

POLYVALENT VACCINES AND THERAPEUTICS AGAINST VIRAL PATHOGENS

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POLYVALENT VACCINES AND THERAPEUTICS AGAINST VIRAL PATHOGENS

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Dedicated to:

My inspiration & strength who taught me to always look for the silver lining:

My parents, Rosen John & Elizabeth Rosen

My little brother, I'm always proud of: Reuben Rosen

My eternally supportive fiancé, my happiness: Tharun Cheriyan

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LIST OF ABBREVIATIONS AND SYMBOLS

NT	Nanotubes
IC ₅₀	Half maximal inhibitory concentration
HA	Hemagglutinin
SWNT	Single walled carbon nanotubes
NA	Neuraminidase
HT	Head-tagged hemagglutinin (with AviTag for biotinylation)
TT	Tail-tagged hemagglutinin (with AviTag for biotinylation)
BB	Biotin-blocked
HT-NT	Head-tagged hemagglutinin – Nanotube conjugates
TT-NT	Tail-tagged hemagglutinin – Nanotube conjugates
BB-NT	Biotin blocked - Nanotubes
PEG	Polyethylene Glycol
PEG-NT	Polyethylene Glycol coated nanotubes
STREP-NT	Streptavidin coated nanotubes
NT-COOH	Carboxyl group functionalized nanotubes
PGA	Poly Glutamic Acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS	N-Hydroxysuccinimide
S-NHS	Sulfo- N-Hydroxysuccinimide
DBCO	Dibenzocyclooctyne group
RNA	Ribonucleic Acid
DNA	Deoxy Ribonucleic Acid
PCR	Polymerase Chain Reaction
LB	Luria Bertani Broth
R13	RoAb13 antibody
R14	RoAb14 antibody

ELISA	Enzyme linked immunosorbent assay
TMB	3,3',5,5'-Tetramethylbenzidine
HRP	Horseradish Peroxidase
LukE	Leukotoxin E
LukD	Leukotoxin D
HB	Hemagglutinin-binding
HIV	Human Immunodeficiency Virus
COOH	Carboxyl functional group
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
CH5/1	Chimeric hemagglutinin: H5 – head region and H1 – stalk region
CD4	Cluster of Differentiation 4
CCR5	C-C Chemokine receptor
FACS	Fluorescence Activated Cell Sorting
FLSC	Full Length Single Chain
GPCR	G- protein coupled receptor
IMAC	Immobilized Metal Affinity Chromatography
SEC	Size Exclusion Chromatography
Ni-NTA	Nickel- Nitrilotriacetic acid (NTA)
CD	Circular Dichroism
AMB	Amber codon
LAV	Live attenuated virus
TIV	trivalent influenza virus (inactivated)
Ab	antibody
mAb	monoclonal antibody
bnAbs	broadly neutralizing antibody
PR8 H1 HA	Hemagglutinin subtype 1, strain: Puerto Rico, number 8
HS	Hot spot

gp41 or gp120	glycoprotein 41 or glycoprotein 120
ECL	Extra cellular loop
BPA	Biotin-PEG2000-amine
MES	2-(N-morpholino)ethanesulfonic acid
PBS	Phosphate Buffer Saline
BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
XG	Xtremegene reagent
PEN-STREP	Penicillin-streptomycin
SF9	Spodoptera frugiperda 9
PMSF	phenylmethylsulfonyl fluoride
IgG	Immunoglobulin G
Poly I:C	Polyinosinic:polycytidylic acid
IACUC	Institutional Animal Care and Use Committee
RBC	Red blood corpuscle
PDB	Protein Data Bank
SOC	Super Optimal Broth
IPTG	Isopropyl β -D-1-thiogalactopyranoside (IPTG)
C _{eff}	Effective Concentration
RMS	Root Mean Square
aaRS	Aminoacyl tRNA synthetase
mRNA	Messenger RNA
BSL	Biosafety Level
HAART	Highly Active Anti-Retroviral Therapy
OD	Optical Density
SUMO	Small Ubiquitin-like Modifier

Summary

Viral infections caused by HIV or influenza viruses have killed millions of people worldwide. The capability of these viruses to easily mutate their envelope glycoproteins is what makes treating both infections so hard. Currently available drugs against such viruses do combat the infections to some extent but are not the best long-term solutions; drug toxicity, drug resistance and viral persistence are just some of the issues that are yet to be overcome. In this work, the concept of polyvalency was used to design viral inhibitors and vaccines.

Hemagglutinin is an envelope glycoprotein in the influenza virus, whose head region is highly prone to mutation but has certain conserved epitopes in the stalk region. These conserved epitopes in the stalk region are usually not easily accessible to the immune system, in a native influenza virus. For the first part of my thesis, a universal vaccine has been developed by controlling the antigen (hemagglutinin) presentation to the immune system to enhance the accessibility of the conserved epitopes in the stalk. Hemagglutinin-biotin was conjugated onto the surface of streptavidin coated nanotubes to yield conjugates with two different orientations of hemagglutinin; one with the stalk region facing outwards (proposed vaccine design, called head tagged-nanotube conjugates) and the other as the control with the head region facing outwards (tail tagged-nanotube conjugates). In preliminary *in-vivo* challenge experiments with a chimeric H5/1N1 virus, the head tagged-nanotube conjugates (proposed vaccine) showed better protection than controls. We have subsequently expressed biotinylated hemagglutinin using baculoviral infection of insect cells and have optimized the purification scheme by adding a size exclusion chromatography step after the affinity chromatography step. The resulting conjugates will be tested *in-vivo* against a homosubtypic (e.g., cH5/1N1) and heterosubtypic (H5 or H6 viruses) challenge in the future.

For the second part of my thesis, polyvalent influenza therapeutics have been designed based on ‘hemagglutinin binding’ proteins that bind to conserved epitopes. Our goal was to characterize the efficacy of polyvalent versions of these hemagglutinin binding (HB) proteins relative to the monomers. We demonstrated that the dimers of both the HB proteins studied were more effective at inhibiting the binding of stalk-binding antibodies to hemagglutinin than the corresponding HB monomers.

For the third part of my thesis, we designed polyvalent molecules that bind to multiple sites on CCR5 (C-C chemokine receptor, type 5). CCR5 plays a key role in HIV’s entry into host cells. We synthesized homodimeric, heterodimeric and multimeric versions of Leukotoxin E (LukE) (a CCR5-binding protein) and characterized their ability to inhibit the binding of a gp120-CD4 fusion protein (called FLSC) to CCR5+ 293T cells. Of these, Leukotoxin E – RoAb14 heterodimers, in which LukE and another CCR5-binding single chain variable fragment (scFv) version of the antibody, RoAb14, were separated by 75- and 100-amino acid linkers showed over 85% inhibition of FLSC binding at concentrations as low as 78nM, as compared to Leukotoxin E which showed 50% inhibition at ~ 1.3µM. Polyvalent conjugates in which multiple copies of LukE were attached to polyglutamic acid scaffolds also showed an enhancement in avidity relative to LukE monomers. Our collaborators will test the ability of these inhibitors to neutralize HIV *in-vitro*.

1. Introduction:

Polyvalency or multivalency involves the simultaneous binding of multiple copies of ligands to their corresponding receptors [1], [2]. Polyvalency exists abundantly in nature; some examples include viral attachment to host cells, antigen/antibody interactions, antigen presenting cell–T cell interactions, binding of bacterial toxins to host receptors, etc. [2]. The concept of polyvalency aids in reducing the half maximal inhibitory concentration (IC_{50}) of ligands or molecules by several orders of magnitude, by increasing the avidity of the molecule/ligand for its receptors. Half maximal inhibitory concentration (IC_{50}) is defined as the concentration of inhibitor at which the maximum response is reduced by 50%. We have extensively used the concept of polyvalency in our research in the design and synthesis of various inhibitors and vaccines [3]–[10].

The goal of this work is to use polyvalency to combat HIV and influenza viruses. Viral diseases caused by influenza or HIV have wreaked havoc in the lives of many over the last century or so. Influenza has reached pandemic proportions multiple times in the past century and has claimed over 30 million lives [11]. HIV has killed about 39 million people since the beginning of the epidemic (early 80s) by eventually destroying the body's capability to fight off infections. Currently about 35 million people are infected with HIV [12]. Many previous attempts to counter both HIV and influenza infections face steep challenges, some of which include drug toxicity (e.g. certain HIV therapeutics) or the ability of the viruses to mutate its envelope proteins, to evade detection by the hosts' immune system. Simply using an inactivated or attenuated virus is far from being the solution; by the time the immune system bolsters a response against such a threat, the virus would have already mutated its surface glycoproteins, thereby rendering the immune repertoire useless.

1.1. Influenza Virus

The envelope of the influenza virus has two key glycoproteins: hemagglutinin (HA – a mutation-prone protein that helps the virus to attach itself to host cells) and neuraminidase (NA - an enzyme that helps the budding virions to be released from the host cell). The host receptor for HA is an α -2,3 (avian) or an α -2,6 (human) sialic acid [13], [14].

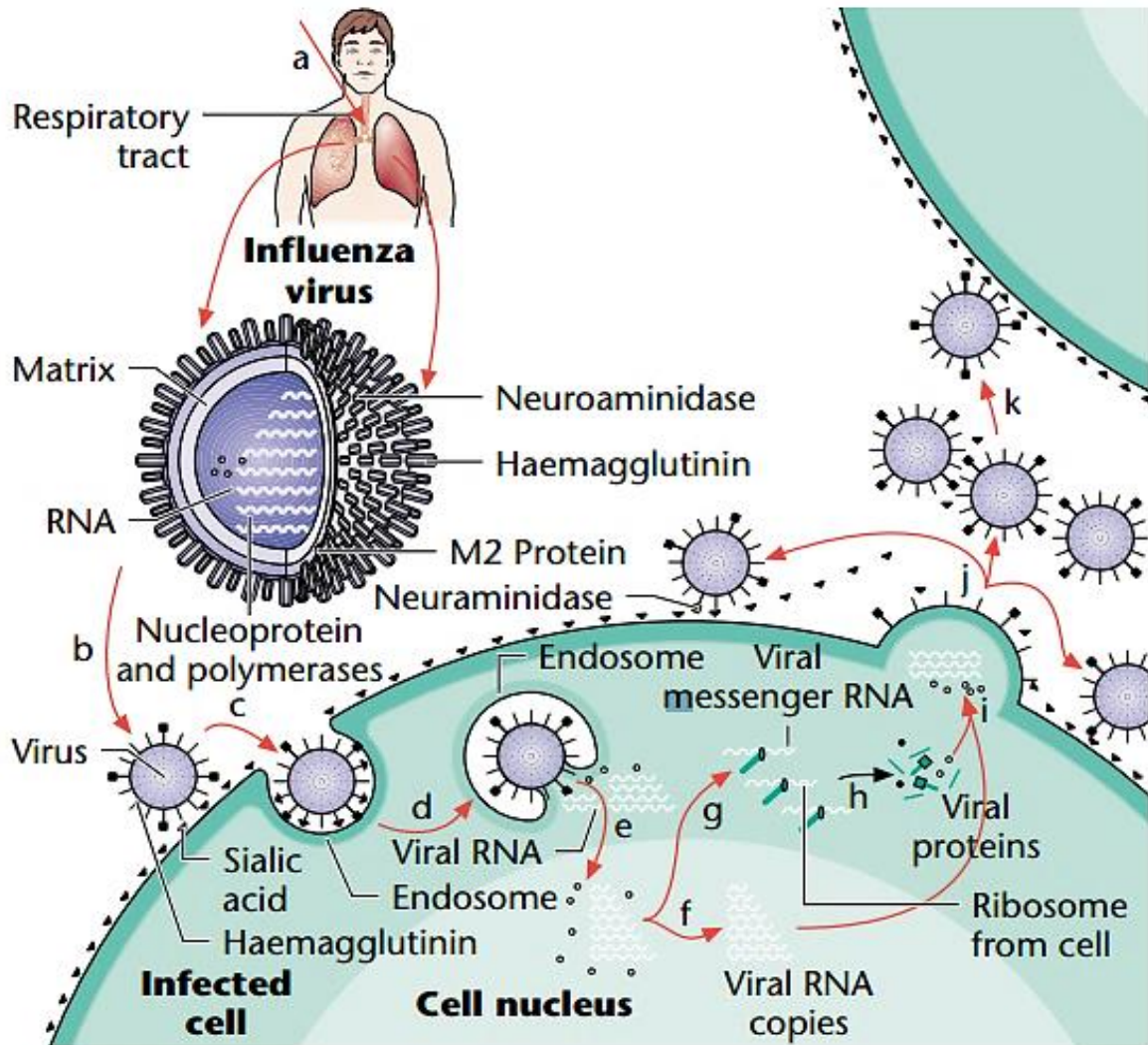


Figure 1: Mechanism of host cell entry of Influenza; figure adapted from Lucas et al. 2010 [66]

HA0 is the pre-cleaved form of HA, before cleavage by host cell proteases into HA1 (primarily contains the head region) and HA2 (contains stalk region). Once the virus has latched onto the sialic acid receptors on the host cell, it is endocytosed into the cell. As the endosomal vesicle moves towards the host nucleus, the pH within the vesicle drops. Once the pH reaches ~ 5.0, the HA undergoes a conformational rearrangement, which results in the exposure of a highly-conserved region, a hydrophobic fusion peptide at the N terminal region of HA2. This peptide inserts itself into the endosomal membrane, thereby helping release the viral RNA into the host cell's cytoplasm for multiplication [14].

Several antiviral molecules have been developed that target different stages of the viral replication cycle, some of which are FDA approved. Although these drugs have been shown to curb infection, a number of drug-resistant strains are appearing [15], [16]. Another issue is that these drugs can only be administered post-infection. Vaccines, on the other hand, offer an advantage: they help elicit antibodies against the virus even before the onset of infection. Currently available influenza vaccines primarily include live, attenuated (LAV) or (TIV - trivalent influenza viruses) inactivated viruses, whose composition is based on those strains that are currently circulating both nationally and internationally, past trends, and how well the current strains work against newly identified viruses. The data on virus strains that is collected for vaccine design needs to be regularly updated so that we are prepared for the next flu season [17]. The problem with the current approach is that there is no guarantee that the vaccine will boost an immune response against the seasonal influenza viruses that end up being prevalent the next flu season. This leaves room for improvement in the design of a more “universal” vaccine [18].

Based on similarities and differences in their sequence and structure, HA is classified into different subtypes, labelled H1 through H17, and more broadly into two phylogenetically different groups:

group 1 (includes HA subtypes 1, 2, 5, 6, 8, 9, 11, 12, 13, 16, 17) and group 2 (includes HA subtypes 3, 4, 7, 10, 14, 15) [19]. Even if the currently available vaccine successfully inhibits infection, the virus can easily mutate the immunodominant surface epitopes of HA so as to escape detection by the hosts' immune system (or undergo 'antigenic drift') [18]. The antibodies generated against the annual vaccines are primarily against the head region of HA and work by preventing the binding of the virus to the host cell and egress of the budding virion from the host cell [20]. So, once the exposed residues on the head are mutated, the anti-head antibodies generated against the previous set of epitopes are rendered ineffective.

Even though the HA head is highly prone to mutation, there are some conserved residues in the stalk region. These conserved residues consist mainly of the N terminal region of HA2 (fusion peptide) and the hydrophobic groove region that surrounds it. The virus is unable to mutate these conserved regions as they are key residues required for successful membrane fusion. Broadly neutralizing antibodies (bnAbs) have been generated against such conserved epitopes of the influenza viral proteins. They hamper viral replication by preventing successful viral-host membrane fusion [20]. BnAbs have been shown to exhibit cross-reactivity to different HA subtypes within a group [21]. Wu et al. have studied the protective nature of bnAbs being injected *in-vivo* right after the onset of infection; they have shown that the bnAbs boost passive immunity [22]. In addition to the use of recombinant broadly neutralizing antibodies for prophylaxis, the design of antigens that generate broadly neutralizing antibodies is a promising avenue for combating influenza [23]–[26].

In this work, the conserved stalk region of HA is targeted for influenza vaccine and therapeutic design. Currently, two projects are being pursued in the area of influenza vaccine/inhibitor design and production. Firstly, a universal carbon nanotube-based influenza vaccine was synthesized to

study the effect of the presentation of PR8/H1 HA displayed to the immune system. The HA was conjugated to single walled carbon nanotubes (SWNT) in two orientations: one with the HA head towards the solution (away from the nanoscale support) with the tail attached to the nanotubes (NTs) and the other, with the stalk towards the solution. The vaccine was tested *in-vivo* for broadly neutralizing activity by our collaborators at the Icahn School of Medicine at Mount Sinai which gave some very encouraging preliminary results.

Secondly, multivalent conjugates of hemagglutinin binding proteins were designed and synthesized; these will be tested as a therapeutic against influenza. Monomeric ‘hemagglutinin binding (HB) proteins’ were designed by David Baker’s group at the University of Washington. The HB proteins were designed to bind to the conserved HA stem-binding sites utilized by other bnAbs such as CR6261 and F10 [27], [28]. Once the overlapping ‘hotspot’ binding sites were mapped out, they searched the protein data bank for proteins that would have a shape complementary to the binding sites in the hydrophobic groove on HA. They modified the selected scaffold so as to incorporate the sites that would interact with the hotspot binding sites [27], [28]. We designed polyvalent copies of these HB molecules; on peptide-linker-based scaffolds of appropriate linker length, with the aim of making anti-influenza therapeutics with an activity significantly greater than that of the monomer alone.

1.2. HIV

HIV, like influenza, first binds to host cell receptors using its surface glycoproteins (gp120) before entry. HIV uses CD4 as the primary receptor and CCR5 or CXCR4 as the co-receptor for entering host cells (CD4+ T cells, macrophages and dendritic cells) [29]. Based on the type of co-receptor

used by the virus, it is called either an R5 (uses CCR5 as co-receptor) or an X4 virus (prefers CXCR4). The envelope glycoproteins consists of gp160 ‘precursor’ units which consists of gp120 (required for the initial viral-cell binding) and gp41 (contains the viral fusion peptide) [30]. The two subunits are cleaved by host proteases. First, the gp120 binds to CD4 receptor on host cells [30]–[32]. This binding triggers a conformational change in gp120 and is followed by the binding of gp120 to the co-receptors CCR5 or CXCR4. Further conformational changes in the viral envelope allows for the insertion of the fusion peptide in gp41 into the host membrane, resulting in the release of viral RNA [30]–[32].

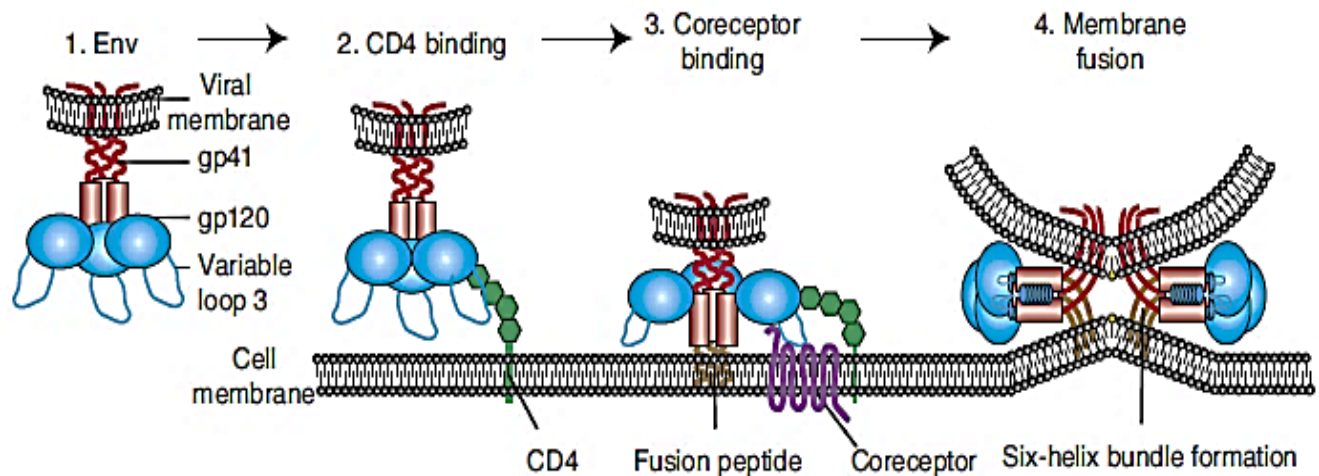


Figure 2: Mechanism of host cell entry of HIV; figure adapted from Wilen et al. 2012 [32]

HIV has one of the highest mutation rates among viruses which prevents it from being detected and easily eliminated by the immune system. Other than using antigenic drift to evade the immune system, the virus can also switch its tropism. R5 tropism is dominant in the ‘acute and asymptomatic’ initial phases of infection, but the virus has the capacity to mutate to X4 tropism as the disease progresses (called viral shift) [29]. So, if CCR5 was targeted in the initial phase of the disease, there are better chances of preventing disease progression than using CCR5 inhibitors in

the advanced stages. In the latter stages, the virus gets a selective advantage to switch to using the CXCR4 receptor [33].

Currently available treatments involve combinational therapy (called HAART or highly active retroviral therapy) which do show marked improvement in preventing disease progression [30]. It involves using a combination of agents that target different receptors or steps of infection, some of which include nucleoside reverse transcriptase inhibitors, fusion inhibitors, protease inhibitors and CCR5 inhibitors/entry inhibitors. However there are serious side effects to the available drugs such as toxic side effects, high cost, difficulties in adhering to treatment and drug resistance [30].

Targeting the CCR5 receptor for anti-HIV therapy does have its advantages. Firstly, CCR5 is amenable to drug development, as it is not prone to antigenic drift as are the HIV envelope glycoproteins [30]. Secondly, the CCR5 receptor is an 'expendable' receptor; it has been shown that individuals having a 'CCR5 delta32 mutation' or the deletion of a set of 32 base pairs in the sequence of the extracellular loop 2 in their CCR5 (which causes a misfolded CCR5 to be expressed on the cell surface) are either resistant to HIV infection or the disease progresses extremely slowly in such individuals [34][35].

Leukotoxin E (LukE) is a protein derived from staphylococcal aureus, and from the name itself, suggests that it is toxic to leukocytes. LukE by itself is harmless to cells, but when paired with Leukotoxin D (LukD), can cause cell death through pore formation [36]. LukE has recently been shown to bind to the HIV co-receptor CCR5 [37]. Our goal was to design polyvalent conjugates presenting multiple copies of LukE alone, as well as conjugates presenting both LukE and other proteins (e.g., scFvs) that bind to alternate sites on CCR5 (as shown in the schematic in figure 3), and test their activity *in-vitro* [38].

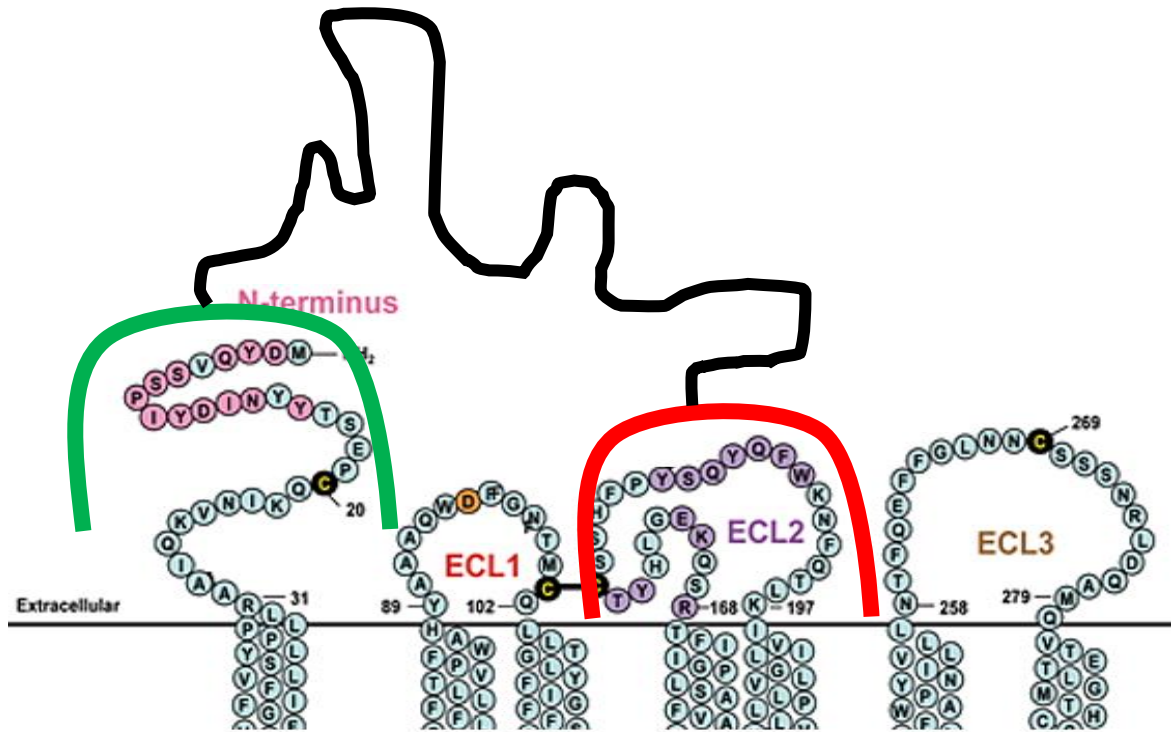


Figure 3: Schematic of a heterodimeric CCR5 binder; CCR5 schematic adapted from Lopalco et al., 2010 [67]

1.3 Research Objectives:

- I. To develop a novel universal influenza vaccine by controlling antigen presentation and to characterize its structure and activity *in-vivo*.
- II. To synthesize multivalent conjugates of hemagglutinin-binding proteins and characterize their activity *in-vitro*.
- III. To synthesize multivalent conjugates that bind to the HIV co-receptor CCR5 and to characterize their activity *in-vitro*.

