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Nanococcons for Anti-HIV

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ABSTRACT-

In this overview, the most significant and recent development in bioengineering is using insect as a model for studying essential mammalian processes such as immune responses to pathogen. Silk from coccons is a promising biomaterial for controlled delivery system of therapeutic molecules. Silk has a unique protein chemistry and also structure that can be tuned to form different carrier formats. The protein has been studied for injectable or implantable sustained release depot systems for the targeted or localized delivery of drugs for treatment of diseases.

Several protein-based HIV inhibitors show great promise as microbicides as it is highly specific and not expected to lead to resistance that would affect the efficacy of current antiretroviral treatments. There are four potent protein HIV inhibitors - 5P12-RANTES, 5P12- RANTES-L-C37, Grft, and Grft-L-C37 can be formulated into silk fibroin (SF) disks and remain functional for 14 months at 25, 37, and 50 °C.

KEYWORDS- Insect natural product, AntiHIV, Silk fibroins, Coccons, Nanoparticles.

INTRODUCTION-

Silk fibroin has been widely employed for biomedical applications such as tissue regeneration, drug delivery, and bioactive coating due to favorable biocompatibility, controlled biodegradability, low in vivo immune response, high drug loading capability and versatile formulations into hydrogels, sponges, fibres, films and tubes. Bacterial and viral infections present a high risk for people's healthcare. Although many antibiotics or other antimicrobial agents are widely used in clinical or nonclinical healthcare, antibiotic or drug resistance has gradually become one of the most significant threats. Among severe infection diseases, HIV-1 infection has become one of the greatest threats to human health. Since the

advent of the AIDS pandemic, efforts have been directed to the search of viral inhibitory agents. Unprotected heterosexual intercourse is the most common mode of HIV-1 transmission worldwide. Spider silks have received considerable attention not only 21 because of their exceptional toughness, which is superior to any 22 other synthetic high-performance fibers, but also due to their 23 biocompatibility and tunable biodegradability, wherefore the 24 spider silks are proposed to be the ideal substances for 25 biomedical applications.

MATERIALS AND METHODS

1.Silk Fibroin Isolation-

Silk fibroin was isolated from Bombyx mori cocoons. Cocoons were cut into pieces and boiled for 30 minutes in 0.02 M Na2CO3 for degumming and then rinsed with distilled water to remove sericin proteins. Then kept of an overnight for air-drying. 1 g of the silk fibroin was dissolved in 4 mL of 9.3 M lithium bromide for 4 hours at 60ºC. This solution was dialyzed (Pierce 3.4 kDa MWCO dialysis cassette; Fisher Scientific, Pittsburg, PA) against distilled water for 2 days to remove the salt. The final concentration of the silk fibroin solution (silk) was calculated by weighing before and after drying and 5 diluted to 3% and 6% with ultra purified water to prepare formulations. Silk solutions were stored at 4°C until use.

2.Etoposide – A Silk Wafer Preparation-

The 3% and 6% silk solutions were prepared by diluting the 30 minutes extracted silk stock. A 100 mg/mL etoposide stock solution was prepared in DMSO and diluted with the 3% and 6% sterile filtered silk solution to obtain 1 mg/mL etoposide concentration with 1% DMSO. Then 100 µL of the prepared solutions were cast into each well of 96 well plates and frozen at -80 °C before lyophilization overnight. The silk foams that were obtained were compressed using a bench-top press to form wafers (3mm diameter & 1mm thickness) and the wafers were then water vapor annealed to increase crystalline structure to obtain water insoluble implants.

One group of the 6% silk wafers was coated with 20 layers of glycerin:6% silk solution (glycerin:silk ratio was 3:10 wt/wt) to evaluate the effects of the coating on etoposide release. Wafers were dipped into the coating solution individually and then left to air-dry to form each layer of coating. The process was repeated 20 times to obtain 20 layers.

