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USING GLYCOSAMINOGLYCAN/CHEMOKINE INTERACTIONS FOR THE LONG-TERM DELIVERY OF 5P12-RANTES IN HIV PREVENTION

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Abstract

5P12-RANTES is a recently developed chemokine analog that has shown high level protection from SHIV infection in macaques. However, the feasibility of using 5P12-RANTES as a long term HIV prevention agent has not been explored partially due to the lack of available delivery devices that can easily be modified for long-term release profiles. Glycosaminoglycans (GAGs) have been known for their affinity for various cytokines and chemokines, including native RANTES, or CCL5. In this work, we investigated used of GAGs in generating a chemokine drug delivery device. Initial studies used surface plasmon resonance analysis to characterize and compare the affinities of different GAGs to 5P12-RANTES. These different GAGs were then incorporated into drug delivery polymeric hydrogels to engineer sustained release of the chemokines. In vitro release studies of 5P12-RANTES from the resulting polymers were performed and we found that 5P12-RANTES release from these polymers can be controlled by the amount and type of GAG incorporated. Polymer disks containing GAGs with stronger affinity to 5P12-RANTES resulted in more sustained, and longer term release than did polymer disks containing GAGs with weaker 5P12-RANTES affinity. Similar trends were observed by varying the amount of GAGs incorporated into the delivery system. 5P12-RANTES released from these polymers demonstrated good levels of CCR5 blocking, retaining activity even after 30 days of incubation.

Keywords
HIV; drug delivery; prevention; microbicide; chemokine; CCL5; CCR5; glycosaminoglycans; heparin

Introduction

With 2.6 million new infections per year\(^1\), the spread of the Human Immunodeficiency Virus (HIV) is a global epidemic. One strategy to stop the spread of HIV is to develop ways to prevent person to person transmissions. Due to societal reasons, proven methods such as condom usage\(^2\) and circumcision\(^3\) are often not adopted\(^4\). Application of topical agents to decrease HIV transmission when applied to the genital mucosa before intercourse is a strategy that has been shown to be plausible in non-human primate models\(^5-8\) and has shown utility in one large clinical study\(^9\). Currently, the delivery of low molecular weight pharmaceutical HIV preventatives using vaginal gels has shown the most clinical progress\(^5, 10, 11\). Results from a recent clinical trial delivering tenofovir using a gel based on hydroxyethylcellulose (HEC) have yielded especially encouraging results, as HIV acquisition was reduced by an estimated 39\(^9\). A major concern with the large scale use of reverse transcriptase inhibitors (RTIs) is that if these agents are simultaneous used in HIV therapy there is the potential for the development of resistant strains\(^6, 7, 12\). Use of these therapeutics in undiagnosed HIV-positive women, could potentially promote the selection
for resistant viruses\textsuperscript{5–7, 12}. Of additional significance is the increasing prevalence of viruses resistant to classes of antiretrovirals widely used in a community\textsuperscript{13}. Thus application of antiretroviral agents for topical prevention of HIV-1 acquisition that have similar resistance patterns to agents used widely for therapy may limit the utility of these strategies\textsuperscript{14}. The development of agents that do not share resistance patterns with current antiretroviral therapeutics is therefore desirable as is the exploration of combination strategies for prevention of infection\textsuperscript{15–17}.

RANTES (CCL5) is a chemokine that binds to the chemokine receptor CCR5, which also serves as a co-receptor for HIV cellular entry. The binding of chemokine to CCR5 subsequently leads to internalization of the receptor, and as a result, prevents HIV binding and infection\textsuperscript{18, 19}. Within hours after RANTES exposure in vitro, however, receptor cycling occurs and unoccupied CCR5 is available on the cell surface again and susceptibility to infection returns\textsuperscript{20}. This receptor cycling is the most important reason for continued presence of RANTES in order to prevent HIV binding. More potent RANTES analogs with more durable effects on CCR5 availability have been developed by our team to try to address this problem\textsuperscript{20}; however, receptor cycling is only prolonged from few hours to few days, indicating a need for sustained delivery over multiple days. Some of these analogues can also occupy CCR5 without inducing receptor internalization or signaling, thereby avoiding potential induction of cellular activation and inflammation\textsuperscript{21}. While a single report suggests that low level resistance to PSC-RANTES could be found in a SHIV isolate found in a rhesus macaque that was not protected by a relatively low dose of analog before virus challenge, it has since been clearly demonstrated that evolution of resistance to both PSC-RANTES and 5P12-RANTES (a more recently developed analog) is disfavored by a high fitness cost\textsuperscript{22}. Both 5P12-RANTES and PSC-RANTES have provided complete protection against SHIV infection in the rhesus vaginal challenge model, but the challenge took place no more than 30 minutes after topical application of the analog\textsuperscript{23}. Delivery methods that can provide sustained delivery of these agents would be desirable: strategies that provide durable protection against HIV acquisition are more likely to be effective than those heavily dependent upon timing of application relative to coitus. While sustained delivery of RANTES analogs has been explored in a microparticulate system\textsuperscript{24}, the long-term sustained dosage of 5P12-RANTES needed for HIV protection is still unclear. A sustained, long-term (weeks-months) delivery system where delivery can be modified is needed to further explore the use of RANTES analogs as a topical HIV prevention strategy.

