# Structure-guided SCHEMA recombination of VRC01-class antibodies for reduced polyreactivity

Thesis by

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# ABSTRACT

The therapeutic administration of monoclonal antibodies (mAbs) has revolutionized treatment options for many diseases over the last decade. Recent findings from clinical trials have demonstrated that broadly neutralizing antibodies (bNAbs) could have a potential role in the future treatment and prevention of HIV-1. There is a group of broad and potent bNAbs that target the CD4-binding site (CD4bs) on the envelope glycoprotein gp120. These VRC01-class antibodies are notable for both their breadth and potency. The Bjorkman lab has designed a remarkably broad and potent bNAb, 45-46m2, that unfortunately cannot currently be used clinically due to its increased polyreactivity and short *in vivo* half-life.

In this study we designed a SCHEMA-guided recombination library composed of sequence fragments of the VRC01-class bNAbs 45-46m2 and 3BNC117, aiming to create a bNAb that binds strongly to gp120 but is not polyreactive. We endeavored to maintain the strong binding of 45-46m2 while gaining the low polyreactivity of 3BNC117.

Our analysis of this family shuffled library of chimeric antibodies revealed the sequence elements that led to strong binding to gp120 for the chimeras in our library. We also identify three key framework regions that can be modified to significantly reduce polyreactivity. Furthermore, we report three novel chimeras from the family shuffled library that bind as strongly to gp120 as 45-46m2 but are significantly reduced in polyreactivity.

# PUBLISHED CONTENT AND CONTRIBUTIONS

**Chapter 4 :**

Keeffe, J.R., Gnanapragasam, P.N., Gillespie, S.K., Yong, J., Bjorkman, P.J., Mayo, S.L. (2011). Designed oligomers of cyanovirin-N show enhanced HIV neutralization. PNAS; 108(34): 14079-14084. doi: 10.1073/pnas.1108777108.

*S.K.G. conducted the experiments and analyzed the data showing that $CVN_2L0$ is domain swapped in solution. S.K.G. created the original figure 4 (showing that the anti-HIV activity of $CVN_2L0$ correlates with the number of functional binding sites), as well as supplemental figure S3 (showing that the partial binding site knockout mutants do not significantly affect the neutralization potency). S.K.G. wrote a draft to describe the experiments and results that led to figure 4 and S3. This draft was later rewritten and improved by J.R.K. J.R.K. also improved S.K.G.'s original figures (changed the original circles to squares) for publication.*

**Chapter 5:**

Wannier, T.M., Gillespie S.K., Hutchins N., McIsaac R.S., Wu S.Y., Shen Y., Campbell R.E., Brown K.S., Mayo S.L. (2018). Monomerization of far-red fluorescent proteins. PNAS; 115(48): E11294-E11301. doi: 10.1073/pnas.1807449115.

*S.K.G. assisted with characterizing the important red fluorescent protein variants in the library. S.K.G. assisted with collecting absorbance scans, fluorescence excitation scans, fluorescence emission scans, measurements of quantum yield, measurements of extinction coefficients, and thermal stability measurements. S.K.G. also assisted with oligomeric determination by analytical ultracentrifugation.*

Manuscripts in preparation (working titles):

**Chapter 2 :**

Gillespie, S.K., Sievers, S.A., West, A.P., Keeffe, J.R., Bjorkman, P.J., and Mayo, S.L. Structure-guided SCHEMA recombination of VRC01-class antibodies for reduced polyreactivity. [Manuscript in preparation].

*S.A.S., A.P.W., and P.J.B. conceived of the project. J.R.K., P.J.B, and S.L.M. advised on experiments and analysis, and provided helpful discussion. S.A.S. used SCHEMA to design the family shuffled library in silico. S.A.S. used Gibson cloning to create chimeric $V_H$ gene DNA from gene block DNA. S.A.S. also used Gibson cloning to insert the 128 chimeric $V_H$ genes into the vector backbone.*

*S.K.G. conducted all other experiments, analyzed the data, prepared the figures, and wrote the paper.*

**Chapter 3 :**

Gillespie, S.K., Vielmetter, J.G., Keeffe, J.R., and Mayo, S.L. Using high-throughput surface plasmon resonance (SPR-32) to determine the concentration of monoclonal antibodies in crude transfected cell supernatants. [Manuscript in preparation].

