A NOVEL PLATFORM TO GENERATE SYNTHETIC VACCINE CANDIDATES

APPROVED BY THE SUPERVISORY COMMITTEE

_____

Ellen S. Vitetta, Ph.D., Professor of Microbiology and Immunology, Director of the Cancer Immunobiology Center

_____

Philip Thorpe, Ph.D., Professor of Pharmacology, Committee Chairman

_____

Ronald Zuckermann, Ph.D., Senior Scientist at Lawrence Berkeley National Laboratory

_____

Robert Eberhart, Ph.D., Professor Emeritus, Department of Surgery

_____

Liping Tang, Ph.D., Adjunct Professor, Department of Surgery

This dissertation is dedicated to the memory of Dr. John M. White, whose advice to "put my oar in the water" launched my scientific ship.

Press on.

A NOVEL PLATFORM TO GENERATE SYNTHETIC VACCINE CANDIDATES

By

Allison Carroll Case

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May 2012

ACKNOWLEDGEMENTS

Foremost, I thank my mentor, Dr. Ellen Vitetta. She is the epitome of scientific excellence, focus, and attention to detail. With her equipping, I will move forward successfully with a firm foundation in science.

I thank my committee members Dr. Robert Eberhart, Dr. Liping Tang, Dr. Philip Thorpe, and Dr. Ronald Zuckermann for their time and willingness to serve on this committee. I thank Dr. Eberhart for his aid in beginning my training at UT Southwestern and for serving as a source of sound advice and support throughout my studies. I thank Dr. Tang for making such a strong impression on me as a student in tissue engineering. I thank Dr. Thorpe for being such a wonderful example of balance and level headedness in science and life. I also thank him for serving as my committee head. Finally, I thank Dr. Zuckermann for demonstrating that science is improved by the sharing of ideas. Under his guidance, the Molecular Foundry serves as a haven for learning, training, and exploration. I thank him for the demonstrating to all he meets that science is fun, that there is room for balance in a successful scientific career, and what a blessing it is to do what you love for a profession.

I thank the members of the “peptoid group”, both past and present, in order of arrival, Kate, Angela Collins, Angela Desmond and Joan Smallshaw. This work was a true collaboration. The tireless efforts and, at times, ingenious thinking of my fellow group mates made working on this project challenging, rewarding and fun.

I thank Drs. Xiaoyun Liu and Laurentiu Pop for sharing their wisdom and their time to train me as a scientist. Our discussions were as much a part of my training as my didactic coursework. In addition, my thanks to Dr. Pop for his unique perspective and interest in others.

My sincere thanks to the Molecular Foundry personnel who taught me everything I know about the practical application of peptoid synthesis. My thanks to Michael Connolly for traveling to Texas to install and modify our peptide synthesizer, to

Helen Tran for her guidance and training, to Rita Garcia for her coordination efforts, and all of the Molecular Foundry employees.

I thank Drs. Maria-Ana and Victor Ghetie for their support and genuine interest in my progression professionally and personally. The memories and experiences from our lunches will remain with me for the rest of my life. I am so please to have met and work alongside you both.

My thanks to Linda Berry who has championed my success over the years, and whose guidance and compassion has helped me achieve my goals.

My thanks to Catherine Holloway, Shea Johnson, and Drew Ivey without whom this project and my graduation would have been impossible.

My thanks to Sara Chirayil and Kevin Luebke for their collaboration, generosity, and manual synthesis of two libraries.

My thanks to Kay Emerson and Peter Antich for keeping the biomedical engineering program going for the years before my arrival and for many years while I attended UT Southwestern. They fought an uphill battle for the duration of their tenure. UT Southwestern, biomedical engineering, and the students are better for your efforts.

My thanks to Dr. Livingston for accepting the challenge to grow the biomedical engineering program into a strong and productive department.

My thanks to Nancy Street for wisdom when it was needed most.

My thanks to Iliodora Pop for her kind words and interest in my success. I thank John Astle for his aid in getting this project off of the ground.

I thank my fellow students in the laboratory, Andrew Bitmansour as a source of humor and diversion; Angela Desmond for the most inspiring and intellectually challenging discussions I've had while at UT Southwestern, and as a hearty travel mate for our two week, sleepless stay at the Molecular Foundry. I thank Dr. Lydia Wu Tsai for her advice and lifelong friendship; Dr. Kate Kim for her ingenious inventions and revelations at the bench; Megan Wachsmann for her insights and

encouragement; Dr. Pavitra Chakravarty for her friendship and constant encouragement; and to Dr. Kim Brooks, Dr. Praveena Selvaduray Marconescu, Angela Collins, Shihong Ma, and Jonathan Tran for their friendship and support.

I thank Kelly Mapes, Ayesha Ahmed, John Gu, Steve Ruback, Jue Yang, Phyllis Barron, and Kelly Dye. It's been said that definition of crazy is doing the same thing twice and expecting different results; in our case we called it research. Thank you for your willingness, perseverance and positive attitude.

I thank my family and friends without whom I would not have succeeded. To my parents, the love and training you instilled in me when I was young equipped me to succeed. The example you set in my youth and today inspire me to do what is right, what is hard, and what is worthwhile. Your loving and unwavering support made this work possible. To the Burke's for their humor, good food, and loving support- I am thankful. To the Case's who endured with patience and hope my pursuit of this degree: thank you for your loving support. My thanks to the Wilson's for encouraging words and genuine interest in my success.

I thank my children for insisting that I stop and smell the roses and for their love. I am thankful for their forgiveness for all of the late nights and for always welcoming me home with hugs, kisses and a laugh.

Finally, I thank my husband, James Case. His tirelessness, selflessness, supportiveness and love enable and empower me to achieve the goals I have for myself and the purposes for which God created me. In a very big way, he has given me all that I've wanted in my life- marriage, children, a PhD, happiness, love and a lifelong ministry together. I look forward to our future together.

A NOVEL PLATFORM TO GENERATE SYNTHETIC VACCINE CANDIDATES

Allison Carroll Case

The University of Texas Southwestern Medical Center at Dallas, 2012

Supervising Professor: Ellen S. Vitetta, Ph.D.

Vaccination remains the optimal means to prevent infectious disease by inducing antibodies that confer protective immunity against the pathogen in question [1-3]. However, there remain viruses against which no effective vaccines exists including human immunodeficiency virus (HIV), West Nile Virus (WNV) and hepatitis C virus (HCV). These viruses and others evade the immune response by undergoing rapid mutations in immunodominant epitopes [4-6]. In addition, although they usually express conserved epitopes that are important for inducing neutralizing antibodies, in many cases these are not immunodominant. Traditional techniques in vaccine development have not been able to overcome these barriers for these and other viruses. Subunit and peptide vaccines are very safe but it is often difficult to identify the key epitopes needed to make them effective.

New approaches to developing safe vaccines that induce broadly neutralizing antibodies are needed. Therefore, the long term goal of this project was to generate vaccine candidates for any virus for which a neutralizing antibody existed or could be made without prior knowledge of the protective epitope(s). Furthermore, we desired a way to administer these vaccine candidates safely and before exposure so as to

induce neutralizing antibodies. To accomplish these goals, we began with the development of a platform to generate synthetic vaccine candidates. This platform consisted of 1) libraries of B cell epitopes or "shapes" prepared by displaying peptoid sequences on beads, 2) neutralizing monoclonal antibodies (MAbs) to select the peptoids that bound to the antibody's antigen-combining site, and 3) protein G dynabeads (PGDs) and a magnet to bind and isolate antibody bound peptoid beads. Any sequences identified in the platform as potential B cell mimetics were further evaluated in two validation assays. The first consisted of a "color screening" assay to determine that the isolated on-bead peptoids were bound by antibody. The second confirmed that these peptoids would fail to be bound by antibody if an excess of the native antigen was added (i.e. that peptoid sequences were bound by the antibody's binding sites).

The major accomplishments to emerge from this study were 1) the creation of an optimized magnetic screening platform for the isolation of peptide B cell epitopes from an on-bead library, 2) a magnetic screening platform optimized for the isolation of peptoid B cell epitopes from a peptoid library, and 3) the identification of potential peptoid B cell epitope mimetics of FLAG peptide from a peptoid library using a MAb. Taken together, a sensitive, specific, and reproducible platform to identify vaccine candidates from a peptoid library was created. This platform is particularly important for viruses like HIV, HCV, and WNV where mutation makes foreknowledge of conserved, neutralizing epitopes difficult.