Affinity-based drug delivery is a class of delivery systems that has recently gained popularity. These systems intentionally incorporate affinity moieties that interact with the agent of interest to control and manipulate its loading and release. Drug release rates from these systems are governed by the kinetic parameters in binding and release between the drug and the affinity moieties within the delivery systems, not by diffusion alone. As a result, release can be tailored based on the strength of these interactions. Affinity-based drug delivery systems have been used in the delivery of antibiotics, chemotherapy agents and growth factors\textsuperscript{25, 26}. RANTES and its derivatives are known to have affinity interactions with various glycosaminoglycans(GAGs), with heparin having the strongest affinity, followed by various chondroitin sulfates\textsuperscript{27} and uncleaved heparan sulfates\textsuperscript{28–31}. These interactions have been explored in the application of biosensor and diagnostic development for cytokines\textsuperscript{32, 33}. In this study, we characterized the affinity strength of different GAGs to 5P12-RANTES. We designed solid delivery implants exploiting these different affinity strengths and examined release of 5P12-RANTES from materials made from these GAGs. We also found that incorporating different amounts and types of GAGs into a sustained delivery system can determine the pace and magnitude of long-term drug delivery. Eventual use of these observation will be in generating short term drug delivery gels similar to HEC gels, or long term drug delivery inserts similar to other HIV microbicide delivery inserts.
currently under investigation. This represents the first use of GAGs to prolong the delivery of either HIV microbicides or chemokines.34

**Experimental Section**

**Materials**

Heparin sodium, chondroitin sulfate A (CSA) and chondroitin sulfate B (CSB, also known as dermatan sulfate) were all purchased from Thermo Fisher Scientific (Pittsburgh, PA). 5P12-RANTES was provided by the Mintaka Foundation for Medical Research, Geneva, Switzerland). Duoset Human ELISA kits were purchased from R&D Systems. Fluorochrome labeled monoclonal antibodies (3A9 APC, APC isotype, 2D7 FITC, FITC isotype, CD8 PE-Cy7, and CD3 PerCP) were all purchased from BD biosciences (Franklin Lakes, NJ). Surface Plasmon Resonance (SPR) supplies were purchased from General Electric (GE Healthcare, Piscataway, NJ). All other reagents used in this study were purchased from Thermo Fisher Scientific (Pittsburgh, PA) unless otherwise stated.

**Surface Plasmon Resonance (SPR) Analysis**

SPR was used to study the affinity between different GAGs and 5P12-RANTES using the Biacore 3000 system (GE Healthcare). A CM5 sensor chip (GE Healthcare) was used for all SPR experiments. The BIA evaluation software (version 4.0.1, GE Healthcare) was used for all post experiment analyses, including curve fitting and kinetic parameter determination. The optimum protein immobilization condition was determined to be pH 5.5 after pH scouting. Immobilization of 5P12-RANTES onto the chip was carried out by amine coupling. Specifically, the surface was activated by injecting 0.4M 1-ethyl-3-(3-dimethylpropyl)-carbodiimide (EDC)/0.1M N-hydroxysuccinimide (NHS), 5P12-RANTES (50ug/ml in acetate buffer (pH 5.5)) in was then injected for the actual immobilization. Inactivation of excess groups was carried out by injection of 1M ethanolamine-HCl (pH 8.5). The specific rate and time of injection for each step was automated to reach the targeted immobilization level of 3000 response units (RU) units. After washing the surface with 10mM HEPES buffer (pH 7.4), association and dissociation studies were carried out by injecting samples at 10ul/min for 90secs (association) and then washing the surface with acetate buffer (pH 4.5), also at 10ul/min for 4.5mins (dissociation). Heparin sodium, CSA and CSB samples at various concentrations (ranging from 0.25µM to 20µM) were examined for real-time affinity interaction with 5P12-RANTES. Dissociation constants were calculated by using the simultaneous kinetics ka/kd fit in the BIAevaluation software.

