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Viruses as a tool in nanotechnology and target for conjugated polymers

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Chapter 4 Application of conjugated polyelectrolytes as enhancers and inhibitors of retroviral infection

4.1 Introduction

The Human Immunodeficiency Virus (HIV) is a lentivirus and part of the retroviridae family. Although HIV currently is considered as a “human” virus, it originates from African primates and crossed the species around hundred years ago¹. Two main factors play a significant role in bringing HIV to a global pandemic. First of all, humans have not yet developed a sufficient immune response to fight this pathogen. Broadly neutralizing antibodies against HIV are present only in 10-30% of HIV-positive patients and they are still not able to stop the virus from spreading². Secondly, HIV targets the immune system of the host. It infects and thereby gradually depletes CD4+ helper T cells that are coordinating host immune functions³. As a result an organism cannot stop HIV from spreading and, in the same time, becomes extremely vulnerable to other pathogens that could potentially enter. This condition is known as the Acquired Immunodeficiency Syndrome (AIDS). In fact, the foremost cause of death in patients suffering from AIDS is not the presence of HIV itself but the encounter of any other pathogen that is not lethal to healthy individuals like cytomegalovirus, candida or pneumocystis pneumonia⁴.

A typical feature of the lentivirus genus is that it causes chronic illness with a long lasting incubation period. Aside from ongoing replication in

infected T-cells, HIV forms reservoirs located in the mucous membranes of the alimentary canal, the respiratory and genital tracks and in the central nervous system⁵. These reservoirs of the latent virus cause a systemic infection and are the biggest challenge in virus eradication.

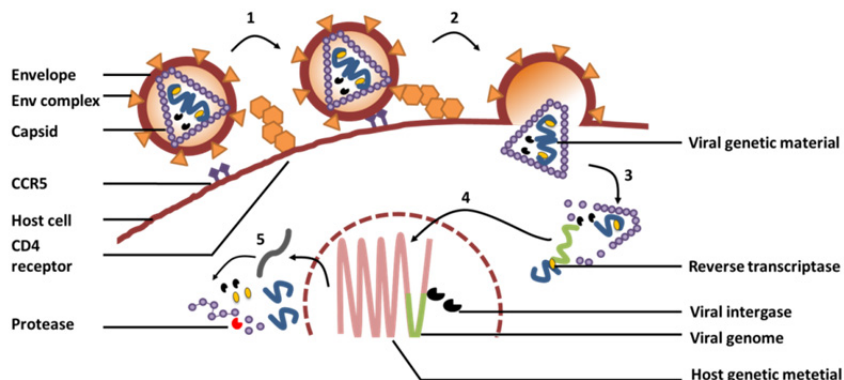


Figure 4.1. Stages of the HIV life-cycle which are targeted in Antiretroviral Therapy. Virion docking and entry is dependent of recognition of CD4 receptor and CCR5 (or CXCR4) co-receptors (1); Next, the HIV envelope fuses with the host membrane (2) and viral RNA and proteins are released from the capsid (3) to the intracellular space; The viral reverse transcriptase then uses the RNA of the virus to create a viral DNA which is integrated in a cellular genome by the HIV integrase (4); Finally, the viral mRNA is translated by cellular ribosomes to yield protein components of new virions (5).

Despite three decades of extensive efforts, a vaccine against HIV has still not been developed⁶. The current treatment is based on Antiretroviral Therapy (ART) which is aimed at maintaining the virus in the latent state and suppressing further spreading. The ART therapeutics inhibit the enzymes crucial for HIV replication in the host cells (Figure 4.1) and are divided in the following drug classes⁷. The first group of ART drugs are nucleoside/nucleotide reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors that block transcription of the HIV's RNA to a coding DNA strand. The second class are integrase inhibitors which are employed to prevent incorporation of the viral DNA with the host genome. A third group comprises inhibitors of HIV protease that suppresses production of the functional virion capsid proteins. Finally, there are two additional ART classes that cover virus entry inhibitors and target either the HIV co-receptors located on the cell (CCR5/CXCR4) or the viral transmembrane glycoprotein (gp41) unit responsible for fusion with the host membrane⁸.

For the best treatment of HIV positive patients, ART drugs have to be administered systemically as a combination of a few antiretrovirals from different classes in a strict dosing regimen. Although there are serious side effects associated with such intense treatment, the ART regimen efficiently suppresses virus proliferation and prevents rapid advance of AIDS for those who adhere to the therapy.

A second approach to fight HIV is termed as Pre-Exposure Prophylaxis (PrEP) and applies to healthy individuals that are at high risk of exposure to HIV. Like in the treatment of HIV positive patients, it is also based on administration of ART drugs. However, as the infection did not take place yet, the drugs can be administered both systemically and locally. Here, the systemic life-long intake of these agents raises questions about the safety of the preventive therapy and, more importantly, about the possible evolution of HIV to an ART-resistant mutant. As such, there is only one oral PrEP agent approved for HIV-prevention and local administration is strongly preferred⁹. Topical PrEP treatment is performed with microbicides that locally prevent sexually transmitted infection. In case of HIV, a remarkable 80% of all infections are the result of sexual intercourse¹⁰. Thus, topically applied PrEP formulations appear to be a simple and effective way of protection against HIV.

The first generation of microbicides was a group of surfactants acting as broadly effective membrane destabilization agents (Figure 4.2). The compound Nonoxynol-9 is the first PrEP agent tested in patients but it was quickly described as highly irritating to the vaginal epithelium and therefore abandoned. Unexpectedly, Nonoxynol-9 promoted HIV entry by disrupting natural protective barriers even when used in polymeric formulations to ease mucosal irritations^{8,11}. A following strategy that reached clinical trials was based on the finding that a pH below 4.5 is harmful for HIV virions. Despite being harmless to the patient, acidic buffering gels also proved to lack efficacy⁸. Subsequently, focus in the field of microbicides moved to polyanions (PAs).

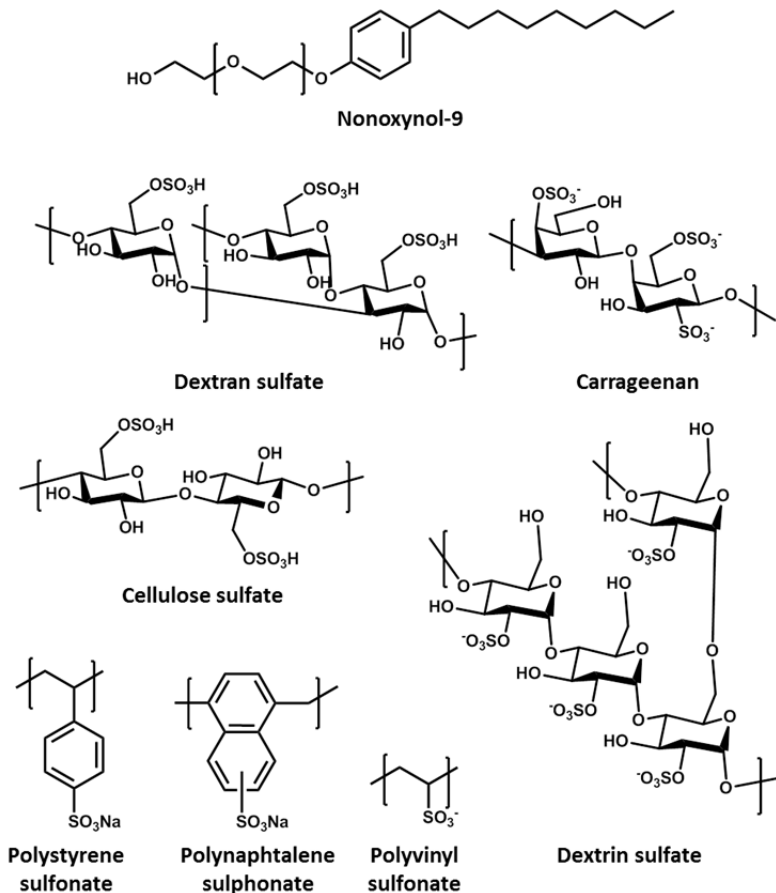


Figure 4.2. Structures of polyanions exhibiting antiviral activity.

These compounds were identified as effective but unspecific inhibitors of virion entry. The majority of PAs screened for anti-HIV properties were polysaccharides and consisted of natural scaffolds such as carrageens, sulphated linear cellulose as well as branched dextran and dextrin¹². Unfortunately, synthetic polymers were investigated to a lesser extent. For instance only few easily accessible PAs such as polystyrene sulfonate and polyvinyl sulfonate, have been tested and showed comparable level of activity to the anionic polysaccharides^{12,13}. Another example of a synthetic PA is polynaphtalene sulfonate (PRO2000), which has even advanced into clinical trials¹⁴. Most of the conducted studies concluded that the polyanionic microbicides block the binding of viral glycoprotein to the CD4+ receptor located on the

mammalian membrane. However, a detailed mechanism has not been revealed. When polyanionic biopolymers such as carboxylated Human Serum Albumin¹⁵ or poly(A)-poly(U) strands¹⁶ also proved to inhibit HIV infection, it seemed that the character of the polymer backbone (polysaccharide, peptide, hydrocarbon chain) and the type of anionic group did not play a crucial role in for the activity. Many PAs were found to be safe and very active in-vitro and in-vivo, but did not yet offer enough protection against infection as was found in Phase III clinical trials of cellulose sulphate, carrageenan (CarraguardTM) and PRO2000⁸.

