.

**Experimental Section**

1. Syntheses

All chemicals used for synthesis were of analytical grade. NMR spectra were recorded with a Bruker-Avance-250 (250 MHz) spectrometer, δ in ppm. MS: Agilent-HP-1100 spectrometer operating in the API-ES mode; in m/z (rel %).

1,3,5-Tris-(bromomethyl)-benzene was purchased from Aldrich. The synthesis of 1-(3,5-dihydroxymethyl-phenyl)-4,4′-bipyridinium-hexafluorophosphate (1) is described by Kathiresan et. al.\cite{23}. The synthetic procedures for the hexa-(hydroxymethyl)- (p_{1}OH) and hexa-(bromomethyl)- (p_{1}Br) precursors and the according precursors (p_{2}OH) and (p_{2}Br) are described by Heinen.\cite{24} The periphery unit, 1-(2-hydroxyethyl)-4,4′-bipyridinium-hexa-fluorophosphate (5) is described by Léon\cite{18}.

General procedure for the counter ion exchange to the chloride salts:

5 – 30 mg of the hexafluorophosphate salts were dissolved in 1 ml acetonitrile, and dropped in 1 ml 0.5 M tetrabutylammonium-chloride / acetonitrile solution. The precipitates were filtered off and dried in vacuo to obtain 50 - 77% of the hygroscopic products.
Scheme 1

**Synthesis of 1-(2-Carboxyethyl)-4,4'-bipyridinium-hexafluorophosphate (2)**

(1 g, $6.4 \times 10^{-3}$ mol) 4,4-bipyridine was dissolved in 25 ml acetonitrile. (0.91 g, $6.0 \times 10^{-3}$ mol) 3-bromo-propionic acid, dissolved in 15 ml acetonitrile, was added at 40°C. After stirring under reflux for 16 hours the precipitation was filtered off and dissolved in 30 ml water. After extraction with 15 ml dichloromethane the aqueous solution were evaporated to dryness. Yield: 0.92 g ($3.0 \times 10^{-3}$ mol), 47%.

The crude product was dissolved in 3 ml water and dropped in the aqueous solution of 2 ml ammonium-hexafluorophosphate (3 molar). The precipitation was collected and dried to yield 0.68 g (61.3%) of a white powder, m.p. 257°C (decomp.).

$\delta_H$ (250 MHz, $D_2O$): 8.95 (d, $^3J = 6.6$ Hz, 2H, Vio); 8.76 (d, $^3J = 6.3$ Hz, 2H, Vio); 8.32 (d, $^3J = 6.6$ Hz, 2H, Vio); 8.06 (d, $^3J = 6.4$ Hz, 2H, Vio); 4.79 (t, $^3J = 6.4$ Hz, CH$_2$, 2H); 2.97 (t, $^3J = 6.4$ Hz, CH$_2$, 2H).

$\delta_C$ (63 MHz, $D_2O$): 173.7 (1C, COOH); 151.0 (1C, Cq, Vio); 144.9 (2C, CH, Vio); 143.8 (2C, CH, Vio); 143.3 (1C, Cq, Vio); 124.7 (2C, CH, Vio); 122.53 (2C, CH, Vio); 56.2 (1C, CH$_2$); 36.7 (1C, CH$_2$).

API-ES MS: m/s: 229.1 (100%); 229.9 (10%)

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**Synthesis of 1-(6-Hydroxyhexyl)-4,4'-bipyridinium-hexafluorophosphate (3)**

0.814 g ($5.21 \times 10^{-3}$ mol) 4,4'-bipyridine was dissolved in 10 ml acetonitrile. 1 g ($5.52 \times 10^{-3}$ mol) 6-bromo-1-hexanole dissolved in 5 ml acetonitrile was added within 30 minutes at 40°C. After 18 hours under reflux the precipitate was filtered off, three times washed with ether and dried under vacuo to yield 0.94 g of a yellow salt.
A subsequently performed anion exchange with 2 ml aqueous 3M ammonium-hexafluorophosphate resulted in 0.83 g (39.8%) of a greyish-white powder.