Once sufficiently large and diverse libraries are created, the B cell epitope mimetics (vaccine candidates) identifiable by this platform will have several advantages over their peptide counterparts. These peptoid-based vaccines are "safe" as there is no potential for reversion, they are less expensive and faster to synthesize than peptides, they are not dependent on the twenty amino acids, and the B cell epitopes identified with this platform can be conjugated to carrier in such a way that the multivalency and immunodominance can be controlled making this platform advantageous both to the generation of new vaccine candidates and in reformulating current vaccines.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
Ahex	Aminohexanoic acid
Alum	Aluminum hydroxide
ARC	Animal Resource Center
BAA	Bromoacetic Acid
BOC	Tert-butyloxycarbonyl
BSA	Bovine serum albumin
CAA	Chloroacetic acid
CNBr	Cyanogen bromide
CHCA	α-hydroxycinnamic acid
CPM	Counter per minute
Da	Dalton
DCE	1,2-dichloroethane
DCM	Dichloromethane
DIC	N', N'-diisopropylcarbodiimide
DMF	N', N'-dimethylformamide
DMSO	Dimethyl sulfoxide
EDT	1,2-ethanedithiol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Eth	O-t-butyl-ethanolamine
Fc	Constant fragment of an antibody

$FLAG^+$	FLAG positive peptide (DYKDDDDK; single letter amino acid code)
$FLAG^-$	FLAG negative peptide (DAKDDDDK; single letter amino acid code)
FMOC	9-fluorenylmethyloxycarbonyl
g	Gravity
g	Gram
h	Hour
H_2O	Water
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate
HCl	Hydrochloric acid
HOBt	N-hydroxybenzotriazole
HRP	Horseradish peroxidase
H_2SO_4	Sulfuric Acid
IACUC	University of Texas Southwestern Medical Center at Dallas Institutional Protocol Review Committee
Ig	Immunoglobulin
Iodogen	Iodo-Gen 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril
IM	Intramuscularly
KLH	Keyhole limpet hemocyanin
L	Liter
M	Molar
M-A-A	FMOC-Methionine- Aminohexanoic acid-Aminohexanoic acid linker
MAb	Monoclonal antibody
MALDI	Matrix Assisted Laser Desorption/Ionization
mcBSA	Maleimide activated BSA
mcKLH	Maleimide activated KLH

MCF	Microcentrifuge
Met	Methionine
mg	Milligram
mg/mL	Milligram per milliliter
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimole
mM/g	Millimole per gram
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N	Normal
N_2	Nitrogen gas
$Na^{125}I$	Radiolabeled iodine
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium bicarbonate
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NMM	N-methylmorpholine
NMP	N-methyl-2-pyrrolidone
NZW	New Zealand white
OD_{280}	Absorbance reading at λ=280
OD_{450}	Absorbance reading at λ=450

OtBu	tert-Butyl ester
OVA	Ovalbumin
PBS	Phosphate-buffered saline, pH 7.4
PBST	Phosphate buffered saline + 0.01% Tween 20
PGD	Protein G dynabeads
PTFE	Polytetrafluoroethylene
QC	Quality control
R5	Five monomer peptoid for rabbit immunizations
RBF	Round bottom flask
RC	Seven monomer peptoid, *in vitro* control for R5 peptoid
RFT5	Mouse anti-CD25 MAb (used as an isotype control antibody)
Rink	Rink amide resin
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RT	Room temperature
Sec	seconds
tBu	tert-butyl
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
Tentagel	TentaGel MB NH_2 resin
TFA	Trifluoroacetic acid
THP	Tetrahydropyranyl
TIS	Triisopropylsilane
TMB	3,3′,5,5′-tetramethylbenzidine
ToF	Time of flight
Tris	Tris(hydroxymethyl)aminomethane

Trt	Trityl group
Tween 20	Polyoxyethylenesorbitan momolaurate
ug	Microgram
uL	Microliter
um	Micrometer
v/v	Volume to volume

I. INTRODUCTION

A. Vaccines

Vaccination saves lives by preventing infection. In the past one hundred years, vaccination was responsible for a 100% decrease in baseline annual deaths attributed to measles, polio, diphtheria, and smallpox [7]. Despite advances in the understanding and treatment of viral infection, in the 216 years since Jenner first vaccinated against small pox, vaccination still remains the optimal means of preventing infectious disease.

Vaccination confers protection against a pathogen *prior* to exposure by inducing the production of neutralizing antibodies that circulate in the blood and are present in the tissues. These neutralizing antibodies prevent the establishment of an infection or can neutralize the toxins made by pathogens. In addition to inducing the production of neutralizing antibodies, vaccination schedules are designed to maintain pools of memory cells to respond to future infection quickly and robustly.

Types of traditional vaccines include those composed of live attenuated pathogens, dead pathogens, pathogen proteins, subunits, carbohydrates or extracts, recombinant proteins or inactivated toxins [8]. **Table 1** summarizes each type including examples, advantages, disadvantages and descriptions.

Although protective, many of these approved vaccines have significant drawbacks [9]. In the case of live attenuated viral vaccines, for example, there is a potential for reversion, which is particularly dangerous to immunocompromised individuals [10]. Dead vaccines require large doses and multiple boosts to confer protection and might only protect against one strain of the virus [9].

Not only are current vaccines suboptimal and potentially dangerous, there are established and emerging pathogens against which no vaccine exists. Examples of these pathogens include human immunodeficiency virus (HIV), West Nile Virus (WNV) and hepatitis C virus (HCV). These viruses and others can evade the immune response by undergoing rapid mutations in immunodominant epitopes [11, 12]. These mutations make vaccination with a dead virus effective against only a few

subtypes of the virus. Vaccination with conserved epitopes responsible for inducing neutralizing antibodies requires prior knowledge of these epitopes.

Table 1: Traditional Vaccines

Vaccine Type	Description	Advantages	Examples	Drawbacks
Live attenuated	Pathogen is attenuated to prevent full-blown infection	Strong immune response because virus replicates in host; strong antigenic stimulation	Polio, mumps, measles, rubella, smallpox, chicken pox and influenza	Insufficient attenuation and reversion are dangerous in immunocompromised individuals
Dead, whole organism	Pathogen is completely inactivated, cannot replicate	--	Cholera, hepatitis A	Large doses with boost and adjuvant necessary Only one viral subtype protected against
Peptide/Protein Subunit Recombinant	Isolation of major antigenic peptides Pathogen proteins expressed in yeast, bacteria or cells	Important epitopes can be isolated and purified	Influenza A and B Hepatitis B	Prior knowledge of protective epitope; Often lacks B cell epitopes; Primes T cells Adjuvant necessary
Carbohydrate / Conjugate Vaccines	Antigenic carbohydrate conjugated to a carrier protein	--	Pneumococcal conjugate	Ineffective in young children Adults make IgM only (no affinity maturation); T_H cell independent activation May require prior knowledge of the epitope
Inactivated Toxin	Inactivated toxin	--	Tetanus	Inactivation can affect immunogenicity

Attempts to overcome these barriers have led to the development of new vaccines such as peptides [13, 14], DNA [15], and anti-idiotypic antibodies [16]. These novel vaccines have disadvantages as well. In the case of peptide vaccines, most are designed to induce T cell responses to aid in killing the infected cells *after* infection [3]. Often peptide vaccines lack B cell epitopes necessary to induce an antibody

response to confer protection *before* exposure. Moreover, small peptides are susceptible to proteases making them less stable than proteins. Lastly, creating peptide vaccines requires prior knowledge of the epitopes that will induce neutralizing antibodies. In the case of anti-idiotypic vaccines, their preparation is labor intensive, difficult, and expensive.

B. Immunogens

To induce the production of immunoglobulin G (IgG) antibodies, a molecule must be *immunogenic* and *contain both T and B cell epitopes.*

Immunogenicity: Immunogenicity is determined by the presence of structural epitopes on the molecule that can be recognized by two different lineages of lymphocytes, B cells, and T cells. The degree of immunogenicity is determined by four factors: foreignness, size, complexity, and degradability. Molecules must be foreign (non-self) to induce an immune response. Errant immune responses against self proteins are the source of autoimmune diseases like Crohn's disease or celiac disease. Secondly, the larger the molecule, the stronger the immune response generated against it. The best immunogens are ~100,000 Da, and molecules less than 1,000 Da are generally non-immunogenic. Thirdly, the complexity of the molecule affects the immune response made against it. In general, immunogenicity increases with structural complexity. Finally, molecules that cannot be degraded are poor immunogens (i.e. peptides of D amino acids).

B and T cell epitopes: B cell epitopes are small "shapes" that that can fit into the antibody binding pocket of a B cell receptor. These epitopes can be linear sequences, but are usually conformational. These conformations can be made by a contiguous sequence of amino acids (continuous epitopes), or dis-contiguous sequences of amino acids (dis-continuous epitopes). Furthermore, in contrast to T cell epitopes, B cell epitopes need not be proteins. They can be carbohydrates, chemicals, small drugs, peptides, etc. B cell epitopes are present on the native molecule, and require no processing to be recognized by the B cell. B cells

recognize B cell epitopes in solution and bind these epitopes as presented on the native antigen.