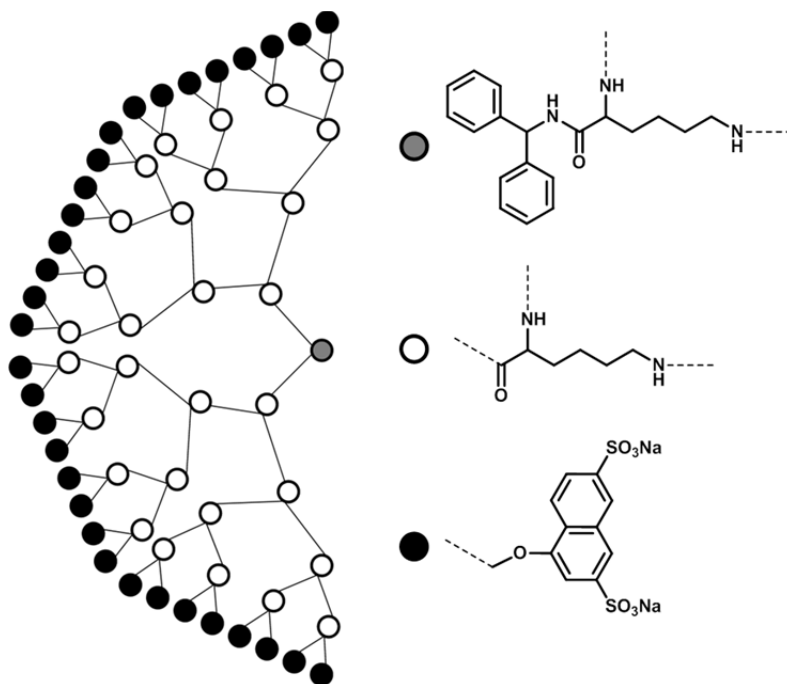


Figure 4.3. Structure of VivaGel dendrimer.

The negative outcome of trials in patients stopped the enthusiasm in the field of anti-HIV PAs until VivaGel was introduced (Figure 4.3). This PA is a nano-sized polylysine dendrimer with a high degree of branching compared to the previously employed polymeric PAs. The dendrimer is functionalized with planar, conjugated naphthalene sulfonate groups, similar in structure to the monomer of PRO2000. In preclinical evaluations, VivaGel showed 85-100% efficacy¹⁷ and it is currently about to enter Phase III clinical trials. The success of VivaGel indicates that structural parameters play an important role and that therefore a

higher synthetic effort might pay off when it comes to potent microbicide design.

Viruses are an undeniable threat to the human health and they still remain a challenging target in medicine. However, in a completely different setting, they should be also recognized as very efficient vehicles that are designed by nature to transport genetic information. Hence, one can pursue the utilization of viruses for therapeutic goals. As already mentioned, lentiviruses cause chronic illnesses and they are typically present in the infected cells for years without causing any disease related symptoms. This happens because they integrate their genome in the DNA of the host to achieve stable and long lasting gene expression. Exactly this property makes them excellent candidates for the treatment of diseases related to gene defects. Likewise, they are a very useful tool to introduce foreign DNA into mammalian cells, called gene transfection, which is widely used technique in developmental biology, stem cell biology, hematology and neuroscience.^{18,19}

Ironically, the retroviruses including HIV are often not infective enough for efficient gene therapy. In general, the entry of the virus to the host cell is facilitated by specialized receptors. However, equally important is the slow process of passive diffusion of the virus towards the membrane and the subsequent override of the electrostatic barrier²⁰. The latter factors are the main limitation of retroviral transduction efficiency. Nevertheless, to overcome these problems and boost infectivity, one can either concentrate the virus or reduce the electrostatic repulsion²¹.

The simplest way to increase the diffusion rate is to have a higher concentration of virions. Current protocols suggest application of viral vector stocks that have been concentrated by ultracentrifugation, ion exchange chromatography or precipitation using polyethylene glycol (PEG6000). Another approach is based on co-localization of viruses with cellular receptors via RetroNectin treatment. This method uses a recombinant human protein composed of domains exhibiting affinity to both cells and (engineered) viruses, thereby greatly enhancing transduction efficiency in-vitro²². Finally, to reduce the electrostatic repulsion between fusing membranes the complexation of the virions with positively charged agents has been widely employed. The state-of-art transduction enhancer is a cationic peptide derived from HIV-1 glycoprotein gp120 which induces up to 34-fold increase of infectivity²³.

Its activity has been also associated with its fibrillar morphology. Unfortunately, it has not been marketed yet.

Several polycations (PCs) have been employed to promote viral infection. High molecular weight cationic substances facilitate fusion by effective reduction of the electrostatic barrier between negatively charged membranes of the host and the virion^{24,25,26,27}. Commonly used PCs include synthetic compounds such as polylysine²⁷, polybrene²⁸ or diethylaminoethyl dextran (DEAE Dextran)^{29,30} (Figure 4.4) as well as natural ones like protamine. Unfortunately, polycations are known to be toxic to cells and their application range is therefore limited. For example, even though polybrene is still very popular in in-vitro procedures, it has not been approved for use in humans due to high cytotoxicity.

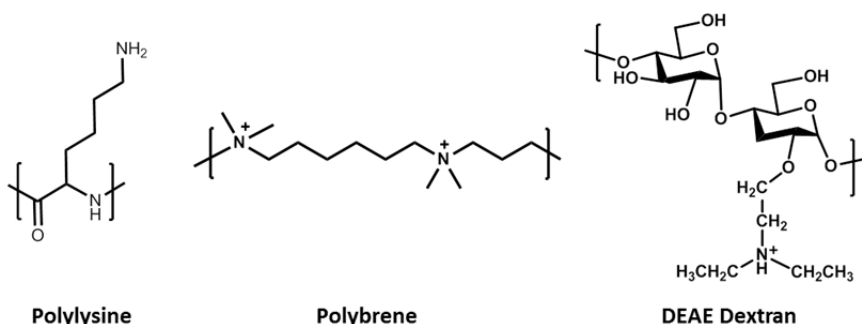


Figure 4.4. Structures of positively charged polymeric transduction enhancers.

Although PCs lack target specificity typical for polypeptide-based agents derived from viral glycoproteins, they form a simpler, cheaper and more stable alternative. They are potent candidates that can rapidly coagulate and concentrate virions in a simple benchtop centrifuge. Moreover, most polymers can be easily tuned to form large macromolecular scaffolds which can further promote clustering of the viruses due to the large surface area.

Up to now, the progress in polymer science has not been reflected in the field of virology. The examples of the HIV-targeting polymers described above represent a limited number of relatively simple structures. Among them, molecules decorated with or consisting of a naphthalene block showed noteworthy antiviral activity. In fact, polyfluorenes share some similarities with naphthalene as both contain planar, rigid and conjugated structures. Therefore we decided to explore polyfluorene as

universal backbone for retroviral infection modulating agents. To investigate the microbicidal use as well as possible application in retroviral gene transfer, two oppositely charged fluorene-based polyelectrolytes were synthesized and their potential in virology was investigated. The highly hydrophobic backbones will be functionalized with short side chains carrying either an anionic phosphonic acid functionality or a positively charged quaternary ammonium group. In order to get more insight in the structure-activity relationship both polyelectrolytes will be compared to their dimeric and monomeric analogues. The biological activity of charged polyfluorenes and their corresponding lower molecular weight counterparts will be investigated using the Human Immunodeficiency Virus in a retroviral transduction assay.

4.2 Results and discussion

4.2.1 Design and synthetic strategies for charged polyfluorenes and their derivatives

The unique properties of fluorene-based polyelectrolytes originate from their highly hydrophobic backbone built of a conjugated system of benzene rings in combination with the side groups ensuring solubility in water. The backbone contributes not only to the rigidity of the molecules but also to their optical characteristics, i.e. exceptional light emitting properties^{31,32}. These compounds have been extensively studied in the field of material science as sensors for chemical and biological targets where their ability to bind to an oppositely charged analyte has been widely exploited^{33,34,35,36}.