*J.G.V., J.R.K., and S.K.G. designed the SPR-32 experiments. J.R.K., J.G.V., and S.L.M. advised on experiments and analysis, and provided helpful discussion.*

*S.K.G. conducted the experiments, analyzed the data, prepared the figures, and wrote the paper.*

# TABLE OF CONTENTS

*Chapter 1*

# INTRODUCTION

It has been over 40 years since the discovery of HIV-1, the virus that causes AIDS. However, HIV/AIDS continues to be a major global public health challenge. By the end of 2023, there were an estimated 39.9 million people living with HIV-1 (UNAIDS, 2024). In 2023, an estimated 1.3 million people became newly infected with HIV-1 (UNAIDS, 2024).

The development of antiretroviral therapy has been a major success in medical research. However, treatment with antiretroviral therapy is lifelong and requires constant access to newer drug regimens (Zaidi, 2024). One reason for this is that antiretroviral therapy does not eliminate latent reservoirs of HIV-1 from the host cells, and the virus can become resistant to the medications (Sun et al., 2024). Another reason is that some patients develop long-term side effects from lifetime usage of these drugs (Chawla et al., 2018). While patients in developed countries have various treatment options, patients in the developing world have access to fewer treatment options (Zaidi, 2024). New methods to treat and prevent infection are urgently needed.

One possible solution might be the use of broadly neutralizing antibodies (bNAbs) for passive delivery to treat and/or prevent HIV-1 infection (Halper-Stromberg et al., 2016). Recent findings from clinical trials have demonstrated the potential role of broadly neutralizing antibodies (bNAbs) in the treatment and prevention HIV-1 (Thavarajah et al., 2024).

There is a group of broad and potent bNAbs that target the CD4-binding site on the envelope glycoprotein gp120. These VRC01-class antibodies are notable for both their breadth and potency, and they share a set of signature features (West et al., 2012). Diskin, Bjorkman and colleagues used structure-based design (Diskin et al., 2011; Diskin et al., 2013) to improve NIH45-46 (Scheid et al., 2011), a more potent clonal variant of VRC01 (Wu et al., 2010; Zhou et al., 2010). This work resulted in the design of NIH45-46$^{G54W}$, 45-46m2, and 45-

46m7, three remarkably broad and potent bNAbs (Diskin et al., 2011; Diskin et al., 2013). Also, the addition of 45-46m2 and 45-46m7 to a mixture of other bNAbs was shown to increase the efficacy of antibody-based therapeutics by restricting potential HIV-1 escape pathways and/or selecting mutant virions with reduced fitness (Diskin et al., 2013).

Unfortunately, NIH45-46$^{G54W}$, 45-46m2, and 45-46m7 cannot currently be used clinically due to their increased polyreactivity and short *in vivo* half-lives. Polyreactivity is correlated with short *in vivo* half-lives because antibodies that bind off-target are eliminated from the circulation by mechanisms involving antigen-mediated clearance (Mager et al., 2006; Wang et al., 2008). There are many unique hypotheses about the biophysical determinants of polyreactivity in antibodies. Some suggestions include: net hydrophobicity (Lecerf et al., 2019), net charge (Rabia et al., 2018), increased prevalence of arginine and tyrosine residues (Kelly et al., 2018; Birtalan et al., 2008), enrichment in glutamine residues (Lecerf et al., 2019), CDR3 length (Prigent et al., 2018), CDR flexibility (Prigent et al., 2018), a shift toward neutrality of the binding interface (Boughter et al., 2020), loop crosstalk in the heavy chain (Boughter et al., 2020), and high isoelectric points (Kelly et al., 2018; Birtalan et al., 2008; Lecerf et al., 2019; Rabia et al., 2018). However, the relative importance of these features is still inconclusive (Harvey et al., 2022; Birtalan et al., 2008). Harvey et al. (2022) recently developed a method for predicting the polyreactivity of a given nanobody sequence based on machine learning models (Harvey et al., 2022). This method also suggests mutations that can be implemented to reduce polyreactivity for the given nanobody (Harvey et al., 2022). However, this method was originally intended only for use in nanobodies (Harvey et al., 2022). A more comprehensive understanding of the sequence features of polyreactive antibodies is still needed. Currently, there is no method that can identify the sequence features of a polyreactive antibody so that the problematic sequences could be modified.