$\delta^1$H (250 MHz, CD$_3$CN): 8.88 (d, $^3$J = 7.5 Hz, 2H, Vio); 8.82 (d, $^3$J = 5.0 Hz, 2H, Vio); 8.36 (d, $^3$J = 7.5 Hz, 2H, Vio); 7.94 (d, $^3$J =5.0 Hz, 2H, Vio); 4.59 (t, $^3$J = 7.5 Hz, 2H, CH$_2$); 3.52 (t, $^3$J = 5.0 Hz, 2H, CH$_2$); 2.77(s, 2H, CH$_2$); 1.49 (t, $^3$J = 7.5 Hz, 4H, CH$_2$).

$\delta^1$C(63 MHz, CD$_3$CN): 153.37 (Cq, 1C, Vio); 149.45 (Cq, 1C, Vio); 145.01 (CH, 2C, Vio), 143.44 (CH, 2C, Vio); 126.28 (CH, 2C, Vio); 123.24 (CH, 2C, Vio); 61.96 (CH$_2$, 1C); 61.69 (CH$_2$, 1C); 32.48 (CH$_2$, 1C); 31.24 (CH$_2$, 1C); 25.76 (CH$_2$, 1C); 25.33 (CH$_2$, 1C).

API-ES MS: m/s: 256.96 (100%), 257.9

**Synthesis of 1-(5-Carboxypentyl)-4,4'-bipyridinium-hexafluorophosphate (4)**

(1 g, 6.4 × 10$^{-3}$ mol) 4,4'-bipyridine was dissolved in 25 ml acetonitrile and (1.17 g, 6.0 × 10$^{-3}$ mol) 6-bromo-capronic acid solved in 15 ml acetonitrile were added in 2 hours. After 20 hours stirring at 90°C the precipitation was filtered off, washed three times with 10 ml ether and dissolved in 10 ml water. The anion exchange with 2 ml 3 molar ammonium-hexafluorophosphate give 0.66 g (2.43 × 10$^{-3}$ mol), 40.5% of a white powder.

$\delta^1$H(250 MHz, CD$_3$CN): 8.76 (d, $^3$J = 4.93 Hz, 2H, Vio); 8.68 (d, $^3$J = 6.11 Hz, 2H, Vio); 8.22 (d, $^3$J = 5.73 Hz, 2H, Vio); 7.70 (d, $^3$J = 4.91 Hz, 2H, Vio); 4.46 (t, $^3$J = 7.34 Hz, 2H, CH$_2$); 2.22 (t, $^3$J = 7.08 Hz, 2H, CH$_2$); 1.93 (q, $^3$J = 7.88 Hz, 2H, CH$_2$); 1.55 (q, $^3$J = 7.38 Hz, 2H, CH$_2$); 1.35 (t, $^3$J = 7.4 Hz, 2H, CH$_2$).
\[ \delta_C(63 \text{ MHz, CD}_3\text{CN}): 174.04 \ (1\text{C, COOH}); 154.03 \ (1\text{C, Cq, Vio}); 151.04 \ (2\text{C, CH, Vio}); 144.91 \ (2\text{C, CH, Vio}); 141.39 \ (1\text{C, Cq, Vio}); 126.04 \ (2\text{C, CH, Vio}); 121.91 \ (2\text{C, CH, Vio}); \]
\[ 61.22 \ (1\text{C, CH}_2); 32.72 \ (1\text{C, CH}_2); 30.51 \ (1\text{C, CH}_2); 24.93 \ (1\text{C, CH}_2); 23.72 \ (1\text{C, CH}_2). \]

API-ES MS: m/s: 271.2 (100%); 272.2 (20%)

Scheme 2

**Synthesis of 1,3,5-Tris-(((2-hydroxyethyl)-4,4′-bipyridinium)-methyl)-benzene-hexakis-hexafluorophosphate \( G_0\)-OH**

