Electrospun polystyrene fibers for HIV entrapment

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The high versatility and ease of electrospinning of polymer solutions have recently resulted in electrospun fibers, which are of interest for a wide variety of chemical and biomedical applications. This is partially due to the high surface area of the fibers, which is attractive for the detection and capture of (bio)chemicals. In the present work, polystyrene (PS) fibers were electrospun and coated with cationic poly(allylamine hydrochloride) (PAH) or anionic dextran sulfate sodium (DSS). The fibers were physicochemically characterized. Upon incubation in a dispersion of inactivated HIV-1, avid binding of HIV to all types of fibers occurred. By atomic force microscopy and spatial selective photobleaching, the binding of the inactivated HIV-1 particles to the fibers could be confirmed. Interestingly, all fibers, especially the DSS-coated and PAH-coated ones, resulted in a significant reduction of infection of CD4\textsuperscript{+} TZMbl cells by replication-competent HIV-1. On top, DSS-coated PS fibers were not toxic for vaginal epithelial cells, which may make these fibers of potential interest to inhibit HIV infection in the context of topical prevention. Copyright © 2014 John Wiley & Sons, Ltd.

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INTRODUCTION

Electrospinning is gaining widespread attention as it is generally considered as one of the most facile methods to produce a wide range of nanosized and microsized polymeric fibers.\textsuperscript{[1]} The basic process of electrospinning consists of a polymer solution or melt, which is pumped through a needle that is connected to a high voltage source (Fig. 1A). Due to the electrostatic forces generated, whipped polymer jets will be created at the needle tip. These jets can then be caught on a grounded collector, and the remaining solvent evaporates and leaves naked electrospun fibers (diameter commonly ranging from 100 nm to several micrometers with lengths of several millimeters) can be easily adapted to better suit the envisaged applications. As there are no stringent requirements regarding the type of polymer used, the process of electrospinning can be used to generate fibers consisting of a wide array of polymers, either natural or synthetic ones.\textsuperscript{[3,4]} Furthermore, more advanced setups are also available, such as coaxial spinning, to produce core/shell fibers, and the use of a rotating drum collector to generate highly aligned fibers.\textsuperscript{[5,6]}

The high number of variables made possible by the electrospinning process has resulted in a wide variety of applications, primarily in the biomedical field: (i) electrospun fibers are often investigated as scaffolds for tissue engineering applications\textsuperscript{[7–9]}; (ii) the wide array of polymers used, including stimulus-sensitive ones, conjoined with the ease of encapsulation of a wide variety of compounds, has resulted in a high interest in electrospun fibers for advanced drug delivery applications\textsuperscript{[10–12]}; (iii) furthermore, the ability to control fiber alignment has resulted in the use of electrospun fibers as tools to guide stem cell differentiation\textsuperscript{[13–15]}; (iv) the high porosity and specific surface area of electrospun fibers result in a high availability for compounds in the surrounding medium to binding sites on the fibers.\textsuperscript{[16]} This property has been exploited to develop chemical sensors, with high sensitivity and very fast reaction times,\textsuperscript{[16,17]} and suggests the possible use of...
fibers for binding and removal of toxins or pathogens; (v) other applications include the encapsulation of living cells and single cell organisms, the labeling of pharmaceutical tablets against counterfeiting with encoded electrospun fibers and the design of scaffolds for wound dressing.

We became interested to test the ability of electrospun fibers to capture HIV viral particles as it could become an attractive strategy to prevent HIV infection. In a first series of experiments we observed, by co-incidence, avid binding of HIV viral particles to the walls of polystyrene (PS) and polypropylene (PP) Eppendorf tubes; this presumably non-specific binding of inactivated HIV, dispersed in a simulated vaginal fluid (SVF) with a pH of 4.2, to PS and PP Eppendorf tubes was quantified using an enzyme-linked immunosorbent assay (ELISA) against the viral protein p24. After 2 h of incubation, about 25% of the viral particles became bound to the PP tubes, whereas binding to the PS tubes was about 35%. This (unexpected) observation inspired us to test the ability of PS and PP electrospun fibers to bind inactivated HIV viral particles. Being aware of the fact that polyelectrolytes (such as PS sulfonate) are under investigation for the PS tubes was about 35%. This (unexpected) observation inspired us to test the ability of electrospun fibers to bind HIV under conditions mimicking the healthy vaginal environment. Finally, we evaluated whether the bound HIV particles were no longer able to infect CD4+ target cells.