**Polymer Synthesis and Washing**

Hydrogels are networks of crosslinked hydrophilic polymers commonly used in drug delivery. The polymeric hydrogels used in this study were firm, solid disks synthesized by crosslinking bovine serum albumin (BSA) with different GAGs using carbodiimide chemistry. BSA was selected as a relatively inert base material of high natural biodegradability and with readily available amines for crosslinking chemistry. Separate mixtures totaling 100mg of 50:50, 25:75, 15:85, 5:95 and 2.5:97.5 heparin to BSA, 25:75 CSA to BSA and 25:75 CSB to BSA ratios were weighed and individually dissolved in 500ul of 0.1M 2-(N-morpholino) ethanesulfonic acid (MES) solution (pH adjusted to 5.2 using 0.1N NaOH). Separately, 40ul solutions of 1mg/ml of EDC in 0.1M MES were prepared. On ice, each 500ul GAG/BSA solution was thoroughly mixed and vortexed with the 40ul EDC crosslinker solution in a 15mm scintillation vial. The solutions were left to reach room temperature and allowed to cure overnight to form the firm, solid disks. After 24hrs, the disks were removed from the vials and washed 3 times. Each wash cycle consisted of swelling the disks in 10ml of PBS and leaving them in gentle agitation for 1
hour. At the end of the 3 wash cycles, the disks were left in gentle agitation overnight before air drying in the hood.

**Polymer Characterization - Fourier Transform Infrared Spectroscopy (FTIR)**

To prepare the samples for FTIR, dried polymers were ground into powder using a mortar and pestle. The powdered polymers were washed in 5ml of deionized (DI) water by vortex. The GAG/BSA powders were collected by centrifuging at 150g. This wash cycle was repeated 3 times and the samples collected by freeze drying. FTIR of the freeze dried powder was performed along with unmodified BSA and GAGs to ensure that BSA and GAG was chemically conjugated, not just physically mixed or entangled. FTIR of the freeze dried powder was performed on the Excalibur FTS 3000 Fourier-Transform Infrared (FTIR) Spectrophotometer (BioRad, Hercules, CA, USA)

**Gel Swelling**

Polymer swelling was determined by incubating the dried polymer disks in pH 4.5 and pH 7.4 buffers overnight. Swelling ratio was calculated using the formula below.

\[
\text{Swelling} = \left( \frac{\text{Swollen Weight} - \text{Dry Weight}}{\text{Dry Weight}} \right) \times 100\%
\]

**Preparation of Simulated Vaginal Fluid (SVF)**

To mimic release in a vaginal vault, release occurred in SVF, which was prepared by a slight modification of a published formula\(^35\). To 900 mL of distilled water, NaCl (3.51 g), KOH (1.4 g), Ca(OH)\(_2\) (0.22 g), bovine serum albumin (10 g), lactic acid (2.00 g), acetic acid (1.00 g), glycerol (0.16 g), urea (0.4 g) and glucose (5.00 g) were added and stirred mechanically until complete dissolution. More BSA was added than in the published formula to counter adsorption of protein to glass. In addition to being used in crosslinked form as a polymeric hydrogel material in this study, BSA is a typical stabilizing protein used in many release studies to prevent protein loss due to adsorption onto glass- or plastic ware. The pH of the mixture was then adjusted to 4.5 using HCl, and the volume was adjusted to 1 L. The SVF was filtered using a 0.2µm pore filter before used in the release study.

**Drug Loading of Polymers**

5P12-RANTES was loaded into the GAG/BSA polymers by hydrodynamic loading. The 5P12-RANTES loading solution was prepared by dissolving freeze-dried 5P12-RANTES in SVF to reach a final concentration of 500ug/ml. Each dried gel was incubated in 250ul of 5P12-RANTES solution in wells of a 24 well plate (1.5cm diameter). The wells were sufficiently large to accommodate the size of the fully swollen gel. The disks were allowed to swell for 48hrs to allow the disks to fully load. The fully loaded disks were weighed before the start of the release study.

**In Vitro Drug Release**

5P12-RANTES release profiles from the GAG/BSA polymers were characterized by an in vitro release study. The release study was carried out by incubating each loaded gel (wet) into 1mL of SVF solution in a 1.5 cm diameter well. The disks were incubated at 37°C under gentle agitation using a incubator shaker (Lab Line incubator-shaker model 3525). At each time point, each gel was carefully removed from its release solution using tweezers and was subsequently placed into a new well containing fresh SVF. The aliquots containing released 5P12-RANTES were then aspirated and stored in 400ul aliquots at −20°C for later analysis. Daily samples were taken for the first 10 days, followed by sampling every other
day for 4 weeks. Levels of 5P12-RANTES in each aliquot were measured by RANTES ELISA. Daily and cumulative release profiles were plotted against time.

Characterization of the authenticity of receptor binding by released 5P12-RANTES

While the human ELISA kit was used to quantify 5P12-RANTES in the release aliquots, a human T-Cell receptor binding bioassay was used to confirm authentic receptor binding of the released 5P12-RANTES. This was done by comparing detection levels of the 3A9 and 2D7 epitopes of CCR5 in the presence of the 5P12-RANTES samples. The murine monoclonal antibody 2D7 specifically targets the second loop of CCR5, while another, 3A9, targets the receptor’s amino-terminal loop. Binding of RANTES to CCR5 has been shown to block antibody binding to the second loop, but not to the amino-terminal loop.

