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**Removing human immunodeficiency virus (HIV) from human blood using
immobilized heparin**

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Abstract We report herein that heparin covalently attached to a water-insoluble resin suspended in HIV-infected aqueous buffer or whole blood captures the virus; subsequent physical separation of the immobilized heparin reduced the viral titers by over 80% and 50%, respectively. The detoxification concept has been validated by both circulating an HIV-1 solution through a column packed with the heparin-Sepharose beads and successively mixing an HIV-1 solution with fresh beads.

Keywords Human immunodeficiency virus · HIV removal · Immobilized heparin · Detoxification
· Adsorption

Introduction

HIV-1 is the world's leading infectious disease killer due to the lack of effective and affordable treatments, absence of vaccine, and unreliable blood supplies (WHO and CDC data 2010). Even the current highly active antiretroviral therapy (HAART) is plagued by its toxicity leading to severe side effects (De Clercq 2007), high cost limiting its implementation in poor communities (Gebo et al. 2010), and not being a cure (Nakamura et al. 2011). One alternative therapeutic design can be an extracorporeal device that selectively binds HIV in the blood. Immobilized anti-HIV antibodies in hollow-fiber dialysis columns have been proposed for that purpose (Tullis et al. 2002), but a limited availability and high cost of such virus-capturing immunoadsorbents are a concern. Additionally, blood donations in underdeveloped countries suffer from a high rate of infections leading annually to tens of thousands of HIV cases (Blood supply and demand 2005). Current proactive pathogen inactivation techniques, including solvent detergent and heat treatments and using photosensitive chemicals, can alter normal blood composition leading to post-transfusion side effects (Bryant and Klein 2007).

Since HIV-1's surface proteins, exterior-gp120 and transmembrane-gp41, naturally attach themselves to the negatively charged proteoglycans on the host cell surface, such soluble polyanions as heparin are capable of blocking virion attachment (Qian et al. 2009). Moreover, selectivity in the interaction of immobilized heparin with hepatitis B and C viruses, as compared to plasma proteins, was established (Zahn and Allain 2005). Immobilized heparin has been shown to bind to herpes simplex virus (type 2) (O'Keeffe et al. 1999) and bioactive VSV-G pseudotyped retrovirus (Segura et al. 2005), although not from the whole blood. To the best of our knowledge, until now HIV has not been investigated in this regard.

In the present study, we have explored the use of heparin covalently attached to a resin to capture and physically remove HIV-1 from infected buffer solutions and from whole human blood. Potential advantages of this concept are: (i) eliminating the systemic spread of treatment chemicals; (ii) that heparin binds HIV-1's Tat (transactivating factor) and gp120 (surface protein) which are responsible for AIDS-associated pathologies (Bugatti et al. 2007); and (iii) using readily prepared starting materials.

Materials and methods

Materials. Sepharose® 4B200, 1,1'-carbonyldiimidazole (CDI), poly(ethylene glycol) (PEG) diamine (1,500 Da), and pH 8.0 borate buffer (0.98% di-Na tetraborate·10 H₂O and 0.59% boric acid in water) were from Sigma Aldrich. Roswell Park Memorial Institute (RPMI) medium was Gibco number 31800 and comprised 0.3 g/l L-glutamine, 2.2 g/l NaHCO₃, and pH 7.15, using 0.2 µm sterile filter. Heparin Na (5,000 Da) was from Santa Cruz Biotechnology. A human osteosarcoma cell line containing green fluorescent protein (GHOST cells) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, and the 293FT cell line was obtained from Invitrogen. The accession number of the NL4-3 HIV-1 strain is AF324493 (National Center for Biotechnology Information). Fresh whole human blood was supplied by Research Blood Components, LLC.

Immobilized heparin synthesis and quantitation. Heparin's covalent attachment to Sepharose using CDI followed a modified procedure for heparinization of hydroxyl-rich surfaces (Hinrichs et al. 1997). Sepharose was rinsed with deionized water and re-suspended in the latter targeting a 5 wt% concentration. The resulting wet gel (2.3 g) was suspended in 20 ml of a pH 8.0 borate buffer along with 0.05 g of CDI for 2 h before the addition of 0.23 g of heparin Na. The suspension was then stirred overnight at room temperature, followed by filtering and rinsing the formed heparin-Sepharose resin with deionized water. The same general attachment chemistry was employed to prepare heparin-PEG-Sepharose. Wet Sepharose gel (0.5 g) was reacted with 0.1 g of PEG-diamine in the presence of 0.01 g of CDI in pH 8.0 buffer. The H₂N-PEG-Sepharose was re-suspended in the fresh buffer and 0.01 g of CDI and 0.05 g of heparin Na were added. After stirring overnight, the final suspension was filtered and rinsed with deionized water. In both functionalized resins, the amount of immobilized heparin was determined by the toluidine blue dye assay (Smith et al. 1980).

HIV-1 (Clade B) virus solution preparation. Briefly, 293FT cells were transfected with NL4-3 strain of HIV-1 using Mirus reagent (Mirus, TransIT-LT1). After transfection, cells were washed with PBS before the addition of RPMI. At 48 h post transfection, the RPMI supernatant was centrifuged, filtered, and frozen at -80°C. The viral titer therein was quantified by tissue culture infectious dose (TCID₅₀) (Knipe and Howley 2001) following incubation with GHOST cells (Cecilia et al. 1998).