T cell epitopes are short, linear stretches of amino acids sitting in the grooves of major histocompatibility complex class II (MHC class II) molecules on antigen presenting cells (APC). T cells recognize T cell epitopes only after the native antigen has been degraded and the T cell are epitopes presented on the surface of an antigen presenting cell. In contrast, B cell epitopes are native structures on the outside of a molecule. It is important that the T cell epitopes are long enough to be loaded into grooves of the MHC class II molecule to anchor the peptide. Once loaded into MHC class II, the peptides are presented to the T cells.

T cell help: T cell help is provided to the B cell to stimulate the B cell to proliferate and differentiate into Ig class-switched, antibody secreting plasma cells or memory B cells. In order for a molecule to induce T cell help, a T cell epitope must be physically attached to (or already part of) the molecule.

In the case of a molecule that lacks both B and T cell epitopes, antibodies will not be induced. However, molecules containing B cell epitopes but lacking T cell epitopes ("haptens": small molecules that on their own cannot induce antibody production) can be made immunogenic by attachment to a carrier protein (a rich source of T cell epitopes). Molecules containing T cell epitopes but lacking B cell epitopes (peptides that are not "shapely" enough to contain B cell epitopes) are weak immunogens.

Immune response to an immunogen: Antibody production is induced when a B cell epitope and T cell epitope are present on the same molecule. B cells recognize structures on the molecule and T cells recognize small peptides derived from the molecule. Sometimes, the B and T cell epitope are one-and-the-same. Usually, however, the B cell epitope and T cell epitope are two separate entities located on the same molecule. Regardless, when both B and T cell epitopes are found on the same molecule, the B cell receptor binds the B cell epitope on the immunogen, and the immunogen is internalized, degraded, and recycled to the cell surface in the context of MHC class II molecules discussed above. A T cell, which has already

been activated by an antigen-presenting dendritic cell expressing the same MCH class II-peptide, then recognizes this same MHC class II-peptide on the B cell and binds it. Through a series of costimulatory events between the T and B cell, the former secretes cytokines to aid the B cell in proliferation and differentiation (T cell help). With T cell help, B cells will differentiate into antibody secreting plasma cells and long-lived memory B cells.

Plasma cells confer immediate protection against the immune insult and are specific for the shape bound by the original B cell receptor. The first time an antigen is presented to the immune system, referred to as the primary response, seven to ten days will pass between antigen exposure and the secretion of antibodies from a plasma cell.

Memory B cells, created during the primary response, are also specific for the shape bound by the B cell. These cells are “long lived”, reside in the bone marrow, lymph nodes, and spleen and await re-exposure to the antigen [17]. Memory B cells are stimulated by lower levels of antigen as compared to naïve B cells. When the antigen is re-encountered, memory B cells bind the antigen and respond rapidly. Instead of seven to ten days, antibody is secreted in three to four days. This response is more robust and long lived memory responses are often sufficient to prevent infection and symptoms.

Along with a more rapid and robust immune response following re-exposure to the antigen, a secondary response also produces antibodies with higher affinity for the antigen than those secreted in the primary response. The mechanism responsible for this increased affinity with re-exposure is due to small errors occur in the coding sequences of the immunoglobulin hypervariable region coding genes that occur each time the B cell differentiates and proliferates. Referred to as “somatic hypermutations”, some of the errors yield B cells with higher affinity than the parent B cell; other errors yield clones with lesser affinity than the parent B cell. When the immune system re-encounters the antigen, B cell receptors with productive errors that fit the shape of the antigen bind more effectively and bind more avidly to the native antigen and are rapidly activated by memory T cells. Hence after each new

encounter with the same antigen, the B cell clones with the best fit are selected and the affinity increases.

C. Vaccination-Induced immune responses

All current FDA-approved vaccines induce a protective immune response by presenting the immune system with shapes found on the native antigen. Upon vaccination, B cells that bound such shapes are selected from the vast B cell repertoire. These B cells bind the epitopes on the exterior of the antigen (vaccine), internalize and degrade it, and present degraded vaccine peptides in MHC class II on the B cell surface. T cells bind these peptides in MHC class II, secrete cytokines, and stimulate the B cells to proliferate and differentiate into antibody secreting plasma cells and memory B cells. These antibodies are specific to the shape presented by the vaccine but cross react with the shapes (B cell epitopes) presented on the native antigen (vaccine or pathogen).

Most types of vaccine require booster shots. Booster shots are designed to stimulate memory B cells and select high affinity B cell clones resulting from somatic hypermutation. Each booster shot increases the affinity, speed, level and duration of the immune response. Over-boosting can have a negative impact on anti-pathogen protection since the selected B cell clones are of such high affinity (and specificity) that they no longer cross react with the native epitopes on related strains of a pathogen. Live attenuated vaccines do not require booster shots. Instead, the virus is able to remain within the host, usually dormant but sometimes undergoing proliferation, providing repeated antigenic stimulation over a period of time.

When the vaccine is live attenuated, or dead whole organisms, the shapes presented in the vaccine are also present on the pathogen. Conjugate, subunit and recombinant proteins present pathogen-related shapes out of context of the native pathogen. In the unique case of toxin vaccines, the shapes presented are not those of the infecting bacteria or virus, but of the toxin these secrete. The toxin is modified

such that it is no longer lethal, but retains some big epitopes. This is called a toxoid. Anti-toxoid antibodies neutralize the native toxin.

D. Mutating vs. Non-Mutating Epitopes

When an epitope is non-mutating, each exposure of the immune system to the epitope serves to re-stimulate the immune response by binding to receptors in memory B cells and selecting the highest affinity clones.

In the case of mutating epitopes, each exposure induces a primary immune response. The *Influenza* virus is one example of viral mutations. Each year, predictions are made as to the mutations undergone by the virus over the course of the year. Based on these predictions, a vaccine is made [18]. If the predictions were accurate, vaccination induces a primary response. When the *Influenza* virus is encountered, memory B cells are stimulated and higher affinity B cell clones are selected. The resulting three day antibody response prevents infection and associated symptoms. If, however, the predictions for the mutations undergone by the virus are inaccurate, despite vaccination, infection with the *Influenza* virus induces a primary response, requiring seven to ten days to induce antibodies. In this time, cells are infected and symptoms are experienced.

In the case of infection with a virus in which viral replication is error prone (mutations are prevalent during replication) to the extent that errors are made within the host over the course of a *single* infection (i.e. each time the virus replicates), a primary antibody response is induced. Over the seven to ten days required to induce antibodies in a primary response, the virus continues to mutate. When these antibodies are finally secreted, the epitopes against which they were raised are no longer present; the epitopes have mutated to an extent that they are not recognized by the antibodies. Viruses like HIV and HCV evade the immune response using such mechanisms. These viruses continually mutate, presenting “new” antigens to the immune system and the pathogen is never eliminated.

E. Epitope conservation, immunodominance and the induction of neutralizing antibodies

Even error-prone viruses like HIV and HCV contain epitopes that do not mutate, or are less prone to mutation. Regions or epitopes on a viral surface not prone to mutation are considered “conserved”. All variants of the virus within a host contain these conserved epitopes. In many cases, variants of the virus across virus sub-classes also contain these epitopes. It seems logical to target these conserved epitopes in creating a vaccine against viruses like HIV and HCV. But, in order to understand the difficulty in vaccine design, the concept of immunodominance and neutralization must also be considered.

Immunorecessive epitopes are those epitopes to which an antibody response is not induced or not robust. Factors including chemical composition, conformation, and location can make some regions of the viral surface “immunorecessive”. In the case of HIV, the envelope protein thought to be responsible for inducing antibodies that prevent infection is a trimer of gp120 and is coated with carbohydrate [19]. Thus, much of the surface is masked from antibodies by these non-immunogenic sugars. Secondly, any vaccine that does not reproduce the trimeric structure of gp120 is also likely to induce the production of antibodies to portions of the protein that are not normally exposed. Finally, although key parts of the viral envelope that bind to CD4 are conserved, they are deeply recessed making it difficult for antibodies to bind [20].

Finally, not all antibodies are created equal. Only some of the epitopes on the pathogen surface induce antibodies that prevent infection. Therefore, a successful vaccination against HIV and other viruses requires the production of antibodies against a conserved, immunodominant, neutralizing epitope. In this light, the vaccines currently available for use in the United States become more impressive. Each of these vaccines, and the pathogens they protect against, contain epitopes that are immunodominant, conserved, and neutralizing.

In summary, many traditional vaccines have disadvantages and furthermore fall short in preventing infection against rapidly mutating pathogens like HIV and HCV.

F. Our Novel Vaccine Platform

To overcome the shortcomings of traditional and peptide vaccines and to create vaccines against viruses for which the neutralizing epitope is unknown, the following were needed:

- a platform to discover broadly neutralizing viral epitopes without prior knowledge of their location, prevalence, immunogenicity, or structure

- a safe and robust vaccine formulation which presents these epitopes to the immune system without risk for reversion, and

- the ability to administer the epitopes prior to exposure such that broadly neutralizing antibodies are present in the blood and tissues *before* exposure.