After provision of written informed consent through a protocol approved by the institutional review board (IRB) of University Hospitals of Cleveland/Case Medical Center, peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Paque density sedimentation from the blood of healthy adult volunteers. PBMCs ($2 \times 10^6$ cells/well in 24 well plates) were incubated in medium (RPMI supplemented with 10% FBS, L-glutamine and antibiotics) with or without aliquots containing previously released 5P12-RANTES. As a positive control, fresh stock solutions of 5P12-RANTES were used to establish standard bioassay values. After 1 hr of incubation, cells stained with fluorochrome-conjugated antibodies that recognize CD3, CD8 and CCR5 (3A9 and 2D7) for 15 minutes on ice prior to analysis on a BD LSRII flow cytometer (BD, Franklin Lakes, NJ).

Results

Surface Plasmon Resonance (SPR) Analysis

The SPR analysis of heparin/5P12-RANTES interaction measures the real-time interaction between immobilized 5P12-RANTES and free heparin (or other GAGs) in solution. The analysis consists of a set of sensorgrams obtained by measuring the refractive indices on the chip surface as GAG solutions of different concentrations flow over a 5P12-RANTES immobilized chip. The refractive index changes (represented as response units (RU)) as GAG molecules bind (specifically or non-specifically) to the chip surface. An increase in RU units generally reflects increased binding interaction (specific or non-specific) between the immobilized molecule (5P12-RANTES) on the chip and the analyte (GAG) in solution flowing above the chip.

SPR Analysis of GAG Interactions with 5P12-RANTES

The sensorgrams (Figure 1) from the GAGs/5P12-RANTES SPR study showed heparin had the fastest association (sharpest increase in RU after injection of GAG), and the slowest dissociation (slowest decrease in RU relative to max RU). CSB showed slower association and faster dissociation compared to the heparin. CSA, however, showed almost no interaction as the RU level returned to baseline almost immediately after wash.

SPR Analysis of GAG-5P12-RANTES Affinity with Concentration Sensorgrams

In general, all the sensorgrams (Figure 2) in the heparin concentration study showed similarly shaped characteristic profiles. The association step consists of a rapid initial response followed by a slower gradual increase and the dissociation (wash) step showed an immediate rapid drop in response followed by gradual decrease over time. Closer examination of the association and dissociation phases of the sensorgrams does show minor differences in both association and dissociation phases with varying concentration. This may reflect multiple interactions possible with higher heparin concentrations, leading to a slight overestimation of Kd at higher concentrations, however its effect appears to be minor.
The results from the SPR analysis of the CSB/5P12-RANTES interactions with different CSB concentrations (Figure 3) confirmed the initial findings that CSB had weaker affinities to 5P12-RANTES when compared with heparin. As was found with heparin, all the sensorgrams in the CSB/5P12-RANTES study showed a very rapid initial response followed by a more gradual increase in the association phase, and a short rapid drop in response followed by very gradual decrease over a long time. One difference is that the CSB/5P12-RANTES study did not exhibit the saturation effect observed in the heparin study. During the dissociation phase, drops in response level corresponded with all CSB concentrations tested.

Using the BIAevaluation software, simultaneous 1:1 ka/kd fit was performed on the four unsaturated heparin concentrations and the calculated dissociation constant (Kd) was 25.5nM. This result is similar to the published Kd (32.1nM) of unmodified RANTES with heparin. A similar SPR concentration study of CSB/5P12-RANTES interactions was performed and the calculated Kd was 1.07mM.

Polymer Formation and Characterization

The 50:50 heparin:BSA mixture did not form materials that stayed intact after drying and swelling, thus it was not analyzed further in these studies. All of the remaining mixtures (25:75, 15:85, 5:95 and 2.5:97.5 heparin to BSA, 25:75 CSA to BSA and 25:75 CSB to BSA ratios) did form materials that stayed intact after removal from the vial, 24hrs following synthesis.

FTIR of Heparin/BSA Polymers

The conjugated heparin/BSA spectra (Figure 4) contained a combination of characteristic peaks of unmodified BSA (amide I and II bands from the amide backbone) and characteristic peaks of unmodified heparin (symmetrical and asymmetrical stretching of S-O from the SO$_3$ groups in heparin). In addition, however, conjugated heparin/BSA showed a small decrease in OH-stretching and in OH-bending when compared with just a physical mixture of heparin and BSA.

Polymer Swelling

Polymer swelling (Table I) in pH 7.4 buffer for all materials tested (BSA only, 25% heparin and 25% CSB) was not significantly different, regardless of the type of GAG used. However, when swelling was performed in a slightly acidic environment (pH 4.5), swelling was significantly decreased for gel systems containing relatively large CSB (25%) and heparin (25%, 15%) content, whereas swelling for BSA only, CSA (25%) and materials with lesser heparin content (5% and 2.5%) all remained relatively high.