**MATERIALS AND METHODS**

**Materials**

Dimethylformamide (DMF), tetrahydrofuran (THF), PAH, and DSS were purchased from Sigma-Aldrich BVBA/SPRL, Pegasuslaan 5, B-1831 DIESGEM, Belgium. PS (Mw 100,000 g/mol) was purchased from Alfa Aesar (Karlsruhe, Germany). PS electrospun fibers were donated as a kind gift from Prof. Martin Möller (DWI an der RWTH, Aachen, Germany).

**Virus**

HIV-1 subtype B, CCR5 co-receptor using reference strain Ba-L (NIH AIDS Research & Reference Reagent program, Rockville, MD, USA), was used. A stock of cell-free virus was prepared by culturing HIV-1 Ba-L in PHA/IL-2 stimulated mononuclear cells and titrated at 10,000 TCID 50 per ml. For some experiments, the virus was inactivated by incubation with 200 μM Aldrithiol-2 (AT2 in dimethyl sulfoxide) for 1 h at 37°C. Subsequently, virus was filtered over a 100 kDa cut-off membrane (Millipore, Billerica, MA, USA) to remove AT2 and was aliquotted and stored at –80°C. This treatment modifies the essential Zn fingers in the nucleocapsid protein but leaves the viral envelope intact, allowing normal binding.

**Preparation and functionalization of fiber webs by electrospinning**

PS fibers were obtained through the electrospinning of a PS solution (Fig. 1A) as described previously. Typically, 0.3 mg PS was dissolved in 1 ml of a solvent mixture (DMF/THF, 1/3) and subsequently electrospun into fibers. A high voltage power supply (up to 40 kV), a syringe, a flat-tip needle, and a grounded collector were used in the electrospinning device. Typically, electrospinning of a 1 ml PS solution was conducted at a 2.0 ml h⁻¹ feeding rate using a syringe pump (NE300-Prosense Single Syringe Pump München, Germany). The applied voltage was kept at 8.4 kV. PS fiber webs were deposited on the grounded collector.

To make functionalized fibers, electrospun PS fibers (approximately 10 mg) were immersed in concentrated sulfuric acid (9.8 M, 10 ml) and stirred for 2 min. The fibers were then removed from the acid solution and washed in distilled water until the pH of the solution was approximately 7. To make positively charged fibers, sulfonated PS fibers (approximately 10 mg) were immersed in 5 ml of a PAH solution (2 mg PAH/ml in 0.5 M NaCl) for 15 min and then extensively washed in water. Negatively charged fibers were made by coating PAH fibers with DSS (0.2 mg/ml in distilled water). At the end of the procedure, all types of fibers were thoroughly washed in distilled water. Before being used for experiments, the fibers were air-dried on a glass slide.

**Scanning electron microscopy**

A PS fiber web of 0.1 mg was deposited onto a silicon wafer and dried under a nitrogen stream, followed by sputtering with gold. Scanning electron microscopy (SEM) images were recorded with a Quanta 200 FESEM scanning electron microscope (North America NanoPort Hillsboro, Oregon 97124 USA) operated at an acceleration voltage of 15 kV.
ELECTROSPUN POLYSTYRENE FIBERS

Contact angle measurements
Contact angles were measured with a Kruss Drop Shape Analysis System G10/DSA10 (Krüss GmbH, Hamburg, Germany) from video images, using the tangent method. The focus of the camera lens was set at 2. The image was calibrated with a Hamilton calibration needle (Bonaduz, GR, Switzerland) with a known diameter. A water droplet of 10 μl was dosed (with a speed of 200 μl/min) on the surface of the fibers.