2. Universal Influenza Vaccine

2.1. Introduction

The goal of this project was to develop a strategy for designing a novel influenza vaccine with orientation-controlled antigen presentation that will elicit a broadly neutralizing response *in-vivo*, and provide complete protection against other group 1 viral challenges such as viruses with HA subtypes H5 and H6. Broadly neutralizing antibodies target certain conserved epitopes on HA, which the virus requires for replication and prevents infection by inhibiting membrane fusion [20]. Here, the PR8/H1 HA (hemagglutinin subtype H1 of the Puerto Rico strain 8) was presented in two orientations; one where the head of HA was towards the solution (away from the nanoscale support) and the tail was the point of attachment to the nanotube (NT) scaffold, and the other where the tail was towards the solution. The viral envelope has a dense packing of proteins (HA and NA) which might restrict the accessibility of the conserved epitopes in the stalk region to the immune system. We hypothesized that by modifying the orientation of HA, we would enhance the accessibility and recognition of these conserved epitopes. Therefore, we expected a “broader” neutralizing response from the conjugates with the HA stalk towards the solution. The vaccine was designed as follows: Raw single walled carbon nanotubes were treated with a concentrated acid mixture for both length reduction and formation of –COOH groups (to serve as reactive handles) on the cut surfaces of the tubes (called NT-COOH). This scaffold then had biotin-PEG2000-amine (BPA) chemically attached to the –COOH groups, using EDC-NHS [1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide – Sulfo-N-Hydroxysuccinimide] chemistry. Subsequently, the PEGylated nanotubes had streptavidin linked to the free biotin on the nanotube surface, to which HA (head-tag biotin or HA (HT)) or HA (tail-tag biotin or HA (TT)) was attached to give

two different conjugates; one with the tail towards the solution (away from the nanoscale support) or HT-NT and the other with the head towards the solution or TT-NT. The “tag” here refers to the insertion of an AviTag sequence either in the head or stalk region of hemagglutinin which acts as a site for enzymatic biotinylation (during protein expression in insect cells).

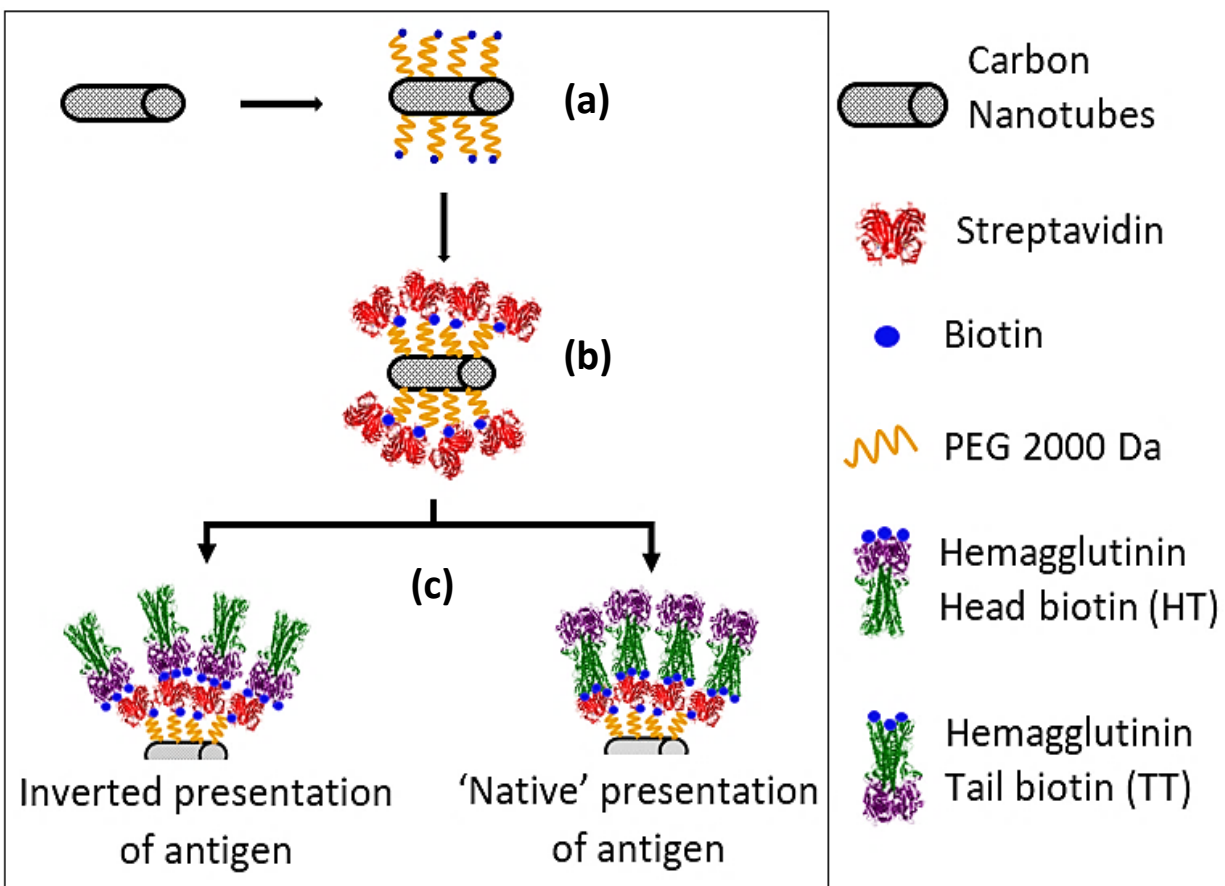


Figure 4: Schematic illustrating the preparation of nanotube-hemagglutinin conjugates: (a) PEGylation of NTs; (b) addition of streptavidin; (c) addition of HA (HT) or HA (TT)

The expression of the antigen, hemagglutinin, was carried out using insect cell culture [39]. Insect cells offers the advantage of producing correctly folded and glycosylated protein. Expression in insect cells using the baculoviral system (pFastBac dual vector) gives high yields because it driven by the strong polyhedron promoter [39].

For this, two different cell lines were used: 1. SF9 insect cells were used to both generate and scale up baculoviruses and 2. High Five insect cells were infected with baculoviruses for the expression of hemagglutinin. The hemagglutinin sequence was cloned into the pFastbac dual vector. The advantage was that this vector also allowed for the insertion of the sequence of BirA, a biotinylation enzyme. So, biotinylation would occur *in-vivo* at the AviTag sequence and HA would be secreted from the High Five cells. The hemagglutinin sequence lacked the native membrane-anchoring domain and was followed by a C terminal trimerization and a hexa-His tag to aid in purification [39]. Since hemagglutinin tends to aggregate, a second round of purification using size exclusion chromatography was found to be necessary.

2.2. Materials and Methods:

2.2.1. Cutting and COOH functionalization of single walled carbon nanotubes (SWNT):

- i) 20mg of SWNT was weighed out (single walled carbon nanotubes, Unidym) and thoroughly dispersed into a concentrated acid mixture of sulfuric acid and nitric acid in the ratio of 3:1, where the total volume of acid was 4 ml acid/mg NT (i.e. 80mL).

Note on safety measures:

- Make sure you have personal protective equipment on (lab coat, gloves, and safety glasses).
- Make sure that the weighing out of NTs is done in a fume hood.
- Before weighing, remove any static on the container the NTs are to be weighed into (using a static gun); static disperses NT particles.
- Clean up the area with a wet wipe after weighing the NTs.

- Wear a double layer of nitrile gloves while measuring out acids.
 - Try to avoid acid spills. In case of spill: (a) prevent it from entering drains (b) dilute it with water, and then neutralize with soda ash (sodium carbonate) or calcium oxide. Absorb neutralized spill with absorbent material and subsequently discard.
- ii) The nanotubes were sonicated in this acid mixture in an ice bath for a total of 21-22 hours, to reduce their length from $>1\mu\text{m}$ to 100-500nm. The ice in the sonicator bath was not allowed to completely melt and was replenished every 20-30 mins.

Note: The sonication should be done under a fume hood too, to minimize the formation and dispersion of aerosols.

- iii) After the acid-based NT cutting was complete, the reaction was quenched in 2L of chilled water. The COOH-NTs were filtered and resuspended in water. Repeated washes of NTs were performed till the filtrate was neutral in pH (~3-4 washes).
- iv) Finally, a concentrated solution of COOH-NTs was made and freeze dried to remove the water. This will help determine the NT yield.

Note: When NTs in the dried form require to be weighed, make sure it is done under the fume hood.

Note: The SWNTs are purchased 'raw', and are cut with concentrated acid mixture of sulfuric and nitric acid. Acid "Cutting" is useful not just to size down the tubes but also to ensure that there are -COOH groups that are generated over the NT surface. The -COOH group improves solubility and provides for a site for further functionalizing the NTs. 100-500nm would seem like an optimum size range for the NTs so that the antigen-coated NTs will be immunogenic enough, yet small enough (i.e. $< 500\text{nm}$) so as to not be easily engulfed and eliminated by the reticulo-endothelial system.

2.2.2. PEGylation of COOH-functionalized NTs:

- i) The NTs were resuspended in 25 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH 4.7) by making a 1 mg/mL solution and the solution was sonicated for 1-2 hrs. The cut NTs should appear more soluble than the raw NTs.
- ii) 9.1 mg of sulfo-NHS (N-hydroxysulfosuccinimide) per mg of NT were added to the well-suspended NTs and sonicated for 5 min.
- iii) This was followed by the addition of 30.5 mg of (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) EDC per mg of NT and briefly sonicated. Then, a stir bar was added to the reaction mix and the reaction was vigorously stirred for ~20 mins. This is the stage where the COOH groups are being activated for the attachment of biotin-PEG2000-amine groups.
- iv) In the meantime, a (>20mg/ml) concentrated solution of biotin-PEG2000-amine (20 mg of BPA/mg of NT) was prepared in 100 mM phosphate buffer (pH 8.0), such that the volume of the BPA solution was the same as the NT solution in MES. This is so that the final pH is ~ 7.4-7.5 which would allow the reaction to go to completion. Increasing final pH is not recommended as this will cause the activated ester group to get quickly hydrolyzed and will prevent successful conjugation of BPA. The reaction was allowed to go to completion at RT for at least 3 hrs, or overnight at 4 °C.
- v) After reaction completion, the solution was given a 3 min spin at 1000xg to remove any larger aggregates that should be discarded. The supernatant was carefully collected and washed to remove the unreacted BPA. This was done by spinning the solution at 504,000xg (70,000rpm) using the 70Ti fixed angle rotor in an ultracentrifuge for an hr. or till the PEG-NTs pellet. The supernatant will contain the excess free BPA. The

supernatant was discarded carefully without disturbing the pellet and then the pellet was resuspended in fresh buffer (de-ionised water). The washes were repeated ~ 5 times so that the tubes are sufficiently washed.

Note: Make sure that the tubes that will be put in the ultracentrifuge rotors are precisely balanced upto +/- 10mg.

- vi) The pellet was collected and resuspended in sterile water. The PEG-NTs can be stored at 4°C. To calculate the yield, the absorbance of a dilute solution was taken at 808 nm such that the absorbance scales linearly. The $A_{808\text{nm}}$ can be multiplied by the dilution factor and then divided by $0.0465\text{mg l}^{-1}\text{ cm}^{-1}$ (Extinction coefficient for SWNT) for calculating the NT concentration [40].

Note: Addition of biotin-PEG2000-amine helps in solubilizing the tubes further and the free biotin is useful for further conjugations. Besides, the toxicity of SWNTs has been shown to be dependent on their surface functionalization [40], [41]. PEG functionalization has shown to reduce the systemic toxicity of raw SWNTs [42]–[44]. The unreacted BPA must be separated from the NTs, else they will hamper with the conjugation steps ahead (i.e., the streptavidin addition stage).

2.2.3. *Conjugation of Streptavidin and HRP-biotin (test molecule):*

- i) For 1 mg of biotin-PEG-NTs, large excesses (10 mg) of streptavidin was used to saturate the surface biotin on NTs. The 1 mg of biotin-PEG-nanotubes (at 200µg/ml) was mixed in small batches to a solution of streptavidin.

Note: This mixing must not be done all at once to avoid crosslinking and aggregation/clumping of NTs. The ideal case is where the NT-PEG is well covered

- with streptavidin, such that as many streptavidin sites are kept free as possible to allow maximum loading of HRP or HA.
- ii) The excess streptavidin was removed using a sucrose gradient of 4%, 10% and 30% sucrose which was spun at 175,000xg or 32000rpm (using a swinging bucket SW32Ti rotor) in an ultracentrifuge. The free streptavidin is held within the layers of the gradient, whereas the streptavidin coated NTs (strep-NT) form a pellet. This spin was allowed to go up to ~ 18 hrs so that the nanotube yield of NT-Strep is ~ 50-60%. The pellet was resuspended in 20 mM phosphate buffer (pH 7.4). The NT concentration can be calculated by taking the absorbance at 808 nm, which should be multiplied by the dilution factor and then divided by $0.0465 \text{ mg l}^{-1} \text{ cm}^{-1}$ (Extinction coefficient for SWNT) [40].
 - iii) The strep-NT batch was split into two; to one batch (sample), biotinylated HRP (horseradish peroxidase – a test molecule here, to assess whether loading is possible) was mixed and to the other batch (control), a 2 mg/ml solution of biotin (at high excess of biotin, to block all the free sites of streptavidin) was added. The samples were kept on a rocking platform. After half an hour, biotinylated HRP was added to the second batch (control).
 - iv) The sample and control batches were put through a sucrose gradient of 4%, 10% and 30% sucrose and spun at 32000 rpm in an ultracentrifuge to remove the excess HRP-biotin that can interfere with ELISAs for HRP. The NT-HRPs (sample) or the NT-BBs (biotin-blocked controls) were collected as a pellet.
 - v) A TMB (3,3',5,5'-tetramethylbenzidine)-based ELISA was performed to assess loading of HRP onto NTs. For this ELISA, serial dilutions of both the sample and control were

loaded onto 96 well, clear flat bottom plates. Each well had no more than 100 μL of solution. Similarly, serial dilutions (of known mass/concentrations of HRP) of HRP-biotin (for the standard curve) were also loaded onto the plate. To these wells, 100 μL of TMB solution was added and the absorbance was taken in a plate reader at 652nm. If HRP is present, it would give a blue coloration with TMB. The amount of HRP in the sample and control could be estimated from the plotted standard curve.

2.2.4. *TEM imaging with gold-biotin:*

A small fraction of the strep-NT samples was mixed with a solution of excess of gold biotin for TEM imaging. The imaging was done by my colleague, Chad Varner.

2.2.5. *Insect culture – Expression of hemagglutinin (antigen) [39]:*

All mutants of hemagglutinin (HT, TT, chimeras) were expressed using the following protocol:

- i) The baculoviral transfer plasmid was transformed into DH10Bac bacteria, which were midiprepmed to yield bacmids.
- ii) SF9 cells were seeded in 6-well plates at a density of 2×10^5 cells/cm². The bacmids were transfected into SF9 cells using Cellfectin II transfection reagent. For this, 2 μg of the bacmid was diluted in serum free TNM-FH (with 1% Pen-Strep and 0.1% Pluronic F68) and mixed with 6 μL of Cellfectin II. This was mixed and allowed to incubate for 20 mins at room temperature.
- iii) The SF9 cells would have stuck to the bottom of the plate by this point. The supernatant of the seeded SF9 cells was removed and replaced with serum free TNM-FH. The

- bacmid-reagent complex was added to this mix, and the plate was gently agitated for 5 mins to facilitate mixing. The media was changed after 6 hours to full TNM-FH (10% FBS, 1% Pen-Strep and 0.1% Pluronic F68). The plate was incubated at 28°C without CO₂ for 6 days, and was occasionally checked to observe signs of infection (cells appear larger, enlarged nuclei, easily detach from the plate).
- iv) The media was harvested after 6 days (centrifugation at 2000xg, 5mins) and this contained the “P1” or the initial baculoviral stock. Such a stock of baculoviruses can be stored at 4°C.
 - v) This P1 stock was used to generate subsequent ‘amplified’ baculoviral stocks. The SF9 cells were seeded in large T175 flasks at a density of 2×10^5 cells/cm², by allowing them to settle for 20 min and subsequently replacing the media with TNM-FH with reduced serum (3% FBS). To this, 400 µL of P1 was added, and the flask was swirled to allow even distribution of the baculoviral inoculum. The flask was incubated at 28°C without CO₂ for 6 days, while occasionally checking for signs of infection.
 - vi) The media was harvested by centrifugation at 2000xg for 5 min to yield the “P2” stock. The baculoviruses could be amplified as required and be used for expression.
 - vii) Hemagglutinin expression: For a 50mL expression, ~ 25 – 30 million High Five cells were harvested from a pass (centrifugation at 100xg for 7 mins). These cells were resuspended with 4.5 mL of baculoviral stock of P3 and the cell suspension was incubated at room temperature for 20 min. In the meantime, 46 mL of fresh SFX medium was placed into a disposable 125 mL Erlenmeyer flask and 500 µL of a 6 mM stock solution of biotin (stock solution made in DI water, sterile filtered, with the

minimum amount – a few drops – of 10N NaOH to enhance solubility of biotin) was added to this flask (final biotin concentration was 50 μ M).

Note: Always test well-in-advance (before beginning expression) that the pH of the biotin stock does not alter the pH of the insect media. For this: Mix the 500 μ L of a 6 mM stock solution of biotin solution to 46 mL of SFX media and compare the pH of this mixture vs. that of the SFX media without the biotin solution added. This is a crucial step as the pH of the media is close to the pI of hemagglutinin and increasing the pH of the media could cause loss of protein due to precipitation at the pI.

- viii) After the 20-minute incubation, the cell suspension was added to the SFX media, mixed well, and incubated at 28°C without CO₂ for 72 – 96hrs.
- ix) The media was harvested by centrifuging the suspension at 2000xg for 10 min. The supernatant contains the hemagglutinin since the plasmid had a signal peptide to trigger secretion into the media. Hemagglutinin was purified using Ni-NTA resin.

2.2.6. *Protein Purification:*

Hemagglutinin has a His tag at the C- terminus, which was used to purify it by affinity chromatography using a Ni column/resin. This step was followed by size exclusion chromatography to separate the trimer fraction (desired) from larger protein aggregates.

- i) A fresh Ni Agarose column must be used per protein. The column was washed with plenty of water to remove the 20% alcohol storage buffer and equilibrated with ~ 5 column volumes of equilibration buffer (chilled PBS, pH 8.0).

- ii) The media supernatant was incubated with the Ni resin for 2-4 hours at 4°C on a rocking platform, after which the mixture was poured through an empty column and the resin was allowed to settle in it while the supernatant flowed through.
- iii) The column was washed with copious amounts of wash buffer (15mL x 4 times) (50 mM Na₂HCO₃, 300 mM NaCl, 20 mM imidazole, pH 8) after which the bottom of the column was capped with a stopper.
- iv) 3 mL of elution buffer (50 mM Na₂HCO₃, 300 mM NaCl, 300 mM imidazole, pH 8) was added to the column and allowed to incubate for 5 min, after which it was collected. This was repeated three times to collect a total of three fractions.
- v) In the meantime, a spin filter was washed with copious amounts of de-ionized water and chilled PBS was added to it, and was spun at 3000xg for 20 min at 4°C.
- vi) The fractions were pooled, aside from keeping 20 µL of each for characterization by SDS PAGE, and were concentrated/washed three times with chilled PBS (spun at 3000xg for 20 min at 4°C).
- vii) This concentrate was passed through a size exclusion chromatography (AKTA, Superdex 200 10/300 GL increase). The trimer eluted at ~ 9 mL whereas the aggregate eluted at ~12 mL.

2.2.7. *Conjugation of HA, followed by Western blots:*

- i) The steps outlined in section 2.2.3 were repeated to make more strep-NTs. This strep-NT batch was split into two; to each batch of 500 µg of streptavidin-NTs, 750 µg of HA (head biotinylated or tail biotinylated) was mixed. The biotinylated HA (HT or TT) was expressed and purified as mentioned in sections 2.2.5 and 2.2.6. The excess/free

HA was removed using a sucrose gradient of 4%, 10% and 30% sucrose by spinning at 175,000xg in an ultracentrifuge. It is essential that the sucrose solutions be removed carefully and discarded without disturbing the NT pellets. The pellet was resuspended in 20mM phosphate buffer (pH 7.4). Again, the nanotube concentration of HA-NT was calculated by repeating the above procedure of taking the absorbance at 808 nm and repeating above-mentioned calculations.

- ii) A western blot was performed to estimate loading of HA onto NT: a range of known amounts of HA-NT was taken (in terms of weight of NTs in ~100s of ng). Also, varying known amounts of HA alone were also used (these served as standards to help estimate the amount of HA loaded onto NTs). All samples were boiled in SDS at 80 °C in a PCR machine for ~ 30-40 min after which they were characterized by gel electrophoresis. The proteins in the samples entered the gels, while the nanotubes remained in the wells itself. The proteins were transferred from the gels onto nitrocellulose membranes (for the western blot) and blocked in 5% BSA solution using a blot transfer apparatus. The primary antibody (1:2000 dilution in 1% BSA solution) used for the blot was an influenza HA anti-head antibody (PY102) obtained from our collaborators from the Icahn School of Medicine at Mount Sinai. The secondary antibody used was an anti-mouse goat HRP-conjugated IgG (1:10,000 dilution in 1% BSA solution). The relative densities of the bands were estimated using the ImageJ software and plotting the linear range of HA standards loaded onto gels. Once we estimated the ng amounts of loaded HA, we could calculate the loading by dividing the HA amount (ng) by the NT amount (ng) loaded per well on the gels.

2.2.8. *Addition of Adjuvants and dilutions before vaccination:*

The HA-NTs (HT-NT and TT-NT) were diluted to make aliquots such that there would be sufficient HA loaded onto the tubes for each mouse to receive 5 µg of HA. The volume injected per mouse per round of injection was 50 µL, out of which 45 µL was the vaccine and 5µL was the adjuvant, poly I:C. A quick vortexing before and after poly I:C addition was helpful in ensuring proper mixing.

Note: Apart from the conjugates with HA oriented in two ways, a required control required for the *in-vivo* testing of a vaccine is the “vehicle-only” control which should ideally lack the antigen or in our case, HA. The aim here is to show that the vehicle alone does not trigger the broadly neutralizing response. I decided to use PEG-NTs at this stage and not Strep-NTs for the “vehicle-only” control. Streptavidin being ‘more accessible’ to the immune system in the case of the Strep-NTs might not make for an appropriate control because the streptavidin in the case of the HA-NT conjugates would be within the ‘inner layers’ i.e. sandwiched between PEG on one side and HA on the other and closely stacked together on NTs; therefore, streptavidin would not be as easily “seen” by the immune system. Hence, I felt that a more appropriate control would be PEG-NT.

2.2.9. *In-vivo cH5/IN1 challenge:*

The immunizations and viral challenge experiments were carried out by our collaborators at the Icahn School of Medicine at Mount Sinai. For each type of sample

(whether HT-NT, TT-NT, or NT-PEG ‘vehicle-only’ control) five mice were vaccinated, which yields a total of fifteen vaccinated mice. The HA-NT conjugates was administered twice, as one prime and one boost, to BALB/c mice, with a gap of 3 to 4-weeks. After both rounds of vaccination, blood samples were collected for ELISAs to assess anti-stalk antibody titers. After the second round of vaccination, another 4 weeks were allowed to pass after which the mice were challenged with a ‘cH5/1N1’ virus. CH5/1N1 is a chimeric virus, modified to have the same stalk region as that of H1 hemagglutinin, but a different HA head region (belonging to H5 subtype). After the challenge, the mice were monitored for the loss in their body weight; if the loss exceeded more than 25% of their body weight, they were euthanized.

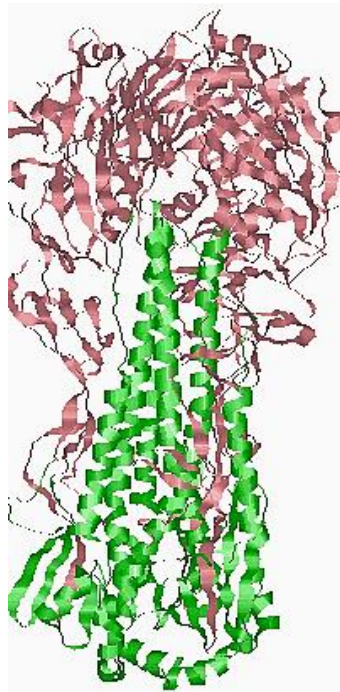


Figure 5: Schematic representation of a Chimeric H5/1 hemagglutinin: with the head of H5, stalk of H1; adapted from PDB ID: 1RD8

2.3. Results:

2.3.1. SEM imaging of Pristine SWNT:

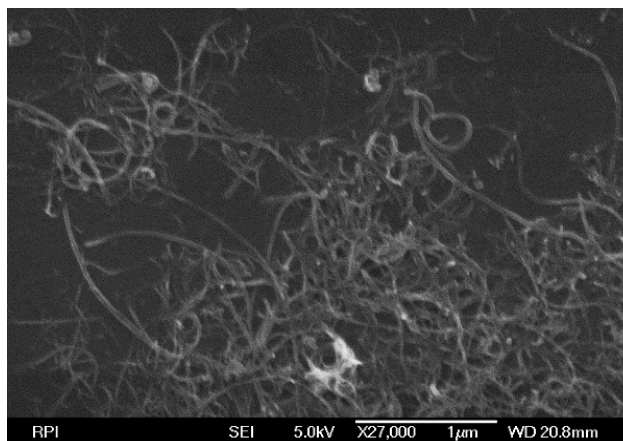


Figure 6: SEM images of raw single walled carbon nanotubes

From the SEM image shown above, the size of raw/pristine SWNTs are easily above $1\mu\text{m}$, which is too large for use in an in-vivo system. These SWNT were cut with the help of a mixture of concentrated sulfuric and nitric acids.