In Vitro 5P12-RANTES Release from GAG/BSA Gels

The 5P12-RANTES release in all GAG/BSA polymers appeared to consist of two phases – a burst phase over the first few days in which relatively large amounts of 5P12-RANTES are released and a slower release phase thereafter. The amount and type of GAG content within the polymers had a significant impact on both phases and these effects were explored in more detail in two different studies. Results from each release study are presented both as release at each time point when the disks were moved to fresh SVF solution and cumulative release, normalized to essentially 100% total release by day 33 (by which time daily release had become negligible).

This calculation is less accurate for affinity-based systems as they still were releasing approximately 50,000 pg/day by day 33, however this represents only a small fraction of the
1 to 10 million pg released in early time points. We conclude therefore that this 100% estimate is sufficient, and any error would represent even more constant, sustained release.

**Effect of Different GAG Types on Release**

The release profiles from the different GAG/BSA polymers (Figures 5 & 6) reflected the affinity strength of GAG/5P12-RANTES within the gel. Gels containing GAGs with stronger affinity to 5P12-RANTES generally resulted in more sustained release profiles. In detail, in the burst phase, release from the CSA/BSA disks resulted in a drop by four orders of magnitude in the amount of 5P12-RANTES released when comparing the first day of release to the fifth day of release. In heparin/BSA and CSB/BSA disks, this burst effect was reduced: a drop of only 1.5 orders of magnitude was observed. After 5–7 days, the release profiles of the latter two materials appear to transition into a more constant release phase. This phase consisted of more constant levels of 5P12-RANTES released from the polymer systems, although the levels of 5P12-RANTES released decreases with time, but at a much slower rate than during the initial burst phase. In this more constant release phase, the release level seems related to GAG/5P12-RANTES affinity strength within the polymer disks. The heparin/BSA disks (highest affinity) exhibited higher proportions of sustained release compared to the disks with lesser affinity (CSB/BSA followed by CSA/BSA).

**Effect of Heparin Content on 5P12-RANTES Release**

The release profiles from the polymers with different heparin (Figures 7 & 8) content showed a relationship between heparin content and release profile, with disks containing more heparin generally resulting in more sustained release profiles. In the burst phase, release from the BSA-only disks resulted in a drop by four orders of magnitude in the amount of 5P12-RANTES released when comparing the first day of release to the fifth day of release. In disks containing conjugated heparin, this burst effect was reduced as the heparin content increased. This reduced burst effect was due to a combination of both decreased 5P12-RANTES release initially and higher 5P12-RANTES release at the end of the burst phase. In the case of disks containing 25% heparin, comparing the first day and fifth day of release, a drop of only 1.5 orders of magnitude was observed. After 5–7 days, the release profiles appear to transition into a more constant release phase. In this phase, the release dose seems to relate to the amount of covalent heparin within the polymer disk. The disks with higher heparin content exhibited higher levels of 5P12-RANTES release compared to the release from disks with lesser heparin conjugation or the no heparin control.

**CCR5 Blocking Assay**

In preliminary studies, we confirmed that 5P12-RANTES blocks the binding of monoclonal antibody 2D7 to the second extracellular loop of CCR5 while binding of 3A9 is mostly unaffected by 5P12-RANTES binding to CCR5.

CD8+ T-cells were analyzed in a receptor blocking assay. The 2D7% presentation and 3A9% presentation were analyzed to determine CCR5 receptor blocking efficacy of 5P12-RANTES which had been incorporated and released from the delivery system. Successful 5P12-RANTES blocking of the CCR5 receptor is expected to result in a decrease in 2D7 % presentation while not affecting levels of 3A9% presentation. Binding of 5P12-RANTES to the CCR5 receptor occupies the 2D7 antibody binding site, thus decreasing the level of 2D7% presentation detected by the antibody. Conversely, 5P12-RANTES binding does not occupy the 3A9 antibody binding site, thus the levels of 3A9% presentation is largely unaffected by 5P12-RANTES occupancy of CCR5 receptors.

The 5P12-RANTES CCR5 blocking efficiency of 5P12-RANTES at different stock concentrations dissolved in SVF was studied and plotted in Figure 9. At stock 5P12-
RANTES concentrations of 100ng/ml or greater, the blocking assay showed good CCR5 blocking as demonstrated by 2D7% binding (Figure 9) of less than 1%; and 3A9% binding (data not shown) near 20%. At concentrations of 0.8ng/ml or less, the blocking assay showed little to no CCR5 blocking with 2D7% binding (Figure 9) and 3A9% binding (data not shown) both near 20%. These results were used to strategically dilute the released aliquots for the bioactivity assay.