We hypothesized that we could develop a novel platform using B cell epitope mimetics *without prior knowledge of the neutralizing epitope*. This hypothesis was based on the knowledge that neutralizing monoclonal antibodies (MAbs) would bind molecules with shapes that mimicked the native antigen (the shapes against which the antibodies were raised). Once identified, these molecules, when properly formulated and injected *in vivo*, would induce the production of protective, cross-reactive antibodies against the virus 1) without posing any chance for viral reversion, 2) without knowledge of the protective epitope, 3) and before exposure to the virus.

To create such a vaccine platform, we began by obtaining broadly neutralizing MAbs against HIV, HCV, WNV, and norovirus. These MAbs were induced against conserved and neutralizing epitopes despite the fact that the identity of these epitopes remains unknown. By using these broadly neutralizing MAbs to screen large synthetic libraries, potential epitope mimetics (mimotopes) should be identified from the library without prior knowledge of the native protective epitopes.

Mimotopes [21] are structures that mimic the structure of an epitope. The work of several groups [12, 22-24] has demonstrated the concept, which is key to our approach, that a mimotope need not resemble the sequence of the native B cell binding epitope. Instead what matters is that the conformation of an epitope bound

by the MAb is similar and that it binds with relatively high affinity. Furthermore, for a structure to be a true mimotope, not only must the structure mimic the antigen by binding to the antibody that recognizes the native epitope, it must elicit a humoral immune response against the native epitope that it mimics [25]. Smith *et al* [26] described a ribonuclease S-peptide antagonist whose amino acid sequence did not resemble any part of the amino acid sequence of the S proteins natural ligand but displayed similar biological function. Lam *et al* discovered discontinuous epitopes by screening one bead one compound L-amino acid libraries with anti-insulin MAb [25]. The resulting mimotopes isolated from the screen had distinct sequence motifs amongst the retained sequences, but no sequence homology with the native peptide.

We then created large, diverse libraries of B cell epitope mimetics that would be screened with the MAbs. To create these vaccines we chose a peptoid-based platform. Peptoids (described below) have characteristics that make them advantageous as compared to peptides for use in combinatorial libraries as well as for use in creating diverse shapes.

To isolate bead-bound peptoid sequences (i.e. “on-bead”) bound by the MAbs from a bead-based library, we chose protein G, which has a high affinity for the Fc portion of most antibodies [27]. Since MAbs of interest all have Fc portions, the choice of protein G to retain antibody bound sequences made this platform applicable to many MAbs from different species. With the exception of human IgA, IgD, IgE, IgM, mouse IgM, and some rat, dog and guinea pig antibodies, protein G has a strong affinity for many species and isotypes and particularly for IgG [27]. IgG antibodies are ideal screening antibodies as they are made by activated B cells (i.e. secondary plasma cells) and are therefore class switched and affinity matured[28]. Furthermore, IgG is monomeric instead of dimeric or pentameric (like IgA and IgM) which could complicate binding and affinity measurements.

Because we would be screening large libraries for a presumably small number of mimetic sequences, we chose to use protein G attached to iron oxide beads (dynabeads; PGDs) to physically isolate the peptoid beads bound by MAb using a

magnet. We also devised two secondary validation assays to validate the resulting sequences of the magnetic screen. The first on-bead assay determined if the MAb re-bound the selected sequence. Identification of these positive sequences was synthesized using a species specific horse radish peroxidase conjugated secondary antibody. Sequences identified as positive in this assay were then sequenced and made in bulk for testing in a blocking enzyme linked immunosorbent assay (ELISA) which determined if the selected sequences were bound by the antibody's binding site.

Together PGDs, MAbs, and combinatorial peptoid libraries comprise our magnetic *screen*. The magnetic screen in conjunction with two secondary assays which validate the binding and affinity of the MAbs binding site for the retained sequences make up the novel peptoid based *platform* by which potential vaccine candidates can be generated and validated without foreknowledge of the key protective epitopes.

The reagents used to develop this platform are introduced and briefly discussed.

G. Peptoids

Structure. Peptoids are oligomers of N-substituted glycines developed by Simon and Zuckermann *et al* [29] in the 1980's in an attempt to create peptidomimetics that were as close to polypeptides as possible, but engineered to remove the protease sensitive sites [30]. They succeeded in creating protease resistant [31] peptidomimetics by translocating the side group from the alpha carbon (where it resides in amino acids) to the neighboring nitrogen (see **Figure 1** below).

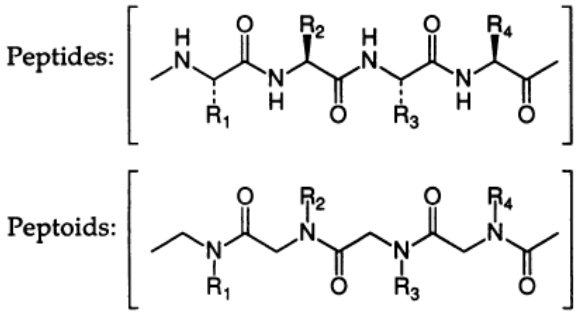


Figure 1: Peptide vs. Peptoid Structure. Modified from Simon *et al* [29]. A comparison of the structure of peptides vs. peptoids reveal a common backbone, but a translocated side chain (R1 – R4 in the figure) from the alpha carbon in peptides to the nitrogen in peptoids. This translocation impacts the structure, behavior, and characteristics of peptoids as compared to peptides.

In addition to affording proteolytic stability, this translocation had other effects on the resulting oligomers including the loss of the chiral center and a hydrogen bond donor. The loss of the chiral center greatly increases the flexibility of the backbone since the amide bonds can isomerize to a *cis* or *trans* conformation [32]. Without the hydrogen bond donor, peptoids are "incapable of forming the same types of hydrogen bond networks that stabilize peptide [secondary structures]" [33]. The lack of hydrogen bonding also makes the *de novo* design of peptoids more difficult as the laws governing peptide secondary structure cannot directly be applied [34]. Chirality and folding *can* be induced, however, with the inclusion of chiral side chains [35]. These structural differences are summarized graphically below in **Figure 2.**

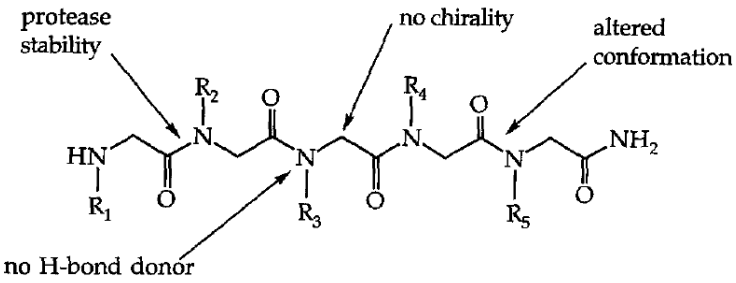


Figure 2: Structural differences between peptoids and peptides. Structural differences between peptoids and peptides. Modified from Figliozzi *et al* [36], structural features of peptoids and some major differences as compared to peptides.

Synthesis. Peptoids are most efficiently synthesized by the sub-monomer [37] method. With this method, a peptoid monomer is added in a two step process of acylation and displacement. The acylation step adds the monomers that make up the peptoid backbone and the displacement reaction then adds the side chain group (**Figure 3)**. As reviewed by Zuckermann [30], the sub-monomer method was a major breakthrough in peptoid synthesis because it replaced a method that was laborious, slow, resulted in low yields and required potentially difficult chemistries for main chain protection. In contrast, the sub-monomer method does not require main chain protection, although reactive side chain groups do; these include, but are not limited to carboxyl, thiol, amino, and hydroxyl groups [38].

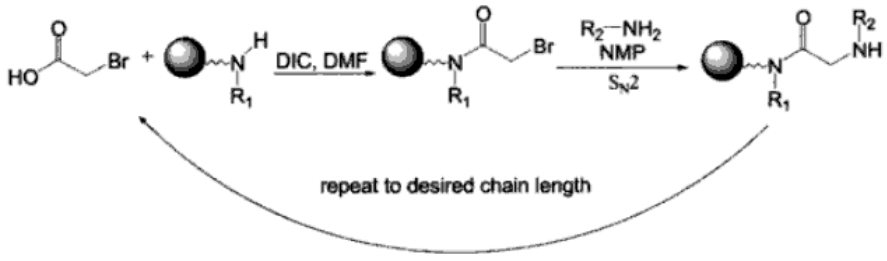


Figure 3: Sub-monomer synthesis. Taken from Zuckermann *et al* [37], this figure shows the two step reaction of acylation and displacement. Together, this two step addition adds a single peptoid monomer to the peptoid chain.

Using the sub-monomer method, peptoids could be synthesized more cheaply and more easily than peptides. Furthermore, side chains need only have a reactive primary amine, thereby allowing the incorporation of hundreds or even thousands of side chains. As compared to the twenty naturally occurring amino acids used in peptide synthesis, peptoids have the potential for incredible monomer diversity [31, 36, 39].