2.3.2. TMB-based ELISA for HRP loading:

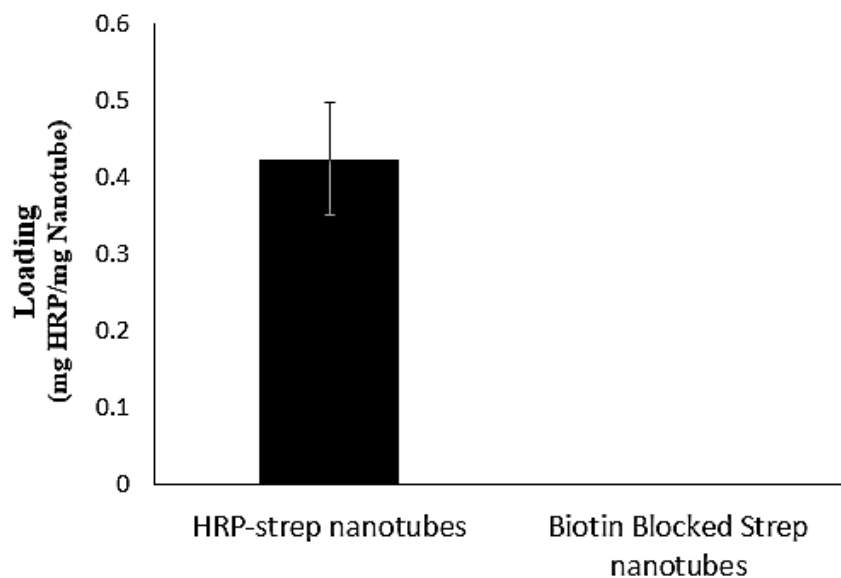


Figure 7: TMB based ELISA data for HRP-NT (sample) and BB-NT (biotin blocked control), averaged over three different batches; the signal for the BB-NT is very low.

HRP was a model protein, loaded onto nanotubes to test the methodology and to quantify the loading of HRP on NTs. The ELISA data was analyzed and plotted in Figure 7. The samples (and corresponding controls) were taken from three different batches of conjugation reactions. From the figure, we see that the HRP-NT samples had a loading ranging from 0.35 - 0.5 mg HRP/mg NT. The loading for the biotin blocked controls (BB-NT) was significantly lower, consistent with a biospecific biotin-mediated immobilization of HRP onto the streptavidin-coated nanotubes with minimal non-specific adsorption.

2.3.3. TEM imaging of biotin-gold coated NTs:

Figure 8 shows TEM images taken of 5nm gold nanoparticle coated nanotubes (NTs themselves are not easily visible). The streptavidin coated NTs were mixed with an excess of biotin-gold to saturate the streptavidin sites. This was imaged by my colleague, Chad Varner.

These images show that the nanotubes are well covered by the gold nanoparticles and corroborate the ELISA results, implying that the nanotubes can be loaded with hemagglutinin. These images also show that the NT length has been reduced after the cutting them with acid. The length of the NTs was found to be between 50-250 nm, which is optimum for using in an *in-vivo* system.

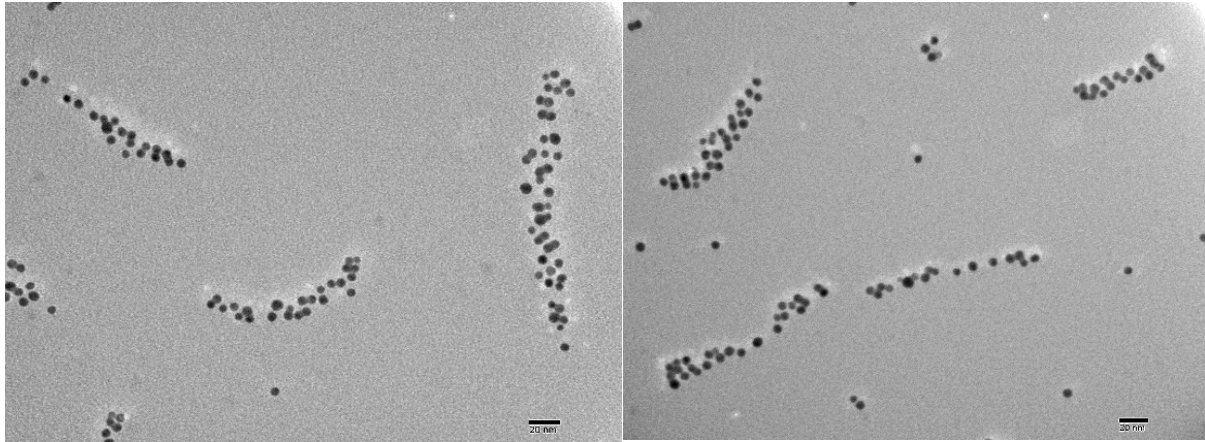


Figure 8: Transmission electron microscopy images of 5nm gold coated nanotubes (scale bar reads 20nm)

2.3.4. *Western Blots:*

Western blots were done to quantify the amount of HA that was loaded onto the nanotubes.

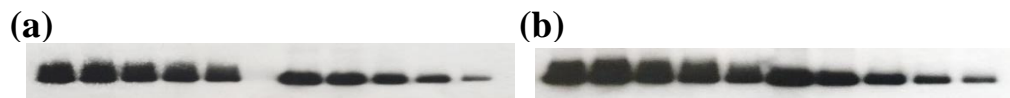


Figure 9: Western blots to semi-quantitatively assess loading of HA on NTs (a) 200-50ng of HT-NTs in the first five lanes, 80-40ng of HT standards in the next five lanes, (b) 200-50ng of TT-NTs in the first five lanes, 80-40ng of TT standards in the next five lanes

ImageJ was used to analyze the images to quantify the amount of HA using densitometry. First the standard curve was plotted with the ‘HA standards’ data. This was used to estimate the amounts of HA loaded onto the HA-NT conjugates. The loading for HT-NT was 1.1 mg HA per mg NT and for TT-NT was 1.2 mg HA per mg NT.

2.3.5. *In-vivo results of cH5/IN1 challenge:*

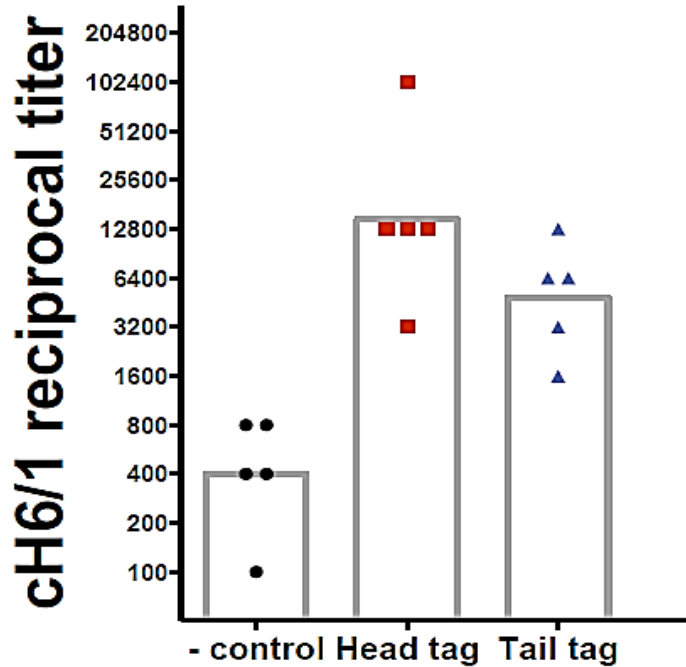


Figure 10: Measure of anti-stalk antibody titers from BALB/c mice sera collected after two rounds of vaccination; tested samples include vehicle-only NT-only: negative control (●), Head tag NTs (■) and Tail tag NTs (▲). Tested for binding against chimeric H6/1 hemagglutinin – with the head region of H6 and tail region of H1

The anti-stalk antibody titers (reported by our collaborators at the Icahn school of Medicine at Mount Sinai) were measured by performing an ELISA on the mice sera collected after both rounds of vaccination (figure 10). The sera were tested for antibodies that would bind to a chimeric H6/1 hemagglutinin with the head region of H6 and stalk region of H1. We see a relatively higher stalk-binding antibody titer in the sera from the mice receiving the head tag HA – NT (HT-NT) where the stalk region faces outwards as compared those seen either in the negative vehicle-only control and the TT-NT. Therefore, at the end of two rounds of vaccinations, we concluded that there was a difference in the stalk antibody titer triggered by the HT-NT vs. TT-NT.

This experiment was followed by a cH5/1N1 viral challenge for the mice receiving the vaccines and control. We observed that after two immunizations rounds, the difference between the protection offered by our proposed vaccine HT-NT was more pronounced vs. that provided by our control vaccine TT-NT.

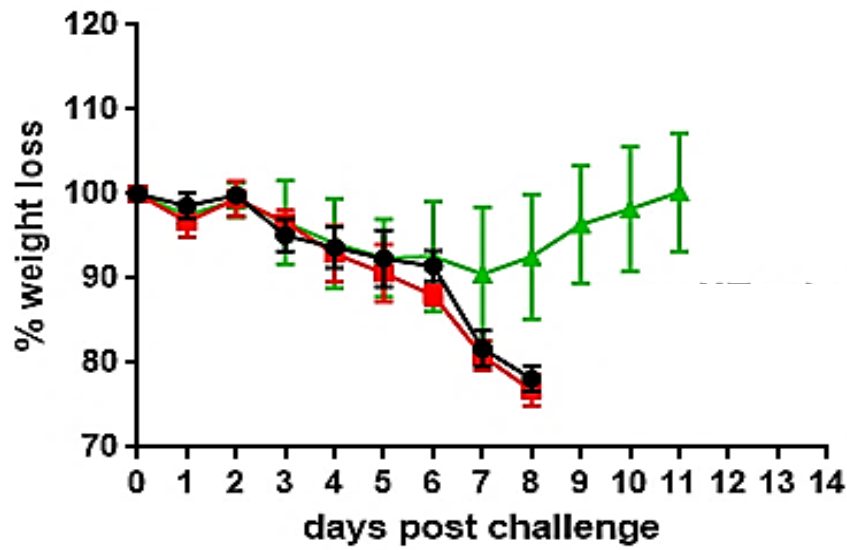


Figure 11: Percentage weight loss in BALB/c mice; monitored for 14 days' following a chimeric viral H5/1N1 challenge after two rounds of immunization with head tagged NT or HT-NT (-▲-), tail tagged NT or TT-NT (-■-), and vehicle-only control (-●-)

Figure 11 shows the % weight lost by the BALB/c mice for 14 days after infection with a chimeric cH5/1N1 virus. Here, we see that the mice receiving the HT-NT recovered from the infection, but the mice receiving both TT-NT (control vaccine) and the vehicle-only control lost more than 25% of their weight and had to be euthanized per the standards set by IACUC. This proves that the protection offered by the novel vaccine is due to the orientation of hemagglutinin on the vaccine scaffold.

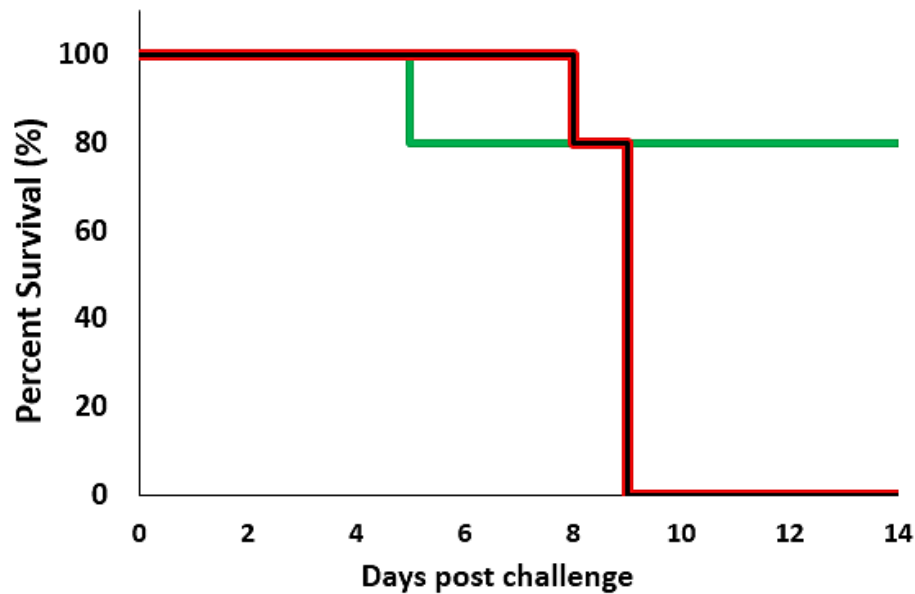


Figure 12: Survival data for the cH5/1N1 viral challenge in BALB/c mice after two rounds of immunization; HT-NT (—), TT-NT (—), and vehicle-only control (—)

Figure 12 corroborates the data shown in figure 11 as it shows the survival data for the BALB/c mice after two vaccination rounds followed by the viral cH5/1N1 challenge. Most of the mice receiving our proposed vaccine (HT-NT) survived the challenge but those receiving the other two constructs (TT-NT and vehicle-only control) required euthanization.

2.3.6. Characterization of hemagglutinin (antigen):

Although the preliminary results to assess the orientation dependence *in-vivo* had been encouraging so far, antigen characterization was crucial to this study before we could attempt the heterologous viral challenge (against whole H5 and H6 viruses). The PR8/H1 hemagglutinin that we had used for our initial immunization experiments was expressed in insect cells (High Five cells) and purified using affinity chromatography

(Ni resin based) by our collaborators and shipped to us on ice. We characterized this protein using Transmission electron microscopy (TEM) and size exclusion chromatography (SEC).

Figure 13 shows the Transmission electron microscope image of ‘free’ hemagglutinin; it reveals rosettes of trimers suggesting that hemagglutinin can form large soluble aggregates.

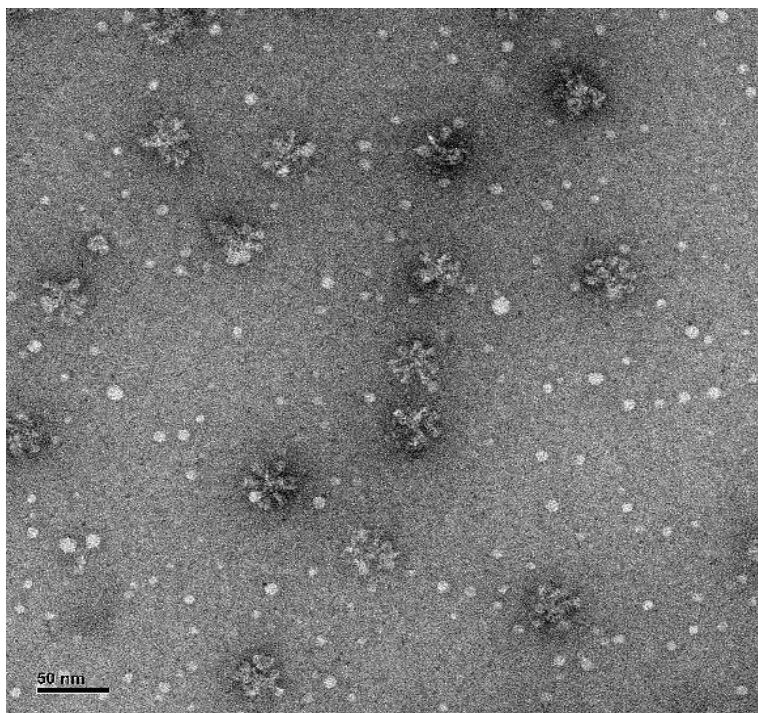


Figure 13: Transmission electron microscopy image of hemagglutinin aggregates forming ‘rosettes’

Size exclusion chromatography data for HA (HT) and HA (TT) (Figures 14a and 14 b, respectively) confirmed that both proteins were a mixture of trimers and larger aggregates. While the initial immunization results were encouraging, we reasoned that a size exclusion chromatography step that separated the trimer from the aggregate was essential before conjugating the hemagglutinin onto NTs to study the orientation dependence.

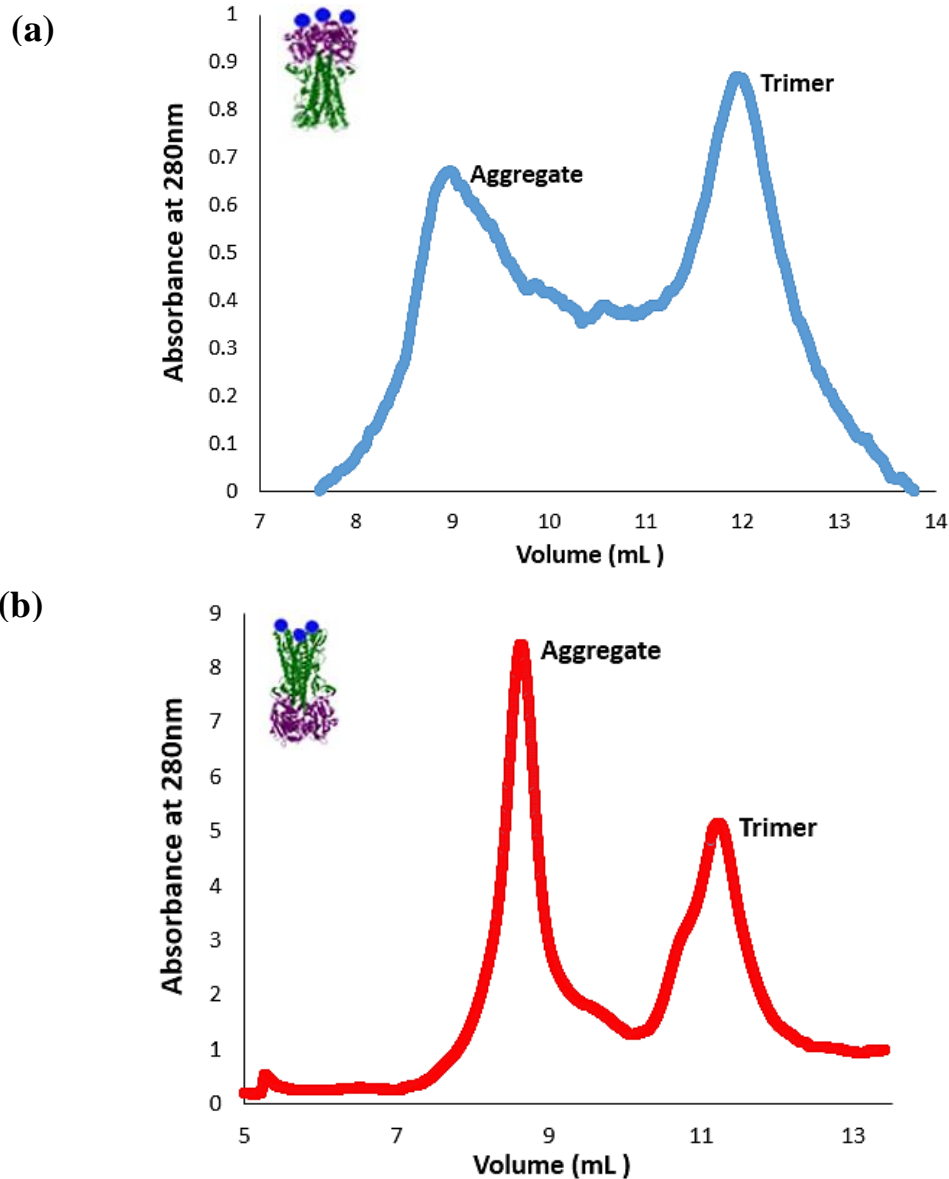


Figure 14: Size Exclusion Chromatography plot for hemagglutinin variants (a) HA HT and (b) HA TT – shipped to us on ice

We therefore decided to express and purify hemagglutinin in our lab so that we could better control the purification processes. Another advantage was that we could avoid the overnight shipping and that the conjugation of hemagglutinin onto nanotubes could be done the very same day.

2.3.7. *Expression, Purification and Characterization of Hemagglutinin:*

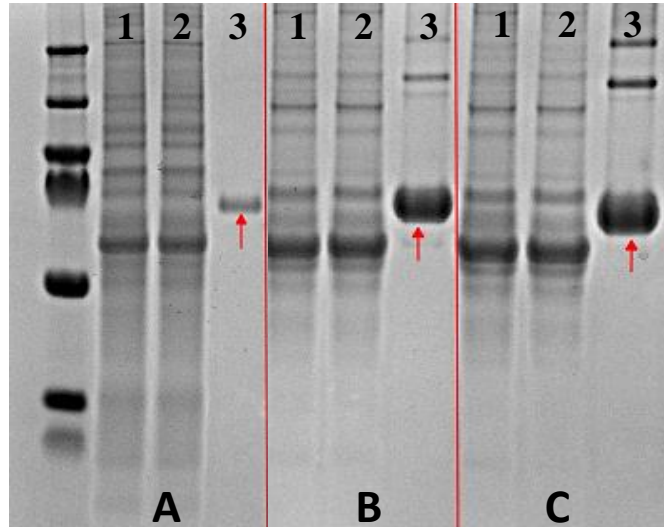


Figure 15: SDS PAGE gel image, Set A: HT HA, B: TT HA, C: H1 HA wild type. Lane order: 1. Expression Media with protein 2. Flow-through from Ni NTA affinity chromatography 3. Eluted fraction. The red arrow indicates purified protein

After multiple attempts in mammalian cell culture, hemagglutinin was successfully expressed in insect culture. Two insect cell lines were considered during the optimization process: SF9 and High Five cells. Using a commercial transfection reagent, Xtremegene HP to express these proteins, SF9 cells gave better yields ~ 0.5-1mg HT/L culture and 2.5-3.5 mg TT/L culture. As shown in figure 15, H1 HA wt., HT and TT were expressed and purified using Ni NTA affinity chromatography.

The HT and TT were run through size exclusion chromatography, and the results are as shown below in figure 16 (a) and (b). Although the proteins were successfully expressed and purified, we observed a larger amount of aggregate relative to trimer. Also, both HT and TT had the AviTag sequence inserted in the head and tail region respectively and needed to be biotinylated *ex-vivo*. When this was attempted, a large

amount of protein precipitated irreversibly. This accounted for about 70 - 80% protein losses in that step.

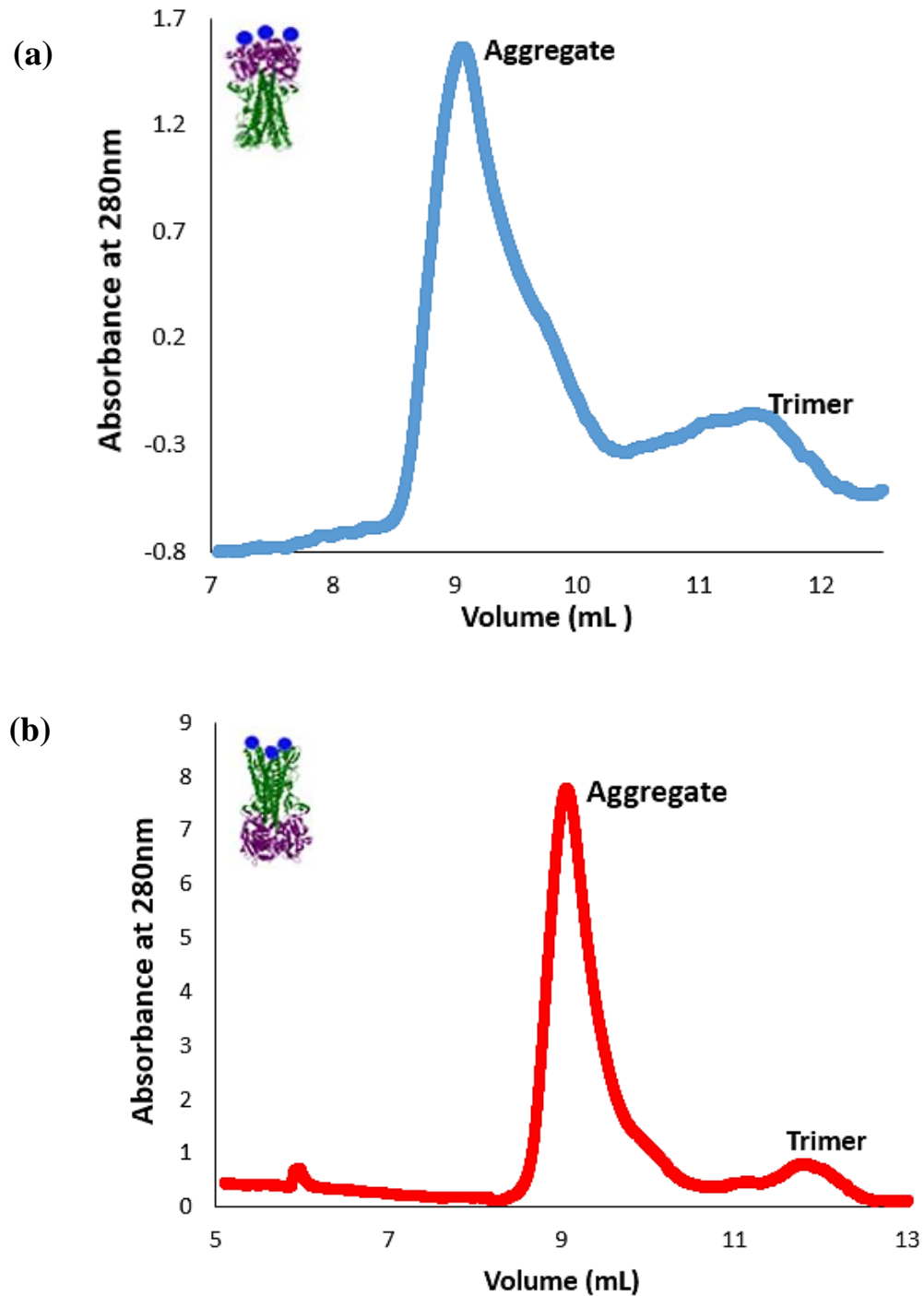
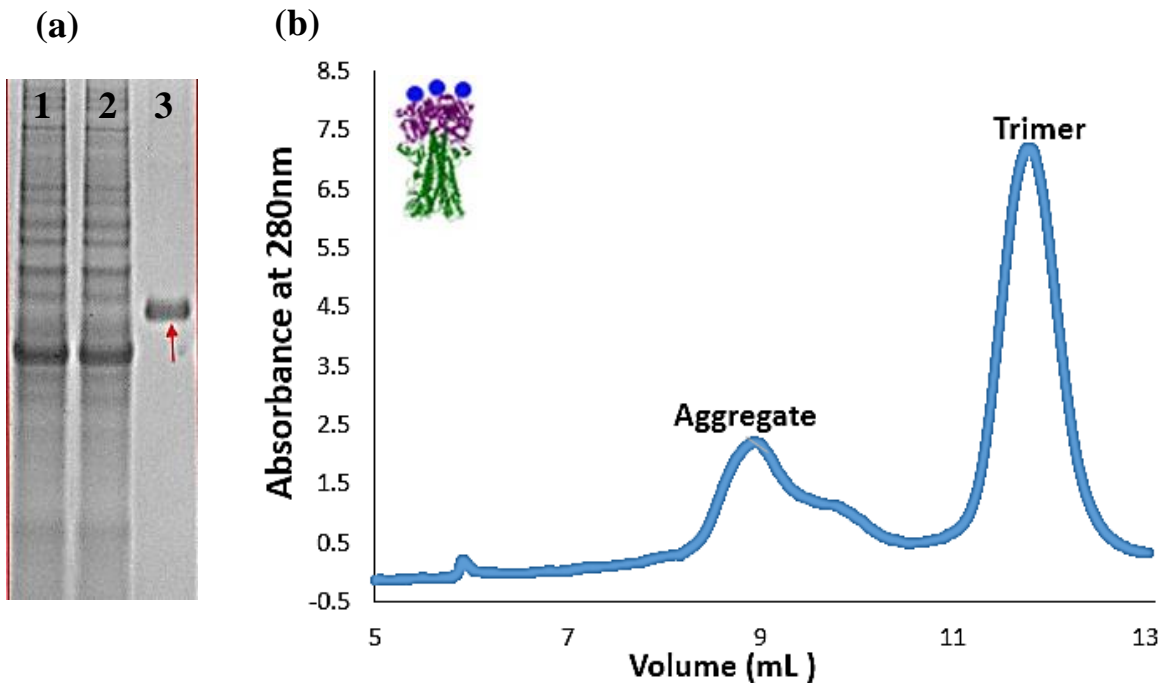


Figure 16: Size Exclusion Chromatography plot for hemagglutinin variants (a) HA HT and (b) HA TT – expressed using transfection reagent in insect culture

To overcome these challenges, we next attempted baculoviral expression of HA, which gave us yields comparable to that of the transfection reagent (Figure 17 (a) below). The baculoviral vector pFastBac Dual allowed for the insertion of two genes which could be expressed simultaneously, and this was advantageous, because hemagglutinin could be expressed alongside an *in-vivo* biotinylation enzyme, BirA. This allowed for the *in-vivo* biotinylation of both HT and TT, when biotin was supplemented into the media.