Based on this capacity to detect CCR5 blocking with a competitive antibody binding assay, we examined the blocking efficiency of select released aliquots to determine whether incorporated and released drug maintained its bioactivity following incorporation, long-term aqueous incubation, and drug release. Samples from three release time points (6h, 265h and 650h) were examined. Each released aliquot was strategically diluted based on its measured concentration (from ELISA) such that one dilution was at a concentration expected to result in good CCR5 blocking, a second dilution would be expected to result in a concentration with little CCR5 blocking and a third dilution would be expected to result in a concentration with little to no blocking (based on stock 5P12-RANTES blocking results from Figure 9).

The CCR5 receptor blocking efficiency of samples released from the BSA disks and the 25% heparin disks are presented (Figure 10). The results from the blocking assay corresponded with the expected activity of each sample based on its 5P12-RANTES concentration previously determined by ELISA. This indicates that blocking ability of the delivered drug was not lost, even after as much as 650 hours of aqueous incubation of the loaded drug delivery device. As an example, one 6h release aliquot from the 25% heparin gel with a measured concentration of 10 µg/ml was diluted 250, 1250 and 6250 times, resulting in samples with expected concentrations of 40ng/ml, 8ng/ml and 1.6 ng/ml respectively. The blocking efficiency of these diluted samples was comparable to the blocking activity of stock 5P12-RANTES samples. More specifically 250x diluted release sample (expected concentration of 40ng/ml) showed good blocking; the 1250x diluted sample expected concentration of 8ng/ml) showing some blocking; and the 6250x diluted sample (expected concentration of 1.6ng/ml) showing little or no CCR5 blocking. The CCR5 blocking assay was also performed for select release aliquots of the 25% CSA disks, 15% heparin, 5% heparin and 2.5% heparin disks. Release samples from all tested polymers showed the expected levels of blocking (data not shown).

Discussion

SPR Analysis of GAG Interactions with 5P12-RANTES

Based on the relative rate of association and dissociation (the shape of the sensorgrams during the association and dissociation phases) and the dissociation constants obtained from the simultaneous ka/kd curve fitting (BIAevaluation software) of concentration sensorgrams, heparin appeared to have the strongest affinity for 5P12-RANTES, followed by CSB, then CSA. This ranking was consistent with published results from another study where interaction between unmodified RANTES and GAGs were examined and corresponds to the relative number of sulfated groups per repeat unit (3, 2 and 1 for heparin, CSB and CSA respectively).

SPR Analysis of 5P12-RANTES to Varying Heparin Concentration

The shapes of the sensorgrams from the heparin/5P12-RANTES concentration study appear to be due to a combined effect of specific non-covalent heparin/5P12-RANTES binding and non-specific interactions. The RU response at the end of the association phase was related to the heparin concentration as increasing concentrations provided more heparin to interact (specifically and non-specifically) with the immobilized 5P12-RANTES. During the buffer wash (the dissociation step), the initial rapid drop is likely due to loss of heparin non-
specifically interacting with the chip surface, while the subsequent gradual decrease in RU over a long time is likely due to dissociation of heparin bound to 5P12-RANTES on the chip surface. At a concentration of 2.5µM (and higher), saturation is reached and no additional heparin can have specific interactions with the immobilized 5P12-RANTES on the chip surface. As a result, all additional increases in response at higher heparin concentrations at the end of the association phase were due to non-specific interactions. The non-specifically bound heparin molecules do not have strong binding and were quickly washed away at the beginning of the dissociation phase. After those heparin molecules were washed away, similar amounts of specifically bound heparin remain on the surface; thus, the subsequent response (in the gradual dissociation phase) for the more concentrated heparin solutions (2.5µM or above) were almost identical. For more dilute heparin concentrations (1.0µM or less), since saturation was not reached, the amount of specifically bound heparin is less than that of the higher concentration solutions; thus, responses are at lower RU levels and correlate with the initial solution concentration.

Polymer Characterization

FTIR of Heparin/BSA Polymers—The FTIR spectrum of the washed and ground up conjugated heparin/BSA materials confirms successful conjugation between BSA and heparin. Since BSA and heparin are both water soluble, the repeated washing of the conjugated heparin/BSA should have removed any free unconjugated BSA or heparin molecules from the crosslinked polymer. The FTIR spectrum of the heparin/BSA powder was a combination of characteristic peaks observed in neat BSA and neat heparin, indicating presence of BSA and heparin in the newly conjugated molecule. In addition, conjugated BSA-heparin also showed a slight decrease in OH bending and stretching when compared to a scan of physically mixed BSA and heparin. While the observed decrease is possibly within the range of detection, this would be how a reduction in the number of carboxylic acid groups, as they are being consumed in the conjugation reaction, would be manifested. Similar analysis was performed on CSA/BSA and CSB/BSA materials to confirm successful conjugation (results not shown).