3.Production of Inhibitor Infused SF Disks-

Aliquots were taken from the sterilized SF solution and their dry weights were determined to calculate the w/v percentage of the SF stock. The four protein inhibitors were made into solution with 20 mM HEPES pH 8 buffer and sterile filtered, and their concentrations were determined by absorbance at 280 nm. For the temperature stability study, protein inhibitors were mixed with the SF to make the final solutions that contained 3 µM of an inhibitor with 7% (w/v) of SF.

As a control a PBS solution of each protein inhibitor was prepared. Except for the PBS solution control set, the inhibitor-SF solutions were aliquoted 200 µL/well into sterile 96-well plates, frozen and lyophilized. All samples were then incubated in forced air incubators at either 25, 37, or 50 °C. For the sustained release study using Grft, the final solutions were prepared to contain 10 µM Grft, and 1–5% SF, then aliquoted 1 mL/well into sterile 24-well plates, frozen and lyophilized.

4. Scanning Electron Microscopy-

SEM was used to evaluate the morphology of the SF disks using a Zeiss EVO MA10 electron microscope. The SF disks were cut to exposure the cross sections, mounted onto SEM stubs and sputter coated with gold.

5. Fourier Transform Infrared Spectroscopy Analysis-

FTIR was performed with a Jasco FT/IR6200 spectrometer (JASCO, Tokyo, Japan) equipped with a MIRacle attenuated total reflection (ATR) Ge crystal cell in reflection mode. For each sample, 32 scans of 4 cm−1 resolutions were coadded and Fourier transformed using a Blackman- Harris apodization function. The amide I region (1585 to 1720 cm^{-1}) was deconvoluted and peak fitted using Opus 5.0 software (Bruker, Billerica, MA) to characterize the secondary structure content (side chains, β-sheet, random coil, a helix and β-turns) as previously described.

6. Viral Plasmids-

All viral and pseudoviral DNA were obtained from the NIH AIDS Research & Reference Reagent Program (http://www.aidsreagent.org/). These include full-length, replication and infection-competent proviral HIV-1 clone, pYU.2[8,9]

7. Cell and Virus Culture Conditions-

All cell cultures were maintained at 37 °C in an atmosphere containing 5% CO2. HEK-293T cells and TZM-bl cells were grown in Dulbecco's Minimal Essential Medium (DMEM; Sigma-Aldrich, Inc., St. Louis, MO) containing 10% fetal calf serum (FCS), 10 mM HEPES, and antibiotics (100 U of penicillin/mL, 100 µg of streptomycin/mL). PBMC were isolated from multidonor buffy coats from healthy HIV-seronegative donors, by centrifugation onto Ficoll-Hypaque, mitogen stimulated as previously described and maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, antibiotics (100 U of penicillin/mL, 100 µg of streptomycin/ml), and 100 U of interleukin-2/mL.

The laboratory-adapted isolate HIV-1 was passage through activated PBMCs for 11 days. Pseudovirus stocks of PVO4 and CAP210 were obtained by cotransfection of HEK-293FT cells with PVO4 or CAP210 plasmid, and subsequently the culture media supernatants containing the viral particles were harvested 48 h post-transfection. The viral solutions were sterile filtered and stored in −80 °C until use.

8.Preparation of AgNPs-

Sericin Silk sericin protein, silver nitrate, and sodium borohydride were purchased from Sigma-Aldrich (USA). Briefly, a series of silver nitrate (0.8 mM) solution with different concentrations of sericin protein were prepared. The solutions were then gently stirred and titrated with 0.8 mM sodium borohydride solution at room temperature. The molar ratio of Ag+:BH4- was maintained at 1:1. The mixture was stirred constantly overnight, and the final AgNP solution was transparent and yellow to brown. AgNPs were isolated using Millipore ultrafiltration tubes (15 ml, 30 kD). The precipitates were harvested and washed with deionized water twice. The samples were stored at 40^0 C for further research.