Baculovirally expressed HT was run through size exclusion chromatography and relatively larger fraction of trimer was observed over aggregate. 70 – 80% of the biotinylated HT expressed was in trimer form and was extracted after SEC and used to conjugate to streptavidin coated nanotubes (Figure 17(b)).



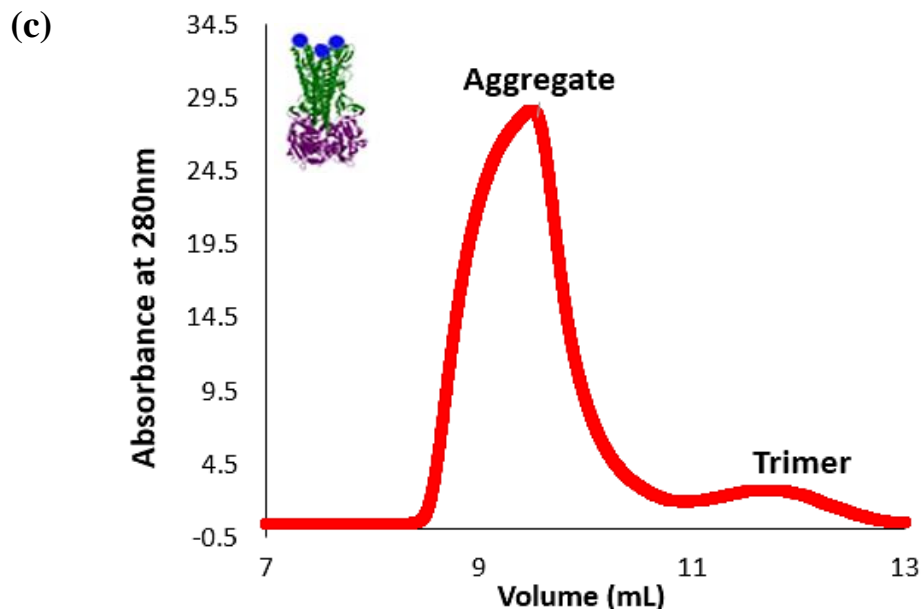


Figure 17: (a) Expression and affinity based purification of baculovirally expressed HT-biotin, Lane order: 1. Expression Media with protein, 2. Flow-through from Ni NTA affinity chromatography, 3. Eluted fraction. (b): Size exclusion chromatography on baculovirally expressed HT-biotin, (c) Size exclusion chromatography on baculovirally expressed TT-biotin

Baculovirally expressed TT was still predominantly aggregate (figure 18 (c)), but the expression yield of the TT variant was higher (~ 3 mg/L) than that of the HT variant (~1mg/L); this enabled scale up of expression to yield sufficient “trimer only” fraction for our conjugation experiments.

Circular Dichroism was also used to characterize the expressed hemagglutinin (H1 HA wt. at concentration at 0.3 mg/ml and 0.1 cm path length) as shown in figure 18 (b); the CD spectra for HA and was similar to one published in the literature (figure 18 (a)) [45]. The CD spectrum in Fig. 18 (b) indicates a rich beta-sheet structure, with a minima appearing close to ~ 215nm. It has been shown that the globular head region of HA is rich in anti-parallel beta-sheets, and the rest of HA1 which runs along the stem region

has a beta-sheet structure. The HA2 region, predominantly consisting of the stem, is folded into a helical coiled-coil structure [46].

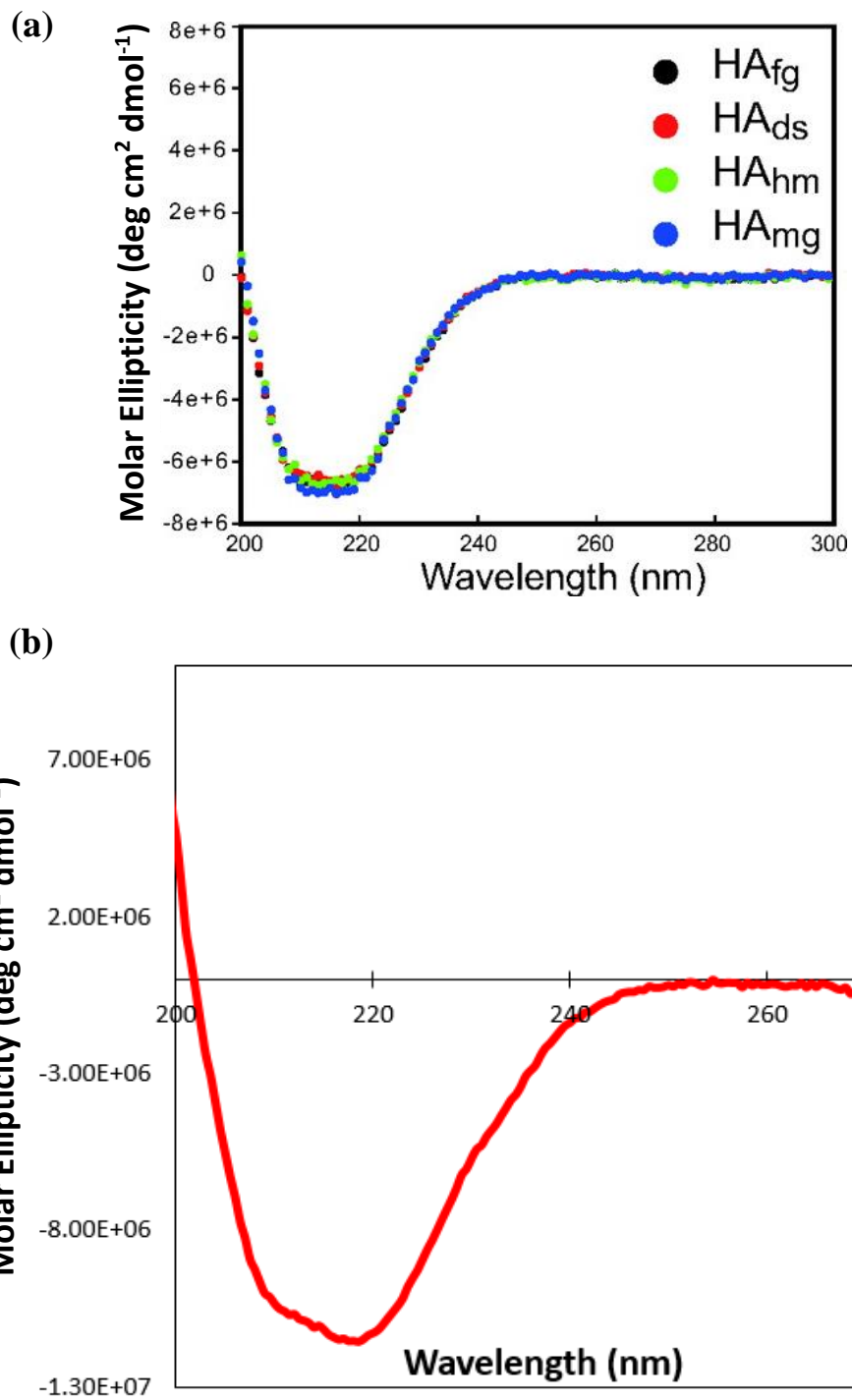
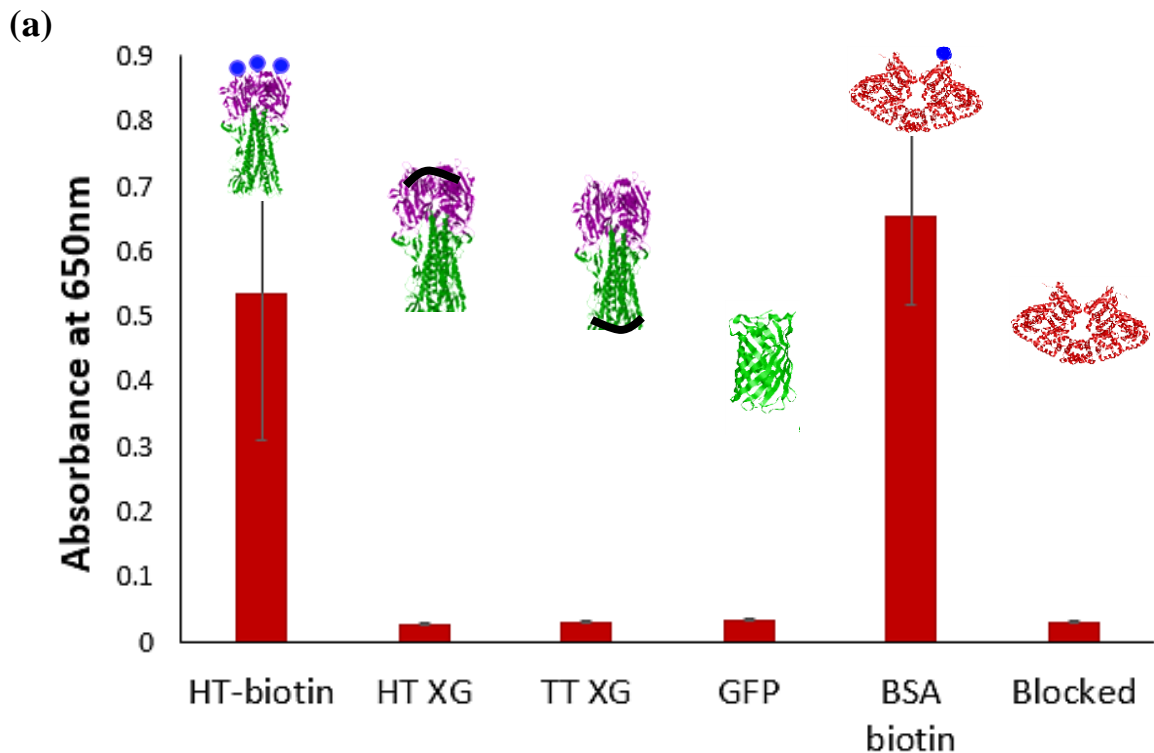


Figure 18: (a) Circular Dichroism spectra adapted from Wang C. et al., 2009 [45], (b) Circular Dichroism on expressed H1 HA wt.

To test if the hemagglutinin was being secreted after *in-vivo* biotinylation, an ELISA was done to probe for biotin, using streptavidin-HRP for detection. As shown in figure 19 (a), the HT expressed using baculoviruses gave a significant signal for biotin, comparable to commercially purchased biotinylated BSA. The HT and TT expressed using transfection reagent (labelled as “XG” in the plot) which was not *in-vivo* biotinylated or a negative control, GFP, gave minimal signal for biotin. A “gel-shift” assay was done too, to verify the presence of biotin as shown in figure 19 (b). When an excess of streptavidin was mixed with the biotinylated HT, we see a disappearance of the HT band (decrease in intensity) since it forms larger molecular weight complexes. A similar trend was seen with commercial biotin-BSA. No such decrease in band intensity was observed when HT was used (using transfection reagent, not biotinylated).



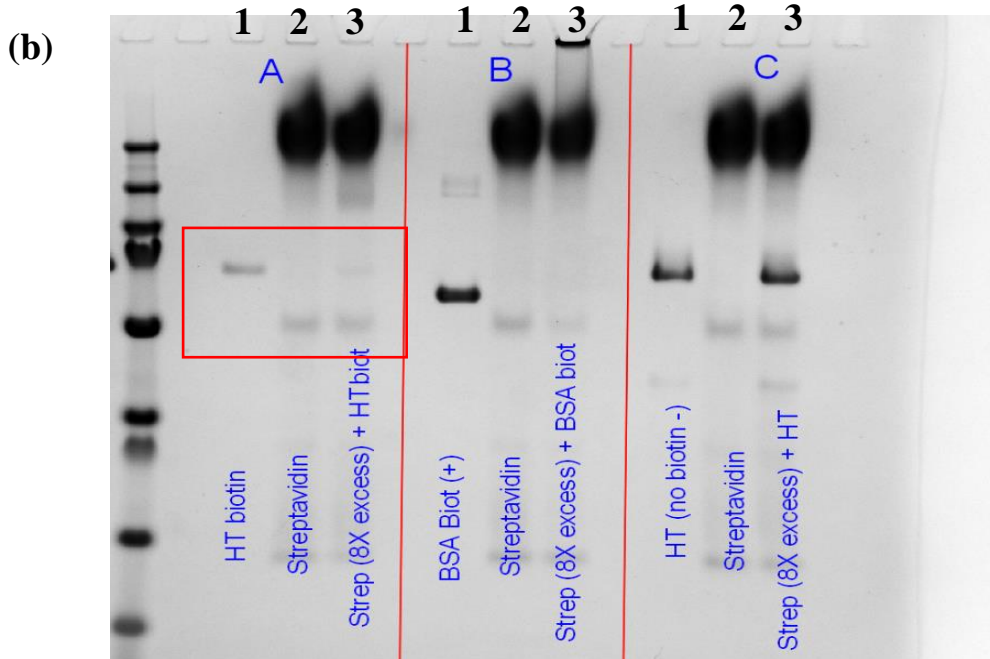


Figure 19: Probing for successful *in-vivo* biotinylation (a) ELISA using streptavidin-HRP to detect biotinylated protein, the controls HT and TT are not biotinylated (expressed with transfection reagent ‘XG’) with the AviTag represented in black, ‘blocked’ represents wells blocked with Bovine Serum Albumin (BSA), GFP and ‘blocked’ both negative controls (b) ‘Gel-shift’ assay to assess shift in band or reduction in original band intensity due to formation of higher MW complexes via biotin-streptavidin interaction. Set A: HT-biotin expressed baculovirally, B: Commercial BSA-biotin, C: HT with AviTag, but not biotinylated, Lane order 1: protein alone, 2: streptavidin (large excess), 3: Protein and streptavidin mixed; equal amounts of protein (being probed for biotin) were used in lanes 1 and 3

2.3.8. *Relative binding of HA antibodies onto hemagglutinin conjugated to Streptavidin:*

The relative affinities for two different HA targeting antibodies; one against the head region (Ab PY102) and the other against conserved epitopes in the stalk (Ab C179) were tested to see if there was a difference in their binding when HT was oriented differently (figure 20). In one case, HT was allowed to coat 96-well plates freely i.e. no specific conformation was enforced whereas in the second case, the wells were first coated with streptavidin and HT was allowed to bind in the presence of a blocking agent (BSA). We see higher binding of the anti-stalk antibody to HT than the anti-head

antibody, when HT was immobilized in a specific orientation, with the stalk facing outward and head conjugated onto streptavidin.

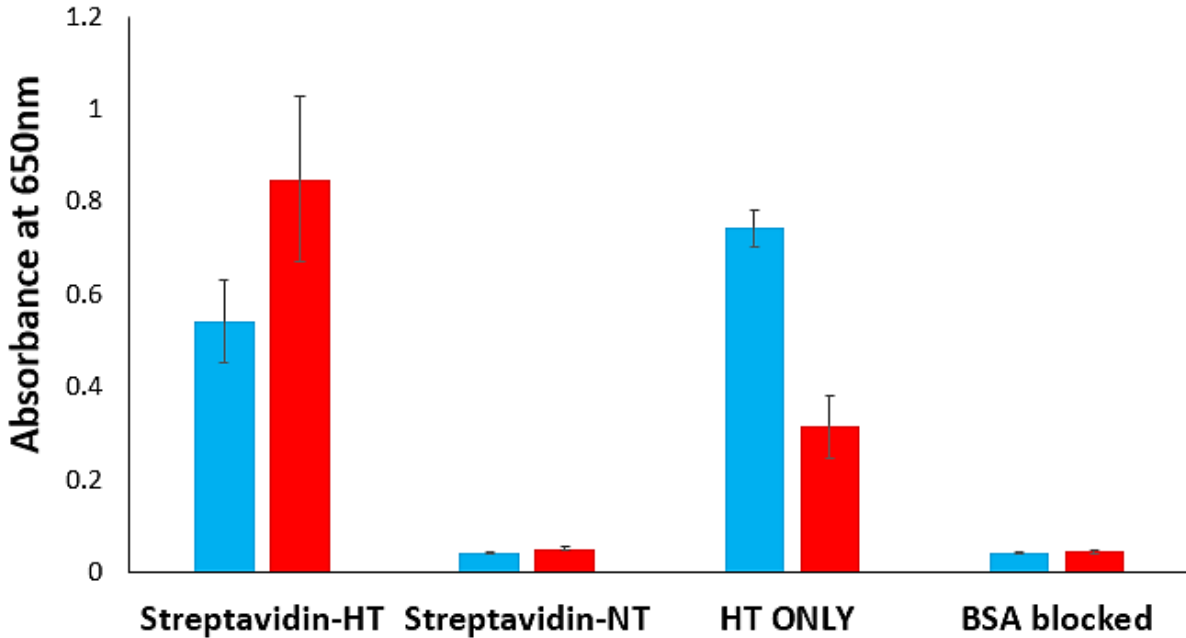


Figure 20: ELISA to probe relative binding of head-binding antibody PY102 (■) vs. stalk binding antibodies C179 (■) – to see the effect of orientation of HT; ‘HT only’ is where HT was freely allowed to coat wells – arbitrary orientation, whereas ‘streptavidin-HT’ is where HT is made to orient itself with the tail facing outward by binding to streptavidin coated wells.

2.4. Future work:

2.4.1. *Heterosubtypic in-vivo challenges after two rounds of vaccination:*

Although encouraging preliminary results were seen after two rounds of immunization, where the HT-NT provided complete protection after a chimeric H5/1 viral challenge, the antigen used may have been in a partially aggregated form. The same experiment therefore must be repeated with conjugates functionalized with purified HT and TT trimers (using an additional round of size exclusion chromatography to extract

hemagglutinin trimers), followed by a cH5/1N1 challenge (to monitor whether the antibody levels elicited after 2 vaccine rounds are sufficient to mount a neutralizing response) and a heterosubtypic challenge with H5 viruses (to assess the breadth of the neutralizing response). Another experiment that could be done is using H1 HA for the first round of vaccination, but use another subtype of HA (within group 1, say H2) for the second round. Since the stalk regions are similar, this might help amplify the orientation dependence. The viral challenge could be repeated with H5 viruses.

2.4.2. *Hemagglutination assay:*

Hemagglutination assays are traditionally performed to measure the levels of viral titers in sera. It exploits the ability of an influenza virus to attach itself to the surface of RBCs and cause clumping or hemagglutination [47]. A hemagglutination assay will be performed as another means to assess the orientation of the HA loaded onto the NTs. In the conjugates with the head of HA towards the solution (away from the nanoscale support), we expect to see hemagglutination (RBC clumping). In the case where the tail of HA is towards the solution, we should ideally see little to no agglutination. This will help verify that the HAs are oriented correctly on the NTs.

2.4.3. *Broadly neutralizing vaccine against both groups of Influenza:*

So far, we have attempted to generate bnAbs against group 1 viruses. We could attempt to elicit bnAbs that neutralize both groups. Another experiment that would be done is using a group 1 viral HA (H1) to prepare the first round of vaccines, but use a group 2 viral HA (say H3) to make the second round of vaccine. The breadth of the neutralizing

repertoire can be studied with ELISAs to measure anti-stalk titers and performing a heterosubtypic mice survival challenge (with viruses from both groups, say an H5 (group 1) and an H7 (group 2) challenge).

2.5. Conclusion and Discussion

In conclusion, we have designed a vaccine that has shown protection against a chimeric virus with the hemagglutinin having the same stalk region as that used in the vaccine (H1) but a foreign head (H5). The construct was tested for successful protein loading and estimated the hemagglutinin loading onto the nanotube scaffold to be ~ 1mg HA/mg nanotube scaffold. The antigen, hemagglutinin was also well characterized, using Transmission Electron Microscopy and Size exclusion chromatography. It was observed that HA was aggregated so we took on the expression and purification from our collaborators to better control the purification process.

Hemagglutinin was expressed using insect cell culture that was infected with baculoviruses that expressed the head tagged biotin or tail tagged biotin version of hemagglutinin. Baculoviral expression provided a twofold benefit: 1. HT was found to aggregate a lot less than in the case when it was expressed using a transfection reagent and 2. In-vivo biotinylation allowed for us to have more protein to work with since ex-vivo biotinylation caused ~75% of the protein to precipitate out. A size exclusion chromatography step was required to follow the affinity chromatography step in close succession so as to be able to quickly extract the trimer fraction and minimize the extent of aggregates formed. The hemagglutinin trimer was then conjugated onto the streptavidin coated nanotubes. In future work, we will test the efficacy of these well-characterized vaccine candidates and their ability to protect mice from a heterosubtypic viral challenge.

3. Multivalent conjugates of hemagglutinin-binding proteins

3.1. Introduction

The goal of this project was to design polyvalent therapeutics against influenza. Our collaborators (Baker Lab) at the University of Washington computationally designed proteins that bind to conserved sites on hemagglutinin [27], [28]. The binding sites of these proteins were ‘chosen’ based on the conserved binding sites of some broadly neutralizing antibodies such as CR6261 and F10 [27], [28]. Three key binding ‘hotspots’ (called HS) were selected; one (HS1) was the TRP21 residue which made aromatic stacking interactions with the Phe side chains on the ‘hemagglutinin-binding proteins’ or HB proteins, another (HS2) was the van der Waals interactions made by the hydrophobic groove (in which the fusion peptide resides) and HS1, and thirdly (HS3), hydrogen bonding interactions on the alpha helical region of the HA2 chain (THR41 and ILE45) [27], [28]. The structure of H1 HA0 was obtained from the protein data bank (PDB 1RD8) and was modified using RASMOL to highlight the binding sites for the HB proteins (in red in figure 21). The two HB protein versions that were used for this study were HB 36.6 and HB 80.4.

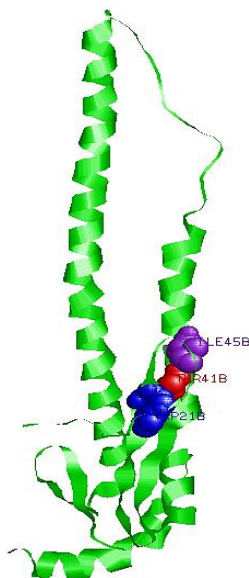


Figure 21: Structure of stem of HA monomer (PDB 1RD8) in green, with binding sites of HB proteins highlighted in red, blue and purple (reported binding ‘hotspots’)

3.2. Materials and Methods:

Initially, monomeric versions of the two HB proteins were expressed, purified and tested so as to determine their IC₅₀ values. The goal was to express/synthesize multimeric versions of HB proteins that will have IC₅₀ values orders of magnitude lower than that of the monomer alone. The design of homodimeric versions of the HB proteins was attempted, for which the optimum linker lengths had to be computed. Some of the protein expression and purification techniques used are listed below:

3.2.1. Protein Expression:

- i) The plasmid containing the HB protein sequence was transformed into the NiCo21 (DE3) cell line (catalog# C2529H) according to the protocol suggested on the NEB (New England BioLabs) website.
- ii) After transformation, the bacteria was allowed to grow in SOC (super optimal broth) media (without antibiotic) for 1 hr at 37°C. 500µL of this culture was used to inoculate a fresh 5 mL pre-autoclaved LB (Luria Bertani) media stock (with antibiotic: kanamycin) near a flame, and was incubated at 37 °C with shaking, overnight.
- iii) 1L LB media was prepared that evening (by dissolving 25g of LB media powder in 1L culture flasks with distilled water). The media was autoclaved and set aside.
- iv) Scale up: The following morning, 1 mL of antibiotic (kanamycin) was added to the scaled-up media near a flame, to maintain sterile conditions. Subsequently, 1 mL of inoculum (that was left overnight at 37°C) was added to the 1L flask.
- v) The flask was placed in a shaker incubator and left at 37°C for 4 to 5 h. This is when the bacteria are allowed to grow until they reach logarithmic phase.