The synthesis of fluorene-based polyelectrolytes can be performed using a direct or indirect approach. In the direct synthesis route, charged monomers form a polymer during a polymerization reaction. However, when employing this method, the polymerization conditions must be carefully chosen to be compatible with the ionic side chains of choice to yield a high degree of polymerization. In the indirect approach, the final product is synthesised from a neutral polymer precursor in a polymer-analogues reaction. The charges are being introduced in the last synthetic step and therefore, a reaction needs to be employed that proceeds with excellent conversion. Otherwise, not all monomers in the polymeric chain will be charged, leading to a heterogeneous species that might differ from the fully charged species. Especially aggregation

effects in aqueous environments might affect further studies. Although a greater variety of charged groups can be achieved by following the direct approach, the indirect route is more convenient regarding the characterization of the polymer because problems with aggregation, hygroscopicity and identity of the counter-ion can be avoided.

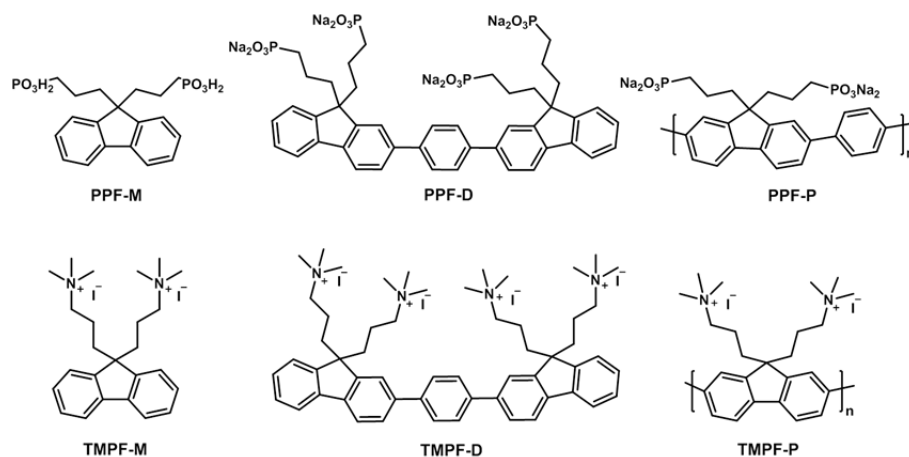


Figure 4.5. Overview of anionic and cationic fluorene based compounds investigated in this chapter: Phosphonato Propyl Fluorene- Monomer, Dimer and Polymer (PPF-M, PPF-D and PPF-P, respectively); TriMethylaminoPropyl Fluorene-Monomer, Dimer and Polymer (TMPF-M, TMPF-D, TMPF-P, respectively).

To investigate the potential of a fluorene-based conjugated backbone as biologically active material two sets of oppositely charged compounds were chosen (Figure 4.5). Detailed reaction schemes are included in the experimental section 4.4.1 and 4.4.2.

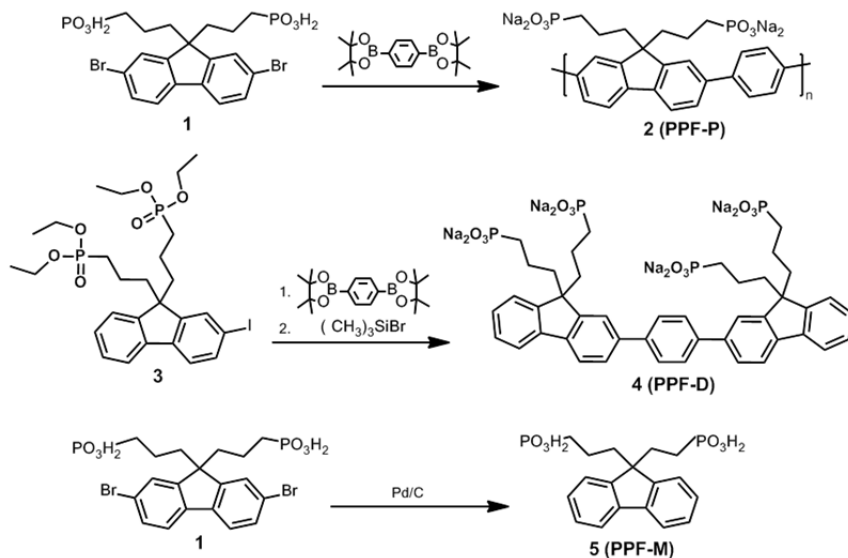


Figure 4.6. Synthetic routes of anionic fluorene derivatives

The copolymer carrying a negatively charged phosphonate group, poly(9,9-bis(3'-phosphonic acid propyl)fluorene-2,7-diyl-*alt*-1,4-phenylene sodium salt) (PPF-P), was chosen as example of an anionic polyfluorene. As shown in Figure 4.6, to obtain PPF-P the charged compound 2,7-dibromo-9,9-bis(3'-phosphonic acid propyl)fluorene (**1**) was directly co-polymerized with a dibronic pinacol ester-functionalized benzene employing a Suzuki coupling reaction. The crude PPF-P was purified by dialysis against ultra-pure water and subsequent precipitation in acetone to yield yellowish fibers with 45% yield. The molecular weight (MW) of the PPF-P polymer was determined to be 3.2 kDa, which corresponds to 6 fluorene units. However, it must be noted that the determination of the molecular weight of this polymer was challenging due to strong ionic interactions with chromatographic resins as reported before³⁷. In order to obtain polymer with higher MW, an attempt was made to synthesise a neutral precursor carrying the esterified phosphonic acid groups and hydrolyse it in the final step. However, due to rapid changes of polymer solubility during the cleavage reaction this approach resulted in formation of insoluble aggregates containing partially hydrolysed precursor.

As a reference for biological activity experiments, the lower molecular weight monomer and dimer analogues were also synthesised (Figure 4.5). The dimer (PPF-D) was obtained by coupling of neutral moniodinated monomers (**3**) to a phenylene unit also employing the Suzuki reaction. A subsequent hydrolysis of ethyl ester group on neutral precursor yielded the charged dimer with 89% efficiency. Finally, the negatively charged monomer PPF-M (**5**) was synthesized by dehalogenation of compound **1** and it was obtained with 85% yield. Both low molecular weight compounds were purified using reverse phase HPLC.

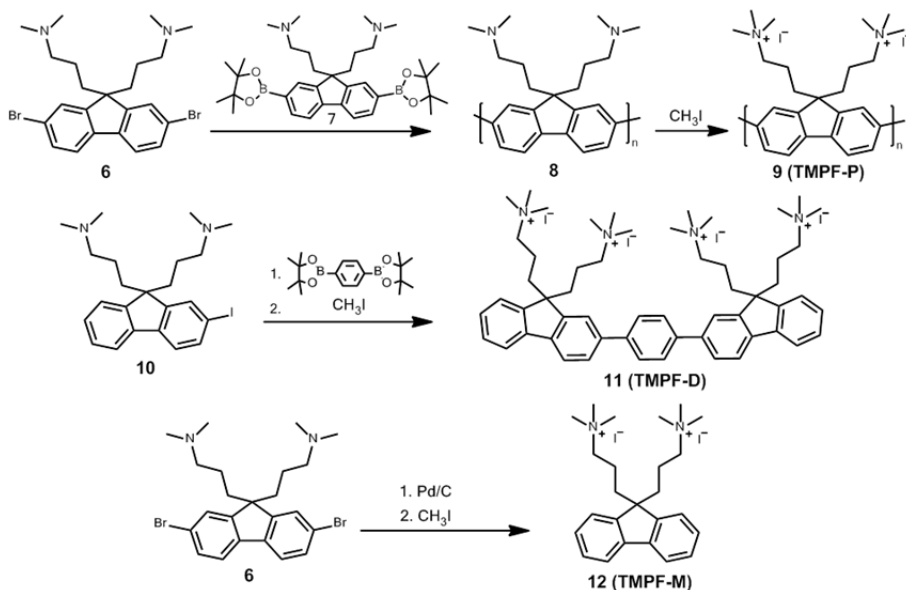


Figure 4.7. Synthetic routes towards cationic fluorene derivatives

The cationic polyfluorene poly(9,9-bis(3'-(N,N,N-trimethylamino)propyl)fluorene-2,7-diyl-*alt*-1,4-phenylene) iodide (**9**) was obtained by quaternization of pendant amine groups on a neutral precursor employing a polymer analogous reaction (Figure 4.7). The neutral variant of TMPF-P (**8**) was synthesized using Suzuki coupling reaction between dibromo- and diboronic pinacol ester derivatives of 9,9'-bis(3'-(N,N-dimethylamino)propyl)fluorene monomers (**6** and **7**, respectively). The neutral precursor was soluble in organic solvents what facilitated its characterisation and determination of molecular weight with gel permeation chromatography. The degree of polymerization was found to be seven, which corresponds to a MW of

4.3 kDa. Quaternization proceeded quantitatively and the final water soluble polymer **9** was obtained in 77% yield. The determined degree of polymerization is comparable to PPF-P. Similarly to the anionic compound, TMPF-D (**11**) as a model dimer was obtained by coupling of monoiodinated neutral monomers (**10**) to a phenylene unit and subsequent quaternization with methyl iodide. Compound **11** was obtained with 73% yield. Dehalogenation of a neutral monomeric precursor of TMPF-M (**6**) followed by quaternization with methyl iodide furnished the cationic fluorene monomer TMPF-M (**12**) in a yield 95%.