9.Patients and Tissue Explants -

Surgically resected specimens of cervical and colorectal tissues were collected at St Mary's Hospital, Imperial College, London, UK. All tissues were collected after receiving signed informed consent from all patients and under protocols approved by the Local Research Ethics Committee. All patients were HIV negative. On arrival in the laboratory, resected tissue was cut into 2–3 mm3 explants comprising both epithelial and muscularis mucosae as described previously. Tissue explants were maintained with DMEM containing 10% fetal calf serum, 2 mM L- glutamine and antibiotics (100 U of penicillin/mL, 100 µg of streptomycin/mL, 80 µg of gentamicin/mL) at 37 °C in an atmosphere containing 5% CO2.

RESULTS AND DISCUSSION-

1. Encapsulation and Characterization of HIV-1 Entry Inhibitors in SF Disks-

The four proteins, 5P12- RANTES, 5P12-RANTES-L-C37, Grft, and Grft-L-C37, were produced recombinantly from E. coli. Structural integrity was monitored by nuclear magnetic resonance (NMR). A cyclization step was performed in the preparation of the RANTES derivatives, because 5P12-RANTES contains an N-terminal Glutamine residue that is expected to spontaneously cyclize in solution and convert to a pyroglutamate moiety. [13] To ensure homogeneity, we dissolved purified recombinant 5P12-RANTES and 5P12- RANTES-L-C37 in acidic solution and incubated at elevated temperature of 50 °C to promote cyclization. The cyclization of these proteins was monitored by NMR, with the cyclized version being considered as the mature form. 5P12- RANTES is virtually fully cyclized after incubation at pH 2.5 for 22 h, and Figure S1C, D similarly shows 5P12-RANTES-L-C37 in its uncyclized and cyclized forms. Each spectrum shows a homogeneous, pure, folded protein. SF was prepared as previously described, resulting in a concentrated solution of fibroin that was then combined with each HIV inhibitor. For temperature stability studies, each protein was dissolved and then thoroughly mixed with a solution of SF stock.

The final solutions were cast into round, disc-shaped materials, frozen and lyophilized. The resulting SF disks were cut to expose the internal structure and visualized via scanning electron microscopy. No obvious visual differences were observed among inhibitor-loaded SF disks. Additionally, FTIR was performed to characterize the protein secondary structure of the inhibitor-loaded SF disks.

Increases in β-sheet content have been previously associated with loss of material solubility. The resulting secondary structure content of all the five SF disks (control and the four inhibitor-loaded groups) was not statistically different. All materials had statistically higher random coil content compared to β-sheet and were found to completely dissolve over the experimental time course.

2.Stability of HIV Inhibitors in SF Disks-

Stabilization of the four HIV inhibitors by SF was tested at various temperatures. First, each inhibitor was encapsulated in SF disks, such that upon dissolution and complete release the concentration of inhibitor would be 3 μM, which in turn would be diluted by 10-fold, and further diluted by 5 fold as part of the assay protocol, corresponding to a final concentration of 60 nM in the pseudovirus assay. A solution of each inhibitor at 3 μM was made using PBS. Each group was incubated at three different temperatures: 25, 37, and 50 °C. At various time points, each of the four HIV inhibitors along with an SF disk control was tested for its ability to inhibit HIV single-round pseudoviruses from two different R5-tropic HIV isolates, namely clade B PVO4 and clade C CAP210. 5P12-RANTES encapsulated in an SF disk provided full protection against both HIV pseudoviruses in vitro, even after 14 months of storage at 50 °C.

This is in contrast to the protein incubated in PBS, which gradually lost its inhibitory potency over time. This protein shows no loss of inhibition after 14 months at 25, 37, or 50 °C in the SF-encapsulated format. In contrast, the inhibitor solution in PBS gradually lost activity when incubated at elevated temperatures. The loss in inhibitory potency is unlikely due to passive adsorption of the proteins to the vials during the incubation time, as passive adsorption is a fast process that tends to occur at low concentrations of protein; also, the loss of activity did not occur for Grft-based inhibitors. Grft and Grft-L-C37 encapsulated in SF disks are fully protective against both pseudoviruses tested upon incubation at all temperatures for 14 months.