- vi) 400 μ M of freshly prepared IPTG solution was added per liter of inoculated media. The temperature of the shaker incubator was reduced to 16°C, and was incubated overnight.
- vii) The next day, the cells were pelleted by spinning at 6000xg for 1 h, while the supernatant was discarded.
- viii) The pellets were made to undergo repeated freeze-thaw cycles first to lyse cells. 50 mL of lysis buffer (50 mM Phosphate, 500 mM NaCl, 25 mM imidazole, 0.2 mg/ml DNase, 1 mM PMSF, pH 8.0) was added to a 1g pellet.
- ix) The pellet was homogenized in the lysis buffer and then sonicated (to completely lyse cells) for 5 minutes in an ice bath to ensure that proteins do not get denatured due to over-heating.
- x) After cell lysis, the cell debris was spun down at 20,000xg for 1h. The supernatant (contains the proteins) was collected. The pellet should be discarded only if the protein is not expected to be present in inclusion bodies.

3.2.2. *Protein Purification:*

The proteins being expressed have a His tag on either the N- or C- terminus, which can be used to purify them using a Ni column/resin using the principle of affinity chromatography.

- i) A fresh Ni Agarose column must be used per protein. The column was washed with plenty of water to remove the 20% alcohol storage buffer and equilibrated with ~ 5 column volumes of equilibration buffer (50 mM Phosphate, 500 mM NaCl, 25 mM imidazole, pH 8.0).

Note: A high NaCl concentration was maintained i.e. 500mM to minimize non-specific adsorption of irrelevant proteins onto the Ni-NTA resin.

- ii) The lysate was passed through the column, while the flow through was collected.
- iii) The column was washed once again with ~ 3 column volumes of equilibration buffer.
- iv) 10 column volume washes were performed with 50 mM and 100 mM imidazole buffers, pH 8.0. All washes were collected. The 100 mM wash was collected as fractions.
- v) Finally, the protein was eluted with 10 column volumes of 400 mM imidazole wash, while collecting fractions.
- vi) Gels were run on the fractions to assess which fraction had the protein of interest with desired purity. The absorbance at 280 nm was taken to determine the protein concentration.

3.3. Results:

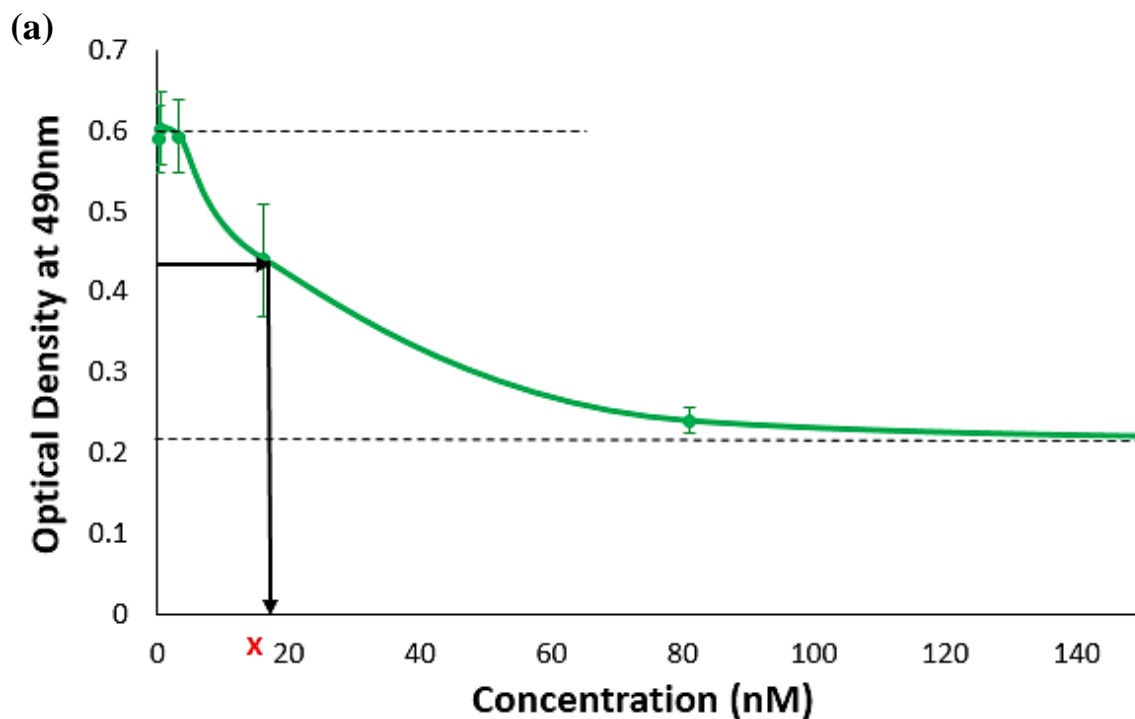
3.3.1. *Rationale behind optimum linker length calculations:*

The linker length analysis was based on a paper on the thermodynamic analysis of the effect of linker length on the avidity of multivalent interactions [48]. This analysis showed that the avidity of a homodimeric protein can be enhanced by optimizing the linker length connecting the two proteins. The parameter that is crucial for consideration is called the effective concentration ($C_{\text{eff}}(r)$), which is a function of “ r ” (the distance between the adjacent binding sites on the receptor) [49], [50]. C_{eff} is a function of the probability that the linker ends are at a distance “ r ” apart [48]. Another crucial parameter to be considered is the r_{RMS} distance, that is, the root mean square distance between the ends of the flexible linker. The optimum r_{RMS} distance (where the value of C_{eff} is maximum) is equal to “ r ” [49]. The free energy/avidity of binding has a

weak dependence on linker length. Consequently, linker lengths that are somewhat longer than optimum will also be effective in enhancing the avidity of a multivalent interaction [48]. However, the extended length of the polymeric chain cannot be less than “r”; otherwise simultaneous binding of the dimeric conjugate to two sites on the target receptor would not be possible [48]. The other extreme scenario, where the linker is much longer than the optimal value, would also diminish the benefit of polyvalency.

3.3.2. *Inhibitory activity data for HB monomers:*

Our collaborators at Emory University tested both HB monomers (HB36.6 and HB80.4) for their ability to inhibit influenza infection. The results for the inhibitory activity tests are shown in Figures 22 (a) and (b); the IC_{50} was calculated to be 17 nM for HB36.6 and 0.8 nM for HB80.4.



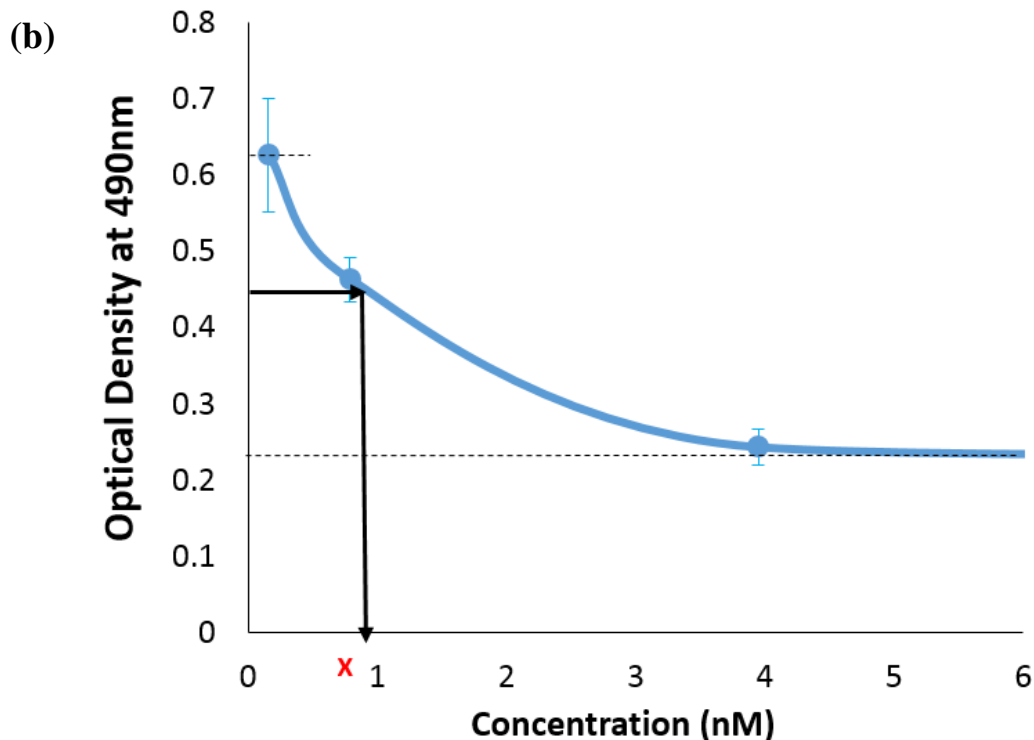


Figure 22: Inhibitory activity plots - averaged over experiments performed in triplicates (a) for HB36.6 monomer ($IC_{50} \sim 17nM$) and (b) HB80.4 monomer ($IC_{50} \sim 0.8nM$)

3.3.3. *Optimum peptide linker length calculations for HB homodimers:*

For calculating optimum linker lengths, the PDB (protein data bank) structures 3R2X (HB36.3 bound to HA) and 4EEF (HB80.4 bound to HA) were studied. The binding sites being targeted by the homodimers are those present on adjacent monomers in a hemagglutinin trimer. The peptide linker connects the C-terminus of one HB protein bound to hemagglutinin to the N-terminus of an adjacent one. The distance between these termini (i.e., the first estimate of the “end-to-end” distance for the linker), was 8.3 nm for HB36.3 and 5.2 nm for HB80.4. A linear path from the C-terminus of one HB protein to the N-terminus of an adjacent one would, however, “go through the HB

protein”, and this constraint would be expected to further increase the optimal RMS length of a designed linker.

To ascertain how many repeats of the –GGSGG- linker are required to span this arc length, we assume that i) the -GGSGG- linker fits a worm-like chain model, ii) the peptide bond contour length (l = the distance between adjacent alpha carbons on a peptide chain; the distance between two amino acids = 0.38nm) [51], iii) persistence length (P = measure of stiffness/flexibility of polymer) = 0.45nm [51], the following formula can be used to calculate the RMS distance $\langle R^2 \rangle$ [51] :

$$\langle R^2 \rangle = 2Pl \left[1 - \frac{P}{l} \left(1 - e^{-l/P} \right) \right]$$

From these calculations, we can plot:

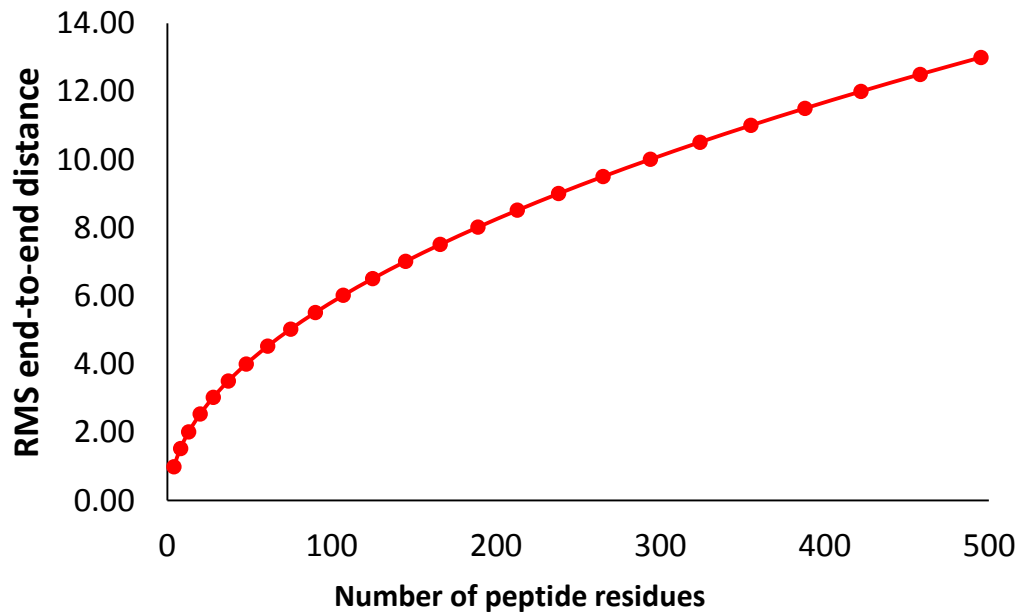


Figure 23: Number of peptide residues vs. RMS end-to end distance

From the above plot (figure 23), we see that the ideal RMS linker lengths would be ~ 200 amino acids (or 40 repeats of GGSGG linker) for HB 36.3 and 80 amino acids (or 16 repeats of GGSGG linker) for HB 80.4. A range of GGSGG repeats were chosen around these values for testing.

3.3.4. *Expression, Purification and activity testing:*

Due to difficulty with the bacterial expression of dimers with large repeats of GGSGG, we decided to test the concept of polyvalency using 75 amino acid linkers of GGSGG (15 repeats of the GGSGG unit) for both monomers, HB36.6 and HB 80.4 (figure 25); to assess the improvement in the avidity of conjugate for the receptor over the affinity of the monomer.

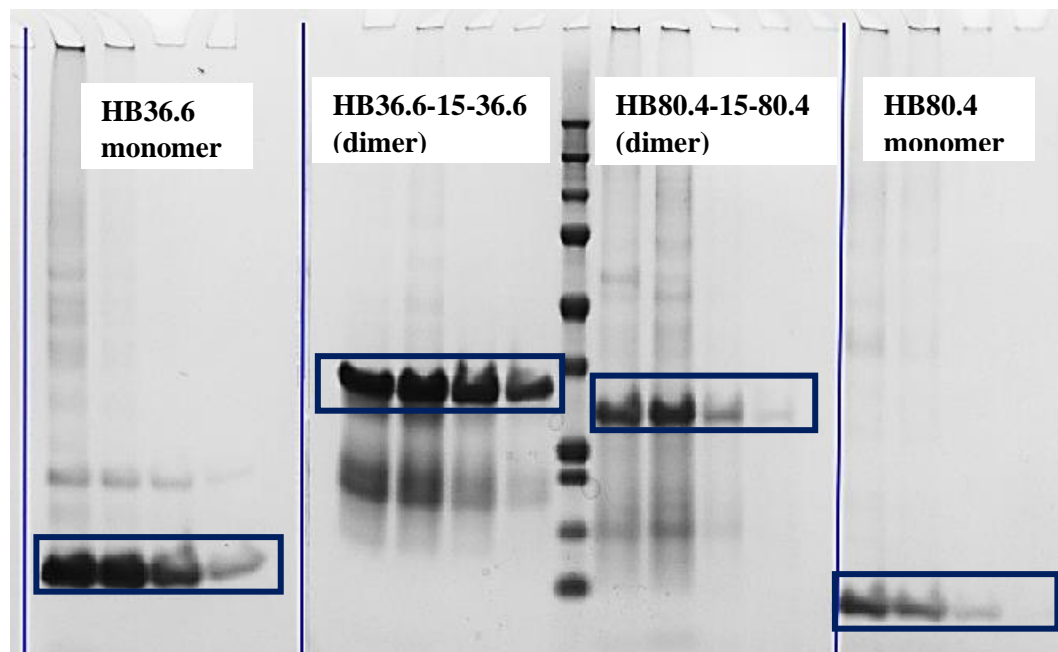


Figure 24: Gel image of IMAC purified fractions of bacterially expressed monomers HB36.6 & HB 80.4, and dimers HB36.6-15-36.6 and HB80.4-15-80.4, where 15 signifies the linker with 15 repeats of GGSGG units

These HB monomers and dimers were tested in an ELISA to assess how effectively they can inhibit the binding of hemagglutinin-head and stalk targeting antibodies. The hemagglutinin used for this inhibition assay was H1 PR8/34 HA, which was first immobilized onto 96-well plates, and subsequently blocked with 5% BSA. After the blocking was complete, the HB monomers or dimers was added to each well in the presence of 2% BSA. This step was followed by the addition of either the anti-head or anti-stalk antibody – which was detected with a secondary mouse Ab linked to HRP. Also, both monomers and dimers were tested at the same “per HB concentration” or effective concentration of 0.1mg/ml. The results are indicated in figure 25 below. Neither the monomers nor the dimers seem to affect the binding of the hemagglutinin-head binding antibody, PY102 vs. the ‘no inhibitor’ control (No HB protein was added to these hemagglutinin-coated wells); this is to be expected since the HBs are hemagglutinin-stalk binding peptides.

Both HB monomers and dimers successfully inhibit the hemagglutinin-stem binding antibody, C179 vs. that indicated in the ‘no inhibitor’ control. At the same effective concentration, both the dimers inhibited the binding of stem targeting antibody, C179 to a greater extent than the monomers. More specifically, a fivefold decrease in signal was observed with the HB36.6 dimer (15 repeats of GGSGG) vs. that seen with HB36.6, and a fourfold decrease in signal was observed with the HB80.4 dimer (15 repeats of GGSGG) vs. that seen with HB80.4.

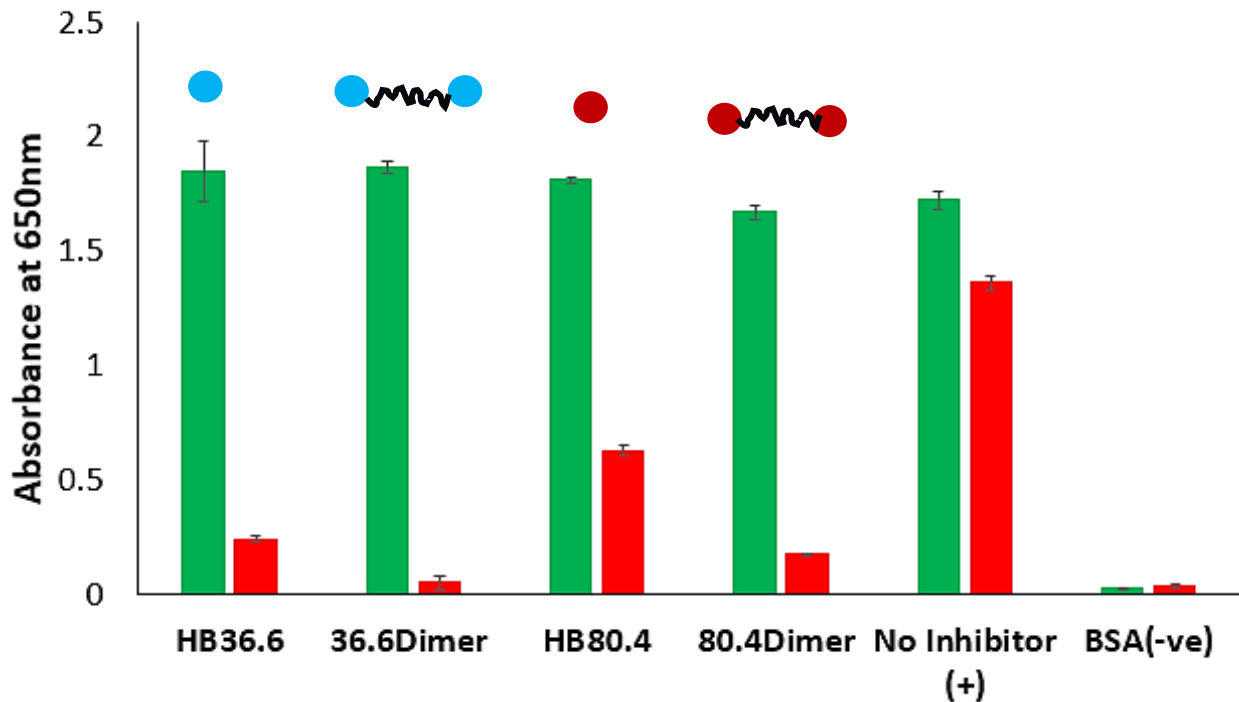


Figure 25: ELISA data showing enhanced inhibition of stalk-binding antibody C179 (■) by dimeric constructs at the same “per-HB” concentration (of 0.1mg/ml) as monomeric peptides. Anti-head antibody PY102 (■) binding is not affected by the presence of either the HB monomers or dimers.

3.4. Future Work and Conclusion:

Dimers of both HB proteins with 15 repeats of -GGSGG- linkers were bacterially expressed. The optimum linkers for both HB36.6 and HB80.4 were found to be 60 repeats and 20 repeats of GGSGG respectively. Although 15 repeats of GGSGG were the less than optimum for both constructs based on optimum linker length calculations, they were used to prove the concept that polyvalency provides for the making of more potent inhibitors that bind to hemagglutinin with higher avidity, and that can block the binding of competing antibodies more effectively than the monomeric HBs could. Polyvalent constructs with a larger number of repeats of -GGSGG- were difficult to express.

In future work, we will

- i) Perform a dose-response study to assess and compare the IC_{50} of the dimers vs. that of the monomers.
- ii) Compare the ability of the homodimers and monomers to neutralize influenza viruses *in-vitro* and *in-vivo*.

4. Multivalent conjugates that target an HIV co-receptor, CCR5

4.1. Introduction:

The goal of this project was to make polyvalent antagonists that bind to different sites on CCR5 as potential inhibitors of HIV infection. CCR5 is a G-protein coupled receptor, which has a key role to play in the cellular entry of HIV. The crucial sites on CCR5 for HIV entry are the N-terminus and the second extra-cellular loop (ECL2). The N-terminus of CCR5 aids in gp120-CCR5 binding; antibodies against the N-terminus inhibit this process [52], [53]. ECL2 plays a vital role in the process of HIV infection as it allows for conformational changes in the *env* to occur so as to permit membrane fusion; anti-ECL2 mAbs have successfully prevented HIV infection [52], [53].

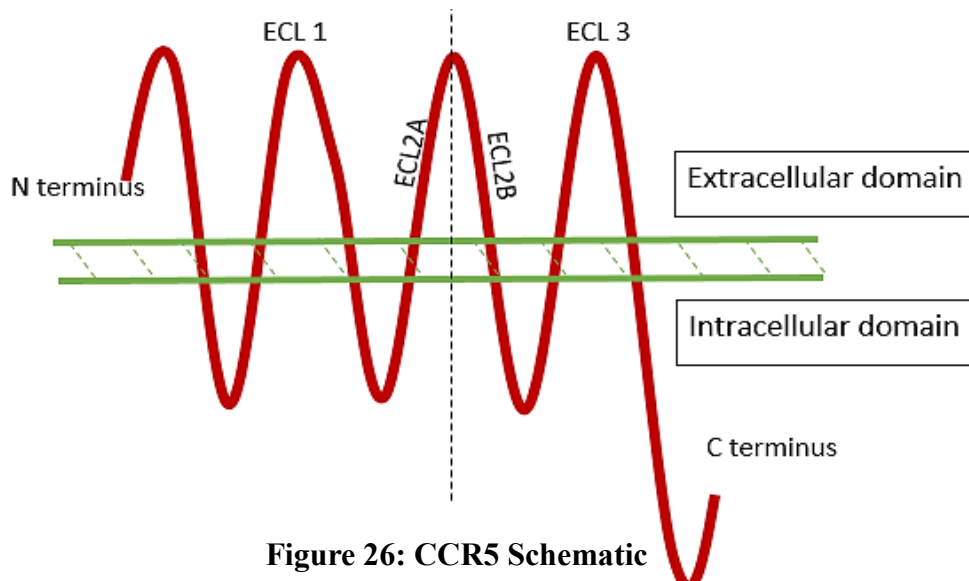


Figure 26: CCR5 Schematic

Leukotoxin E (LukE) interacts directly with the second extracellular loop (ECL2) loop of CCR5 [53], [54]. The interaction of LukE with CCR5 was inhibited by CCR5 ligands, Maraviroc (anti-HIV drug), and antibody 45531 directed against ECL2 (C-terminal side) [37]. Literature was studied to identify other CCR5 binders that would not hinder the binding of LukE to CCR5.



Figure 27: Structure of CCR5 receptor (PDB 4MBS) with the RoAb13 (at N term, in red) and RoAb14 (2 points on ECL2, min yellow) binding sites highlighted

Two antibodies were selected; RoAb13 and RoAb14. RoAb13 binds to the first 8-12 residues on the N term of CCR5 (figure 27), whereas RoAb14 binds to two sites on ECL2 which lies on either side (N-terminal and C-terminal) of the ECL2 receptor [38]. RoAb13 was expressed using mammalian cell culture to ensure that it shows binding to the N terminus of CCR5.

To design polyvalent molecules comprising Luke, RoAb13 and RoAb14, -GGSGG-, polyglutamic acid (PGA) chains or PEG would make appropriate linkers, as they can be made to span a large range of possible RMS end-to-end distances. The dimers that we designed were: Luke-RoAb13, Luke-RoAb14 and Luke-Luke and Luke multimers with PGA linkers. Since attaching whole length antibodies would make the entire construct bulky and difficult to bacterially express, single chain fragment variable (scFv) versions of the same were used. ScFvs are fusion proteins consisting of a variable heavy chain and a variable light chain of an antibody, which are connected by a GGSGG linker [54]. Both Leukotoxin E and the heterodimers, Luke-RoAb13 and Luke-RoAb14, were expressed bacterially [55]–[57]. The heterodimers were linked with a GGSGG

linker with 15, 20 and 30 repeats of the -GGSGG- unit, while the LukE homodimer was made using 5000Da PEG linker via azide-DBCO (Dibenzocyclooctyne) conjugation chemistry.