(1.04 g, \( 3.04 \times 10^{-3} \) mol) 1-(2-hydroxyethyl)-4,4′-bipyridinium-hexafluorophosphate was dissolved in 30 ml acetonitrile, (0.180 g, \( 0.5 \times 10^{-3} \) mol) 1,3,5-tri-(bromomethyl)-benzene was added in three portions and the reaction mixture was stirred under reflux for 20 hours. The yellow product precipitates after 2 hours. By the end of the reaction the precipitate was filtered off, washed 3 times with acetonitrile to yield 0.234 g (33.6%) Br-/PF\(_6\)- salt. The crude product was solved in 5 ml water and dropped in the aqueous solution of 3 mol ammonium-hexafluorophosphate. The precipitate was filtered off, dried to give a greyish-white powder. Yield: 0.204 g (25.7%) , mp. 236 °C (decomp.) , MW (\( C_{45}H_{48}N_{6}O_{3}P_{6}F_{36} \)): 1590.66 g / mol

\[ \delta_H(250 \text{ MHz, CD}_3\text{CN}): 8.94 \ (\text{dd}, \ ^3J = 6.4 \text{ Hz, } ^2J = 2.5 \text{ Hz, } 12\text{H, Vio}); 8.42 \ (t, \ ^3J = 6.9 \text{ Hz, } 12\text{H, Vio}); 7.69 \ (s, 3\text{H, aromat. H}); 5.86 \ (s, 6\text{H, CH}_2); 4.72 \ (t, \ ^3J = 4.6 \text{ Hz, } 6\text{H, CH}_2); 4.02 \ (t, \ ^3J = 4.6 \text{ Hz, } 6\text{H, CH}_2); 3.49 \ (t, \ ^3J = 5.3 \text{ Hz, } 3\text{H, OH}). \]
δC(63 MHz, CD$_3$CN): 151.14 (3C, Cq, Vio); 150.38 (3C, Cq, Vio); 146.51 (6C, CH, Vio); 146.20 (6C, CH, Vio); 135.32 (3C, Cq, aromat.); 132.27 (3C, CH, aromat.); 127.88 (6C, CH, Vio); 127.23 (6C, CH, Vio); 64.44 (3C, CH$_2$); 64.03 (3C, CH$_2$); 60.62 (3C, CH$_2$).

**Synthesis of G$_1$-COOH - octadecakis-(hexafluorophosphate)**

(0.070 g, 3.06 × 10$^{-5}$ mol) (p$_1$Br) was dissolved in 8 ml acetonitrile and (0.085 g, 2.27 × 10$^{-4}$ mol) 1-(2-carboxyethyl)-4,4'-bipyridinium-hexafluorophosphate (2) was added. After stirring for 4 days at 70°C the yellow precipitate was collected and dissolved in methanol / water (1:1). The solution was dropped in an mixture of 1 ml aqueous 3 molar ammonium-hexafluorophosphate and 4 ml water. The white precipitation was filtered off and dried to yield 0.031 g of product. The filtrate of the reaction mixture was evaporated to dryness and the residue was treated by the same procedure to obtain 0.029 g product. The overall yield: 0.060 g (1.22 × 10$^{-5}$ mol), 39.8%. MW (C$_{144}$H$_{138}$F$_{108}$N$_{18}$O$_{12}$P$_{18}$): 4903.14 g/mol

δH(250 MHz, CD$_3$CN): 8.88 – 8.83 (m, CH, arom., Vio, 36H); 8.41 – 8.30 (m, CH, arom., Vio, 36 H); 7.60 (s, CH, arom., 9H); 7.55 (s, CH, arom., 3H); 5.83 (s, CH$_2$, 12H); 5.74 (s, CH$_2$, 12H); 4.75 – 4.69 (b, CH$_2$, 12H); 2.99 (b, CH$_2$, 12H).

δC(63 MHz, CD$_3$CN):  δ(ppm): 150.98 (6C, Cq, Vio); 150.36 (6C, Cq, Vio); 146.81 (12C, CH, Vio); 146.15 (24C, CH, Vio); 135.50 (6C, Cq, arom.); 135.44 (6C, Cq, arom.); 132.43 (9C, CH, arom.); 127.81 (24C, CH, Vio); 127.09 (12C, CH, Vio), 64.06 (12C, CH$_2$); 58.42 (6C, CH$_2$); 35.11 (6C, CH$_2$).
Synthesis of Tris-[(6-hydroxyhexyl)-4,4′-bipyridinium-1,3,5-methyl]-benzene-hexafluorophosphate (p₂OH)

0.110 g (2.73 x 10⁻⁴ mol) 1-(6-hydroxyhexyl)-4,4′-bipyridinium-hexafluorophosphate (3) was dissolved in 10 ml methanol and 0.017 g (4.55 x 10⁻⁵ mol) 1,3,5-tris-bromomethyl-benzene solved in 3 ml methanol was added. After 36 hours stirring under reflux the precipitate was collected four times washed with ether and vacuo dried. After anion exchange the greyish-white product was dried again to give 0.017 mg (9.7 x 10⁻⁶ mol), 21 % of the hexafluorophosphate salt.