While D amino acids can also be incorporated in peptide synthesis, this increase in diversity is small as compared to the number of monomers available for synthetic peptidomimetics libraries. Furthermore, although D-amino acids are structural analogs of L-amino acids, research has shown that **not** all retro-inverso D-amino acid analogs of L-amino acid epitopes induce antibodies with the same biological activity. This is speculated to be due to the enantiomer (each member of a pair of molecules that are mirror images of each other) specific nature of some antibodies and the requirement for both structural mimicry and the mimicry of the molecular interactions important for binding [40, 41].

Taken together, peptoids lend themselves to use in combinatorial libraries because they are stable, easy to synthesize in bulk [42], the monomers have a wide variety of functional groups which can be added as side chains, the linking chemistry used to build peptoid chains resulting in high yields, and the monomers are achiral [29].

Peptoids can be synthesized as linear structures, as cyclic structures [43, 44], or as hybrids (sequences of peptoid monomers and amino acids) [45, 46]. Cyclized and hybrid peptoid sequences have the advantage of increased conformational stability, as both cyclization and the inclusion of peptide monomers in the peptoid monomer chain serve to stabilize the otherwise flexible nature of peptoid sequences. However, both floppy and constrained peptoids have been identified as biologically relevant ligands [47].

Peptoids can also be synthesized as single sequences or in large combinatorial libraries. For single sequence synthesis, peptoids are commonly synthesized on a resin bead with an acid labile linker (Rink Amide linker derivatized solid supports), since this resin results in the high yield cleavage of the sequence from the support using acid. Alternatively, large combinatorial libraries can be quickly and easily created by combining the sub-monomer method for peptoid synthesis with the split and pool technique described by Furka *et al* [48], Lam *et al* [49], and Huebner *et al* [50]. In split and pool synthesis, depicted in **Figure 4**, the resin is first evenly distributed by weight into each of the reaction vessels. The submonomers (here A, B or C) are then added to the resin and incubated. The resin is then washed and pooled together into a common vessel (here Recombine and Mix).The pooled resin is mixed well to randomize the resin, and then redistributed again to the reaction vessels in preparation for the next submonomer addition. In this way, libraries of millions of compounds made from a relatively small set of monomers can be made quickly and with relatively little equipment.

Peptoid libraries can be synthesized on microarrays [51-54] or on resin beads [55]. Microarray libraries present fewer than 10,000 compounds at a time whereas on-bead libraries can present in excess of 1×10^6 compounds. For the success of our platform, we chose to synthesize peptoids on-bead in an effort to increase library size.

On-bead peptoids are commonly synthesized using TentaGel resin, a grafted copolymer resin of polystyrene and polyethyleneglycol [56]. TentaGel is advantageous to on-bead screening because it is compatible with both the organic solvents required for peptoid synthesis and the aqueous buffers often associated with biological applications. Furthermore, the poly(ethylene glycol) chains provide a surface that resists nonspecific protein binding [57, 58]. The use of TentaGel resin together with a cleavable linker [59] makes possible the on-bead evaluation of the bead- bound sequence followed by cleavage with acid (i.e. cyanogen bromide) in preparation for sequencing.

Analysis. Following synthesis, peptoids can be analyzed by high performance liquid chromatography, and depending on the application, by nuclear magnetic resonance, and circular dichroism [60]. They can be sequenced by Edman degradation [61] or tandem mass spectrometry [24].

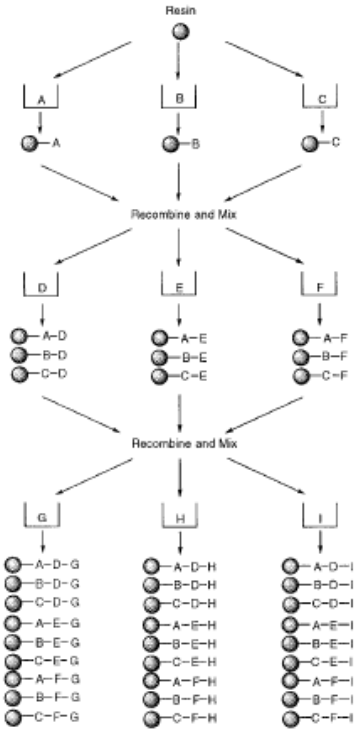


Figure 4: Split and pool synthesis (from Thompson *et al* [62]). Depicted here is the split and pool method for one bead, one compound libraries developed by Furka *et al* [48] and Lam *et al* [49]. In split and pool synthesis, the resin is first evenly distributed by weight into each of the reaction vessels. The submonomers (here A, B or C) are then added to the resin and incubated. The resin is then washed and pooled together into a common vessel (here *Recombine and Mix).*The pooled resin is mixed well to randomize the resin, and then redistributed again to the reaction vessels in preparation for the next submonomer addition.

Biological Applications. Peptoid libraries have been proven by us and others to be rich sources of ligands for a variety of different proteins [29, 31, 39, 47, 63-68] with affinities ranging from micromolar [69] to nanomolar [70]. Examples include peptoid

analogs of lung surfactant, as antimicrobials, and G-protein coupled receptor ligands.

Additionally, peptoids can bind antibodies. Hoffmann *et al* [71] have reported a single example of a peptoid capable of binding to an antibody that also recognized a linear peptide. However, to the best of our knowledge there has been no demonstration or suggestion that peptoids can mimic discontinuous, "three-dimensional" B cell epitopes of the type found in natural proteins.

Peptoids are Immunogenic. In a small pilot study [72] we determined that peptoids were immunogenic when coupled to a typical carrier protein, and hence were "haptens". Seven random 6 to 10-mer peptoids were synthesized. These sequences were chemically coupled to one of two carrier proteins, keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA), adsorbed onto the commonly used vaccine adjuvant, aluminum hydroxide (alum) and injected into mice. For controls, we also injected peptoids only or carrier only, both adsorbed to alum.

Mice received four vaccinations, given six weeks apart. Blood was collected from each mouse before the vaccination and two weeks following the second, third and final boosters, as well as five months after the final vaccination, and the serum titer of peptoid-specific antibodies were measured. A peptoid-specific response was induced in the mice immunized with peptoid-carrier but not with either alone, indicating that the peptoids were immunogenic but only when conjugated to a carrier protein.

Peptoid-based vaccines would be "safe" since there is no potential for reversion and the B cell epitopes could be conjugated to carrier proteins in such a way that peptoid multivalency and immunodominance can be controlled. By controlling the valency and immunodominance, high affinity antibodies should be induced and these should cross react with immunorecessive but important epitopes on the native antigen, resulting in neutralization of a pathogen. Thus, this platform is advantageous for both

the generation of new vaccine where neutralizing epitopes are unknown candidates and for the reformulation of current vaccines whose protective epitopes are immunorecessive.

H. Combinatorial Libraries

Library screening is one approach to the identification of unknown ligands. Other fields of research including drug discovery and molecular biology use library screening as a tool in identifying peptides, small molecules, drugs, and biopolymers of interest [73] both with and without knowledge of the ligand of interest. These libraries, range in size from thousands to billions of compounds. (For a chart comparing library size for each library technique, see Gordon *et al* [74]).

Combinatorial libraries are made by preparing compounds from a set of building blocks. These libraries, can be biological or synthetic, can be synthesized on solid-support or in-solution, and can be naïve or immune (sub-libraries) [39]. **Figure 5** summarizes the types of combinatorial libraries and classifies each as biological or synthetic.

An example of a biological combinatorial library is phage display. Phage are viruses that infect bacterial cells [75]. Such libraries were impossible to make before the 1980's. However, the work of Smith *et al* [76] displaying peptides on filamentous phage and expounded upon in key works by Winter and McCafferty *et al* [77], and Lerner and Barbas *et al* [78] made large, biological libraries successful and widely implemented. Phage display uses standard recombinant DNA technology to associate peptides with replicating viral DNAs that include the peptides' coding sequences. When the phage vector is used to infect bacteria, the peptide is expressed on the coat, making it accessible for screening.