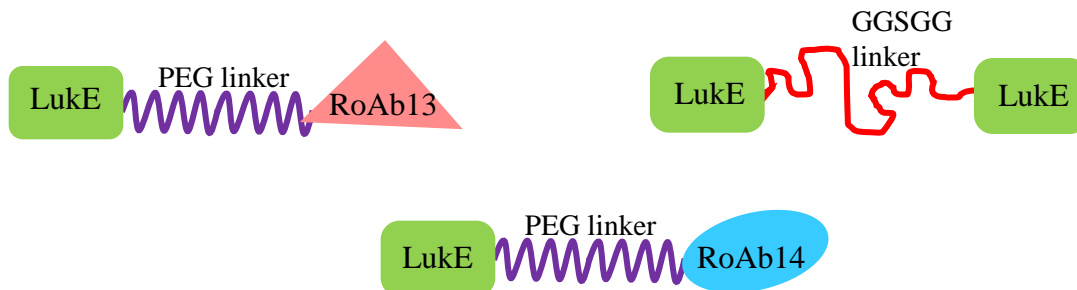


Figure 28: Schematic of CCR5-binding dimers

To prepare PEG based homodimers of LukE, we chose to use ‘azide-DBCO’ copper free Click chemistry, since it has been reported that this reaction gives good yields, is highly selective, has good physiological stability and can be carried out in a wide range of buffer conditions and pH values [58]. PEGs containing two reactive DBCO (Dibenzocyclooctyne) groups were commercially purchased.

To introduce the reactive azide into Leukotoxin E: an unnatural amino acid was incorporated into the protein, on the C-terminal end where the stop codon was replaced with a ‘TAG’ codon or the ‘amber stop codon’, which can be made to code for the unnatural amino acid (p-Azido Phenylalanine) [59], [60]. The unnatural amino acid was supplemented to the medium during bacterial expression of Leukotoxin E. For the successful incorporation of this amino acid, an orthogonal translation system was used; the vector ‘pEVOL’ was co-transformed along with the pET28b(+) vector that contained the gene insert for Leukotoxin E[60]. The pEVOL vector coded for both aminoacyl-tRNA synthetase(s) (aaRS) and suppressor tRNA [59]. Aminoacyl tRNA

synthetases aid in the conjugation of the amino acid to the corresponding tRNA, only after which it is shuttled to the respective site on the mRNA [61]. The suppressor tRNA is unique in that it is only aminoacylated by this specific synthetase encoded in the pEVOL vector, thus allowing for orthogonality, and the incorporation of a specific amino acid.

The overall Leukotoxin E sequence was modified from ‘LukE-GGSGG-Hisx6-STOP’ to ‘LukE-GGSGG-**Amb**-Hisx6-STOP’. The incorporation of the Amb codon between the hexahis tag and the GGSGG linker was crucial as it was a check for us to ascertain if the unnatural amino acid was truly incorporated into the protein sequence as we expected. If the ‘TAG’ codon was read as an unnatural amino acid codon, p-Azido Phenylalanine (F*) would be successfully inserted and the translation would continue to incorporate the hexahistidine tag, only after which the translation would terminate – such a protein would selectively bind to a Ni resin during affinity purification. If the ‘TAG’ codon was read as a STOP codon instead, translation would cease before translating the hexahistidine tag and such proteins would not bind to the Ni resin in the purification process and would be eliminated in the flow through.

PGA (polyglutamic acid) linkers as the name suggests, contain multiple copies of glutamic acid. The free -COOH groups of the glutamic acid act as a reactive site for conjugation to amino-ethyl DBCO via EDC-NHS chemistry; this step is called “PGA activation”. At the end of the activation step, the PGA chain had multiple DBCO groups randomly attached to the scaffold, since chemical conjugation techniques made it difficult to control spacing and valency of reactive groups on the scaffold. 0.5% and 2% molar ratios of LukE-F* to monomer of glutamic acid were mixed to generate polyvalent LukE conjugates.

4.2. Materials and Methods:

4.2.1. Bacterial Protein Expression:

- xi) The plasmid containing the Leukotoxin E or the dimer sequence was transformed into the NiCo21 (DE3) cell line (catalog# C2529H) according to the protocol suggested on the NEB (New England BioLabs) website.
- xii) After transformation, the bacteria were allowed to grow in SOC (super optimal broth) media (without antibiotic) for 1 h at 37 °C. 500µL of this culture was used to inoculate a fresh 5mL pre-autoclaved LB (Luria Bertani) media stock (with antibiotic: kanamycin) near a flame, and was incubated at 37 °C with shaking, overnight.
- xiii) 1L LB media was prepared that evening (by dissolving 25g of LB media powder in 1L culture flasks with distilled water). The media was autoclaved and set aside.
- xiv) Scale up: The following morning, 1 mL of antibiotic (kanamycin) was added to the scaled-up media near a flame, to maintain sterile conditions. Subsequently, 1 mL of inoculum (that was left overnight at 37°C) was added to the 1L flask.
- xv) The flask was placed in a shaker incubator and left at 37°C for 4 to 5 hours. This is when the bacteria are allowed to grow until it reaches logarithmic phase.
- xvi) 400 µM of freshly prepared IPTG solution was added per liter of inoculated media after which the temperature of the shaker incubator was reduced to 16°C, and the culture was allowed to incubate overnight.

Note: To express a protein with an unnatural amino acid (p-azido phenylalanine) a similar expression protocol was followed except for the following changes: the pEVOL vector (chloramphenicol resistance) had to be co-transformed along with the expression vector. Also, other than the 1 mM IPTG that was added to the culture, both 0.02% w/v

- of arabinose and 1 mM of p-azido phenylalanine (dissolved initially in DMSO to facilitate dissolution) was added alongside.
- xvii) The following day, the cells were pelleted by spinning at 6000xg for 1 h, while the supernatant was discarded.
 - xviii) The pellets were made to undergo repeated freeze-thaw cycles first to lyse cells. 50mL of lysis buffer (50 mM Phosphate, 500 mM NaCl, 25 mM imidazole, 0.2 mg/ml DNase, 1mM PMSF, pH 8.0) was added to a 1g pellet.
 - xix) The pellet was homogenized in the lysis buffer and then sonicated (to completely lyse cells) for 5 minutes in an ice bath to ensure that proteins do not get denatured due to over-heating.
 - xx) After cell lysis, the cell debris was spun down at 20,000xg for 1h. The supernatant (contains the proteins) was collected. The pellet should be discarded only if the protein is not expected to be present in inclusion bodies.

4.2.2. *Mammalian culture – Protein expression:*

Antibody fragments were expressed using mammalian cells, more specifically 293F cells. These cells were grown in suspension culture and seeded at a density of 3×10^5 to 5×10^5 cells/mL for each pass to maintain the cell line.

Note: All cell culture was done in strictly aseptic conditions, in a BSL-2 cabinet.

The protocol for transfection has been taken from the literature for the product sold by Thermo Fisher Scientific called ExpiFectamine 293. The protocol has been re-iterated below for the sake of clarity. Also, mammalian suspension cell culture was performed in disposable 125mL Erlenmeyer flasks.

- i) The transfections were done in batches of 30 mL. For each transfection/batch, on the day before the transfections were carried out: 60 million cells were seeded in 30 mL of Expi293 serum-free media (or at a density of 2×10^6 cells/mL) and incubated at 37°C in a humidified atmosphere of 8% CO₂ in air on an orbital shaker rotating at 125 rpm. The viability must be above 95% for effective transfection and protein yields.
- ii) After 24 hours, the cell count/viability was assessed with Trypan blue stain.
- iii) For each transfection, 75 million cells were seeded in 25.5 mL of Expi293 media (the volume was made up to 25.5 mL with fresh media) in a 125 mL Erlenmeyer flask.
- iv) The DNA-transfection reagent complexes were made as follows:
 - a. 30µg of plasmid DNA was diluted using OPTI-MEM reduced serum media (Cat. no. 31985-062) to a final volume of 1.5 mL.
 - b. 81 µL of transfection reagent was diluted using OPTI-MEM media to a volume of 1.5mL and was incubated for 5 minutes. Increasing the incubation time reduces transfection efficiency.
 - c. The diluted DNA was added to the diluted transfection reagent to a final volume of 3mL and mixed gently. This solution was incubated at room temperature for 20-30 mins. This allows the DNA to form complexes with the transfection reagent.
- v) The DNA-transfection reagent was added to the seeded flask and mixed gently. This was incubated at 37°C in a humidified atmosphere of 8% CO₂ in air on an orbital shaker rotating at 125 rpm.
- vi) After 16-18 h, 150 µL of ExpiFectamine™ 293 Transfection Enhancer 1 and 1.5 mL of ExpiFectamine™ 293 Transfection Enhancer 2 was added to the flask and returned to the incubator for 6-7 days.

- vii) Since the antibodies were secreted into the media, the media was harvested and proteins were purified using the Ni-NTA agarose resin.

4.2.3. *Protein Purification:*

The proteins being expressed have a His tag on either the N- or C- terminus, which can be used to purify them using a Ni column/resin using the principle of affinity chromatography.

- vii) A fresh Ni Agarose column must be used per protein. The column was washed with plenty of water to remove the 20% alcohol storage buffer and equilibrated with ~ 5 column volumes of equilibration buffer (50 mM Phosphate, 500 mM NaCl, 25 mM Imidazole, pH 8.0).

Note: A high NaCl concentration is maintained i.e. 500 mM to minimize non-specific adsorption of irrelevant proteins onto the Ni-NTA resin.

- viii) The lysate was passed through the column, while the flow through was collected.
- ix) The column was washed once again with ~ 3 column volumes of equilibration buffer.
- x) 10 column volume washes were performed with 50 mM and 100 mM imidazole buffers, pH 8.0. All washes were collected. The 100 mM wash was collected as fractions.
- xi) Finally, the protein was eluted with 10 column volumes of 400 mM imidazole wash, while collecting fractions.
- xii) Gels were run on the fractions to assess which fraction had the protein of interest with desired purity. The absorbance at 280 nm was taken to determine the protein concentration.

4.2.4. *Activity testing using Fluorescence Activated Cell Sorting:*

- i) 100,000 cells (either CCR5+ 293T cells or control 293T cells) were plated per well in a 96-well plate (25µL of a cell suspension at $4 \times (10)^6$).

Note: Make sure to save some cells (both CCR5+ and the control 293T) for optimizing the gate selection and voltage on the Cell Sorter.

- ii) 50µL of known concentration of inhibitor was added to the cells and was incubated at room temperature for 30 minutes.
- iii) This was followed by the addition of 25µL containing 10 nanomoles of FLSC (Full Length Single Chain) to each well and was incubated at 4°C for 30 minutes.

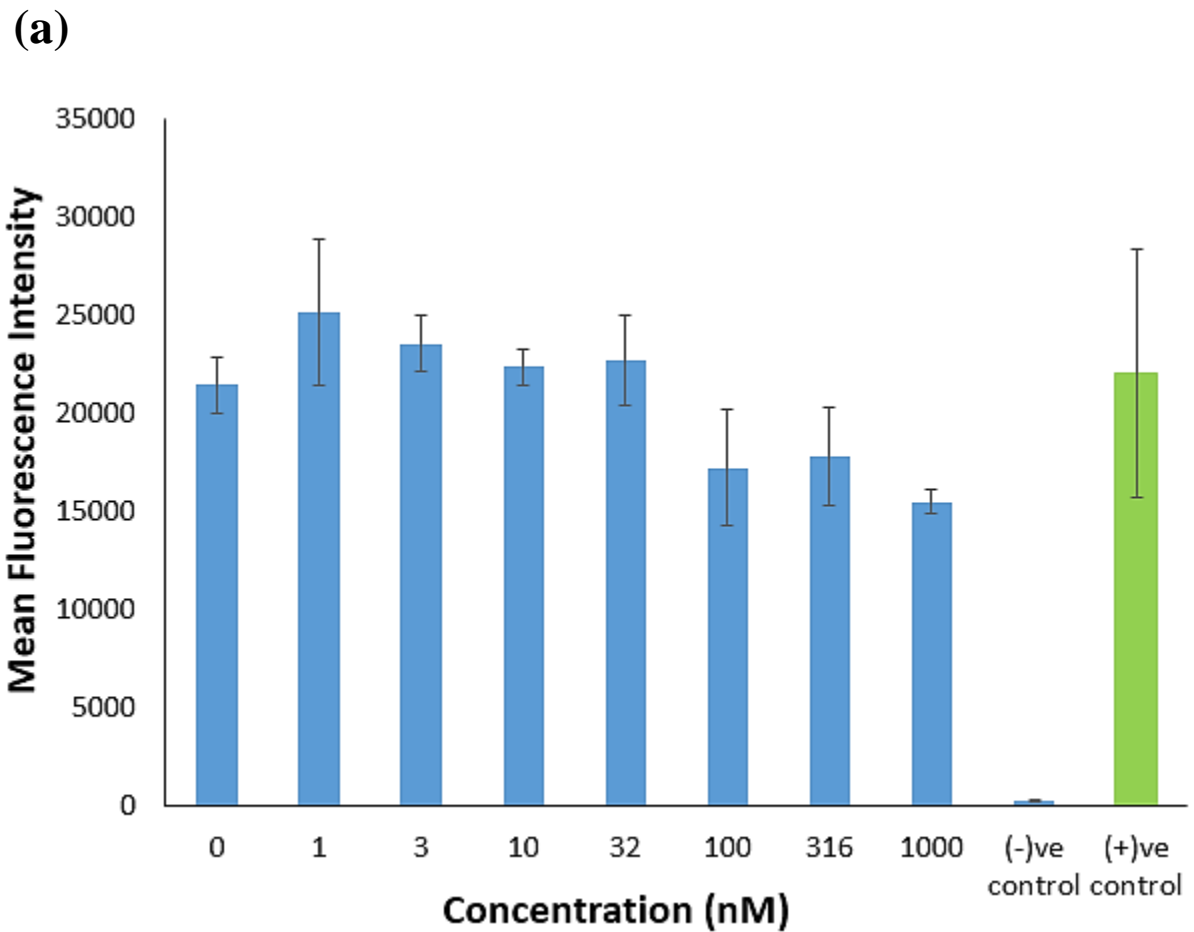
Note: FLSC is a fusion protein consisting of the HIV's envelope glycoprotein gp120 and the human CD4; along with a human Fc region (for detection) [62], [63]. FLSC interacts with CCR5 at similar sites as HIV; at the N terminus and the Extra Cellular Loop 2 (ECL2).

- iv) The cells in the plate were spun at 400xg for 4 minutes, and the supernatant was discarded by gently emptying contents into a sink and tapping the plate dry on a paper towel (carefully to not dislodge the cells by tapping too hard). The cells were resuspended with 100µL PBS.
- v) Step iv was repeated for a wash and the cells were resuspended in 100 µL of PBS. The plate is ready to be run on the Cell Sorter (run on High Throughput Screening mode/Plate reader mode).

4.3. Results:

4.3.1. Expression and Purification:

Leukotoxin E was successfully expressed bacterially and the RoAb13 antibody was expressed in mammalian cell culture. They were initially tested for binding to CCR5+ 293T cells using Fluorescence Activated Cell Sorting. As shown in figure 29, LukeE and RoAb13 showed binding to CCR5 positive cells.



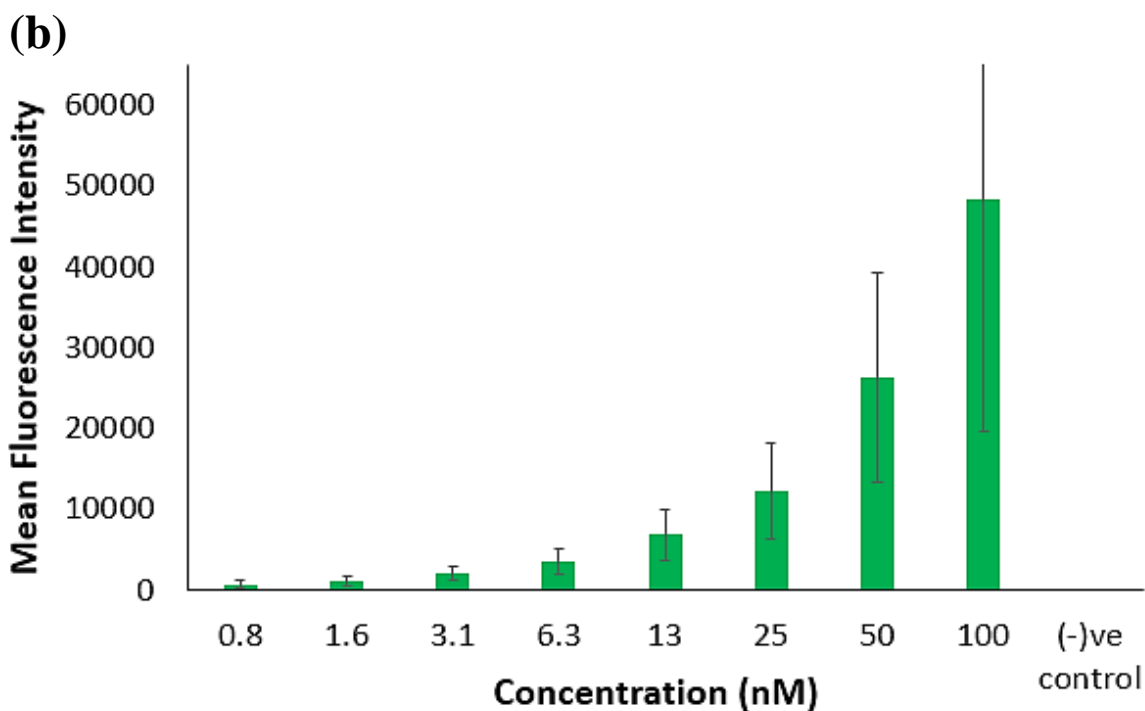
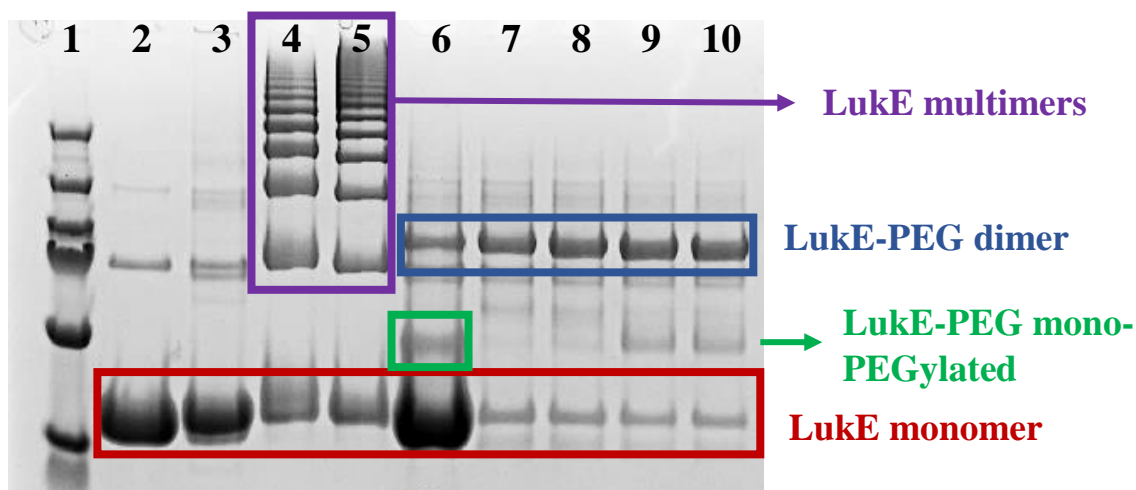


Figure 29: Binding activity of monomeric Leukotoxin E (a) and antibody RoAb13 (b) binding on CCR5+ 293T cells

Subsequently, both LukE monomer and LukE-F* were expressed along with the six heterodimers: LukE-75-R13, LukE-100-R13, LukE-150-R13 (15, 20 and 30 repeats of -GGSGG- respectively), and similarly LukE-75-R14, LukE-100-R14, LukE-150-R14, where R13 and R14 are abbreviated versions of the RoAb13 and RoAb14 scFv. For simplicity, the heterodimers have been referred to in plots ahead (FACS data) without LukE being explicitly mentioned i.e. as 75-R13, 100-R13 and so on.

LukE-F* was used to make conjugates with both di-DBCO 5k PEG and PGA. To prepare the PGA scaffold-based conjugates, two relative concentrations of LukE-F* were used: 0.5% and 2% molar ratios of LukE-F* to monomer of glutamic acid (or DBCO groups, assuming 100% conjugation of the amino-ethyl DBCO and carboxyl groups of glutamic acid) on the PGA scaffold. As shown in lanes 4 and 5 in Figure 30,

the reaction resulted in a mixture of polyvalent LukeE-PGA conjugates differing in valency (i.e., in the number of LukeE proteins per polymer chain). These conjugates were tested as is (without additional purification). To make homodimers with PEG, LukeE-F* and di-DBCO PEG were mixed in a 5:1 molar ratio (i.e. a 2.5:1 molar ratio of LukeE-F* to reactive DBCO groups) to promote preferential formation of dimeric product over that of the mono-PEGylated product. The result is shown in figure 30 in lane 6.



**Figure 30: SDS-PAGE gel; Contents of lanes appear in the order:
 Lane 1: ladder, Lane 2: LukE, Lane 3: LukE-F*, Lane 4: PGA-LukE 0.5%,
 Lane 5: PGA-LukE 2%, Lane 6: mixture of LukeE-F* and di-DBCO PEG (before
 SEC) – homodimer conjugation, Lane 7-10: select fractions after first round of SEC
 on the homodimer conjugation mixture**

For the purification of the homodimers, two rounds of size exclusion chromatography were found to be necessary to extract the dimeric product from the mixture; the first step used a prep-scale ‘superdex 16/600 200pg’ column and the second round used a ‘superdex 200 10/300 GL increase’ column. The SEC plot for the first round is shown in figure 31.

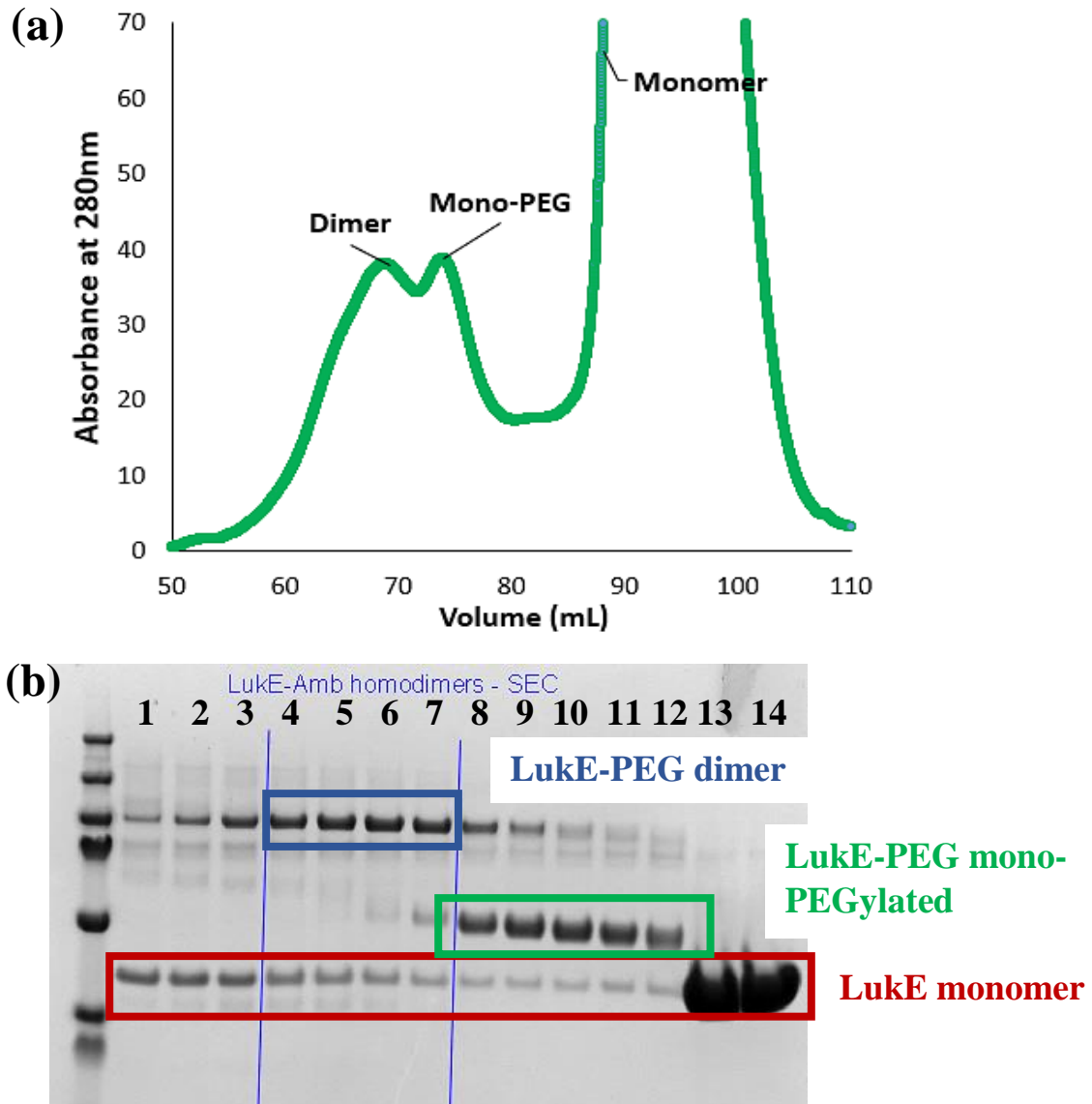


Figure 31: The figure shows (a) an SEC chromatogram for the purification of the LukE homodimer from the mixture of dimeric, mono-PEGylated product and monomer and (b) SDS PAGE gels on select fractions obtained after the first round of SEC purification

The first round of SEC did manage to separate a large amount of mono-PEGylated product and unreacted monomer, although some amount of LukE monomer remained – so another round of SEC was necessary to improve purity. Fractions corresponding to lane number 4-7 in figure 31 (b) were pooled to run through SEC once again.