In order to create new variants of 45-46m2 with lower polyreactivity, Stuart Sievers, a former postdoctoral fellow in the Bjorkman lab, and Anthony West, a senior research scientist in the Bjorkman lab, proposed the use of directed evolution by DNA shuffling of a family of related

genes. Family shuffling permits a greater exploration of sequence space than random mutagenesis and makes changes already demonstrated to be compatible with the fold and function of the protein (Crameri et al., 1998). What makes family shuffling possible in this case is that VRC01-class antibodies all bind to gp120 with the same basic geometry despite large sequence diversity. In addition, this class of antibodies includes naturally occurring antibodies with low polyreactivity (Prigent et al., 2018).

In Chapter 2, we describe the construction of a SCHEMA-guided recombination library composed of sequence fragments of the VRC01-class bNAbs 45-46m2 and 3BNC117. Our goal was to create a bNAb that maintained the strong binding to gp120 of 45-46m2, while gaining the reduced polyreactivity of 3BNC117. In addition, we hoped to gain knowledge about the sequence features that result in decreased polyreactivity so that this knowledge could be applied to newly designed antibodies that are not amenable to family shuffling. We used SCHEMA, a computational approach, to accomplish this goal. SCHEMA uses structure-guided recombination to produce large families of protein chimeras that have high sequence and functional diversity (Otey et al., 2006). Chimeras are made by recombining a set of homologous parent proteins at crossover locations chosen to minimize structural disruption (Endelman, et al., 2004). SCHEMA uses the 3D structures of the parent proteins to identify pairs of amino acids that are interacting. Each chimera is characterized by the value E, the number of residue-residue contacts that are broken upon recombination. SCHEMA uses the recombination as a shortest path problem (RASPP) algorithm to minimize the number of contacts that are disrupted when blocks are swapped to generate new sequences (Endelman, et al., 2004). RASPP minimizes E, which improves the chances a chimera will be folded and functional (Meyer et al., 2006).

A key benefit of SCHEMA is the ability to use the “sample, model, and predict” approach developed by Arnold and colleagues (Li et al., 2007; Heinzelman et al., 2009; Romero et al., 2012; Heinzelman et al., 2013). A small sample set of chimeras is characterized, and then this data is used to construct models that accurately predict the properties of every family member in the library (Heinzelman et al., 2013). This approach has been used successfully

to construct libraries of novel chimeric proteins, including beta-lactamases, cytochrome p450s, arginases, fungal cellulases, channelrhodopsins, adeno-associated virus (AAV) capsids, and fungal laccases (Meyer et al., 2003; Otey et al., 2006; Li et al., 2007; Romero et al., 2012; Heinzelman et al., 2009; Bedbrook et al., 2017; Ojala et al., 2018; Mateljak et al., 2019).

In Chapter 2, we pinpoint the sequence elements that led to strong binding to gp120 for the chimeras in our family shuffled library. We also identify three key framework regions that can be modified to significantly reduce polyreactivity. Furthermore, we report three novel chimeras from the family shuffled library that bind as strongly to gp120 as 45-46m2, but are significantly reduced in polyreactivity.