MW (C₅₋₇H₋₇₂F₃₆N₆O₃P₆): 1759.02 g / mol

δ_H(250 MHz, CD₃CN): 8.95 (t, 3J = 5.81 Hz, 12H, Vio); 8.43 (d, 3J = 7.08 Hz, 12H, Vio); 7.69 (s, CH, arom., 3H); 5.86 (s, CH₂, 6H); 4.65 (t, 3J = 7.43 Hz, CH₂, 6H); 3.52 (t, 3J = 7.3 Hz, CH₂, 6H); 3.39 (t, 3J = 5.6 Hz, 3H, OH); 1.97 (q, 3J = 2.46 Hz, CH₂, 6H); 1.44 (q, 3J = 2.53 Hz, CH₂, 18H).

δ_C(63 MHz, d₆-DMSO): 150.26 (Cq, 3C, Vio); 149.12 (Cq, 3C, Vio); 146.88 (CH, 6C, Vio); 146.65 (CH, 6C, Vio); 136.31 (Cq, arom., 3C); 131.48 (CH, arom., 3C); 127.72 (CH, 6C, Vio); 127.47 (CH, 6C, Vio); 63.55 (CH₂, 3C); 61.86 (CH₂, 3C); 61.30 (CH₂, 3C); 33.01 (CH₂, 3C); 31.73 (CH₂, 3C); 26.20 (CH₂, 3C); 25.81 (CH₂, 3C).
Synthesis of 1,3,5-Tris-((6-bromohexyl)-4,4'-bipyridinium)-methyl)-benzene-hexakis-hexafluorophosphate (p$_2$Br)

0.150 g (7.8 x 10$^{-5}$ mol) tris-[(6-hydroxyhexyl)-4,4'-bipyridinium-1,3,5-methyl]-benzene-hexafluorophosphate (p$_2$OH) was dissolved in 13.5 ml 5.7 M hydrobromic acid and stirred for 2 days at room temperature. The hydrobromic acid was evaporated and the residue dissolved in 5 ml methanol / water (1:1). The solution was dropped in 2.5 ml 3 molar ammonium-hexafluorophosphate. The precipitate was collected and dried under HV to yield 0.135 g (6.9 x 10$^{-5}$ mol), 88.5%. MW (C$_{57}$H$_{69}$Br$_3$F$_{36}$N$_6$P$_6$): 1947.71 g / mol

δ$_H$(250 MHz, CD$_3$CN): 8.95 (t, $^3$J = 7.3 Hz, 12H, Vio); 8.40 (d, $^3$J = 6.9 Hz, 12H, Vio); 7.70 (s, 3H, CH, arom.); 5.87 (s, 6H, CH$_2$); 4.64 (t, $^3$J = 7.5 Hz, 6H, CH$_2$); 3.52 (t, $^3$J = 6.7 Hz, 6H, CH$_2$); 2.25 (q, 12H, CH$_2$); 1.47 (q, 12H, CH$_2$).

δ$_C$(63 MHz, d$_6$-DMSO): 150.19 (Cq, 3C, Vio); 149.27 (Cq, 3C, Vio); 146.90 (CH, arom., 6C, Vio); 146.61 (CH, arom., 6C, Vio); 136.37 (Cq, arom., 3C); 131.60 (Cq, arom., 3C); 127.80 (CH, 6C, Vio); 127.50 (CH, 6C, Vio); 63.51 (CH$_2$, 3C); 61.76 (CH$_2$, 3C); 35.85 (CH$_2$, 3C); 32.71(CH$_2$, 3C); 31.42 (CH$_2$, 3C); 27.72 (CH$_2$, 3C); 25.37 (CH$_2$, 3C).