Preparation of SVF
The preparation of SVF was described earlier.[24] Briefly, NaCl (3.51 g), KOH (1.4 g), Ca(OH)₂ (0.222 g), bovine serum albumin (0.018 g), lactic acid (2 g), acetic acid (1 g), glycerol (0.16 g), urea (0.4 g), and glucose (5 g) were dissolved in 11 of water. The pH of the solution was adjusted to 4.2 with hydrochloric acid.

Cell culture
Immortalized human vaginal epithelial cells were VK2/E6E7, which were kindly provided by Dr R. Fichorova (Harvard Medical School, Boston, MA) and cultured in a humid atmosphere at 37 °C and 5% CO₂. Cells were kept in keratinocyte serum-free medium (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with human recombinant epidermal growth factor (0.1 ng ml⁻¹), bovine pituitary extract (0.05 mg ml⁻¹), and calcium chloride (0.4 mM) (Gibco, Invitrogen). Cells were passaged when reaching 70–80% confluence and split 1/5. TZMbl cells were obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD, USA) and were cultured in Dulbecco’s modified Eagle’s medium (sigmaaldrich Lonza) supplemented with 10% fetal calf serum (Lonza), 50 mg/ml gentamicin (Lonza), and 200 mM L-glutamine (Lonza). Cells were refreshed twice a week.

Toxicity tests
To test the toxicity of PS, PAH, and DSS (and fibers made from these polymers), vaginal epithelial cells (VK2 cells) or TZMbl cells were seeded in 24-well plates (50,000 cells/well) and allowed to settle overnight. The cells were then exposed to solutions of respectively PS, PAH, and DSS and electrospun fibers for 5 h at 37 °C. Cell viability was evaluated 24 h later by an MTT assay according to the manufacturer’s instructions (Roche, Vilvoorde, Belgium) and compared to the viability of untreated control cells.

Adsortion of inactive HIV to fibers
Electrospun PS fibers, naked, PAH-coated, or DSS-coated (at varying concentrations [0–5 mg/ml]), were incubated for 2 h in 200 μl of a dispersion of inactive HIV [solvent respectively SVF or phosphate buffered saline (PBS)]. The concentration of inactive HIV in the dispersion (expressed as HIV-p24 antigen) equaled 220 pg p24/ml, as measured by a commercial ELISA kit (Innogenetics, Zwijnaarde, Belgium). All samples were kept in the dark at room temperature during incubation. After incubation, the p24 concentration in the supernatant (coming from non-adsorbed HIV) was measured.

Labeling of HIV
Inactivated HIV was covalently linked with NHS-Alexa488 (Molecular Probes, Invitrogen) according to the manufacturer’s recommendations. For the labeling of 50 μl inactivated HIV stock dispersion (660 ng p24/ml), 3 μl of NHS-Alexa488 was used. The excess of dye was removed by overnight dialysis against 5 l of distilled water using a Spectra/Por microdialysis device (Spectrum Labs, Rancho Dominguez CA, USA) with a molecular weight cutoff of 25 kDa.

Confocal microscopy
The binding of inactivated HIV to the fibers was further studied by confocal microscopy. To this end, 0.1 mg of PAH-coated fibers was incubated in 1 ml of inactivated (fluorescently tagged) HIV (220 pg p24/ml) for 30 min at 37 °C. Afterwards, the fibers were washed three times with PBS to remove unbound HIV. Subsequently, specific regions of the fibers were photobleached and imaged using a Biorad MRC 1024 confocal system (Japan). An inverted microscope (Eclipse TE300D, Nikon, Japan) equipped with x20 and x40 objective lens was used.