4.2.2 Antiviral properties of anionic polyfluorene and its derivatives

At first, the influence of the negatively charged polyfluorene PPF-P on the infectivity of Human Immunodeficiency Virus was investigated. The monomeric (PPF-M) and the dimeric (PPF-D) variants of the PPF-P polymer were also included in this study to obtain systematic information about structure-activity relations. The TZM-bl indicator cell line was used to determine the HIV transduction efficiency in the well-established β -galactosidase activity assay^{23,26}.

Cells used in this assay (TZM-bl) are very sensitive towards HIV infection since they overexpress receptor (CD4) and co-receptors (CXCR4 and CCR5) essential for HIV entry. They also contain a β -galactosidase reporter gene introduced to facilitate quantification of HIV infection using an enzymatic reaction. The HIV particles carry a gene that encodes a trans-activator of transcription (tat), which is a protein that drastically enhances the efficiency of transcription of viral DNA³⁸. The β -galactosidase reporter gene is tat-inducible and thus upon infection of the TZM-bl cells the β -galactosidase production is upregulated. Hence, the amount of β -galactosidase product is directly proportional to the HIV infection and therefore can be used to quantify HIV infectivity.

For initial screening of inhibition of the viral infection, the cell culture medium was supplemented with PPF-M, PPF-D or PPF-P in concentrations ranging from 0.8-100 $\mu\text{g}/\text{mL}$. Afterwards HIV was added to the cells and the virus infectivity was determined three days after infection by measuring the β -galactosidase activity level (Figure 4.8). It must be noted here that no cytotoxicity was found when the anionic compounds were applied to mammalian cells.

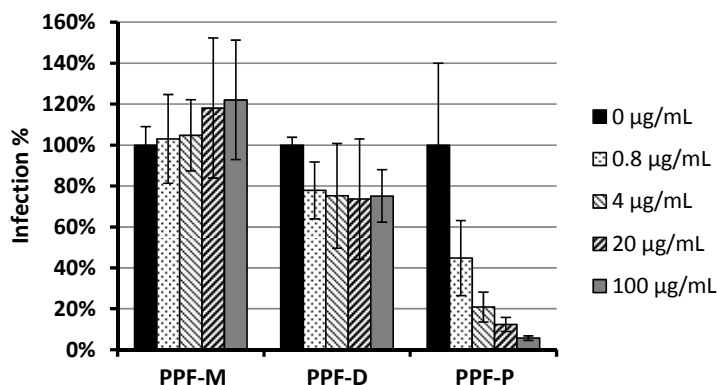


Figure 4.8. The influence of anionic fluorene-based compounds on HIV infectivity.

As observed above, the PPF-P polymer strongly inhibited HIV infection while the monomer and dimer analogue did not exhibit any significant effect. Also, the inhibition appears to be dose dependent and is already visible at the lowest concentration of the polymer (0.8 µg/ml).

Although the virucidal properties of PPF-P are promising, to further evaluate this polymer as a potential microbicide, an additional parameter has to be investigated. It is known that the HIV infectivity is greatly enhanced in the presence of semen. This body fluid contains proteins that are enriched in cationic domains and isolation of those domains resulted in discovery of positively charged amyloid fibrils that substantially increase HIV infectivity. They are termed SEVI (Semen derived Enhancers of Viral Infection)¹⁰. Due to the critical contribution of these fibrils to HIV infectivity, it is important to investigate whether the virucidal properties of PPF-P are maintained in the presence of SEVI. Recently it has been suggested that some PAs might actively promote formation of functional SEVI fibrils and therefore exhibit an opposite activity than in a SEVI-free environment³⁹. Thus we have tested whether PPF-P can efficiently neutralize these agents and further minimize the chance of infection.

To this end, a similar experiment as presented above was performed in presence and absence of SEVI peptides (Figure 4.9). Here, virions were preincubated with the tested compounds in the same concentration range. The pristine compound solutions were employed as well as

solutions supplemented with 100 $\mu\text{g}/\text{mL}$ of SEVI fibrils. To show the drastic change in infectivity of the HIV virions in the presence of SEVI, the raw β -galactosidase activity levels are presented too (Figure 4.9B).

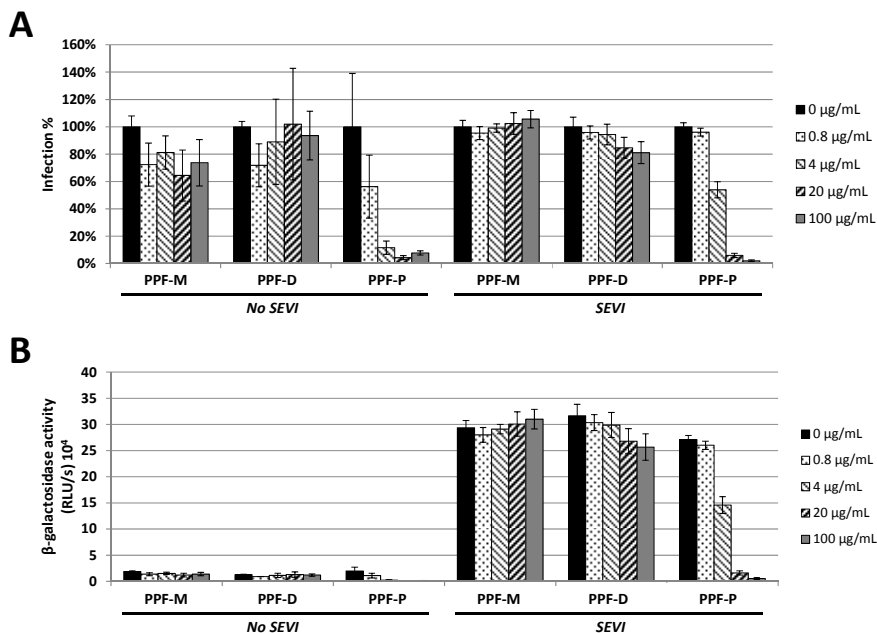


Figure 4.9. The virucidal properties of anionic fluorene-based compounds in the presence and absence of SEVI. (A) PPF-P efficiently inhibits HIV infectivity regardless of the presence of SEVI. Results are normalized to the level of HIV infectivity without addition of a tested compound (set as 100%); (B) For comparison purposes, graph B presents raw data and shows the difference in the level of HIV infectivity in the presence and absence of SEVI.

Figure 4.9A reconfirms the earlier observed antiviral activity of PPF-P. Furthermore, it clearly shows that also in the presence of SEVI, PPF-P greatly reduces the virus infectivity and therefore counteracts the well-known HIV infection promoting factor. In both cases the PPF-M and PPF-D analogues show negligible antiviral activity, which was expected from the results found earlier. Finally, Figure 4.9B demonstrates the scale of enhancement of HIV infectivity upon the addition of SEVI peptides.

From the data obtained above the IC_{50} value of PPF-P preincubated with HIV was determined to be 1.1 $\mu\text{g}/\text{mL}$, which corresponds to the values of other PAs¹². The IC_{50} value in the presence of SEVI peptides was

found to be higher (5.0 $\mu\text{g/mL}$). This was anticipated as the negatively charged polymer is likely partially consumed to neutralize the cationic peptides during the preincubation step, thereby reducing the effective concentration of PPF-P.

After confirming the antiviral effect of the anionic polyfluorene in the absence, but also in the presence of SEVI peptides, it was investigated whether PPF-P can also efficiently target the cellular component and inhibit HIV infection. From previous experiments it is unclear whether the polymer targets the TZM-bl cells or the HIV virion, as all components were present in the incubation solution. Therefore the cells were first pre-treated with the anionic polyfluorene derivatives and the culture medium was exchanged prior infection. Similar to the experiment presented earlier, the experiment was conducted in the presence and in the absence of SEVI (Figure 4.10).

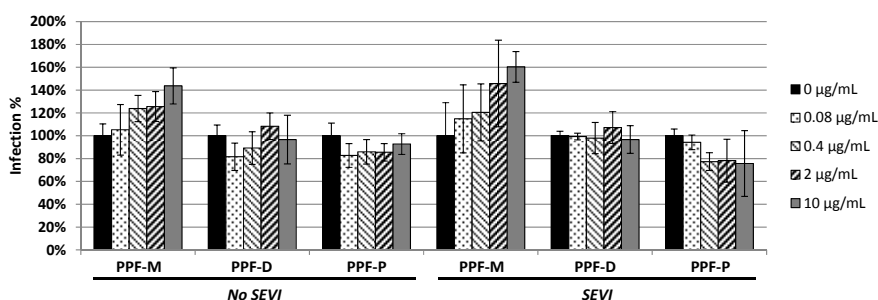


Figure 4.10. The effect of the cell pretreatment with negatively charged fluorene-based compounds on the infectivity of HIV virions. Results are normalized to the infectivity level in the absence of tested compound (100%). The applied concentration range corresponds to the effective concentration of the compounds on cells in virion pretreatment experiment.