Synthesis of the Hexa-hydroxymethyl-precursor (p$_3$OH)

(0.05 g, 2.6 x 10$^{-5}$ mol) 1,3,5-tris-(((6-bromohexyl)-4,4'-bipyridinium)-methyl)-benzene (p$_2$Br) was dissolved in 5 ml nitromethane and (0.093 g, 2.05 x 10$^{-4}$ mol) 1-(3,5-dihydroxymethyl-phenyl)-4,4'-bipyridinium-hexafluorophosphate (1) was added. The reaction mixture was stirred at 100 °C for 24 hours. After cooling the solvent was
evaporated and the residue was dissolved in methanol / water (1:1) and dropped in the solution of 3 ml aqueous 3 molar ammonium-hexafluorophosphate. The precipitate was filtered off and dried under HV to yield 0.092 g, (2.6 × 10^{-5} \text{ mol}), 97% of a brown powder. MW (C_{114}H_{126}F_{72}N_{12}O_{6}P_{12}) : 3499.89 g /mol

{\delta}_{\text{H}}(250 \text{ MHz, CD}_{3}CN): 8.95 \text{ (d, } ^{3}J = 5.9 \text{ Hz, 24H, Vio); 8.42 \text{ (d, } ^{3}J = 6.1 \text{Hz, 24H, Vio); 7.69 \text{ (s, 3H, CH arom.); 7.57 \text{ (s, 3H, CH arom.); 7.41 \text{ (s, 6H, CH arom.); 5.85 \text{ (s, 12H, CH}_{2}); 4.64 \text{ (s, 24H, CH}_{2}); 3.49 \text{ (t, 6H, OH, } ^{3}J = 3.6 \text{ Hz); 1.97 \text{ (m, 12H, CH}_{2}); 1.49 \text{ (t, 12H, 3J = 6.0 Hz).}}

{\delta}_{\text{C}}(63 \text{ MHz, CD}_{3}CN): 150.51 \text{ (Cq, 3C, Vio); 150.3 \text{ (Cq, 3C, Vio); 150.16 \text{ (Cq, 3C, Vio); 150.10 \text{ (Cq, 3C, Vio); 146.23 \text{ (CH, 6C, Vio); 146.11 \text{ (CH, 6C, Vio); 145.95 \text{ (CH, 6C, Vio); 144.24 \text{ (CH, 6C, Vio); 135.30 \text{ (Cq, 6C, arom.); 134.13 \text{ (Cq, 3C, arom.); 132.20 \text{ (CH, 6C, arom.); 131.90 \text{ (CH, 3C, arom.); 129.05 \text{ (CH, 6C, arom.); 127.85 \text{ (CH, 6C, Vio); 127.66 \text{ (CH, 6C, Vio); 126.66 \text{ (CH, 6C, Vio); 126.37 \text{ (CH, 6C, Vio); 64.56 \text{ (CH}_{2}, 3C); 64.03 \text{ (CH}_{2}, 3C); 63.46 \text{ (CH}_{2}, 6C); 62.92 \text{ (CH}_{2}, 3C); 62.29 \text{ (CH}_{2}, 3C); 30.97 \text{ (CH}_{2}, 6C); 25.31 \text{ (CH}_{2}, 6C).}}}

\textbf{Synthesis of the Hexa-bromomethyl-precursor (p}_{3}\text{Br)}

(0.04 g, 1.14 \times 10^{-5} \text{ mol}) (p_{3}\text{OH}) was dissolved in 7 ml 5.7 M hydrobromic acid / acetic acid and stirred for 48 hours at RT. The hydrobromic acid has been evaporated and the solid residue was solved in 5 ml methanol / water and an anion exchange with 2 ml 3 molar ammonium-hexafluorophosphate was performed. After drying of the precipitate a brown powder was obtained, yield 0.055 g (1.42 \times 10^{-5} \text{ mol}), 89%. MW (C_{114}H_{120}Br_{6}F_{72}N_{12}P_{12}) : 3877.28 g / mol.
δ_H(250 MHz, CD_3CN): 8.96 (d, J = 7.1 Hz, 24H, Vio); 8.43 (d, J = 6.5 Hz, 24H, Vio); 8.11 (d, J = 6.8 Hz, Vio); 7.84 (s, 3H, CH arom.) 7.70 (s, 6H, CH arom.); 7.52 (s, 3H, CH arom.); 5.86 (s, 12H, CH_2); 4.67 (s, 12H, CH_2); 4.64 (s, 12H, CH_2); 2.04 (m, 12H, CH_2); 1.44 (b, 12H, CH_2).