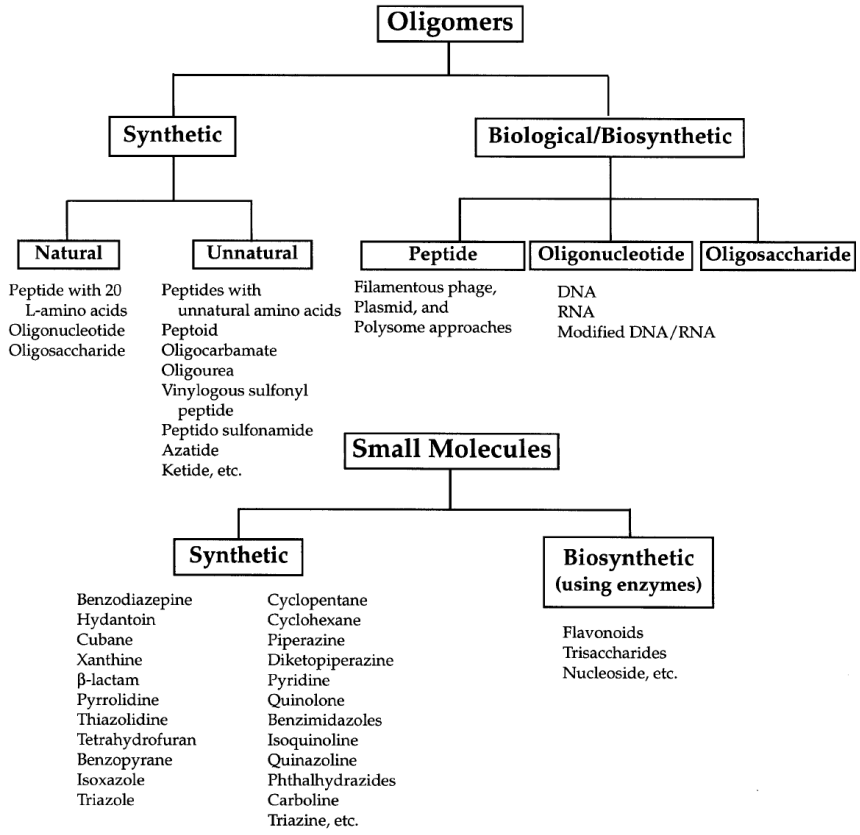


Figure 5: Types of combinatorial libraries. Taken from Lam *et al* [39].

Phage display has been used to express antibody fragments as well as peptides for a variety of applications. Examples of peptides and proteins expressed by phage display include hormones, receptors, toxins, antigens, and antibodies [79]. Humira, a TNF alpha inhibitor for the treatment of rheumatoid arthritis, was the first antibody derived from phage display to be approved by the Food and Drug Administration (FDA) [75].

Bio-panning is the process of screening phage display to select a phage-clone that expresses the protein or peptide of interest. In biopanning, the receptor is tethered to a solid support, and the phage library is passed over the immobilized receptor. Those phages which display peptides specific for the receptor are captured on the surface and the unbound phages are washed away. This is the selection phase of biopanning. Next, the bound phages are eluted, yielding an “eluate” population of phages that is greatly enriched for receptor-binding clones. These clones are then

amplified by infecting a fresh bacterial host. Phage clones from the final eluate are propagated and characterized individually [79].

Phage display has advantages over synthetic peptide biological approaches including greater library size (up to 10^{11} clones), replicability (i.e., ability to make copies of themselves) and mutability (i.e., ability to undergo changes that are passed on to their progeny), and larger scale affinity selection (many orders of magnitude) as compared to combinatorial chemical libraries. Furthermore, phage display is cited as behaving as if the peptides and proteins are not attached [79]. However, diversity is limited to the twenty amino acids, and limits on enrichment affect the size of genetic diversity that is accessible [80].

In contrast, synthetic combinatorial libraries are made by solid-phase or solution-phase synthesis. Based on the work of Merrifield *et al* [81] describing solid phase peptide synthesis, Geysen *et al* [82] was the first to describe the synthesis of peptides on "pins". His work was expounded on by Houghton *et al* [83] and Lam *et al* [84, 85] who employed "tea bags" and resin beads respectively to synthesize libraries on solid supports using the split and pool techniques of Furka *et al* [48]. Together, these advances allowed the development of the biology and chemistry of modern combinatorial libraries.

The biggest advantage to synthetic combinatorial libraries is the ability to include amino acids as well as amines, generating libraries with greater chemical diversity as compared to peptide libraries. Furthermore, synthetic combinatorial libraries can be made quickly, easily, and inexpensively.

Combinatorial libraries can be synthesized on solid phase support or in solution. Solid phase synthesis is advantageous because large numbers of sequences can be rapidly screened, the beads can be used multiple times for binding to different targets, and the beads facilitate the retrieval of the high affinity ligand for future testing or analysis [59]. Solution phase screening is possible but its attributes make it disadvantageous for our use, particularly because individual sequences cannot be retained and isolated [86].

Hit rates as low as 0.000012% [87] and as high as 4.00% [88] have been described using biological and synthetic libraries. Most likely, the hit rate lies somewhere in between these extremes, and closer to that described in the work of Lam [49] *et al* who screened a library of two million peptides with a MAb against B-endorphin, resulting in six lead sequences (0.0003%).

The nature of the library strongly impacts the hit rate observed. Combinatorial libraries can be naïve or "immune/focused" (i.e. sublibraries). Naïve libraries are those constructed without biases for the conformational space or functional groups suspected to play a role in the binding between two proteins. Sub-libraries, in contrast, are those made from monomers known or suspected to participate in the protein-protein (or in our case antibody-peptoid) interaction.

Naïve libraries are advantageous when the nature of the ligand of interest is unknown [89] (i.e. if the monomers important for biological activity are unknown). However, very large libraries (millions) of sequences and up to 1 billion sequences, as proposed by Sidhu *et al* [90*],* are thought to be necessary to identify high affinity binders from a naïve library.

Sub-libraries are ideal when the nature or monomers important for binding are known. Sub-libraries can generate hit rates (the number of positive sequences retained divided by the total number of sequences) and high affinity ligands using far fewer monomers and using smaller libraries [73].

I. Screening Reagents

Combinatorial libraries have been screened with serum [24, 79, 87, 91-93], antibodies prepared from serum [94], cells [80], and other proteins [47, 70]. Antisera are valuable for many biological purposes but they have certain inherent disadvantages that relate to the heterogeneity of the antibodies they contain [1]. Although many groups screen combinatorial libraries with antisera from animals or humans with a known disease vs. sera from healthy controls, the enormous number of proteins and antibodies in the sera can confound screening and lead to artifacts.

One cannot assume that the only differences between sera from individuals with a given disease vs. normal controls will be the antibodies or “biomarkers” of interest.

To obtain a more homogeneous preparation of antibodies, sera can be purified to obtain polyclonal antibodies (PAbs) or MAbs devoid of other serum proteins that might also recognize ligand mimetics. PAbs are a mixture of the antibodies induced against a specific antigen, each recognizing a different epitope or region of the antigen, and each binding to their respective epitopes with a range of affinities. **Table 2** below summarizes three types of purification, the rationale behind using them, and the downsides of each.

As summarized in **Table 2**, PAbs can be purified from sera by affinity or as immunoglobulin (Ig) preparations. Affinity purification results in the isolation of antigen specific proteins, while Ig preparations isolate all Ig from the serum, regardless of their specificity.

MAbs are antibodies generated from a single B cell clone. These MAbs recognize a single epitope with a defined affinity, as compared to polyclonal antibodies which are a collection of antibodies with a range of affinities, specific against many epitopes comprising a single antigen.

Table 2: Methods to purify antibodies from serum

Technique	Mechanism	Resulting Eluate	Downsides
Ammonium Sulfate	At high salt concentrations, protein surface charge is neutralized, allowing protein aggregation and removal	Crude purification of total Ig from other serum proteins	All isotypes retained
Protein A/G Column	Protein A and G have high affinity for the Fc of IgG (and other isotypes)	IgG	All IgG retained (antigen and non antigen related IgG)
Antigen Column	Proteins (IgG or other) specific for the antigen are retained	Proteins (IgG and other) specific for the antigen	In addition to antigen specific antibodies, other non Ig proteins can be retained

MAbs can be generated using hybridomas or phage display. Hybridoma technology involves fusing the B cell of interest to myeloma cells to create hybrid cells that proliferate indefinitely and secrete antibody specific for the epitope on the antigen bound by the receptors on the selected B cell. MAb production by phage display involves the fusion of the immunoglobulin genes to those encoding the coat protein of filamentous phage. When bacteria are infected with the phage, the resulting phage particles have coats that express antibody-like fusion proteins [1, 79]. MAbs are isolated by screening these phage libraries with the antigen of interest.

MAbs have been used in a broad range of biological applications. Currently, there are ten MAbs approved for use by the FDA for the treatment of conditions including rheumatoid arthritis, cancer, and psoriasis to name a few [95].

Both PAbs and MAbs are advantageous in library screening because the protein responsible for library-sequence-binding is more clearly defined.