As can be seen in Figure 4.10, no substantial effect on HIV transduction was found both in the presence and absence of SEVI peptides. This suggests that any adsorption of PPF-P molecules to the cellular membrane does not have a large influence on the virion entry. Therefore, it can be concluded that the mode of action of negatively charged polyfluorene PPF-P most likely is virion-oriented. Surprisingly, the PPF-M analogue caused an increase of HIV infectivity, which could indicate a certain degree of membrane destabilization. This in turn could facilitate fusion of the virion and cell membrane. The PPF-M monomer is a planar molecule that could potentially intercalate between lipid tails of the phospholipid bilayer and thereby influence its

fluidity and stability. This is an important finding as it suggests that viral membrane destabilization properties might contribute to the bioactivity of fluorene based compounds.

To summarize, the transduction efficiency of HIV virions was greatly suppressed in the presence of the anionic polyfluorene. The findings strongly suggest that the polymer directly impairs HIV virion entry either by membrane destabilization or by blockage of glycoproteins responsible for fusion with the host membrane. Surprisingly, the low molecular weight model compounds did not exhibit any activity. Therefore, multivalency of the monomer units in the polymer and a certain size of the polyfluorene amphiphile might be necessary to interact with the viruses and modulate their infectivity.

4.2.3 Cationic polyfluorene as retroviral transduction enhancer

To further study polyfluorenes as a universal polymeric scaffold with tuneable activity regarding viral infections, the effect of the cationic TMPF-P on the HIV virion infectivity was assessed. In this assay the HIV virion was preincubated with different concentrations of TMPF-P for 15 minutes and afterwards added to the cells. Although no cytotoxic effects were found for all positively charged compounds, this procedure was employed as it helps to minimize any cytotoxicity that might be exhibited by a positively charged substance¹⁰. The β -galactosidase activity assay was employed to quantify HIV infection, as described in section 4.2.2.

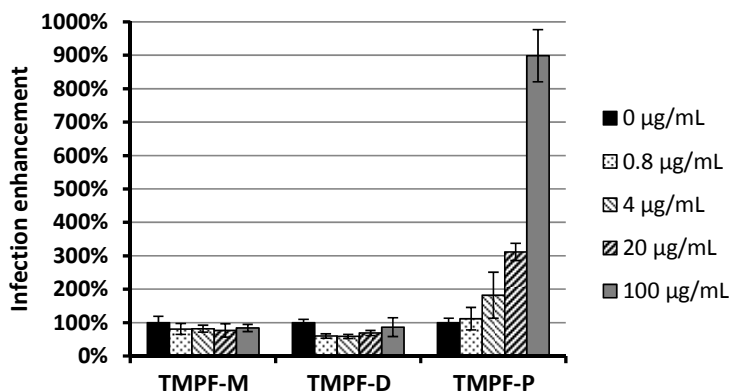


Figure 4.11. TMPF-P, TMPF-D and TMPF-M activity as retroviral transduction modulators at different concentrations. The infection level of untreated virions was set to 100%.

The data presented above shows that the positively charged TMPF-P enhances the viral infection in a dose dependent manner. Strikingly, virus particles incubated in 100 $\mu\text{g}/\text{mL}$ solution of TMPF-P were 9-fold more infective than the untreated control. The virion infectivity was also investigated in the presence of model monomer and dimer, the structural analogues of TMPF-P. It was assumed that any affinity that TMPF-P polymer shows towards the cellular or viral component would also be exhibited by its structural analogues, TMPF-M and TMPF-D. Thus the small cationic molecules were expected to neutralize negatively charged virions in the same manner as the full length polymer. However, as can be seen, these compounds did not show an increased infection activity. This indicates that the high number of charges in the polymer may play an important role in the observed increase in infection efficiency. These observations are in line with the experiments performed with the anionic substances, where lower molecular weight variants were also found to be inactive.

Due to high number of cationic groups on a single chain, the presence of PCs might lead to coagulation of the HIV virions, as multiple virus particles can interact with one polymer chain. This results in formation of nanoscopic aggregates that have a significantly higher molecular weight. It is expected that these clusters precipitate sediment on the surface of the cells, thereby passively increasing the possibility of virus-cell contact and enhancing the effectivity. Additionally, once HIV is coated with the polycation the electrostatic repulsion between virus and cell will also be greatly reduced. This then naturally increases the kinetics of viral glycoprotein and receptor binding and facilitates fusion. Hence, the electrostatically driven clustering and the consequent neutralization of the HIV particles appear as prerequisite conditions for fusion enhancement. However, the latter effect seems to be of lesser influence since TMPF-M and TMPF-D were found inactive despite their similar electrostatic properties.

These results show that the virus treated with the synthesized cationic polymer exhibits a considerably improved infectivity and that the multivalency of PCs plays an important role. Furthermore, the activity of cationic polyfluorenes could further be improved by modification of the polymer structure. There are several possibilities to do this. Firstly, the synthesis of the neutral polymer precursors with a higher molecular weight is feasible. Consequently more charged groups will be available in the final polymer, which might further influence the aggregation

characteristics. Secondly, the side chains can be modified with the different type of charged groups, thereby facilitating either stronger or weaker ionic interactions. Finally, positively charged polyfluorenes have distinct light emitting properties, unlike polypeptide based agents or commercially available PCs. This feature can easily be utilized to facilitate research on the viral docking and fusion processes.

4.3 Conclusion

The conjugated polyelectrolytes described in this chapter are an example of a new polymeric scaffold that was successfully applied as effector in the field of virology. It was demonstrated that the activity of water soluble, charged polyfluorenes is determined by the nature of the side chain modification.

Anionic PPF-P was identified as a potent microbicide that very effectively prevents HIV transduction. The IC_{50} values of this polymer were found to be 1.1 and 5.0 $\mu\text{g}/\text{ml}$ in the absence and presence of SEVI peptides, respectively. The polyfluorene modified with negatively charged phosphonate groups is directly targeting the virus particles as it was shown that cell pre-treatment with the polymer did not affect virus infectivity at any of the tested concentrations. More research is needed to elucidate the mechanism of action of the anionic polyfluorene. However, it is assumed that PPF-P destabilizes the HIV envelope or blocks glycoproteins responsible for fusion with the host cell.

In contrast, the cationic variant of polyfluorene, TMPF-P, facilitated the entry of the virions into the host cells. The TMPF-P mediated enhancement of virus infection up to a factor of nine, which can most likely be attributed to strong electrostatic interactions. Positive charges of quaternary ammonium groups efficiently neutralize the negatively charged virions and, as a result, promote virion-virion and virion-cell interactions.

Surprisingly, the model dimers and monomers did not exhibit any activity comparable to the polymers, regardless of their charge. Hence, it can be concluded that high molecular weight and consequential multiple charges along the hydrophobic backbone are necessary to obtain the observed bioactivity.

4.4 Materials and methods

All chemicals and solvents were purchased from commercial sources (Sigma Aldrich, Acros Organics) and used as received without further purification. Catalysts, used in Suzuki couplings, were purchased from STREM Chemicals (USA). Medium used for cell culture was supplemented with L-glutamine (350 mg/ml), streptomycin (120 mg/ml), penicillin (120 mg/ml) and heat inactivated FCS (10% v/v). Gal-screen Kit was obtained from Applied Bioscience. Solvents used for Suzuki couplings were degassed in a sonication bath and afterwards saturated with argon. Column chromatography was performed using silica gel 60Å (200-400 mesh). Dialysis membrane (RC, 6 Spectra/Por, Spectrum® Laboratories) was obtained from VWR (The Netherlands). NMR spectra were recorded on Varian Mercury (400 MHz) spectrometer at 25°C. High Resolution Mass Spectrometry was performed on LQT ORBITRAP XL instrument (Thermo Scientific). MALDI-ToF spectra were recorded on ABI Voyager DE-PRO MALDI-TOF Biospectrometry Workstation. High Performance Liquid Chromatography was done on Shimadzu VP series HPLC equipped with PDA detector using HPLC-grade acetonitrile, modifier and ultra-pure water. MS-TOF analysis was done on Waters Acquity M-class UHPLC.