Polymer Swelling—The largest difference in swelling at different pHs was observed in polymers with highest heparin and CSB content. One possible explanation for this difference in swelling is that highly sulfated GAGs (heparin and CSB) in neutral pH (7.4) are negatively charged. The electrostatic repulsion between negative charges prevents the heparin molecules from close stacking, resulting in materials with relatively high swelling. However, in slightly acidic environments (pH 4.5), many of the carboxylic acid (unreacted) groups (pKa, typically around 5) are protonated, losing their negative charges and minimizing electrostatic repulsion. This observation has been extensively exploited in enteric drug delivery with polymeric hydrogels that swell with pH change. The decreased repulsion from fewer negative groups conceivable allows the molecules to be closer, leading to decreased swelling. In materials with relatively lower heparin content, this effect is somewhat minimized as there are not as many heparin molecules available to stack, resulting in still relatively higher swelling. Additional experiments like computational methods to model charge density distributions within GAGs would verify the mechanism of the noted difference in swelling.

In Vitro Release of 5P12-RANTES from GAG/BSA Polymers

Effect of Different GAGs on Release—The release profiles from the different GAG/BSA polymers showed a relationship between GAG affinity to 5P12-RANTES and the release profile, with polymers containing GAGs with stronger affinity to 5P12-RANTES resulting in more sustained release profiles. Specifically, from the SPR results, CSA showed the lowest affinity to 5P12-RANTES. Correspondingly, the CSA/BSA polymers showed the

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most significant burst effect and the least sustained release profile. CSB, with greater affinity to 5P12-RANTES than CSA, resulted in a release profile with a lesser burst effect, and a more prolonged and sustained release profile. Finally, disks containing heparin, the molecule with the greatest affinity to 5P12-RANTES had a smaller burst effect and the highest and most sustained prolonged 5P12-RANTES release. These results suggest that the affinity between GAG and 5P12-RANTES can be used to control release by decreasing the burst effect and sustaining release, thus creating a more sustained release profile.

Effect of Heparin Content on 5P12-RANTES Release—The differences in the release profiles of the disks can be attributed to the affinity between the 5P12-RANTES and the heparin within the gel. Initially, during the burst phase, in the BSA-only (no heparin) control disks, 5P12-RANTES diffuses down the concentration gradient and out of the polymers. There are no other mechanisms to slow this diffusion. After most of the 5P12-RANTES has diffused out of these disks, the gel transitions into a more constant release phase, where minimal release is observed and is likely due to some nonspecific interactions between BSA and 5P12-RANTES. In the disks with conjugated heparin, the affinity between the gel and the 5P12-RANTES retains some 5P12-RANTES within the delivery system in the beginning. The initial burst release of 5P12-RANTES from these disks is likely due to the diffusion of unbound 5P12-RANTES out of these polymers. After a few days, most of the 5P12-RANTES that remains within the polymer is bound to the polymer by reversible affinity interactions. The release during this phase is governed by the repeated association and dissociation of 5P12-RANTES to heparin within the polymer. This results in a more constant release. The heparin content incorporated into the polymers seems to determine the 5P12-RANTES released in this phase. The disks that contain more heparin have higher levels of this more constant release than do disks with lesser heparin content.

Blocking Assay—These data indicate that the 5P12-RANTES released from the GAG/BSA delivery systems maintain good biologic activity even after 4 weeks in the gel. The blocking activity of the released aliquots correlated well with the measured 5P12-RANTES concentrations in the aliquots (from ELISA). These results show that this delivery system does not interfere with the activity of the loaded 5P12-RANTES. This also supports the earlier observation that 5P12-RANTES is stable in biological environments, and may even represent further stability bestowed by being incorporated in the drug delivery device.

Conclusions

In summary, we have demonstrated GAGs can be strategically incorporated into a sustained delivery device for the long-term delivery of 5P12-RANTES. We have demonstrated the strength of affinity interaction can be used to modify long-term release profiles from these systems. Finally, we have shown our delivery system does not negatively affect the CCR5-binding activity of 5P12-RANTES. Moving forward, the method of gel formulation allows the delivery device to be made into any desirable shape (such as rings) by crosslinking the gel in a desired shaped mold. Thus, these disks, as currently constituted are ready to be used to study the feasibility of 5P12-RANTES as a long-term HIV preventative in an animal model. Beyond this immediate application, the use of BSA as a base inert material may raise some concerns. Human serum albumin has however been used in many biomedical applications (e.g., coating of medical devices). Our observation that the type and amount of GAG can be used to modify long-term release profiles, and prove useful in other settings. For the delivery of RANTES analogs, our group has been exploring conjugating various GAGs to other polymers (e.g., polyethylene glycol (PEG) or polyethylene–co-vinyl acetate (PEVA)). PEVA is especially interesting because it is the material currently used in some contraceptive vaginal rings. Rings of a similar nature are also under investigation for the
delivery of small molecule HIV microbicides. The systems and principles used in this study also can be applied for the delivery of other proteins or therapeutics.