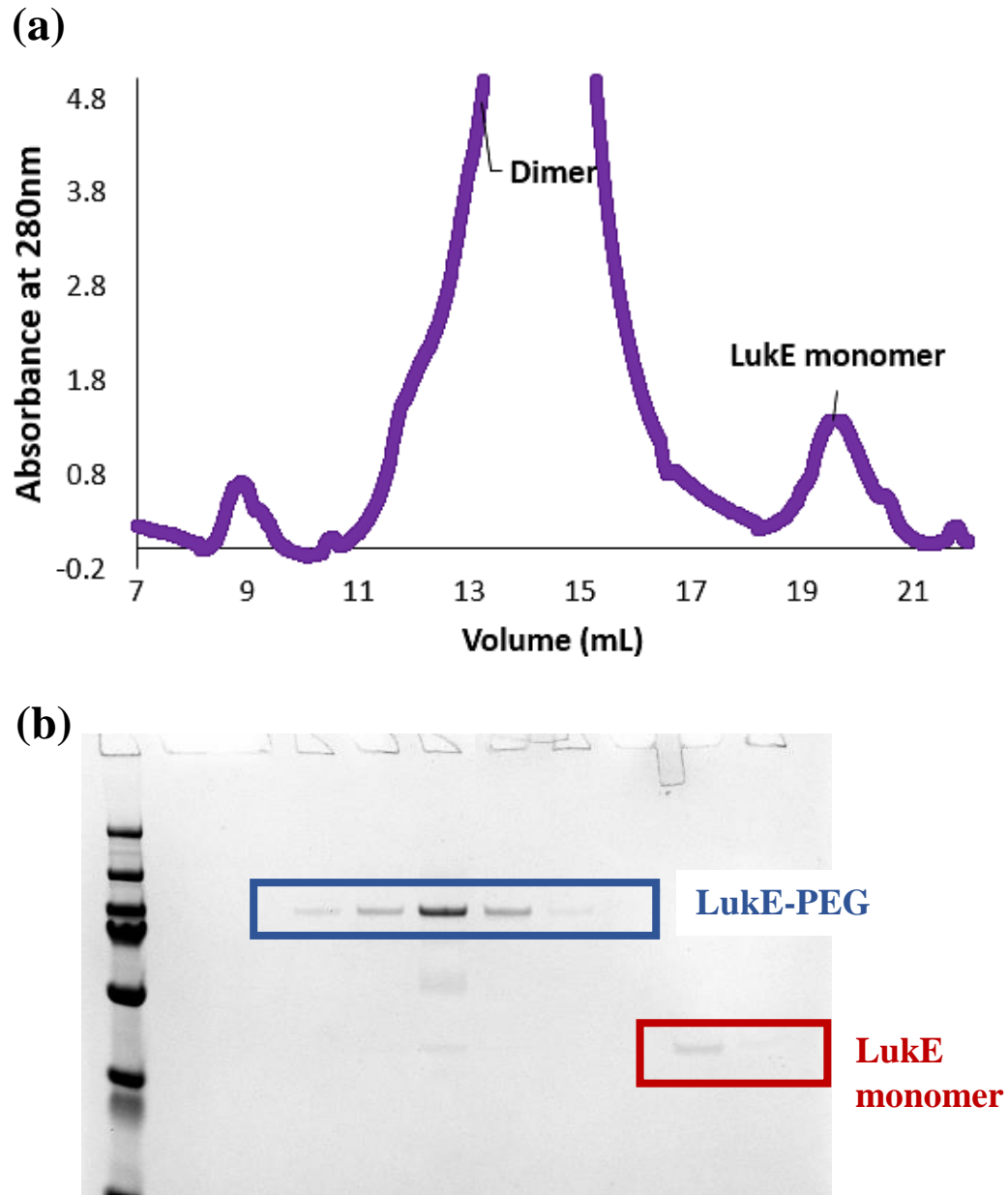


Figure 32: The figure shows (a) an SEC chromatogram for the purification of the LukE homodimer from the mixture of dimeric and monomer and (b) SDS PAGE gels on select fractions obtained after the second round of SEC purification – Pure dimer is obtained

As seen in figure 32 (b), the second round of SEC yielded relatively pure LukE homodimer. The conjugation was started with 10 mg of LukE; after allowing for the reaction to complete and two rounds of size exclusion chromatography with selecting

only certain fractions to carry forward, 300 μ g of homodimer was obtained, which was sufficient for the FACS assay.

4.3.2. FACS assay – Inhibition of FLSC binding:

The nine homodimers, heterodimers and multimers were tested for binding to CCR5 (+) 293T cells by competing with FLSC binding. This was to probe whether we observe enhanced avidity for the receptor with these polyvalent conjugates vs. the monomer (LukE or LukE-F*, depending on the case). The results are shown in figure 33 (a) and (b).

Of the conjugates tested, the most promising candidates were the 0.5% PGA-LukE and all three LukE-R14 heterodimers, of which LukE-75-R14 showed the most inhibition and LukE-150-R14, the least. Based on the inhibition assay, it was determined that LukE had an IC_{50} of $\sim 1.3\mu$ M, whereas the LukE-R14 heterodimers had IC_{50} s below 78nM (lower concentrations need to be tested to determine a more accurate IC_{50}), indicating a more than 17-fold improvement in avidity. Even at 78nM, $\sim 85\%$ inhibition was observed for LukE-75-R14 and LukE-100-R14. They prove to be promising candidates that should be characterized further. Based on the inhibition data for LukE multimers on the PGA scaffold (0.5%), the IC_{50} was $\sim 1.3\mu$ M, as compared to that of LukE-F* (IC_{50} of $\sim 7.5\mu$ M), indicating a 6-fold improvement in inhibitory activity.

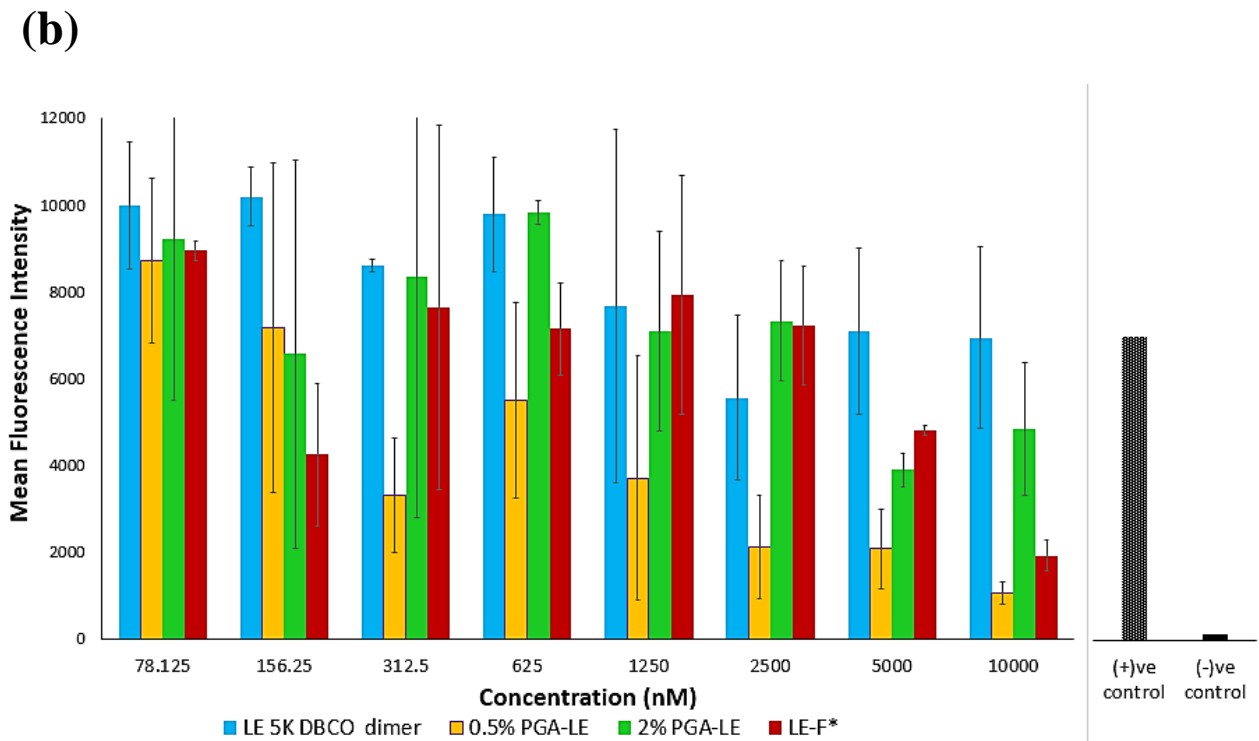
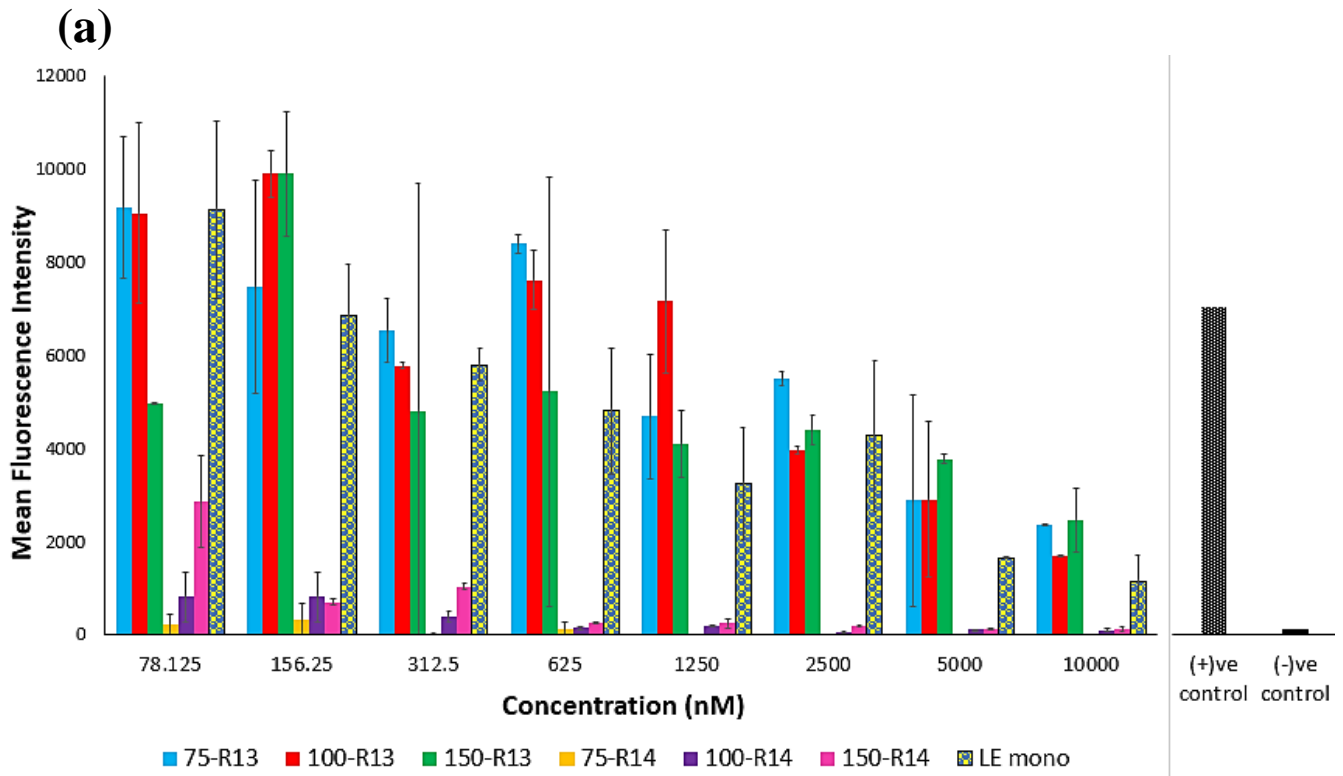


Figure 33: FACS assay – Inhibition of FLSC binding (a) with Luke heterodimers vs. Luke monomer and (b) Luke homodimer and multimers vs. Luke-F*

4.4. Future Work

4.4.1. Repeating the FACS assay:

The Luke-RoAb14 heterodimers should be tested at even lower concentrations on a FACS assay against FLSC to get an accurate estimate of the IC_{50} . Also, my colleague Ammar Arsiwala is currently working on making RoAb14 monomer (SUMO fusion) so as to facilitate successful expression the scFv version of RoAb14 in *Escherichia coli* bacteria [64], [65]. This would be an important control to include in further tests.

4.4.2. Synergy tests – probing for polyvalency:

While repeating the FACS assay, as an additional control, one could test inhibition by an equimolar mixture of LukE and RoAb14. The results would help confirm a role for divalency in enhancing avidity, i.e., test whether joining the two proteins by a linker of appropriate length will enhance avidity for the receptor relative to a mixture of two monomeric proteins.

4.4.3. In-vitro testing against HIV:

The Luke-RoAb14 heterodimers and the Luke-PGA (0.5%) multimers will be tested against HIV by our collaborators at the University of Toronto along with appropriate controls.

4.5. Conclusion and Discussion

In conclusion, the LukE-RoAb14 heterodimers and the LukE-PGA (0.5%) multimers proved to be the most promising candidates in an FLSC inhibition-based FACS assay. The LukE-RoAb14 heterodimers indicated improvement in avidity to more than 17-fold whereas the LukE multimers, 6-fold. The FACS assay will be repeated to better characterize the IC_{50} values of these heterodimers. Also, RoAb14 monomer scFv would also be attempted to be expressed with the help of a SUMO fusion tag. These CCR5 binders would be tested for inhibition of HIV *in-vitro* by our collaborators at the University of Toronto.

5. Conclusion

In this work, polyvalency has been used to design influenza vaccines and inhibitors targeting influenza hemagglutinin and an HIV co-receptor, CCR5. I have developed a strategy for displaying an antigen (hemagglutinin) polyvalently on the surface of an inert scaffold (PEGylated carbon nanotubes) in a controlled orientation. Our goal was to re-direct the immune response towards certain conserved epitopes, with the goal of making a ‘universal’ flu vaccine that would offer protection against a wide variety of viral strains. We have also optimized the antigen purification process by incorporating a size exclusion chromatography step following a metal affinity chromatography step. Constructs presenting these purified antigens polyvalently will soon be tested *in-vivo*.

I have also made polyvalent molecules that targeting hemagglutinin and CCR5, which have shown enhancements in avidity relative to the corresponding monomeric ligands. The dimeric hemagglutinin-binding constructs inhibited the binding of stalk-directed antibodies to hemagglutinin by a significantly greater extent than the monomeric proteins at the same total protein concentration. Heterodimeric and multimeric CCR5-targeting inhibitors have shown some promising initial results; the most promising being Leukotoxin E-RoAb14 heterodimers which were significantly more effective than monomeric Leukotoxin E at inhibiting the binding of a gp120-CD4 fusion protein (called FLSC; Full Length Single Chain) to CCR5. Polyvalency is thus a promising approach to design influenza vaccines and inhibitors of both influenza viruses and HIV.

Appendix: A

Materials and Methods:

A.1. *Cutting and COOH functionalization of single walled carbon nanotubes (SWNT):*

- i) 20mg of SWNT was weighed out (single walled carbon nanotubes, Unidym) and thoroughly dispersed into a concentrated acid mixture of sulfuric acid and nitric acid in the ratio of 3:1, where the total volume of acid was 4 ml acid/mg NT (i.e. 80mL).

Note on safety measures:

- Make sure you have personal protective equipment on (lab coat, gloves, and safety glasses).
 - Make sure that the weighing out of NTs is done in a fume hood.
 - Before weighing, remove any static on the container the NTs are to be weighed into (using a static gun); static disperses NT particles.
 - Clean up the area with a wet wipe after weighing the NTs.
 - Wear a double layer of nitrile gloves while measuring out acids.
 - Try to avoid acid spills. In case of spill: (a) prevent it from entering drains (b) dilute it with water, and then neutralize with soda ash (sodium carbonate) or calcium oxide. Absorb neutralized spill with absorbent material and subsequently discard.
- ii) The nanotubes were sonicated in this acid mixture in an ice bath for a total of 21-22 hours (over a span of two days; ~ 11 hours each day of cutting), to reduce their length from $>1\mu\text{m}$ to 100-500nm. The ice in the sonicator bath was not allowed to completely melt and was replenished every 20-30 mins.

Note: The sonication should be done under a fume hood too, to minimize the formation and dispersion of aerosols. Make sure that 1. The water in the sonication bath is up to the optimum level as indicated within the bath, and 2. The acid level inside the flask matches that of the water level outside the flask to as to have maximum effect of the sonication. After “day one” of cutting, the acid-nanotube was quenched in 2L chilled DI water overnight (DI water was collected and chilled at 4°C ~ 4 hours before. This suspension had to be concentrated down to ~ 5 mL and added to a fresh batch of concentrated acid mixture (prepared as done so previously) to resume cutting the following day.

- iii) After the acid-based NT cutting was complete, the reaction was quenched, by adding the acid mixture to 2L of chilled DI water. The COOH-NTs were filtered and re-suspended in 100 mL of DI water. This was allowed to sonicate for 30 min – 1hr; as much time as it takes to re-suspend the NT clumps before diluting it into 2L of DI water, and the said process is repeated. Repeated washes of NTs were performed until the filtrate was neutral in pH (~3-4 washes, final pH of ~ 6, closely matching the DI water available via the grey tap).
- iv) Finally, a concentrated solution of COOH-NTs was made and freeze dried to remove the water. This will help determine the NT yield.

Note: When NTs in the dried form require to be weighed, make sure it is done under the fume hood.

Note: The SWNTs are purchased ‘raw’, and are cut with concentrated acid mixture of sulfuric and nitric acid. Acid “Cutting” is useful not just to size down the tubes but also to ensure that there are –COOH groups that are generated over the NT surface. The –

COOH group improves solubility and provides for a site for further functionalizing the NTs. 100-500nm would seem like an optimum size range for the NTs so that the antigen-coated NTs will be immunogenic, yet small enough (i.e. < 500nm) so as to not be easily engulfed and eliminated by the reticulo-endothelial system.

A.2. *Freeze-drying protocol for carbon nanotubes:*

- i) An empty glass vial is weighed with and without the cap, and the weight is noted down. The nanotubes obtained after cutting are concentrated down to ~ 10 – 15 mL and stored in a 20 mL glass vial. This is placed at -80°C for ~ 2 – 3 h, until all the liquid freezes.
- ii) The vial is taken out of the freezer and the cap is kept aside, and replaced with an aluminum foil that wraps the mouth of the vial – with the edges pressed against the rim of the vial. One can also use parafilm to seal the rim, over the foil. A couple of fine holes are punches on the top of the vial.
- iii) This vial is placed in the freeze drying container (glass) and sealed off with the rubber top/stopper. Make sure a metal connector is affixed to the top of the rubber stopper (which would be used to connect it to the main drying unit). On the drying unit: Make sure the valve connecting the low pressured unit to the atmosphere are sealed off initially (the triangular notch on the rotating port is 180°(opposite) from the site where the metal connector is to be inserted), after which the metal connector (connected to the glass container) is inserted into one of the closed ports. After the flask is firmly attached in its place, the valve is opened (the triangular notch is brought to point towards the insertion port) and the pressure within the glass container drops and equilibrates with the low pressure of the system. One can observe the pressure suddenly

rise when the unit is just attached and observe the slow dip in pressure as the pressure in the glass container is finally brought to equilibrium with the main system. The drying process will take anywhere between 2 – 4 days depending on how much water was used to re-suspend the nanotubes.

A.3. *PEGylation of COOH-functionalized NTs:*

- i) The NTs were resuspended in 25 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH 4.7) by making a 1 mg/mL solution and the solution was sonicated for 1-2 hrs. The cut NTs should appear more soluble than the raw NTs.
- ii) 9.1 mg of sulfo-NHS (N-hydroxysulfosuccinimide) per mg of NT were added to the well-suspended NTs and sonicated for 5 min.
- iii) This was followed by the addition of 30.5 mg of (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) EDC per mg of NT and briefly sonicated. Then, a stir bar was added to the reaction mix and the reaction was vigorously stirred for ~20 mins. This is the stage where the COOH groups are being activated for the attachment of biotin-PEG2000-amine groups.
- iv) In the meantime, a (>20mg/ml) concentrated solution of biotin-PEG2000-amine (20 mg of BPA/mg of NT) was prepared in 100 mM phosphate buffer (pH 8.0), such that the volume of the BPA solution was the same as the NT solution in MES. This is so that the final pH is ~ 7.4-7.5 which would allow the reaction to go to completion. Increasing final pH is not recommended as this will cause the activated ester group to get quickly hydrolyzed and will prevent successful conjugation of BPA. The reaction was allowed to go to completion at RT for at least 3 hrs, or overnight at 4 °C.

- v) After reaction completion, the solution was given a 3 min spin at 1000xg to remove any larger aggregates that should be discarded. The supernatant was carefully collected and washed to remove the unreacted BPA. This was done by spinning the solution at 504,000xg (70,000rpm) using the 70Ti fixed angle rotor in an ultracentrifuge for an hr. or until the PEG-NTs pellet. The supernatant will contain the excess free BPA. The supernatant was discarded carefully without disturbing the pellet and then the pellet was resuspended in fresh buffer (de-ionised water). The washes were repeated ~ 5 times so that the tubes are sufficiently washed.

Note: Make sure that the tubes that will be put in the ultracentrifuge rotors are precisely balanced upto +/- 10mg.

- vi) The pellet was collected and resuspended in sterile water. The PEG-NTs can be stored at 4°C. To calculate the yield, the absorbance of a dilute solution was taken at 808 nm such that the absorbance scales linearly. The $A_{808\text{nm}}$ can be multiplied by the dilution factor and then divided by $0.0465\text{mg l}^{-1}\text{ cm}^{-1}$ (Extinction coefficient for SWNT) for calculating the NT concentration [2].

Note: Addition of biotin-PEG2000-amine helps in solubilizing the tubes further and the free biotin is useful for further conjugations. Besides, the toxicity of SWNTs has been shown to be dependent on their surface functionalization [2], [3]. PEG functionalization has shown to reduce the systemic toxicity of raw SWNTs [4]–[6]. The unreacted BPA must be separated from the NTs, else they will hamper with the conjugation steps ahead (i.e., the streptavidin addition stage).

A.4. *Conjugation of Streptavidin and HRP-biotin (test molecule):*

- i) For 1 mg of biotin-PEG-NTs, large excesses (10 mg) of streptavidin was used to saturate the surface biotin on NTs. The 1 mg of biotin-PEG-nanotubes (at 200 μ g/ml) was mixed in small batches (200 μ L, added dropwise with intermittent mixing over 2-3 mins) to a 2 mL solution of 5 mg/mL streptavidin (in PBS). This mixture was allowed to incubate on a shaking platform for ~ 30 mins.

Note: This mixing must not be done all at once to avoid crosslinking and aggregation/clumping of NTs. The ideal case is where the NT-PEG is well covered with streptavidin, such that as many streptavidin sites are kept free as possible to allow maximum loading of HRP or HA.

- ii) The excess streptavidin was removed using a sucrose gradient of 4%, 10% and 30% w/v sucrose (or 4g, 10g and 30g respectively in 100mL) which was spun at 175,000xg or 32000rpm (using a swinging bucket SW32Ti rotor) in an ultracentrifuge. (Sucrose gradient preparation: The sucrose was layered from bottom to top; that is, 9 mL of the 4% w/v sucrose solution was added first to the ultracentrifuge tube. This was followed by the addition of 9 mL of the 10% sucrose layer, *under* the 4% sucrose layer with the help of a syringe and long needle. Lastly, 9 mL of the 30% sucrose layer was added below the 10% sucrose layer with the help of the same long needle and syringe. Try to maintain a steady hand while layering the gradients to minimize mixing of two adjacent layers). The free streptavidin is held within the layers of the gradient, whereas the streptavidin coated NTs (strep-NT) form a pellet. This spin was allowed to go up to ~ 18 hrs (less time could affect the yield, while more could allow the free streptavidin to settle along with the pelleted NTs) so that the nanotube yield of NT-Strep is ~ 50-60%.

- It is essential that the sucrose solutions be removed carefully and discarded without disturbing the NT pellets – this can be done by carefully aspirating the sucrose solution from the top, all the way to the bottom of the gradient. If the pellet seems firm (which it usually does), the tube can be tilted to draw out the free sucrose solution. The pellet was re-suspended in 20 mM phosphate buffer (pH 7.4). Sonication in bursts, can be done to aid in the re-suspension, if necessary. The NT concentration can be calculated by taking the absorbance at 808 nm, which should be multiplied by the dilution factor and then divided by $0.0465 \text{ mg l}^{-1} \text{ cm}^{-1}$ (Extinction coefficient for SWNT) [2].
- iii) The strep-NT batch was split into two; to one batch (sample), biotinylated HRP (horseradish peroxidase – a test molecule here, to assess whether loading is possible) was mixed and to the other batch (control), a 2 mg/ml solution of biotin (at high excess of biotin, to block all the free sites of streptavidin) was added. The samples were kept on a rocking platform. After half an hour, biotinylated HRP was added to the second batch (control).
 - iv) The sample and control batches were put through a sucrose gradient of 4%, 10% and 30% w/v sucrose and spun at 32000 rpm in an ultracentrifuge to remove the excess HRP-biotin that can interfere with ELISAs for HRP. The NT-HRPs (sample) or the NT-BBs (biotin-blocked controls) were collected as a pellet.
 - v) A TMB (3,3',5,5'-tetramethylbenzidine)-based ELISA was performed to assess loading of HRP onto NTs. For this ELISA, serial dilutions of both the sample and control were loaded onto 96 well, clear flat bottom plates. Each well had no more than 100 μL of solution. Similarly, serial dilutions (of known mass/concentrations of HRP) of HRP-biotin (for the standard curve) were also loaded onto the plate. To these wells, 100 μL

of TMB solution was added and the absorbance was taken in a plate reader at 652nm. If HRP is present, it would give a blue coloration with TMB. The amount of HRP in the sample and control could be estimated from the plotted standard curve.