These proteins show no loss of activity in any format tested. The stability of Grft has been reported previously for incubations up to three months at room temperature and the current work demonstrates that much longer term incubations at high temperatures do not affect the activity of these proteins. This confirms that Grft and its variants could be suitable as microbicides in a variety of formulations.

3. Inhibitory Activity of Nonformulated and SF-Encapsulated HIV Inhibitors in PBMCs-

The potency of the four inhibitors, 5P12-RANTES, 5P12-RANTES-L-C37, Grft and Grft-LC37, was tested against an R5-tropic isolate, HIV-1, in activated PBMCs. A dose response curve was obtained for all four proteins within the range of concentrations tested. All four inhibitors exhibited subnanomolar activity. The antiviral activity of nonformulated 5P12-RANTES and Grft increased with conjugation to the C37 peptide with a reduction in the IC50 value. Formulation with SF generally reduced the IC50 values compared to the unformulated inhibitors, likely due to the solubilized SF being viscous and retaining the inhibitors, potentially resulting in higher local concentrations. The SF control had no inhibitory activity and importantly, the SF-formulated proteins showed no cytotoxic effect by a 3-(4,5-

dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay at the concentrations tested.

4. SF-Encapsulated HIV Inhibitors Are Functional in Human Tissue Explant Assays-

The inhibitory activity of the four inhibitors was next assessed in mucosal tissue explants. SF-encapsulated proteins and the same proteins unformulated (as lyophilized powder) were tested in nonpolarized colorectal and ecto-cervical tissue explants against HIV-1, and showed inhibition in the nanomolar range. In both models, the SF formulated inhibitors were more potent than the corresponding base compounds in colorectal explants. Similarly, to the results obtained in PBMCs, conjugation of GRFT or 5P12-RANTES to C37 resulted in an increase in potency. SF-encapsulated 5P12-RANTES-L-C37 was the most potent inhibitor in both mucosal models. No inhibition was observed with the SF control. The safety profile of the SF-encapsulated inhibitors was preclinically evaluated in mucosal tissue explant models. Patterns of cytokine release were measured following exposure of tissue explants to SF control or to SF-formulated inhibitors for 3 h (mimicking a pulse exposure) or 24 h (mimicking a sustained exposure to the drug) (Figure 4). Pulse exposure of ecto-cervical and colorectal explants to SF or SF-formulated compounds did not induce a significant change in the levels of cytokines measured in culture supernatants compared to baseline levels of nontreated explants. With sustained exposure, no up-regulation of pro inflammatory markers was observed in either explant model. In colorectal explants, the levels of adaptive cytokine IL-2 were up-regulated by 5P12-RANTES and 5P12-RANTES L-C37 after sustained exposure.

In ecto-cervical explants, sustained treatment with 5P12- RANTES resulted in a statistically significant increase secretion of adaptive cytokine IL-4 and antimicrobial protein P-selectin. Regarding Grft and Grft-L-C37, in colorectal explants, sustained exposure of Grft induced a statistically significant increase of antimicrobial proteins SLP-1, IL-2, and Human β- defensin 3. Meanwhile, sustained exposure of Grft-LC37 induced a statistically significant decrease in the levels of inflammatory cytokine IL-6, chemokines (MCP-1, MCP-2, MIP-1β, SDF-1β and IP-10), growth factor GM-CSF, and significant increase of antimicrobial protein SLP-1 in culture supernatants. In ecto-cervical explant cultures, exposure to Grft- L-C37 for 24 h induced some down-regulation of the chemokine IL-8 and up-regulation of antimicrobial protein L-selectin. The modulation of certain cytokines in this ex vivo model should be interpreted with caution and will be analyzed in planned in vivo nonhuman primate studies to further assess the safety profile of these formulations. No pro-inflammatory effects such as those described by others for Nonoxynol-9 were observed. Hence, our results indicate preliminary suitability of SF materials in this application