While 5P12-RANTES has previously been shown to be stable at vaginal pH\textsuperscript{9}, open questions and subjects which remain to be studied in future work are how changes in vaginal pH, common over the course of the menstrual cycle and during sexual intercourse, could affect the stability and release rate of these materials, and how other compounds present in the vaginal lumen (e.g. chemokines and other cytokines) could affect the dissociation kinetics of the 5P12-RANTES from the polymer.

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Figure 1.
SPR analysis of GAGs (heparin, CSA and CSB) interactions with immobilized 5P12-RANTES. Overlayed sensorgrams show initial background wash (1 min), followed by injection of GAG solution and association of GAGs with the immobilized 5P12-RANTES on the chip surface over the following 1.5 min, and then subsequent washing (dissociation) of GAGs from the surface for the remaining 4.5 min.
Figure 2.
SPR concentration analysis of heparin/5P12-RANTES interactions. Overlayed sensorgrams showing initial background wash (1 min), followed by injection of heparin at 0.25, 0.5, 1.0, 2.5, 5.0, 10 and 20 µM for the next 1.5 min, and then subsequent washing (dissociation) of heparin from the surface for the remaining 4.5 min.
Figure 3.
SPR concentration analysis of CSB/5P12-RANTES interactions. Overlayed sensorgrams showing initial background wash (1 min), followed by injection of heparin at 0.25, 0.5, 1.0, 2.5, 5.0, 10 and 20 µM for the next 1.5 mins, and then subsequent washing (dissociation) of CSB from the surface for the remaining 4.5 mins.
Figure 4.
FTIR of BSA, heparin, heparin mixed with BSA (no conjugation) and crosslinked heparin/BSA. The spectra of crosslinked BSA and heparin occurred after multiple, extensive washings. The presence of both BSA and heparin confirm that crosslinking occurred. Small changes in OH groups indicate that conjugation could be through coupling to the GAG carboxylate.
Figure 5.
5P12-RANTES released at each time point from polymers containing different GAGs. Heparin/BSA (○) disks showed the highest level of sustained release. CSB/BSA (□) disks also showed substantial and sustained levels of release. CSA/BSA (♦) disks resulted in release profiles with the lowest sustained release, similar to the release in BSA-only control disks (not shown). Error bars represent standard deviation of means.
Figure 6.
Normalized cumulative release profile from GAG/BSA polymers. The Heparin/BSA (○) disks resulted in the most sustained release, followed by CSB/BSA (■) disks. The CSA/BSA (♦) disks resulted in the least sustained release. Error bars represent standard deviation of means. The main graph and the insert presented in the figure represent the same data. The main graph is zoomed in to highlight the sustained release.
Figure 7.
5P12-RANTES release from polymers containing incremental heparin fractions - BSA only (no heparin) (♦), 2.5% heparin (■), 5% heparin (▲), 15% heparin (×) and 25% heparin (○). All the release curves are characterized by an initial burst phase, and then followed by a sustained release. In the burst phase, an increase in heparin content appears to decrease the burst effect, whereas in the sustained release, increases in heparin content corresponded with increases in release at each time point. Error bars represent standard deviation of means.
Figure 8.
Normalized cumulative release profiles from heparin/BSA polymers. Gels tested include: BSA only (no heparin) (♦), 2.5% heparin (■), 5% heparin (▲), 15% heparin (×) and 25% heparin (○). The main graph and the insert presented in the figure represent the same data. The main graph is zoomed in to highlight the sustained release. Sustained release from the heparin/BSA disks corresponded with heparin content, more sustained release profiles were observed for polymers with higher heparin content. Error bars represent standard deviation of means.
Figure 9.
CCR5 blocking capacity of stock 5P12-RANTES as determined by monoclonal antibody (clone 2D7) binding. In samples where 5P12-RANTES concentrations were greater than 100ng/ml, 2D7% presentation was less than 1%, indicating good CCR5 blocking. In samples where 5P12-RANTES concentrations were below 0.8ng/ml, 2D7% presentation was at or greater than 20%, suggesting little to no CCR5 blocking (this level is similar to that of no 5P12-RANTES, the negative control). Insets show FACS histograms of 2D7 presentation on the studied PBMCs at low and high 5P12-RANTES concentrations.
Figure 10.
CCR5 blocking activity of the released aliquots from GAG/BSA polymers. Release aliquots from BSA and 25% heparin disks at 3 time points (6h, 265h and 650h) were evaluated. Protein concentration were previously determined using ELISA, and samples were diluted to concentrations both one order of magnitude above and below the CCR5 blocking threshold. Each symbol represents that release sample at all of its tested dilution concentrations. Release samples at or above the blocking threshold concentration showed good blocking, while samples diluted to below the blocking threshold concentration showed poor blocking, comparable to that of stock 5P12-RANTES.
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