4.4.1. Synthesis of negatively charged compounds PPF-M, PPF-D and PPF-P

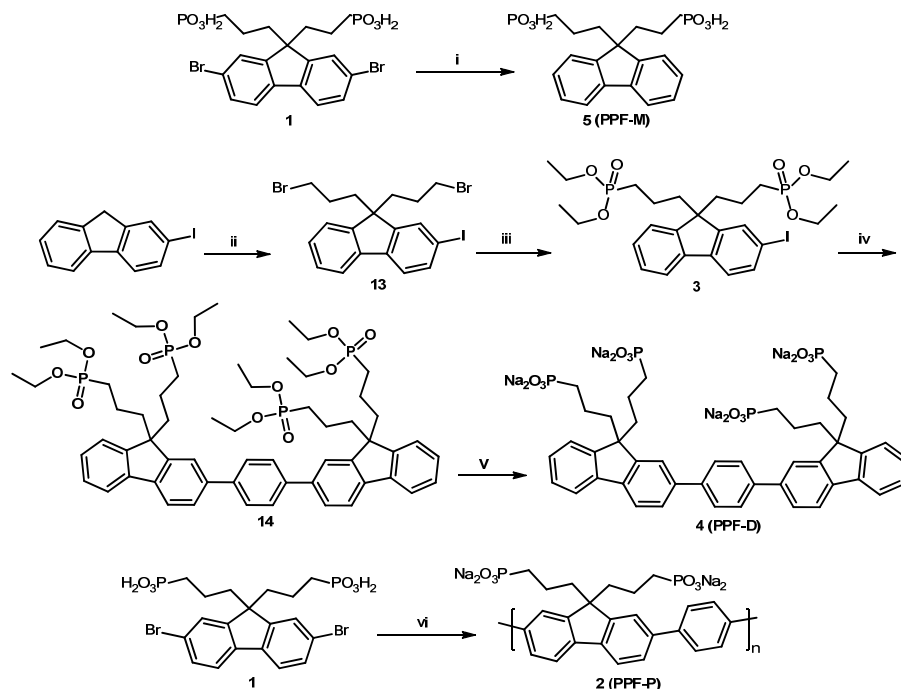


Figure 4.12. Synthesis of negatively charged PPF-M, PPF-D and PPF-P. i: Pd/C; ii: KOH, 1,3-dibromopropane; iii: Triethylethylphosphite; iv: 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)benzene, Pd(OAc)₂; v: Bromotrimethylsilane, MeOH; vi: 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)benzene, Pd(dppf)Cl₂·CH₂Cl₂

Synthesis of 9,9-bis(3'-phosphonic acid propyl)fluorene (5, PPF-M)

2,7-Dibromo-9,9-bis(3'-phosphonic acid propyl)fluorene (**1**) was synthesized according to a previously published procedure³³. In short, 50 mg of **1** (0.088 mmol) and 39.6 mg of potassium t-butoxide (4 eq, 0.352 mmol) were dissolved in 5 mL of isopropanol under argon atmosphere. Next, 10 mg of Pd/C and 1 mL of sodium formate solution (30 mg/mL in water) were added and the reaction mixture was gently heated (30°C) overnight. The solution was filtered using a RC syringe filter to remove insoluble catalyst and the crude product was purified on RP-HPLC using a GraceSmart RP18 C18 column (buffer A: 0.1% TFA and 5% AcN, buffer B: acetonitrile; linear gradient; 40°C). Purification was monitored at a wavelength of 300 nm (Figure 4.13). The product was obtained with 85% yield.

^1H NMR (400 MHz, D_2O): δ 7.87 (bs, 2H), 7.61 (bs, 2H), 7.44 (bs, 4H), 2.10 (4H), 1.13 (4H), 0.79 (4H)

MS-TOF (+) (m/z): found 411.09 $[\text{M}+\text{H}]^+$, calc. 411.34 $[\text{M}+\text{H}]^+$

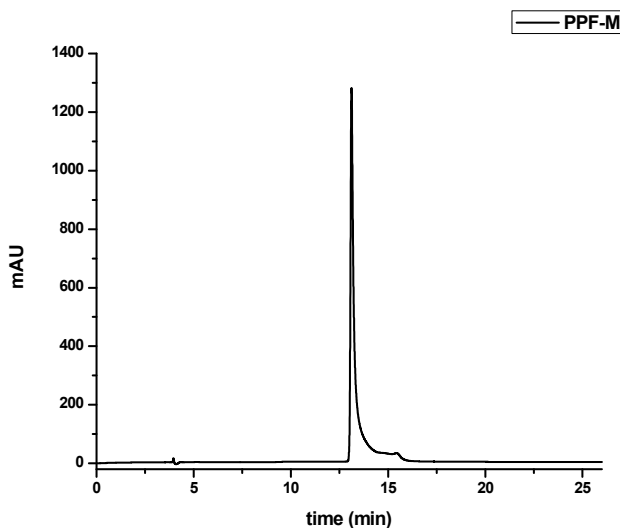


Figure 4.13. Analytical run of reverse-phase purified PPF-M.

Synthesis of 2-iodo-9,9-bis(3'-bromopropyl)fluorene (13)

First, 2-iodofluorene (0.3 g, 1.03 mmol) and 50 mg of tetrabutylammonium bromide were dissolved in 5 mL of 1,3-dibromopropane and 5 mL of 50% w/w KOH and stirred overnight at 80°C. After cooling to room temperature, the reaction mixture was diluted with 30 mL of chloroform and washed twice with water and once with brine. The organic layer was dried over magnesium sulfate and concentrated. The excess of 1,3-dibromopropane was distilled off in vacuum. Product **13** was purified on silica gel column using a hexane:dichloromethane:methanol (10:2:1) mixture as eluent. 252 mg of **13** was obtained as white solid (46% yield).

^1H NMR (400 MHz, CDCl_3) δ (ppm): 7.83-7.58 (m, 1H), 7.45 (d, $J = 7.7$ Hz, 1H), 7.36 (m, 3H), 3.11 (t, $J = 6.6$ Hz, 4H), 2.27-2.00 (m, 4H), 1.23-0.98 (m, 4H).

HRMS (ESI+) (m/z): found 534.994 $[\text{M}+\text{H}]^+$ calc. 534.890 $[\text{M}+\text{H}]^+$

Synthesis of 2-iodo-9,9-bis(3'-diethoxyphosphorylpropyl)fluorene (3)

2-Iodo-9,9-bis(3'-bromopropyl)fluorene (0.2 g, 0.31 mmol) was dissolved in 10 mL of triethylphosphite and heated overnight at 140°C. Then the excess of triethylphosphite was distilled off and the product was purified on silica gel column with ethyl acetate:methanol (100:5) as an eluent. 2-Iodo-9,9-bis(3'-diethoxyphosphorylpropyl)fluorene was obtained as a yellowish oil in 98% yield (2.02 g).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.64-7.62 (m, 3H), 7.41 (d, *J* = 7.7 Hz, 1H), 7.29 (d, *J* = 2.5 Hz, 3H), 3.93-3.84 (m, 8H) 2.07-2.03 (m, 4H), 1.48-1.40 (m, 4H), 1.15 (q, *J* = 7.0 Hz, 12H), 0.87 (m, 4H).

HRMS (ESI+) (*m/z*): found 649.243 [M+H]⁺, calc. 649.133 [M+H]⁺

Synthesis of 1,4-di-[9,9-bis(3'-diethoxyphosphorylpropyl)fluorene-2-yl]phenylene (14)

2-Iodo-9,9-bis(3'-diethoxyphosphorylpropyl)-fluorene (122 mg, 2.05 eq, 0.188 mmol), 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)benzene (30 mg, 1 eq, 0.091 mmol) and 6.24 mg of Pd(OAc)₂ (10 mol%) were placed in a Schlenk flask. Three vacuum-argon cycles were performed to remove all oxygen and a degassed mixture of DMF and 2M K₂CO₃ (6 mL, 1:1 v/v) was added. Reaction mixture was vigorously stirred for 18 h at 80°C and subsequently diluted with ethyl acetate, washed twice with water and once with brine. The organic layer was dried over magnesium sulfate and evaporated to dry. Finally, the crude dimer was purified on a silica gel column with ethyl acetate:methanol (10:1) as eluent. Compound **14** was obtained in 60% yield (102 mg).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.78-7.71 (m, 8H), 7.64-7.61 (m, 4H), 7.38-7.28 (m, 6H), 3.93-3.85 (m, 16H), 2.19-2.16 (m, 8H), 1.52-1.44 (m, 8H), 1.14 (td, *J* = 7.1, 2.9 Hz, 24H), 1.02-0.96 (m, 8H).

MALDI-ToF(+) (*m/z*) found 1119.66 [M+H]⁺, calc. 1119.18 [M+H]⁺

Synthesis of 1,4-di[9,9-bis(3'-phosphonic acid propyl)fluorene]phenylene (4, PPF-D)

An excess of bromotrimethylsilane (100 μL, 0.76 mmol) of was added slowly to 50 mg (0.045 mmol) of dimer **14** dissolved in 10 mL of dry dichloromethane. After 12 h of stirring at room temperature the solvent was removed in vacuum and 5 mL of methanol was added to the flask. After an additional 12 h of stirring, the reaction mixture was concentrated and the dimer **6** was purified on RP-HPLC on a GraceSmart RP18 C18 column (Buffer A: 0.1% TFA, 5% AcN, 95% water, Buffer B: AcN; linear gradient; 40°C) in 89% yield. Purification was monitored at a wavelength of 300 nm (Figure 4.14).