5. Sustained Release of SF-Encapsulated HIV Inhibitors-

Modifications during the SF formulation process were tested to explore the possibility of sustained release of relevant amounts of inhibitor over time. As opposed to the SF disks in the stability studies that were designed to quickly dissolve and fully release all the encapsulated inhibitors, an SF disk for sustained release should stay largely insoluble. In a scenario of sustained inhibitor release, it is envisioned that the user would insert a SF disk and the body's moisture would gradually mediate the release of the inhibitor over the course of days or weeks. In this case, SF disks should act as a scaffold/matrix, while allowing for slow release of the inhibitor in an aqueous/mucosal environment. A process termed "water vapor annealing" (WVA) has been reported to promote β-sheet formation in SF materials, reducing their water solubility. Extensive WVA processing results in a fully insoluble SF scaffold, hindering or even prohibiting drug release. On the other hand, insufficient annealing leads to lack of sustained release capability due to dissolution of the SF.

For time release of macromolecules such as HIV inhibitory proteins, it is important to tailor the formulation parameters for a specific molecule to achieve the desired release profile. To demonstrate the feasibility of HIV protein inhibitor time release, Grft was selected as the inhibitor for testing. Various parameters of the SF disk were tested, including the SF percentage, the size of the disk, as well as the temperature, relative humidity, and annealing duration used in the WVA process. It was experimentally determined that satisfactory Grft release profiles can be achieved by encapsulating 147 µg Grft (10 µM final in-SF. Author Manuscript Author Manuscript Author Manuscript Author Manuscript concentration) into a round, disc-shaped material that is 1 mL in volume (2 cm^2 of bottom surface area, 5 mm in thickness), comprising 1–2% SF, that has been annealed for 3–4 h at 37 °C with ≥75% relative humidity. The annealed SF disks were able to maintain their general structure in the presence of buffer solution, and gradually release Grft over time. To determine the suitability of the time-release SF disks for potential physiological use, we incubated WVA-processed SF disks containing Grft in either PBS or simulated vaginal fluid (SVF,pH 4.2), representing colorectal and vaginal conditions, respectively. At each time point, the incubation solution was removed and replaced with fresh buffer solution and tested for the presence of Grft.

Representing colorectal and vaginal conditions, respectively. At each time point, the incubation solution was removed and replaced with fresh buffer solution and tested for the presence of Grft. Grft release was detected in both buffers throughout the experimental duration (Figure 5). During the first 3 weeks, the amount of released Grft ranged from 550 to 1300 ng in approximately 1 mL fluid (corresponding to 41.3–99.4 nM) in PBS, and from 570 to 1000 ng (corresponding to 43.1– 75.5 nM) in SVF (Figure 5A, C). After 3 weeks, the amount of release decreased, but was still sustained at levels of around 300–400 ng per mL (20 nM for PBS release, and about 30 nM for SVF release; Figure 5B, D). The reported IC50 values for Grft inhibition toward a variety of HIV strains are typically in the subnanomolar range. As such, the amount of Grft released is expected to effectively inhibit HIV infection. No significant difference was observed between the release behavior in PBS and SVF. Cumulatively, a

total of 14.8 ± 1.6 μg of Grft was released in PBS, and 13.3 ± 0.8 μg of Grft was released in SVF, representing ~10% and ~9% of loaded Grft, respectively. These amounts are satisfactory as initial proof of concept from a pharmacological perspective. Recent reports describe intravaginal rings that are manufactured with much higher quantities of small molecule and antibody inhibitors, showing release in the mg range. We are also pursuing larger amounts of protein in the context of larger disks, films, and inserts. Further material development is expected to provide various release kinetics, if desired.