Virus adsorption to immobilized heparin. In all experiments, 1 mg of dry immobilized heparin (and plain Sepharose as a control) was used per 1 ml of viral solution. The latter was a 4:1 (v/v) mixture of the medium (buffer or blood) and HIV-1 in RPMI stock. The immobilized heparin was

suspended in HIV-1 solution for 1 h at room temperature. After centrifugation, the supernatant (in the case of blood samples, the plasma) was isolated and assayed for HIV by TCID₅₀.

Results and discussion

Heparin covalently attached to Sepharose (0.3 µg heparin per mg of the resin) avidly bound HIV-1 in an aqueous buffer: a $81 \pm 5\%$ drop in viral titer was observed after a 1-h incubation at room temperature (left bar in Fig. 1). Importantly, no appreciable removal of the virus from solution was observed with underivatized Sepharose under otherwise the same conditions (middle bar in Fig. 1), pointing to the critical role of the heparin ligand. It is noteworthy that the average pore diameter of wet Sepharose 4B used in these experiments is approximately 30 nm, which far too small to accommodate HIV-1 (120 nm in diameter), thus presumably explaining the lack of virus entrapment in the control experiment.

Fig. 1

To test an alternative virus-capturing method, a HIV-infected aqueous buffer was circulated through a packed heparin-Sepharose bed. Over time, a steady decrease in viral titer was observed, resulting in a 40% viral titer drop after 2.5 h.

Given the incomplete detoxification seen in Figure 1, left bar, we examined the possibility that the binding of the virus to immobilized heparin is restricted by steric hindrances imposed by the resin. To test it experimentally, we incorporated a long and flexible PEG spacer between the matrix and the ligand. As one can see in Figure 1 (right bar), heparin-PEG-Sepharose (0.3 µg heparin per mg of the resin) performed identically, a $85 \pm 3\%$ viral titer drop, to heparin-Sepharose, thus suggesting an unimpeded accessibility of HIV-1 to the immobilized receptor and ruling out the steric hindrance hypothesis.

Next, we investigated the effect of the concentration of HIV-1 in solution on the fraction of the virus adsorbed to heparin-Sepharose. When the initial viral titer was varied between 180 and 2,900 TCID₅₀/50 µl in either PBS or RPMI buffers, the percentage of captured viruses remained unchanged (Fig. 2A). Furthermore, the dependence of the amount of adsorbed HIV-1 on the initial HIV-1 titer is linear (Fig. 2B), which corresponds to an adsorption isotherm at low surface coverage (Nygren 1995). These data support the view that the incomplete detoxification seen in Figure 1 is due to a relatively modest intrinsic affinity of HIV-1 to immobilized heparin.

Fig. 2

The physical removal of HIV-1 from buffer solutions using suspended heparin-Sepharose demonstrated above was then explored in fresh whole human blood. Two 800- μ l samples of the blood were spiked with 200 μ l of two dilutions of HIV-1 in RPMI resulting in 5,900 and 69 TCID₅₀/50 μ l titers. Following a 1-h incubation, the fraction of captured viruses was determined to be $49 \pm 12\%$. This lower percentage compared to buffer solutions (Fig. 2A) is likely caused by other blood components competing for adsorption to immobilized heparin.

We further found that after suspending fresh beads of immobilized heparin in each of the aforementioned two supernatants, another $55 \pm 8\%$ of HIV-1 was adsorbed. Thus it appears that, as in the buffer (Fig. 2B), a linear isotherm applies in the blood as well. It is worth noting that the two successive suspensions combined decreased the viral titer of the blood by nearly 80%.

In conclusion, we have demonstrated that immobilized heparin captures HIV-1 in both buffer solutions and whole blood, whether in a batch or continuous flow system.

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Figure 1. HIV-1 capture by heparin-Sepharose and heparin-PEG-Sepharose in an aqueous PBS buffer at the initial viral titer of 330 TCID₅₀/50 µl. Number of samples (n) tested is 3 (except for heparin-PEG-Sepharose, n = 2); bars ± standard deviations (SD) are depicted

Figure 2. HIV-1 capture by heparin-Sepharose as a function of the initial viral titer in RPMI and PBS aqueous buffers. In the case of x = 184 or x = 2,901, the medium is RPMI and n is 2. In the case of x = 263 or x = 329, the medium is PBS and n is 3; bars ± SD

Figure 1

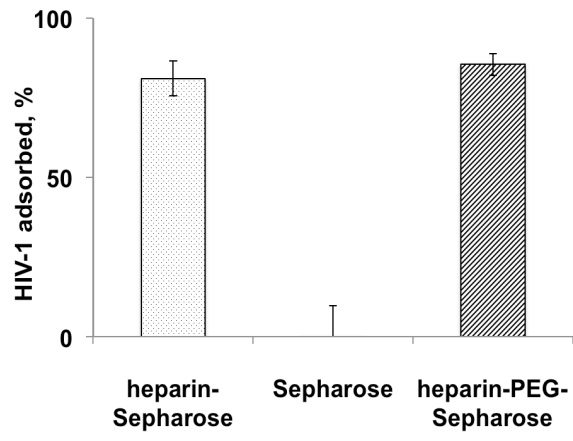


Figure 2