J. FLAG Peptide and MAb Anti-FLAG

"FLAG" peptide [96, 97] (DYKDDDDK) was originally created to aid in the detection and purification of lymphokines from yeast extracts. Now commercially available, the DNA encoding the FLAG-tag (N-DYKDDDDK-C) can be fused to DNA encoding specific proteins by recombinant DNA technology. These expressed conjugates are then used to purify proteins using mouse anti-FLAG MAb ("anti-FLAG"). There are three mouse anti-FLAG MAbs (M1, M2 and M5), each differing in their requirements for epitope binding [97]. Anti-FLAG M1 binds to FLAG peptide in a calcium dependent manner and identifies FLAG peptide only when it is displayed as an N-terminal fusion protein. Anti-FLAG M1 was the original anti-FLAG and was generated by immunization with the purified peptide followed by affinity purification on a synthetic peptide affinity column. Anti-FLAG M2 is calcium independent and recognizes FLAG peptide in more than one configuration (as an N-terminal protein or preceded by additional amino acids). Because M2 is not calcium dependent, elution from an affinity column is accomplished by decreasing the pH [98] or by competition elution with the synthetic peptide [99]. Anti-FLAG M2 was chosen for this platform because it was the most versatile MAb as far as antigen recognition and its binding was calcium independent. Anti-FLAG M5 also binds independently of calcium. M5 exhibits a higher affinity for N-terminal FLAG, in which the FLAG epitope is preceded by a methionine, making M5 ideal for detection of cytoplasmically expressed FLAG fusion proteins [97].

Slootstra *et al* [100] have described, in addition to the FLAG positive peptide (DYKDDDDK), two modified FLAG peptides. The first modified peptide, "FLAG50", (DYKDDADK), is an alanine replacement analog of an aspartic acid residue in FLAG positive peptide. FLAG50 peptide was bound by anti-FLAG M2 with 50% avidity as compared to MAb anti FLAG binding to FLAG positive peptide. The second modified peptide, FLAG negative peptide (DAKDDDDK) also resulted from an alanine substitution which abrogated anti-FLAG binding.

The work described in this dissertation employed FLAG peptides and anti-FLAG to serve as a proof of principle antigen/antibody pair in the absence of a peptoid/anti-

peptoid pair. Specifically, we employed FLAG positive and FLAG negative peptide, as described by Slootstra *et al* [100], as a positive and negative control epitope with which to optimize the novel peptoid-based screening platform

K. Long Term Goal

The long-term goal of this project was to develop a vaccine candidate discovery platform that could overcome some of the drawbacks of traditional vaccine development and to identify vaccine candidates for any pathogen against which a broadly neutralizing MAb exists or could be made. The advantage of this platform was that the foreknowledge of the conserved and/or neutralizing epitopes that would induce broadly neutralizing antibodies did not have to be known or have to be immunodominant. The objective of the work described here was to identify these B cell epitope mimetics from a peptoid library using MAbs. To successfully develop this platform, I first had to learn basic peptoid synthesis, library synthesis, peptoid cleavage and analysis using mass spectrometry. I then devised a model system to test and validate the platform.

II. MATERIALS AND METHODS

REAGENTS

A. Reagent Preparation

All reagents and solvents were purchased from commercial suppliers and used without further purification with the exception of glycine tert-butyl ester hydrochloride (Sigma Aldrich St. Louis, MO and Chem-Impex International, Wood Dale, IL) which was free-based before use, and ethanolamine (Sigma Aldrich, St. Louis, MO), which was protected with tetrahydropyran (THP; Sigma Aldrich, St. Louis, MO) before use.

Glycine Free Base and Ethanolamine Protection with THP

Glycine tert-butyl ester hydrochloride was converted to the free base before use by dissolving 30 grams (g) glycine (0.179 moles) in 90 milliliters (mL) dichloromethane (DCM; Honeywell, Burdick and Jackson, Muskegon, MI), and 7.15 g (0.179 moles) sodium hydroxide (NaOH) (Sigma Aldrich, St. Louis, MO) in 60 mL water (H_2O). Both solutions were then added to a separatory funnel (Fisher Scientific, Pittsburgh, PA) with a capacity of at least twice the fluid volume. The funnel was inverted and shaken by hand, and the pressure relieved intermittently by opening the stopcock. When organic and aqueous layers formed, the bottom layer was drained into a beaker and retained until the protocol was completed. This process was repeated twice more by adding fresh DCM, shaking in the funnel, allowing organic and aqueous layers to form, and collecting the organic layer. The aqueous layer was discarded from the top of the separatory funnel and all the collected organic layers were added back to the empty funnel. Brine [H_2O saturated with sodium chloride (NaCl; Fisher Scientific, Pittsburgh, PA)] was then added to the organic layers in the separatory funnel. The funnel was shaken and the pressure relieved as described above. Both brine addition and layer separation were repeated twice. Finally, sodium sulfate (Sigma Aldrich St. Louis, MO) was added to the organic layers in a beaker and the solution shaken by hand for 30 seconds (sec). The solution was allowed to sit for 3 minutes (min) and the glycine was then pipetted into a round bottom flask

(RBF) (Fisher Scientific, Pittsburgh, PA or Adams and Chittenden Scientific Glass, Berkeley, CA) and placed on a rotary evaporator (Buchi**,** Flawil, Switzerland) until the DCM was removed.

Ethanolamine was protected with THP before use as previously described [101].

B. Project Overview and Strategic Plan

The strategic plan depicted in **Figure 6** was implemented to develop a proof-of-principle platform to generate synthetic vaccine candidates. Briefly, large combinatorial peptoid libraries were synthesized on resin (on-bead), screened with monoclonal antibodies (MAbs) followed by retention with protein G dynabeads ("PGDs"; Invitrogen, Carlsbad, CA) and the peptoids bound by the MAb/PGDs conjugates were isolated on a magnet (Invitrogen, Carlsbad, CA). These potential "hits" (peptoids bound by MAb/PGDs) were validated using a secondary assay, sequenced, and synthesized in bulk for further testing. Blocking enzyme linked immunosorbent assays (ELISAs) were used to determine mimicry of the hit peptoid to native antigen. Validated hits that block antibody binding to native antigen at a fold excess molar concentration of 1.98 will be conjugated to carrier protein, adsorbed to aluminum hydroxide (alum), and injected into animals. The blood will be collected, sera prepared and sera tested in an ELISA to determine the binding of the anti- peptoid antibody for the native antigen. The processes briefly described here are described in detail in the remainder of this dissertation.

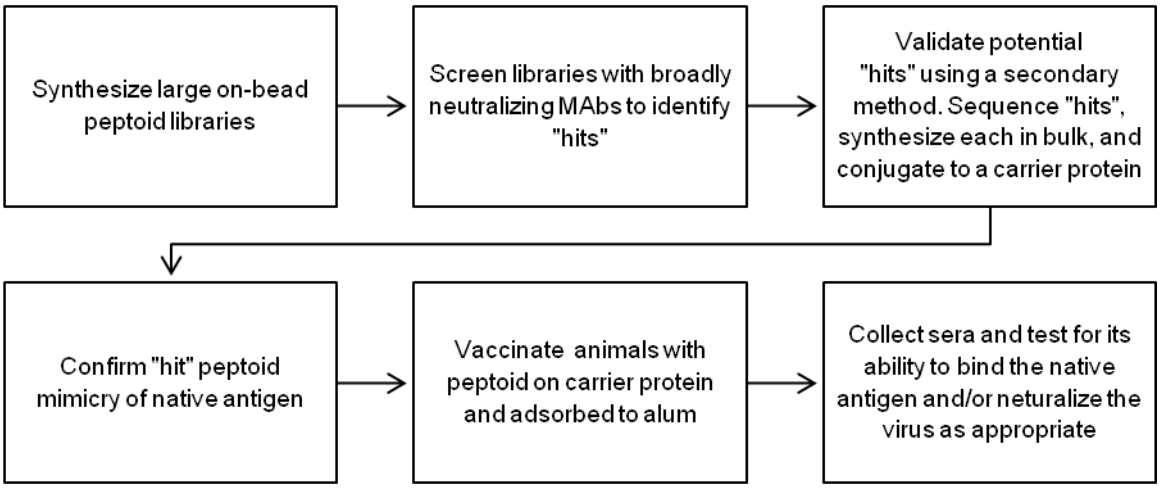


Figure 6: Strategic plan to design and validate a proof-of-principle platform using the FLAG system (see *FLAG peptide synthesis, and Antibodies and Control Proteins*).

C. Resin and Associated Linkers

All sequences used in on-bead assays were synthesized (as later described) on TentaGel MB NH_2 resin ("Tentagel"; Rapp Polymere, Tubingen, Germany) with 0.4-0.6 millimole per gram (mmol/g) loading and 520,000 beads per gram. Sequences synthesized directly onto this resin cannot be removed, so a cleavable peptide linker was first synthesized by N',N'-diisopropylcarbodiimide (DIC) (Advanced Chemtech, Louisville, KY) - mediated peptide couplings (described below) of two or three amino acids / amino acid derivatives (fluorenylmethoxycarbonyl(Fmoc)-cysteine[Trityl(Trt)] , Fmoc-methionine ("Met"), Fmoc-aminohexanoic acid ("Ahex"), O-tert-butyl (OtBu) - ethanolamine ("Eth") or methylamine ("Meth"); see **Table 3** for a linker summary and **Figure 7** for a schematic of a Tentagel-bound peptoid sequence with linker). In preparation for synthesis, Tentagel resin was swollen for 5 min in dimethylformamide (DMF; VWR, West Chester, PA).