**Synthesis of G_1-Hexyl-COOH dendrimer**

(0.02 g, 5.71 × 10^{-6} mol) (p_3Br) was dissolved in 10 ml nitromethane and (0.023 g, 8.57 × 10^{-5} mol) 1-(5-carboxypentyl)-4,4'-bipyridinium-hexafluorophosphate (4) was added. After stirring at 80°C for 36 hours the reaction mixture was evaporated to dryness, the residue resolved in 5 ml methanol / water and an anion exchange with 1 ml ammonium-hexafluorophosphate (3M) solution was performed to yield 0.016 g (2.33 × 10^{-6} mol), 41% of a brown powder. PF_6^- - salt: MW (C_{210}H_{234}N_{24}O_{12}P_{24}F_{144}): 6765.35 g/mol Cl^- - salt: MW (C_{210}H_{234}N_{24}O_{12}Cl_{24}): 4221.34 g/mol

δ_H(250 MHz, CD_3CN): 8.93 (d, J = 5.9 Hz, Vio, 48H); 8.42 (t, J = 5.8 Hz, Vio, 48H); 7.69 (s, CH arom., 12H); 5.86 (s, CH_2, 24H); 4.65 (t, J = 7.1 Hz, CH_2, 24H); 3.48 (q, 6H, COOH); 2.34 (t, CH_2, 12H); 1.95 (m, 24H, CH_2); 1.67 (t, J = 6.7 Hz, CH_2, 12H); 1.44 (t, J = 6.7 Hz, CH_2, 24H).

δ_C(63 MHz, D_2O, Cl^-): 151.19 (Cq, Vio, 12C); 146.03 (CH, Vio, 36 C); 135.43 (Cq, arom., 12C); 131.99 (CH, arom., 12C); 127.75 (CH, Vio, 24C); 127.38 (CH, Vio, 12C); 63.95 (CH_2, 12C); 62.34 (CH_2, 6C); 49.19 (CH_2, 6C); 30.78 (CH_2, 6C); 30.52 (CH_2, 12C); 25.06 (CH_2, 12C).
2. Cyclic voltammetry

The cyclic voltammetric measurements give an insight in the complex formation and offer a method to check the electrolyte stability of the dendriplexes in the presence of the cell growth media. The cyclic voltammetric measurements of figure 2 and 3 were carried out with a three-electrode system under Argon, using a glassy carbon working electrode (0.018 cm$^2$) in a volume of 500 microliter against a 0.1 M Ag/AgCl-reference. The 10 mM TE-buffer was adjusted to pH 7.5. To exclude precipitations the working electrode was polished for every measurement, to assure the observed phenomena occurred in solution. Unless mentioned otherwise, the scan rate was 0.1 V/s. The measurements of figure 4 were performed in 10 ml TE buffer (10mM) with calf thymus DNA (CT-DNA) using a glassy carbon working electrode with 0.07 cm$^2$ at a scan rate of 400 mV/s.

3. Cell culture and transfection experiments

**CT-DNA, Plasmid DNA and Cells**

Calf thymus DNA (CT-DNA) was purchased from Sigma-Aldrich (D4522). The eukaryotic expression plasmid pC1-eGFP contains a eGFP reporter gene, the viral promoter of the cytomegalovirus (CMV), a kanamycin / neomycin resistance marker and is commercially available by Clontech (Mountain View, CA). The viral HSV amplicon plasmid contains the eGFP reporter gene under the control of the viral promoter HSV-1 IE 4/5 and an ampicillin resistance marker as described previously.[17] Plasmids were isolated after transformation from E. coli (DH 5α) according to the “Pure Yield™” plasmid protocol from Promega. CHO cells were grown in α-MEM with 10% fetal bovine serum (FBS), 2 mM glutamine and
50 units / ml penicillin, 50 µg / ml streptomycin. NIH 3T3 cells were maintained in DMEM with 10% FBS, 2 mM glutamine and 50 units / ml penicillin, 50 µg / ml streptomycin. Human terato carcinoma cells (NT2 cells) were grown in DMEM containing 10% FBS, 5% horse serum (HS), 2 mM glutamine and 50 units / ml penicillin, 50 µg / ml streptomycin. Rat pheochromocytoma PC 12 cells were cultured in DMEM supplemented with 10% HS, 5% FBS, 2 mM glutamine and 50 units / ml penicillin and 50 µg / ml streptomycin. When about 70% confluent, cells were splitted and maintained at 37°C in a 5% CO₂ humidified atmosphere.