Atomic force microscopy measurements
For atomic force microscopy (AFM) measurements on free inactivated HIV, 5 μl of a diluted HIV dispersion (6.6 ng p24/ml) was applied onto a silicon wafer. For AFM measurements on fibers, 1 mg of PAH-coated PS fibers was dispersed in 1 ml inactivated HIV dispersion (6.6 ng p24/ml) for 1 h at room temperature after which the fibers were washed three times with PBS to remove free HIV. Subsequently, the fibers were deposited onto a silicon wafer and studied by AFM (Veeco, Mannheim, Germany); the AFM instrument was equipped with an optical microscope, a video camera, a monitor, and AFM tip. Standard V-shaped 115-μm-long silicon nitride cantilevers (with a spring constant of 0.32 N/m) and pyramidal tips (with an estimated tip diameter of 10 nm; DNP-S tips, Veeco) were used. The images were recorded in the tapping mode with 512 lines per screen and at a scan rate of 1.5 Hz.

HIV infection assay
Various amounts (as indicated in the main text) of electrospun PS fibers (naked, PAH-coated, or DSS-coated) were dispersed in 200 μl of active HIV (220 pg p24/ml) in SVF or PBS and incubated in 96-well plates for 2 h. All samples were kept in the dark at 37 °C. The viral efficacy was then determined, making use of CD4⁺ TZMbl cells expressing luciferase under the control of a long terminal repeat promoter, as described in detail elsewhere.[25]

RESULTS
PS and PP electrospun fibers
As explained in the Introduction, the avid binding of HIV particles to PS and PP Eppendorf tubes drove us to test HIV binding to electrospun PS and PP fibers. Representative SEM images of PS and PP fibers are shown in Fig. 2(A and B). Average diameters were calculated to be 4.1 and 1.3 μm for PS and PP fibers, respectively (Fig. 2C and D). Interestingly, whereas PP fibers showed a very smooth surface (Fig. 2B), PS fibers were found to have a high porosity (Fig. 2A), which may be explained by the differences in how the fibers were produced, as PS fibers were electrospun using a solution of PS in THF/DMF, while PP fibers were obtained through electrospinning of a PP melt. As one can expect that the high porosity increases the specific surface area, which may promote HIV binding, in further experiments, only the electrospun PS fibers were used. To evaluate the effect of surface charge on HIV binding, PS fibers were coated with polycationic PAH or polyanionic DSS, as described in the
Materials and Methods section. The amount of PAH or DSS adsorbed onto the fibers could be calculated by measuring the concentration of either compound in solution before and after exposure to the fibers. For instance, for DSS coating, 10 mg of PAH-coated fibers was suspended in a DSS solution (200 μg/ml) for 15 min, which resulted in a total of 2.5 μg of DSS being adsorbed per milligram of PAH-coated fibers.

To further evaluate the binding of PAH or DSS to the PS fibers, water contact angles were measured (Fig. 3E, F, and G), showing a clear decrease of the water contact angle for PAH (11°) and DSS.

Figure 2. (A) SEM image of PS electrospun fibers; the inset is a high-magnification image. Scale bar: 10 μm. (B) SEM image of PP electrospun fibers (as obtained from a melt). Scale bar: 1 μm. (C, D) Histograms indicating the distribution of fiber diameters for PS fibers (C) and PP fibers (D). (E–G) Representative images showing the typical structure of a water drop deposited at the surface of the electrospun fibers. Naked PS fibers (E), PAH-coated PS fibers (F), and DSS-coated PS fibers (G).

Figure 3. The binding of inactivated HIV-1 to three types of electrospun fibers (naked PS fibers [red], PAH-coated PS fibers [black], and DSS-coated PS fibers [green]) at concentrations ranging from 0 to 5 mg/ml in respectively SVF (A) and PBS (B). The results were obtained by measuring the p24 concentration by an ELISA assay. Data are shown as mean ± SEM (n = 3). This figure is available in colour online at wileyonlinelibrary.com/journal/pat.
(25°) coated fibers, when compared to naked PS (130°) fibers. These data confirm the adsorption of PAH and DSS to the PS fibers. The increased hydrophilicity of the fibers may facilitate the binding of HIV viral particles.