6. Sustained Release Grft Inhibits HIV Infectivity in Vitro-

To determine whether the Grft from sustained-release SF disks is capable of inhibitory function against HIV, the activity of SF disk-released Grft over the course of a month (obtained as described in Methods) was tested against HIV-1 pseudo viruses CAP 210 (Figure 6A) and PVO4 (Figure 6B) in TZM-bl cells. Grft released into PBS or SVF at various time points effectively inhibited both viruses, with all the time point samples from the first 3 weeks showing full inhibition, and the day 31 samples showing over 90% inhibition. This long-term inhibition property is particularly desirable in situations where the user prefers, and hence would be more adherent to, a longer-acting inhibitor that does not require daily dosing. Given this and findings from others, SF-mediated sustained release systems could be applicable for a broad range of antiviral molecules.

CONCLUSION-

In this study, we present a silk fibroin-based inhibitor delivery system that not only shows great capability in stabilizing protein-based HIV inhibitors but also shows the feasibility of being developed for sustained release of these macromolecules. The stability of SF encapsulated inhibitors was illustrated with four protein inhibitors, which vary in molecular weight, tertiary structure and charge distribution. Each of these retained potent functionality in HIV pseudovirus assays, even after incubation at 50 °C for over 14 months. In comparison, when stored in solution, some protein inhibitors showed decreased activity. Furthermore, formulated inhibitors were shown to be effective against HIV in both colorectal and cervicovaginal tissues, and in PBMC. This demonstrates the potential utility of SF formulations without refrigeration in areas with extreme temperature conditions such as sub-Saharan Africa. Therefore, this preclinical study describes the feasibility of a SF disk approach as part of an HIV prevention strategy.

ABBREVIATIONS

HIV- human immunodeficiency virus

AIDS- acquired immune deficiency syndrome

PrEP -pre-exposure prophylaxis

SF - silk fibroin

Grft- griffithsin

RANTES- regulated on activation, normal T cell expressed and secreted; RANTES is also known as CCL5
Gln/Q glutamine

DMEM- Dulbecco's modified Eagle's medium

NMR - nuclear magnetic resonance

DSS - 4,4-dimethyl-4-silapentane-1-sulfonate

IPTG - isopropyl β-D-1-thiogalactopyranoside

HSQC - heteronuclear single quantum coherence

TFA - trifluoroacetic acid

PBMC - peripheral blood mononuclear cells

PBS - phosphate-buffered saline

SVF - simulated vaginal fluid

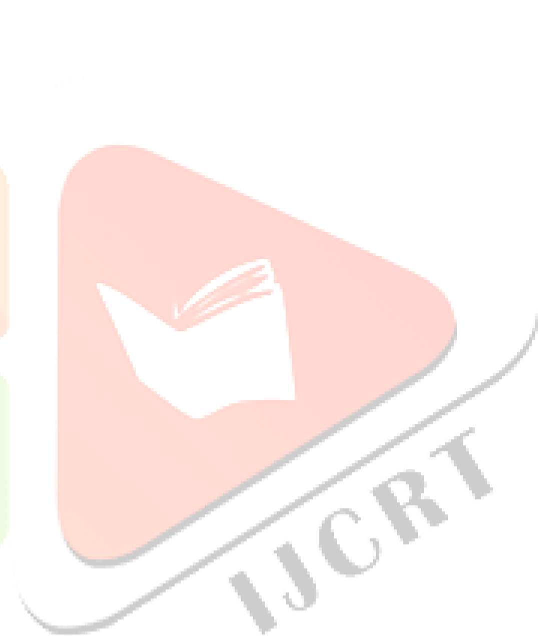
ELISA -enzyme-linked immunosorbent assay

SEM - scanning electron microscopy

FTIR - Fourier transform infrared spectroscopy

SD - standard deviation

WVA- water vapor annealing



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