Transfection experiments

Prior to transfection, cells have been seeded at a density of 2 x 10⁵ cells / well. For transfections, cells were rinsed with PBS, 160 µl aqueous sucrose (0.25 M) and 40 µl MES buffer (10 mM) were added, ensuring a pH of 4.5. A complex was formed with dendriplex solution containing 2 µg plasmid in TE buffer and 2, 4 or 6 µg dendrimer and incubated for 10 min. Then solution was filled up to 500 µl and added to the cells. After 3 hours incubation at 37°C in a 10% CO₂ atmosphere the sucrose solution was substituted with serum-containing medium. The cells were further incubated for 36 hours, fixed with 4 % formaldehyde in PBS for 20 min, rinsed with PBS and incubated with 0.1 M glycine in PBS for 20 min. Nuclei were stained with DAPI.

Acknowledgements

We thank Angelika Hilderink for expert technical assistance. This work was supported by a former fellowship of the Interdisciplinary graduate college 612 of the Deutsche Forschungsgemeinschaft (D.B.)
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Generation-Dependent Intramolecular CT Complexation in a Dendrimer Electron Sponge Consisting of a Viologen Skeleton
Scheme 1

1. R—Br / MeCN
2. 3 M aq. NH$_4$PF$_6$

\[ \text{R} = -(\text{CH}_2)_2-\text{COOH} \quad 2 \]
\[ -(\text{CH}_2)_6-\text{OH} \quad 3 \]
\[ -(\text{CH}_2)_2-\text{COOH} \quad 4 \]
\[ -(\text{CH}_2)_2-\text{OH} \quad 5 \]
**Figure Captions**

**Scheme 1:** Synthesis of mono-alkylated viologens: all counter ions: PF$_6^-$

**Scheme 2:** Synthesis of the hexylene / methylene bridged viologen dendrimers: all counter ions: PF$_6^-$, 2 a.) every reaction step is followed by an anion exchange with aqueous 3 M NH$_4$PF$_6$

**Figure 1:** Schematic depiction of viologen dendrimer detection within a single mammalian cell, viologen dendrimer in oxidative state, and in reducible state

**Figure 2:** CV of G$_1$-COOH (20.4 µmol) / pC1-eGFP revealing time dependence of DNA complex formation, with 1: 30 µg G$_1$-COOH; 2: after addition of 5.95 µg pC1-eGFP, +/-: 0.1; 3: 5 minutes; 4: 15 minutes; 5: 19 minutes; 500 µl cell volume, scanrate: 0.1 V/s.

**Figure 3:** CV of G$_1$-Hexyl-COOH (11) / pC1-eGFP with 1: 20 µg G$_1$-Hexyl-COOH; 2: after addition of 1.4 µg pC1-eGFP, +/-: 0.03; 3: 20 mM sodium chloride; 4: 80 mM sodium chloride, scanrate: 0.1 V/s.

**Figure 4:** CV’s of physiological salt solution, sucrose and cell growth media, G$_0$-OH (4) / calf thymus DNA (CT-DNA) complex, scanrate: 400 mV/s.

a. 1: 39 µmol G$_0$-OH in 10 mmol TE, pH 7.5
   2: addition of 110 µmol DNA; 3: addition of 0.7% sodium chloride;

b. 1, 2 same like a. , 3: addition of 0.25 mol sucrose

c. 1: 9.5 µmol G$_0$-OH in DMEM, pH 8.4 ; 2: addition of 130 µmol CT-DNA

d. 1: 39 µmol G$_0$-OH in OptiMEM, pH 8.5 ; 2: addition of 226.5 µmol CT-DNA
Figure 1
Figure 2

![Graph](image)

**E / V vs Ag/AgCl**

$I / A$

Figure 3

![Graph](image)

**E / V vs Ag/AgCl**

$I / A$
Two types of viologen dendrimers differing in the spacer length between the viologen (4,4'-bipyridinium) units have been investigated in respect for drug and gene delivery tools. Due to the low electrolyte stability only a small cell incubation could be achieved.