Adsorption of inactive HIV to electrospun PS fibers

Next, the adsorption of inactive HIV to naked, PAH-coated, and DSS-coated fibers was measured by means of a viral p24 specific ELISA assay. To this end, various amounts of the fibers were exposed (for 2 h) to SVF containing 220 pg p24/ml of inactive HIV. A concentration-dependent adsorption of HIV viral particles to all types of fibers was observed (Fig. 3A). PAH-coated and DSS-coated fibers had similar binding efficiencies, which were slightly better than those for the more hydrophobic “naked” PS fibers. As both DSS-coated and PAH-coated fibers resulted in similar HIV-adsorption efficiencies, this may suggest that HIV viral particles contain both positive and negative charges. As viruses can have a lipid “envelope” derived from the host cell membrane, an overall negative charge of the particles has been reported, although at lower pH, the overall negative surface charge diminishes.[26] Furthermore, although phospholipid head groups such as phosphocholine (which is often present in natural membranes) have an overall neutral charge, the specific orientation of the negative and positive charges present may lead to local charge heterogeneities. Also, the viral envelope consists of several glycoproteins such as gp120, which includes several regions such as the V3 loop, which is known to contain high numbers of positively charged amino acids, resulting in local positive charges.[27] Taken together, the presence of both positive and negative charges on the HIV surface likely results in the better adsorption of HIV to the coated PS fibers compared to the neutral (non-coated) ones.

As during sexual intercourse slightly alkaline seminal fluid, which has a high buffering capacity, has been described to transiently elevate the pH of the surrounding vaginal fluid,[28] we further tested the binding of HIV to fibers at a higher pH value. Inactive HIV particles were therefore suspended in PBS with a pH of 7.0. The data in Fig. 3(B) again clearly show an avid binding of the HIV viral particles to the various fibers. The binding efficiencies of bare, PAH-coated, and DSS-coated PS fibers seemed rather similar. Interestingly, however, is the observation that in both conditions (SVF and PBS), the adsorption efficiency of the viral particles could be boosted up to nearly 100% upon using higher concentrations of the fibers.

Direct analysis of inactive HIV binding to PAH-coated PS fibers

As PAH-coated fibers resulted in avid adsorption of inactive HIV viral particles in SVF, we tried to further characterize the binding through AFM. The AFM data (Fig. 4A) showed a flat surface with some indentations indicative of the high porosity of the fibers. AFM on (free) inactive HIV viral particles showed an average diameter of approximately 25 nm (Fig. 4B). This value is significantly lower than the size (120 nm) reported by McPherson et al.[29] and might be due to the chemical inactivation of the HIV viral particles, which may result in a collapse of the viral core. Nonetheless, these particles were found to avidly bind to the PAH-coated fibers (Fig. 4C and D).

To test whether the interaction between the HIV particles and the fibers was only transient or long lasting, inactivated HIV was labeled with NHS-Alexa Fluor488 as described in the Materials and Methods section. These fluorescent viral particles were then incubated with PAH-coated fibers at room temperature for 1 h, after which the fibers were washed three times with PBS and placed in SVF. Using an argon-ion laser beam at 488 nm, fluorescent viral particles were bleached by spatial selective photobleaching at precise locations on the fibers (Fig. 4E). These bleached fibers were subsequently kept in SVF for up to 4 days, after which the fibers were again visualized by confocal laser scanning microscopy (Fig. 4F). The data clearly show that the photobleached regions remain clear after 4 days, suggesting that the viral particles did not desorb and were firmly bound to the fibers, as any movement of viral particles along the fiber surface would have resulted in a recovery of the fluorescence in the bleached region.

Cytotoxicity of electrospun fibers to vaginal epithelial cells

For an efficient inhibition of HIV transmission, electrospun fibers should be applied intravaginally. Of great important
for maintaining the healthy vaginal environment is the vaginal epithelial lining. Any loss of cells or disruptions of the epithelial barrier function would greatly facilitate HIV transfer across the epithelial lining into deeper tissues and finally the bloodstream, from where HIV could be spread throughout the body.