In Chapter 3, we describe a new method to measure the concentration of monoclonal antibodies in crude transfected cell supernatants without the need for antibody purification. We used this method to measure the concentration of the engineered chimeric antibodies in our family shuffled library.

The traditional workflow for generating monoclonal antibodies for use in downstream assays is not practical for generating large libraries of hundreds of engineered antibody variants. A new method for measuring antibody concentration that could obviate the need for affinity-purification would be useful. Additionally, a method that would not require large volumes would be significantly advantageous, as our workflow for generating engineered family shuffled antibody variants would include small-scale transfections performed on a liquid handling robot. Furthermore, a method that could quantify only the folded and functional antibodies in a complex mixture of both active and inactive molecules would be useful for many downstream assays involving monoclonal antibodies.

We sought to test whether high-throughput surface plasmon resonance (SPR-32) could be used to measure the concentration of antibodies in crude transfected cell supernatant, a complex mixture of many proteins. SPR is a label-free optical technique that measures molecular interactions in real time (Jason-Moller et al., 2006).

The concept for this new method is as follows. First, we created a sensor chip with immobilized protein A on 16 of the 32 individual detection spots. The remaining 16 spots were reference spots. These spots were used for reference subtraction to compensate for bulk refractive index differences between the buffer and analyte samples. Next, we created a serial dilution of an affinity-purified and SEC-purified IgG of known concentration. We then performed a run on the SPR-32, where the analyte (IgG) was flowed over the 32 detection spots.

The sensorgrams for the known standard samples were used to create standard reference curves (calibration curves) for all 16 spots with immobilized protein A. We selected a report point, and then the resonance units at the report point for each known sample of the standard curve were plotted against the known concentration. This was done for each spot on the chip that had immobilized protein A. We then determined the equation that best fit the standard reference curves. Using this equation, the concentration of an unknown sample could be inferred from the resonance units at the report point for that unknown sample.

This method allowed us to accurately measure the concentration of expressed antibodies in crude transfected cell supernatants in high-throughput. These measurements were critical for the study described in Chapter 2 because it allowed us to normalize the engineered chimeric antibodies in our library to a standard concentration in preparation for the downstream assays.

In the final two chapters of this thesis, we explore the use of other protein engineering methods applied to unique problems. In Chapter 4, we describe the engineering of obligate cyanovirin-N (CV-N) oligomers to determine whether increasing the number of binding sites would have an effect on viral neutralization (Keeffe et al., 2011). A tandem repeat of two CV-N molecules (CVN2) increased HIV-1 neutralization activity by up to 18-fold compared to wild-type CV-N (Keeffe et al., 2011). In addition, the CVN2 variants showed extensive cross-clade reactivity (Keeffe et al., 2011). My involvement in this research was to create a series of binding site mutants of $CVN_2L0$ in order to show that $CVN_2L0$ is domain swapped

in solution. We showed that the anti-HIV activity of $CVN_2L0$ correlates with the number of functional binding sites, and that the partial binding site knockout mutants do not significantly affect the neutralization potency (Keeffe et al., 2011).

In Chapter 5, we explore protein variant libraries targeted at monomerizing far-red red fluorescent protein (RFP) variants and describe a method to monomerize RFPs of interest (Wannier et al., 2018). This method preserved the fluorescence of the molecule throughout its monomerization, which is in contrast to older break-fix methods (Wannier et al., 2018). This new method also resulted in selective enrichment of bright, far-red monomeric variants (Wannier et al., 2018). In addition, we identified four bright monomeric RFPs, which were among the most red-shifted of any monomeric fluorescent proteins (Wannier et al., 2018). My involvement with this research was to assist in the characterization of the important red fluorescent protein variants in our library. I assisted with collecting absorbance scans, fluorescence excitation scans, fluorescence emission scans, measurements of quantum yield, measurements of extinction coefficients, and thermal stability measurements. I also assisted with the oligomeric determination of the important red fluorescent proteins in our library by analytical ultracentrifugation.

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