A.5. *Insect culture – Expression of hemagglutinin (antigen) [7]:*

The cell lines used, SF9 and High Five, were maintained as suspension culture for all passages. When animating frozen cell stocks, the cell count might be too low to begin with suspension culture – so it is better to begin them as adherent culture initially (if there are ~ 1 – 3 million viable cells in the thawed stock) and then switch over to suspension culture when the viable cell count is high enough (more than 9 million viable cells). Maintaining insect cells as suspension culture proves to be relatively easier because adherent culture need to be dislodged from the flasks by tapping the sides (for every passage) – which introduces a lot of shear to the cells and can cause cell death.

Remember to maintain sterile conditions for all cell culture techniques. **Signs of contamination:** Declining cell health or if cell viability is severely hampered in successive passages, either the passage number is too high (i.e. not to exceed passage no. 25, or maintain the same culture for more than 3 months) or the culture could be contaminated. It is a good idea to start a fresh pass from a frozen cell stock during such an instance. Sometimes, contamination can be visualized on a microscope using Trypan Blue stain – the background might appear ‘grainy’ (as opposed to a clear background, when the cells are not contaminated) or you might be able to see smaller cells on the same slide along with the insect cells. Fungal contamination could show up as a small

but visible “floating, fuzzy ball” in suspension culture or will grow to form a “little colorful island” on top of adherent culture. Contamination can also appear to make the cell suspension excessively turbid – more than you would normally see. A color change in the media is also visible at times.

All mutants of hemagglutinin (HT, TT, chimeras) were expressed using the following protocol:

- i) The baculoviral transfer plasmid was transformed into DH10Bac bacteria, which were midiprepmed (PureLink® Genomic DNA Kits, Thermo Fisher Scientific) to yield bacmids.
- ii) SF9 cells were seeded in 6-well plates at a density of 2×10^5 cells/cm². The bacmids were transfected into SF9 cells using Cellfectin II transfection reagent. For this, 2µg of the bacmid was diluted in serum free TNM-FH (with 1% Pen-Strep and 0.1% Pluronic F68) and mixed with 6µL of Cellfectin II. This was mixed and allowed to incubate for 20 mins at room temperature.
- iii) The SF9 cells would have stuck to the bottom of the plate by this point. The supernatant of the seeded SF9 cells was removed and replaced with 2 mL serum free TNM-FH. The bacmid-reagent complex was added to this mix, and the plate was gently agitated for 5 mins to facilitate mixing. The media was changed after 6 hours to 2 mL of full TNM-FH (10% FBS, 1% Pen-Strep and 0.1% Pluronic F68). The plate was incubated at 28°C without CO₂ for 6 days, and was occasionally checked to observe signs of infection (cells appear larger, enlarged nuclei, easily detach from the plate).

- iv) The media was harvested after 6 days (centrifugation at 2000xg, 5mins) and this contained the “P1” or the initial baculoviral stock. Such a stock of baculoviruses can be stored at 4°C.
- v) This P1 stock was used to generate subsequent ‘amplified’ baculoviral stocks. The SF9 cells were seeded in large T175 flasks at a density of 2×10^5 cells/cm², by allowing them to settle for 20 min and subsequently replacing the media with 50 mL TNM-FH with reduced serum (3% FBS). To this, 400 µL of P1 was added, and the flask was swirled to allow even distribution of the baculoviral inoculum. The flask was incubated at 28°C without CO₂ for 6 days, while occasionally checking for signs of infection.
- vi) The media was harvested by centrifugation at 2000xg for 5 min to yield the “P2” stock. The baculoviruses could be amplified as required and be used for expression.
- vii) Hemagglutinin expression: For a 50mL expression, ~ 25 – 30 million High Five cells were harvested from a pass (centrifugation at 100xg for 7 mins). These cells were re-suspended with 4.5 mL of baculoviral stock of P3 and the cell suspension was incubated at room temperature for 20 min. In the meantime, 46 mL of fresh SFX medium was placed into a disposable 125 mL Erlenmeyer flask and 500 µL of a 6 mM stock solution of biotin (stock solution made in DI water, sterile filtered, with the minimum amount – a few drops – of 10N NaOH to enhance solubility of biotin) was added to this flask (final biotin concentration was 50 µM).

Note: Swirl-mix media with biotin well before adding cells to media, to ensure that the pH is uniform throughout.

Note: Always test well-in-advance (before beginning expression) that the pH of the biotin stock does not alter the pH of the insect media. For this: Mix the 500 µL of a 6 mM

- stock solution of biotin solution to 46 mL of SFX media and compare the pH of this mixture vs. that of the SFX media without the biotin solution added. The pH would have barely changed at all. This is a crucial step as the pH of the media is close to the pI of hemagglutinin (pI ~ 6.4) and increasing the pH of the media could possibly cause loss of protein due to precipitation at the pI.
- viii) After the 20-minute incubation, the cell suspension was added to the SFX media, mixed well, and incubated at 28°C without CO₂ for 72 – 96 hrs.
 - ix) The media was harvested by centrifuging the suspension at 2000xg for 10 min. The supernatant contains the hemagglutinin since the plasmid had a signal peptide to trigger secretion into the media. Hemagglutinin was purified using Ni-NTA resin.

*** *Alternate method of Insect cell transfection using transfection reagent:*

- HA expression was also attempted using two transfection agents (i.e. without the use of baculoviruses): 1. Gene Juice (EMD Millipore) 2. XtremeGene HP (Sigma). HA could not be expressed with Genejuice, even after trying to optimize expression conditions. XtremeGene HP was successfully used to express hemagglutinin (wt. HA, HT HA and TT HA) using SF9 cells. High Five cells gave a relatively lower yield when paired with this transfection reagent. The transfection protocol used for Xtremegene HP is as recommended by Sigma. The following protocol has been modified from their suggested protocol from their product info sheet available online:
- i) X-tremeGENE HP DNA Transfection Reagent, DNA and diluent (BacVector media, EMD Millipore, serum free) was allowed to equilibrate to +15 to +25°C. The X-tremeGENE HP DNA Transfection Reagent vial was briefly vortexed.

- ii) The DNA was diluted with appropriate diluent (e.g., serum-free medium) to a final concentration of 1 μg plasmid DNA /100 μL medium (0.01 $\mu\text{g}/\mu\text{L}$) and gently mixed. (Use a minimum of 100 μl of diluent; lower volumes may significantly decrease transfection efficiency)
- iii) 3 μL of the X-tremeGENE HP DNA Transfection Reagent per μg of DNA (3:1 = μL reagent: μg DNA was used, but other ratios such as 2: 1 or 4:1 could also be attempted) was pipetted directly into the medium containing the diluted DNA without coming into contact with the walls of the plastic tubes and gently mixed. (To avoid adversely affecting transfection efficiency, do not allow undiluted X-tremeGENE HP DNA Transfection Reagent to come into contact with plastic surfaces. Do not use siliconized pipette tips or tubes.)
- iv) The transfection reagent: DNA complex was incubated for 20 minutes at +15 to +25°C. (Some reagent: DNA ratios and cell types may require longer incubation (15 - 30 min). Determine this for your particular cell line and the ratio used.)
- v) In the meantime, SF9 cells were seeded at a density of 1×10^6 cells/mL in fresh BacVector media (serum free, EMD Millipore). The transfection complex was added to the cells in a dropwise manner. The wells or flasks were gently swirled during the addition of the complex to ensure even distribution throughout the suspension. (Once the transfection reagent: DNA complex has been added to the cells, there is no need to replace with fresh medium - as may be necessary with other transfection reagents)
- vi) Following transfection, the cells were incubated for 18 – 72 hours before measuring protein expression. The duration of incubation will depend on many factors, including the transfected vector construct, the cell type being transfected, the cell medium, cell

density, and the type of protein being expressed. After the incubation period, the proteins were purified using the Ni-NTA resin.

A.6. *Protein Purification:*

Hemagglutinin has a His tag at the C- terminus, which was used to purify it by affinity chromatography using a Ni column/resin. This step was followed by size exclusion chromatography to separate the trimer fraction (desired) from larger protein aggregates.

- i) A fresh Ni Agarose column must be used per protein. The column was washed with plenty of water to remove the 20% alcohol storage buffer and equilibrated with ~ 5 column volumes of equilibration buffer (chilled PBS, pH 8.0).
- ii) The media supernatant was incubated with the Ni resin for 2-4 hours at 4°C on a rocking platform, after which the mixture was poured through an empty column and the resin was allowed to settle in it while the supernatant flowed through.
- iii) The column was washed with copious amounts of wash buffer (15mL x 4 times) (50 mM Na₂HCO₃, 300 mM NaCl, 20 mM imidazole, pH 8) after which the bottom of the column was capped with a stopper.

Note: The pH of the buffer for washes/elution and all further handling is best maintained at 8.0 and not below 8.0. The pI of hemagglutinin ~ 6.4. Below pH 6, HA tends to undergo conformational change. Physiological pH can also promote aggregation. Swartz et al. (PNAS, 2014) found that basic pH are better to minimize aggregation (they recommend working closer to pH 10).

- iv) 3 mL of elution buffer (50 mM Na₂HCO₃, 300 mM NaCl, 300 mM imidazole, pH 8) was added to the column and allowed to incubate for 5 min, after which it was collected. This was repeated three times to collect a total of three fractions.
- v) Spin filter prep: In the meantime, a spin filter was washed with copious amounts of de-ionized water and chilled PBS was added to it, and was spun at 3000xg for 20 min at 4°C. This can help minimize the amount of protein that non-specifically sticks to the filter.
- vi) The fractions were pooled, aside from keeping 20 µL of each for characterization by SDS PAGE, and the volume was made up to 15 mL, after which it was concentrated using the ‘prepared’ spin filter down to ~ 1 – 1.5 mL. The filtrate was discarded while fresh chilled PBS was added to the retentate to make up the volume to 15 mL and the spin was repeated → this is the “first wash” to lower the imidazole concentrations in the buffer. This wash was repeated twice with chilled PBS (spun at 3000xg for 20 min at 4°C). The final concentrated volume with HA was brought down between 800 µL – 1 mL which was injected into the SEC system (saving ~ 20 µL for an SDS PAGE gel).
- vii) This concentrate was passed through a size exclusion chromatography (SEC, AKTA, Superdex 200 10/300 GL increase). The trimer eluted at ~ 9 mL whereas the aggregate eluted at ~12 mL.
- *** The “trimer” fraction collected after SEC should be handled very carefully. Store at 4°C or chilled conditions continually. Even when holding the fraction in your hand while carrying it from bench to bench try to maintain chilled conditions. If one has to quickly bring it from one point to another – say, AKTA to the fridge/other storage unit: try not

to hold the Eppendorf tube firmly within your palm as that could raise the temperature.
Hold the tube on the very top.

**** While running the SEC, always try to end up collecting most of the trimer within *one* fraction. This is important because, HA has to be as concentrated as possible in that single eluted fraction as is. Concentration at this stage is *strongly discouraged*, as it is observed to promote aggregation.

**** It would be ideal to do the conjugations onto Streptavidin coated nanotubes the very same day as when the SEC is completed. This will prevent the HA from possibly aggregating by letting it sit overnight (even if stored at 4°C). The HA yield was quantified (and compared) by running an SDS PAGE gel on the imidazole fractions, the concentrated fraction and the SEC fractions and comparing their intensity to that of varying concentrations of BSA.

**** To quicken the SDS PAGE staining procedure, the dye can be mixed with the gel and microwaved for 30 s → this will reduce the incubation time from 1 h to 15 min. The same trick can be used to quicken de-staining as well.

A.7. *Conjugation of HA, followed by Western blots:*

- i) The steps outlined in section 2.2.3 were repeated to make more strep-NTs. This strep-NT batch was split into two; to each batch of 500 µg of streptavidin-NTs, 750 µg of HA (head biotinylated or tail biotinylated) was mixed. This mixture was allowed to incubate for 45 min – 1hr. The biotinylated HA (HT or TT) was expressed and purified as mentioned in sections 2.2.5 and 2.2.6. The excess/free HA was removed using a sucrose gradient of 4%, 10% and 30% sucrose by spinning at 175,000xg in an

ultracentrifuge for ~ 15.5 – 16 h (increasing spin time can cause free HA to settle along with the NT pellet). It is essential that the sucrose solutions be removed carefully and discarded without disturbing the NT pellets – this can be done by carefully aspirating the sucrose solution from the top, all the way to the bottom of the gradient. If the pellet seems firm (which it usually does), the tube can be tilted to draw out the free sucrose solution. The pellet was re-suspended in 20mM phosphate buffer (pH 7.4). Again, the nanotube concentration of HA-NT was calculated by repeating the above procedure of taking the absorbance at 808 nm and repeating above-mentioned calculations.

- ii) A western blot was performed to estimate loading of HA onto NT: a range of known amounts of HA-NT was taken (in terms of weight of NTs in ~100s of ng). Also, varying known amounts of HA alone were also used (these served as standards to help estimate the amount of HA loaded onto NTs). All samples were boiled in SDS at 80 °C in a PCR machine for ~ 30-40 min after which they were characterized by gel electrophoresis. The proteins in the samples entered the gels, while the nanotubes remained in the wells itself. The proteins were transferred from the gels onto nitrocellulose membranes (for the western blot) and blocked in 5% BSA solution using a blot transfer apparatus. The primary antibody (1:2000 dilution in 1% BSA solution) used for the blot was an influenza HA anti-head antibody (PY102) obtained from our collaborators from the Icahn School of Medicine at Mount Sinai. The secondary antibody used was an anti-mouse goat HRP-conjugated IgG (1:10,000 dilution in 1% BSA solution). The relative densities of the bands were estimated using the ImageJ software and plotting the linear range of HA standards loaded onto gels. Once we estimated the ng amounts of loaded

HA, we could calculate the loading by dividing the HA amount (ng) by the NT amount (ng) loaded per well on the gels.

A.8. *Addition of Adjuvants and dilutions before vaccination:*

The HA-NTs (HT-NT and TT-NT) were diluted to make aliquots such that there would be sufficient HA loaded onto the tubes for each mouse to receive 5 µg of HA. The volume injected per mouse per round of injection was 50 µL, out of which 45 µL was the vaccine and 5µL was the adjuvant, poly I:C. A quick vortexing before and after poly I:C addition was helpful in ensuring proper mixing.

Note: Apart from the conjugates with HA oriented in two ways, a required control required for the *in-vivo* testing of a vaccine is the “vehicle-only” control which should ideally lack the antigen or in our case, HA. The aim here is to show that the vehicle alone does not trigger the broadly neutralizing response. I decided to use PEG-NTs at this stage and not Strep-NTs for the “vehicle-only” control. Streptavidin being ‘more accessible’ to the immune system in the case of the Strep-NTs might not make for an appropriate control because the streptavidin in the case of the HA-NT conjugates would be within the ‘inner layers’ i.e. sandwiched between PEG on one side and HA on the other and closely stacked together on NTs; therefore, streptavidin would not be as easily “seen” by the immune system. Hence, I felt that a more appropriate control would be PEG-NT.

A.9. *In-vivo cH5/1N1 challenge:*

The immunizations and viral challenge experiments were carried out by our collaborators at the Icahn School of Medicine at Mount Sinai. For each type of sample (whether HT-NT, TT-NT, or NT-PEG ‘vehicle-only’ control) five mice were vaccinated, which yields a total of fifteen vaccinated mice. The HA-NT conjugates was administered twice, as one prime and one boost, to BALB/c mice, with a gap of 3 to 4-weeks. After both rounds of vaccination, blood samples were collected for ELISAs to assess anti-stalk antibody titers.

After the second round of vaccination, another 4 weeks were allowed to pass after which the mice were challenged with a ‘cH5/1N1’ virus. CH5/1N1 is a chimeric virus, modified to have the same stalk region as that of H1 hemagglutinin, but a different HA head region (belonging to H5 subtype). After the challenge, the mice were monitored for the loss in their body weight; if the loss exceeded more than 25% of their body weight, they were euthanized.

A.10. *Protein Expression (Hemagglutinin binding proteins):*

- i) The plasmid (pET 28b (+)) containing the HB protein sequence was transformed into the NiCo21(DE3) cell line (catalog# C2529H) according to the protocol suggested on the NEB (New England BioLabs) website.
- ii) After transformation, the bacteria was allowed to grow in SOC (super optimal broth) media (without antibiotic) for 1 hr at 37°C. 500µL of this culture was used to inoculate a fresh 5 mL pre-autoclaved LB (Luria Bertani) media stock (with antibiotic: kanamycin) near a flame, and was incubated at 37 °C with shaking, overnight.

- iii) 1L LB media was prepared that evening (by dissolving 25g of LB media powder in 1L culture flasks with distilled water). The media was autoclaved and set aside.
- iv) Scale up: The following morning, 1 mL of antibiotic (kanamycin) was added to the scaled-up media near a flame, to maintain sterile conditions. Subsequently, 1 mL of inoculum (that was left overnight at 37°C) was added to the 1L flask.
- v) The flask was placed in a shaker incubator and left at 37°C for 4 to 5 h. This is when the bacteria are allowed to grow until they reach logarithmic phase.
- vi) 400 µM of freshly prepared IPTG solution was added per liter of inoculated media. The temperature of the shaker incubator was reduced to 16°C, and was incubated overnight.
- vii) The next day, the cells were pelleted by spinning at 6000xg for 1 h, while the supernatant was discarded.
- viii) The pellets were made to undergo repeated freeze-thaw cycles first to lyse cells. 50 mL of lysis buffer (50 mM Phosphate, 500 mM NaCl, 25 mM imidazole, 0.2 mg/ml DNase, 1 mM PMSF, pH 8.0) was added to a 1g pellet.

Note: In place of PMSF, we have more recently started using SIGMA's protease cocktail inhibitor tablets (EDTA free – to make it compatible with the Ni-NTA resin) – as it offers a wider range of types of protease inhibitors. For these, one tablet was dissolved in 100mL of lysis buffer.

- i) The pellet was homogenized in the lysis buffer and then sonicated (to completely lyse cells) for 5 minutes in an ice bath to ensure that proteins do not get denatured due to over-heating. Sonication parameters: 3 s ON and 3 s OFF, 20% amplitude, 4-5mins.

- ix) After cell lysis, the cell debris was spun down at 20,000xg for 1h (or 30,000xg for 30 mins). The supernatant (contains the proteins) was collected. The pellet should be discarded only if the protein is not expected to be present in inclusion bodies.

A.11. Protein Purification:

The proteins being expressed have a His tag on either the N- or C- terminus, which can be used to purify them using a Ni column/resin using the principle of affinity chromatography.

- i) A fresh Ni Agarose column must be used per protein. The column was washed with plenty of water to remove the 20% alcohol storage buffer and equilibrated with ~ 5 column volumes of equilibration buffer (50 mM Phosphate, 500 mM NaCl, 25 mM imidazole, pH 8.0).

Note: A high NaCl concentration was maintained i.e. 500mM to minimize non-specific adsorption of irrelevant proteins onto the Ni-NTA resin.

- ii) The lysate was passed through the column, while the flow through was collected.
- iii) The column was washed once again with ~ 3 column volumes of equilibration buffer.
- iv) 10 column volume washes were performed with 50 mM and 100 mM imidazole buffers, pH 8.0. All washes were collected. The 100 mM wash was collected as fractions.
- v) Finally, the protein was eluted with 10 column volumes of 400 mM imidazole wash, while collecting fractions.

- vi) Gels were run on the fractions to assess which fraction had the protein of interest with desired purity. The absorbance at 280 nm was taken to determine the protein concentration.

A.12. Bacterial Protein Expression (protein with unnatural amino acid p-Azido phenylalanine F):*

- ii) The plasmid (pET 28b (+)) containing the Leukotoxin E-F* was co-transformed along with the pEVOL vector (Addgene reference: pEvol-pAzFRS.2.t1 (Plasmid #73546); chloramphenicol resistance) into the NiCo21 (DE3) cell line (catalog# C2529H) according to the protocol suggested on the NEB (New England BioLabs) website.
- iii) After transformation, the bacteria were allowed to grow in SOC (super optimal broth) media (without antibiotic) for 1 h at 37 °C. 500µL of this culture was used to inoculate a fresh 5mL pre-autoclaved LB (Luria Bertani) media stock (with antibiotic: kanamycin) near a flame, and was incubated at 37 °C with shaking, overnight.
- iv) 1L LB media was prepared that evening (by dissolving 25g of LB media powder in 1L culture flasks with distilled water). The media was autoclaved and set aside.
- v) Scale up: The following morning, 1 mL of antibiotic (kanamycin) was added to the scaled-up media near a flame, to maintain sterile conditions. Subsequently, 1 mL of inoculum (that was left overnight at 37°C) was added to the 1L flask.
- vi) The flask was placed in a shaker incubator and left at 37°C for 4 to 5 hours. This is when the bacteria are allowed to grow until it reaches logarithmic phase.
- vii) 400 µM of freshly prepared IPTG solution, 0.02% w/v of arabinose and 1 mM of p-azido phenylalanine (dissolved initially in DMSO to facilitate dissolution and

subsequently added to culture) were added per liter of inoculated media after which the temperature of the shaker incubator was reduced to 16°C, and the culture was allowed to incubate overnight.

- viii) The following day, the cells were pelleted by spinning at 6000xg for 30 mins - 1 h, while the supernatant was discarded.
- ix) The pellets were made to undergo repeated freeze-thaw cycles first to lyse cells. 50mL of lysis buffer (50 mM Phosphate, 500 mM NaCl, 25 mM imidazole, 0.2 mg/ml DNase, 1mM PMSF, pH 8.0) was added to a 1g pellet.

Note: In place of PMSF, we have more recently started using SIGMA's protease cocktail inhibitor tablets (EDTA free – to make it compatible with the Ni-NTA resin) – as it offers a wider range of types of protease inhibitors. For these, one tablet was dissolved in 100mL of lysis buffer.

- x) The pellet was homogenized in the lysis buffer and then sonicated (to completely lyse cells) for 5 minutes in an ice bath to ensure that proteins do not get denatured due to over-heating. Sonication parameters: 3 s ON and 3 s OFF, 20% amplitude, 4-5mins.
- xi) After cell lysis, the cell debris was spun down at 20,000xg for 1h. The supernatant (contains the proteins) was collected. The pellet should be discarded only if the protein is not expected to be present in inclusion bodies.
- xii) Protein was purified as specified in section A.11.

A.13. *Mammalian culture – Protein expression:*

Antibody fragments were expressed using mammalian cells, more specifically 293F cells. These cells were grown in suspension culture (in Expi293 media) and seeded at a density of 3×10^5 to 5×10^5 cells/mL for each pass to maintain the cell line.

Note: All cell culture was done in strictly aseptic conditions, in a BSL-2 cabinet.

The protocol for transfection has been taken from the literature for the product sold by Thermo Fisher Scientific called ExpiFectamine 293. The protocol has been re-iterated below for the sake of clarity. Also, mammalian suspension cell culture was performed in disposable 125mL Erlenmeyer flasks.

- i) The transfections were done in batches of 30 mL. For each transfection/batch, on the day before the transfections were carried out: 60 million cells were seeded in 30 mL of Expi293 serum-free media (or at a density of 2×10^6 cells/mL) and incubated at 37°C in a humidified atmosphere of 8% CO₂ in air on an orbital shaker rotating at 125 rpm. The viability must be above 95% for effective transfection and protein yields.
- ii) After 24 hours, the cell count/viability was assessed with Trypan blue stain.
- iii) For each transfection, 75 million cells were seeded in 25.5 mL of Expi293 media (the volume was made up to 25.5 mL with fresh media) in a 125 mL Erlenmeyer flask.
- iv) The DNA-transfection reagent complexes were made as follows:
 - a. 30µg of plasmid DNA was diluted using OPTI-MEM reduced serum media (Cat. no. 31985-062) to a final volume of 1.5 mL.

- b. 81 μ L of transfection reagent was diluted using OPTI-MEM media to a volume of 1.5mL and was incubated for 5 minutes. Increasing the incubation time reduces transfection efficiency.
- c. The diluted DNA was added to the diluted transfection reagent to a final volume of 3mL and mixed gently. This solution was incubated at room temperature for 20-30 mins. This allows the DNA to form complexes with the transfection reagent.
- v) The DNA-transfection reagent was added to the seeded flask and mixed gently. This was incubated at 37°C in a humidified atmosphere of 8% CO₂ in air on an orbital shaker rotating at 125 rpm.
- vi) After 16-18 h, 150 μ L of ExpiFectamine™ 293 Transfection Enhancer 1 and 1.5 mL of ExpiFectamine™ 293 Transfection Enhancer 2 was added to the flask and returned to the incubator for 6-7 days.
- vii) Since the antibodies were secreted into the media, the media was harvested and proteins were purified using the Ni-NTA agarose resin as specified in section A.11. It is crucial to note that the Expi293 media is not compatible with the Ni resin as it contains agents that could leach Ni off the resin, therefore the media supernatant must be buffer exchanged with PBS before loading onto the column.

A.14. Activity testing using Fluorescence Activated Cell Sorting:

- i) 100,000 cells (either CCR5+ 293T cells or control 293T cells) were plated per well in a 96-well plate (25 μ L of a cell suspension at $4 \times (10)^6$).

Note: Make sure to save some cells (both CCR5+ and the control 293T) for optimizing the gate selection and voltage on the Cell Sorter.

- ii) 50µL of known concentration of inhibitor was added to the cells and was incubated at room temperature for 30 minutes.
- iii) This was followed by the addition of 25µL containing 10 nanomoles of FLSC (Full Length Single Chain) to each well and was incubated at 4°C for 30 minutes.

Note: FLSC is a fusion protein consisting of the HIV's envelope glycoprotein gp120 and the human CD4; along with a human Fc region (for detection) [8], [9]. FLSC interacts with CCR5 at similar sites as HIV; at the N terminus and the Extra Cellular Loop 2 (ECL2).

- iv) The cells in the plate were spun at 400xg for 4 minutes, and the supernatant was discarded by gently emptying contents into a sink and tapping the plate dry on a paper towel (carefully to not dislodge the cells by tapping too hard). The cells were re-suspended with 100µL PBS.
- v) Step iv was repeated for a wash and the cells were re-suspended in 100 µL of PBS. The plate is ready to be run on the Cell Sorter (run on High Throughput Screening mode/Plate reader mode).

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