To evaluate whether the fibers were toxic for vaginal epithelial cells, an MTT assay was performed on vaginal epithelial cells exposed to bare, PAH-coated, or DSS-coated PS fibers (Fig. 5A). The data show that DSS-coated and bare PS fibers did not have significant effects on mitochondrial metabolism. For bare PS fibers, a slight decrease in cell viability was noted at higher fiber concentrations. PAH-coated fibers resulted in a concentration-dependent decrease in cell viability. The toxicity of PAH-coated fibers is likely explained by the polycationic nature of PAH, which may significantly interact with the anionic cell membrane, thereby rupturing the cell membrane. Similar effects have been observed for other polycationic molecules such as poly-1-lysine.

**Reduction of HIV infectivity by adsorption to electrospun PS fibers**

The previous sections showed avid binding of inactive HIV viral particles to the electrospun fibers at different pH. An important question that remains is whether the binding of active viral particles also lowers viral infection. Therefore, active HIV viral particles were exposed for 2 h to electrospun PS fibers dispersed in cell medium. Subsequently, samples of the cell medium were taken, and infectivity (due to remaining HIV) was evaluated using CD4+ TZMbl cells, as described in the Materials and Methods section (Fig. 5B). The data clearly show a fiber concentration-dependent reduction of the HIV infectivity. In line with the binding efficiency of inactive HIV viral particles to the various fibers, the effect was most pronounced for PAH-coated fibers, followed closely by DSS-coated ones. Bare PS fibers also reduced HIV infectivity, but to a lower extent. Interestingly, at the highest concentration of the fibers, infection of the cells by HIV could be almost completely abolished.

**DISCUSSION**

Since HIV is characterized by a very high genetic variability, no vaccine is currently available. For that reason, novel strategies preventing HIV infection are highly required. One strategy that is currently widely investigated is the use of microbicides, which is currently being tested for its potential to prevent sexually transmitted infections, including HIV infection. However, all gel-based microbicides suffer from several practical inconveniences such as the need to clean and reuse the gel applicators and washing away of the gel with urination. Recently, multiple large-scale clinical trials failed to show any effect of currently available microbicides, possibly due to the inability of current drug formulations to form a stable and durable barrier along the whole epithelial lining. In order to try and overcome these issues, researchers are exploring the use of nanotechnology, such as PS nanoparticles coated with lectins, such as concanavalin A, which is reported to have a high affinity for the viral protein gp120. Another study reported on the use of mercaptobenzoic acid modified 2 nm diameter Au nanoparticles, which were conjugated to SDC-1721, which is a known antagonist of CCR5 and may thus be of use to prevent HIV infection. Such nanoparticles, however, still suffer from the same intrinsic problems as the gel-based microbicide formulations, i.e. they do not form a durable barrier along the entire epithelial lining and would need to be reapplied frequently.

Another possible strategy relies on the intravaginal application of effective adsorptive films. An adsorptive film is a sort of a woman's condom that could prevent transmission of HIV by virus adsorption and inactivation. The present work takes a first step in this direction, where electrospun fibers were found to efficiently bind and inactivated HIV viral particles. Moreover, from such fibers, a "web-based" film can be produced, which may be applied as a condom. The high specific surface area and porosity of the electrospun PS fibers allow for a high binding potential, which may offer great potential in the efficient capture
of HIV. The flexibility of the electrospinning process furthermore may allow to easily incorporate antiviral drugs in the fibers to further boost the antiviral efficacy. Another advantage of PS fibers is their relative low cost compared to the expensive antiviral drugs and the ability to upscale production.

CONCLUSIONS

Based on our initial observations that inactivated HIV viral particles avidly bound to PS and PP Eppendorf tubes at low pH, the binding of the HIV to electrospun PS fibers was investigated. The electrospun PS fibers were characterized and showed a highly porous structure. Coating of the PS fibers with PAH or DSS resulted in charged hydrophilicity. Interestingly, the coated fibers affected their viability, while PAH-coated fibers affected their viability, thereby avoiding the infection of CD4+ TZMbl cells.

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