^1H NMR (400 MHz, CD_3OD) δ (ppm): 7.85-7.77 (m, 12H), 7.70 (d, 2H), 7.49-7.35 (m, 4H), 2.32-2.18 (m, 8H), 1.53-1.44 (m, 8H), 1.02-0.98 (m, 8H).

MALDI-ToF(+) (m/z) found 895.06 $[\text{M}+\text{H}]^+$ calc. 895.23 $[\text{M}+\text{H}]^+$

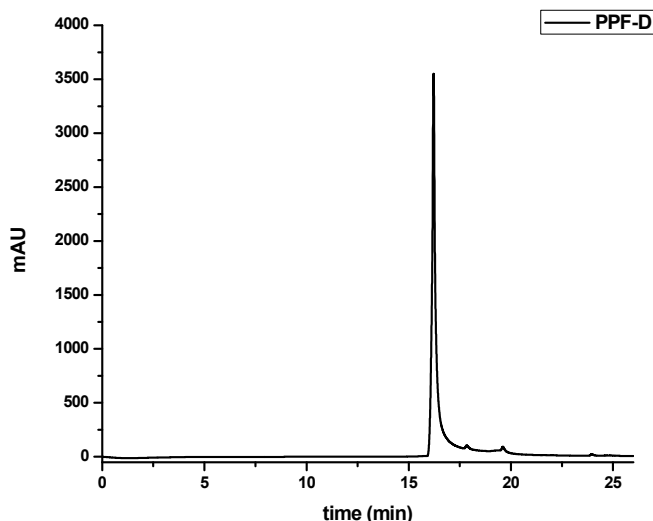


Figure 4.14. Analytical run of reverse-phase purified PPF-D.

Synthesis of poly(9,9-bis(3'-phosphonic acid propyl)fluorene-2,7-diyl-alt-1,4-phenylene) sodium salt (2, PPF-P)

Poly(9,9-bis(3'-phosphonic acid propyl)fluorene-2,7-diyl-alt-1,4-phenylene) sodium salt (**2**) was synthesized according to a previously published procedure³³. In short, 0.2 g of monomer **1** (0.35 mmol), 0.11 g (0.35 mmol) of 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)benzene and 11 mg of $\text{Pd}(\text{dppf})\text{Cl}_2 \cdot \text{CH}_2\text{Cl}_2$ (2 mol%) were placed in a Schlenk flask. Three vacuum-argon cycles were carried out to remove oxygen. Afterwards degassed solutions of 2M Na_2CO_3 (10 mL) and DMF (5 mL) were added and two additional vacuum-argon cycles were performed. The reaction mixture was vigorously stirred at 80°C. After 48 h the reaction mixture was cooled to room temperature. An additional portion of water (5 mL) was added to dissolve the salt and polymer before pouring everything into 150 mL of acetone. The precipitated polymer was filtered off and afterwards dissolved in 10 mL of warm water, filtrated once more to remove any insoluble material

and dialyzed against 4L of demineralized water using 2kDa MWCO membrane. The polymer solution was concentrated and precipitated once more in acetone. The polymer **7** was obtained with 45% yield (140 mg) as yellowish fibers. The degree of polymerization equals six fluorene-phenylene units and was determined with MALDI-ToF mass spectrometry using α -cyano-4-hydroxycinnamic acid as a matrix.

^1H NMR (400 MHz, D_2O) δ 7.95-7.91 (m, 8H), 7.85-7.78 (m, 2H), 7.62-7.57 (m, 2H), 7.44-7.41 (m, 4H), 2.29-2.08 (m, 8H), 1.29-1.17 (m, 8H), 0.86-0.76 (m, 8H).

MALDI-ToF (+) 3,0 kDa (3,2 kDa as a sodium salt) (DP=6)

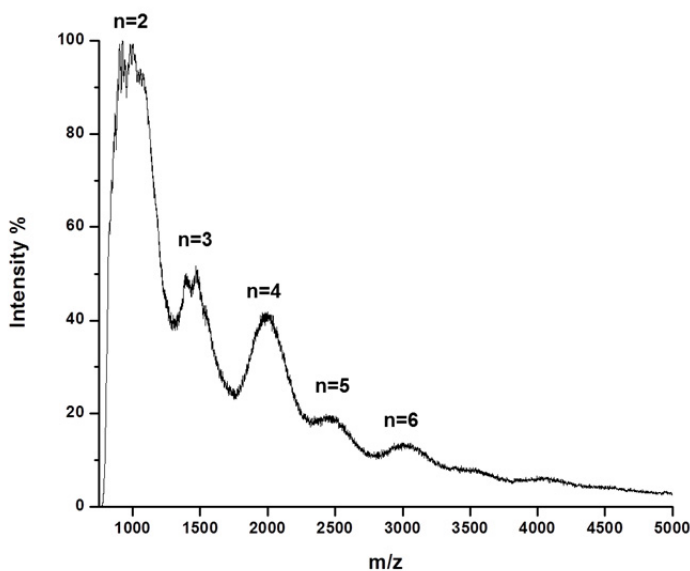


Figure 4.15. MALDI-ToF spectrum of PPF-P

4.4.2. Synthesis of cationic compounds TMPF-M, TMPF-D, TMPF-P

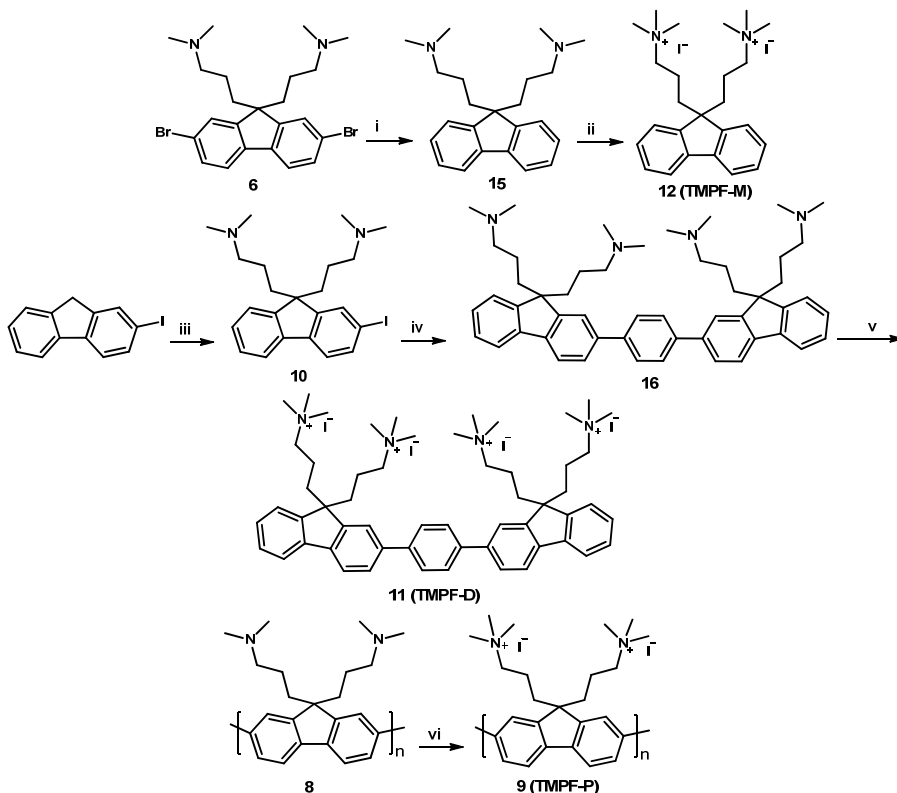


Figure 4.16. Synthesis of TMPF-M, TMPF-D and TMPF-P. i: Pd/C; ii: MeI; iii: KOH/dimethylaminopropylchloride·HCl; iv: 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)benzene, Pd(dppf)Cl₂·CH₂Cl₂; v: MeI; vi: MeI.

Synthesis of 9,9'-bis(3'-(N,N-dimethylamino)propyl)fluorene (15)

2,7-Dibromo-9,9-bis(3'-(N,N-dimethylamino)propyl)fluorene (**6**) was kindly provided by Jur Wildeman and synthesized according to previously published procedures³⁷. Compound **6** (0.25 g, 0.5 mmol) was dissolved in 5 mL of methanol under an argon atmosphere. Afterwards, 160 mg of Pd/C (3 mol %) and 5 equivalents of sodium formate (170 mg dissolved in 1 mL water) were added and the reaction mixture was stirred at room temperature for 48 h. Finally 10 mL of methanol and 5 mL of water were added and the mixture was filtered using RC syringe filter to remove insoluble material. Methanol was evaporated and the crude product was extracted with CHCl₃. Purification was carried out on silica gel column employing chloroform, methanol and triethylamine

(10:0.1:1) as mobile phase. Product **15** was obtained in an amount of 159 mg of product **15** (yield 95%).

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ : 7.68 (d, $J=8$ Hz, 2H), 7.36-7.28 (m, 6H), 2.00 (m, 20H), 0.77 (m, 4H).

HRMS (ESI+) (m/z) found 337.263 $[\text{M}+\text{H}]^+$, calc 337.264 $[\text{M}+\text{H}]^+$

Synthesis of 9,9-bis(3'-(N,N,N-trimethylamino)propyl)fluorene diiodide (12, TMPF-M)

In a round bottom flask, 100 mg (0.3 mmol) of 9,9'-bis(3'-(N,N-dimethylamino)propyl)fluorene (**15**) was dissolved in 5 mL of methanol and 78 μL of methyl iodide was added (179 mg, 1.26 mmol). The reaction mixture was stirred at room temperature overnight, subsequently concentrated and finally precipitated in THF. The crude product was filtered off, redissolved in warm water and precipitated one more time in THF. Product **12** was obtained in 94% yield (175 mg).

$^1\text{H NMR}$ (400 MHz, D_2O) δ : 7.97 (d, $J = 5.9$ Hz, 2H), 7.64 (d, $J = 6.0$ Hz, 2H), 7.54 (m, 4H), 3.03 (4H), 2.78 (18H), 2.23 (4H), 1.02 (4H).

HRMS (ESI+) (m/z) found 493.206 $[\text{M}-\text{I}]^+$ calc. 493.208 $[\text{M}-\text{I}]^+$

Synthesis of 2-iodo-9,9'-bis(3'-(N,N-dimethylamino)propyl)fluorene (10)

2-Iodofluorene (1 g, 3.42 mmol) and 100 mg of tetrabutylammonium bromide were first dissolved in 10 mL of DMSO and then 5 mL of 50% w/w NaOH was slowly added. Next 2.2 eq of N,N-dimethylaminopropylchloride hydrochloride (1.2 g, 7.53 mmol) dissolved in 5 mL of DMSO was added to the mixture dropwise and the reaction was allowed to stir at room temperature. After 24 h the reaction mixture was diluted with 20 mL of water and the product was extracted with diethyl ether (3 x 50 mL). The combined organic fractions were washed with water and brine and dried over magnesium sulfate. Crude mixture was purified on silica gel column with chloroform:methanol:triethylamine (10:1:1) mixture as eluent. Product **10** was obtained as orange oil (1.13 g, 72% yield).

$^1\text{H NMR}$ (400 MHz, cdCl_3) δ 7.67 (d, $J = 1.2$ Hz, 1H), 7.62-7.60 (m, 2H), (d, $J = 8.0$ Hz, 1H), 7.33- 7.28 (m, 3H), 1.97 (m, 20H), 0.74 (q, $J = 8$ Hz, 4H).

HRMS (ESI+) (m/z) found 463.160 $[\text{M}+\text{H}]^+$, calc. 463.160 $[\text{M}+\text{H}]^+$

Synthesis of 1,4-di-[9,9-bis(3'-(N,N-dimethylamino)propyl)fluorene-2-yl]phenylene (16)

0.255 g of monomer **10** (0.55 mmol, 2.1 eq), 0.086 mg of 1,4-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl)benzene (1 eq, 0.26 mmol) and 22 mg of $\text{Pd}(\text{dppf})\text{Cl}_2 \cdot \text{CH}_2\text{Cl}_2$ were placed in a Schlenk flask. Three vacuum-

argon cycles were performed to remove oxygen. Then 10 mL of degassed THF and 5 mL of degassed 2M K₂CO₃ were added to the flask and an additional two vacuum-argon cycles were performed. The reaction mixture was stirred at room temperature for 24 h. After that time THF was evaporated, and the crude product was extracted with ethyl acetate and purified on silica gel column using chloroform:methanol:triethylamine (10:1:1) mixture as eluent. Compound **16** was obtained as a brownish solid with yield 60.3%.

¹H NMR (400 MHz, CD₃OD) δ 7.90-7.81 (m, 10H), 7.74 (d, *J* = 7.8 Hz, 2H), 7.50 (d, *J* = 6.5 Hz, 2H), 7.43 – 7.34 (m, 4H), 2.45 – 2.30 (m, 8H), 2.19 (s, 32H), 0.91 (m, 8H).

HRMS (ESI+) (*m/z*) found 747.535 [M+H]⁺ calc. 747.536 [M+H]⁺

*Synthesis of 1,4-di-[9,9-bis(3'-(N,N,N-trimethylamino)propyl)fluorene-2-yl]phenylene tetraiodide (**11**)*

Dimer **16** (100 mg, 0.134 mmol) was dissolved in methanol and 8 equivalents of CH₃I were added (152 mg, 1.1 mmol). The reaction was stirred for 16 h at room temperature. Finally, the reaction mixture was concentrated and the quaternized dimer **13** was precipitated in diethyl ether yielding a brownish solid (129 mg, 73%).

¹H NMR (400 MHz, D₂O) δ: 8.06- 7.85 (m, 12H), 7.63 (bs, 2H), 7.55 (bs, 4H), 3.04 (bs, 8H), 2.78 (s, 36H), 2.30 (bs, 8H), 1.10 (bs, 8H).

HRMS (ESI+) (*m/z*) found 1187.340 [M-I]⁺ calc. 1187.336 [M-I]⁺

*Synthesis of poly(9,9-bis(3'-(N,N,N-trimethylamino)propyl)fluorene-2,7-diyl-alt-1,4-phenylene) iodide (**9**, TMPF-P)*

Polymer **8** was kindly provided by Jur Wildeman. It was synthesized and characterized as published previously⁴⁰. For quaternization 100 mg of polymer **8** (0.037 mmol) was dissolved in THF and excess (1 mL) CH₃I was added. The mixture was stirred at room temperature for 24 hrs. The formed precipitate was filtered off and washed with diethyl ether to yield a yellow polymer powder (77% yield). Quantitative quaternization was proven by ¹H-NMR. The molecular weight was calculated to be 4,3 kDa (DP=7)⁴⁰.

¹H NMR (400 MHz, D₂O) δ 8.18-7.58 (6H), 3.20- 2.30 (26H), 1.17 (4H)

4.4.3. Biological procedures

Experiments were performed in collaboration with Dr. David Palesch and Prof. Dr. Jan Münch in the Institute of Molecular Virology, University of Ulm, Ulm, Germany.

HIV particles preparation

The CCR5-tropic NL4-3 HIV-1 particles were generated according to previously described procedures⁴¹.

Virion treatment protocol

TZM-bl cells were seeded in a microtiter plate (10^4 cells/well) in 180 μ L of supplemented DMEM medium 24 h before infection. CCR5-tropic NL4-3 HIV-1 virus (40 μ L) was preincubated for 15 min in a solution containing the tested compound in PBS buffer (40 μ L), corresponding to a final concentration at preincubation of 100, 20, 4, 0.8 and 0 μ g/mL, and afterwards transferred to the cells in triplicates. β -galactosidase activity in a luminescence-based assay was measured after 3 days.

Cell treatment protocol

TZM-bl cells were seeded in a microtiter plate (10^4 cells/well) in a supplemented DMEM medium (180 μ L) 24 h before infection. The solution of a tested compound in PBS buffer (40 μ L) was mixed with DMEM medium (40 μ L), applied on the cells in triplicates and incubated for 2 h. The following final concentrations of the compounds on the cells were used: 10, 2, 0.4, 0.08 and 0 μ g/ml. After incubation, supernatant was removed, fresh medium was added and cells were infected with the virus. The β -galactosidase activity in a luminescence-based assay was measured after 3 days.

SEVI treatment protocol

The tested compounds were prepared as described above, but with the following modification. A PBS solution of the tested compound was supplemented with same volume of PBS solution of SEVI peptides at the final concentration of 100 μ g/ml and preincubated at room temperature for 15 minutes. Afterwards, 40 μ L of the mixture was mixed either with 40 μ L of the CCR5-tropic NL4-3 HIV-1 (virion treatment protocol) or DMEM medium (cell treatment protocol). Afterwards the virion treatment or cell treatment protocol was followed as described above.

β -galactosidase assay

The HIV-1 infectivity was quantified using Gal-Screen Kit according to manufacturer protocol (Applied Biosystems). In short, 3 days after infection the cell culture medium was removed and 50 μ L of Gal-Screen reagent diluted with PBS 1:8 was added to the cells. After approximately 30 min of incubation, the cell lysate was transferred to a white

microtiter plate and luminescence was recorded using an Orion microplate luminometer.

4.5 Acknowledgment

I would like to acknowledge Dr. D. Palesch and Prof. Dr. J. Münch for their efforts of in investigating biological activity of substances presented in this chapter. I would like to thank Jur Wildeman for the help with chemical synthesis.

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