Table 3: Two types of resin used for synthesis and their associated linkers

Resin Type	Linker Abbreviation	Linker Details
Rink Amide	Cys	Fmoc-Cysteine(Trt)-OH [A]
Tentagel	Met-Ahex-Ahex ("M-A-A")	Fmoc-methionine ("Met") [B] Fmoc-aminohexanoic acid ("Ahex") [C]
	Met-Eth ("M-E")	Fmoc- methionine ("Met") [B] Ethanolamine ("Eth") [D]
	Met-Meth ("M-Me")	Fmoc-methionine ("Met") [B] Methylamine ("Meth") [E]

[A] NovaBiochem, San Diego, CA; [B] Advanced Chemtech, Louisville, KY; [C] Chem-Impex International, Wood Dale, IL; [D] CSPS Pharmaceuticals, San Diego, CA; [E] Sigma Aldrich, St. Louis, MO.

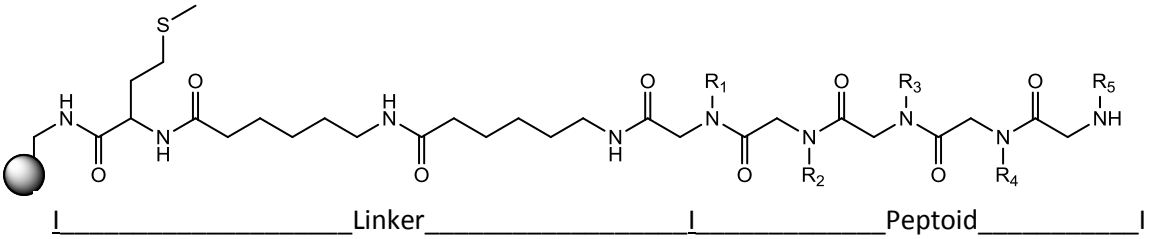


Figure 7: On-bead peptoid structure. This diagram displays a prototypic Tentagel - bound peptoid sequence. The Tentagel resin is depicted here as a grey circle followed by a three monomer linker (M-A-A shown here). Following the linker, five to ten peptoid monomers (Table 4) (here, five are represented, with R1-R5 representing the five side chains) were added in a two step process of acylation and displacement. The peptoid sequence is displayed carbon terminus (C terminus, C') to amine terminus (N terminus, N') as this is the order in which peptoids are synthesized.

All sequences to be conjugated to a carrier protein were synthesized on Rink Amide resin ("Rink resin"; NovaBiochem, San Diego, CA) with 0.6 mmol/g loading, following a cysteine (Fmoc-Cys(Trt)-OH) linker (**Table 3)** added by DIC - mediated peptide coupling (described below). In preparation for synthesis, the Rink resin was swollen in DMF for 5 min and the groups which protect the amine (protecting groups) were removed (a process called deprotection) in 40 mL/mM-of-resin of 20 percent (%) volume to volume (v/v) 4-methyl piperidine (Sigma Aldrich, St. Louis, MO), in DMF for 20 min followed by five DMF washes. This deprotection and wash were repeated once. The deprotection and wash protocol is explained in detail in *Peptoid Synthesis, General Protocol* below.

D. Synthesis

Automated Synthesizer General Protocol

Peptide and peptoid sequence synthesis was carried out on a custom-made automated synthesizer at the Molecular Foundry (Lawrence Berkeley National Laboratory, Berkeley, CA) or on an Aapptec Titan 357 synthesizer (Louisville, KY) at UT Southwestern (UTSW). Before synthesis, the synthesizer was powered on and connected to a pump and cold trap. Reagent solutions were made in glass bottles and loaded onto the synthesizer by attaching synthesizer reagent lines to the solvent bottles. These solutions included 0.6 molar bromoacetic acid (BAA; Sigma Aldrich, St. Louis, MO), in DMF, 0.6 molar chloroacetic acid (CAA; Sigma Aldrich, St. Louis, MO) in DMF, 50% (v/v) DIC in DMF, 65% (v/v) dichloroethane (DCE; Sigma Aldrich, St. Louis, MO) in DMF, and 20% 4-methylpiperidine in DMF. One molar amines in DMF or 0.4 M amino acids in 0.4 M N-hydroxybenzotriazole (HOBt) (Aapptec, Louisville, KY) in N-methyl-2-pyrrolidone (NMP) (Advanced Chemtech, Louisville, KY) were made in 50 mL disposable polypropylene tubes (Falcon, BD Biosciences, San Jose, CA) and loaded onto the synthesizer. Finally, the synthesizer was connected to a waste container, DMF, and a nitrogen (N_2) source. Resin was loaded onto the machine by weighing out the desired amount of resin into a weigh boat on a scale and then pouring it into the synthesizer reaction vessels (Adams and

Chittenden Glass, Berkeley, CA or Aapptec Titan 357, Louisville, KY). Resin was washed to the bottom of the vessel using DMF and a transfer pipette (BD Biosciences, San Diego, CA). The synthesizer was programmed to swell the resin in DMF by automatically delivering DMF, and allowing resin to stay suspended in DMF for 5 min before draining the DMF to the waste. Amines and amino acids were delivered to the reaction vessels automatically ("amine/amino acid addition") by the synthesizer using a robotic arm. All synthesizer washes ("washes", "washing", or "washed" in the remainder of this section) proceeded with the addition of 2 mL DMF which was then N_2 bubbled for 1 min and drained. One DMF wash cycle repeated this process five times. To de-protect the resin or amines, 40 mL/mM-of-resin of 20% 4-methylpiperidine in DMF (v/v) (which removes the Fmoc or other protecting groups) was added to each reaction vessel and incubated with N_2 bubbling for 5 min. The solvent was then drained and 40 mL/mM-of- resin of 20% 4-methylpiperidine in DMF (v/v) was again added to each reaction vessel and incubated with N_2 bubbling for 12 min before being drained and washed five times in DMF.

Peptide Synthesis

General Protocol

Peptide sequences were synthesized on Tentagel or Rink resin following linker addition (**Table 3)**, using standard Fmoc chemistry on a custom-made automated synthesizer at the Molecular Foundry (Berkeley, CA) or on an Aapptec Titan 357 synthesizer (Louisville, KY) at UTSW. Briefly, the resin was loaded onto the synthesizer and prepared (swollen, washed and/or de-protected) as described above in *Resin and Associated Linkers*. For each amino acid coupling, the amino acid was dissolved to 0.4 M in 0.4 M HOBt in NMP in 50 mL disposable polypropylene tubes and loaded onto the synthesizer. Twenty milliliters per millimole-of-resin of the amines were added to the resin by the synthesizer. Next, 2.84 mL/mM-of-resin of DIC (50% in DMF) was added to the resin and incubated for 1 hour (h) at 35 °C followed by five washes with DMF. Fmoc de-protection with 40

mL/mM-of-resin of 20% 4-methyl piperidine in DMF was performed as described immediately above.

Generating a Peptide/Anti-Peptide Antibody Pair: Overview and Peptide Sequence Synthesis

To begin creating and optimizing a novel platform to identify vaccine candidates, a peptide control system was created until we could create a peptoid/anti-peptoid control pair. A FLAG peptide control system was created by synthesizing two peptides, DYKDDDDK ($FLAG^+$; single letter code) and DAKDDDDK ($FLAG^-$) (**Figure 8**) and purchasing a commercially available MAb anti-$FLAG^+$ peptide ("anti-FLAG" in the remainder of this dissertation). This MAb/peptide pair was selected because of the ability to compare the binding of the anti-FLAG to $FLAG^+$ peptide versus $FLAG^-$ peptide containing an alanine substitution that abrogates binding of the MAb as previously described by Slootstra *et al* [100]. $FLAG^+$ and $FLAG^-$ peptide were synthesized on Rink resin or Tentagel resin following the linker addition using standard Fmoc chemistry. FLAG peptide amino acids (Fmoc-Lys (tert-butyloxycarbonyl; Boc)-OH, "K"; Fmoc-Asp (OtBu)-OH, "D"; Fmoc-Ala-OH, "A"; and Fmoc-Tyr (t-Butyl; tBu)-OH, "Y") were purchased from NovaBiochem (Gibbstown, NJ). Each amino acid was dissolved to 0.4 M in 0.4 M HOBt/NMP. The final peptides were de-protected and/or cleaved off of the resin as described below in *Deprotection and Cleavage*. This peptide pair was used to establish optimized screening conditions, and using these optimized conditions, an on bead assay that allowed the retention of $FLAG^+$ peptide beads bound by anti-FLAG and PGDs using a magnet was performed (See *Screen Optimization using the FLAG System)*. Finally, we validated this on-bead assay using this control peptide system by adding $FLAG^+$ peptide into $FLAG^-$ peptide or a peptoid library aliquot and evaluated the retention of the $FLAG^+$ peptides "spiked" into the aliquots (See *Platform Validation using the FLAG System).*

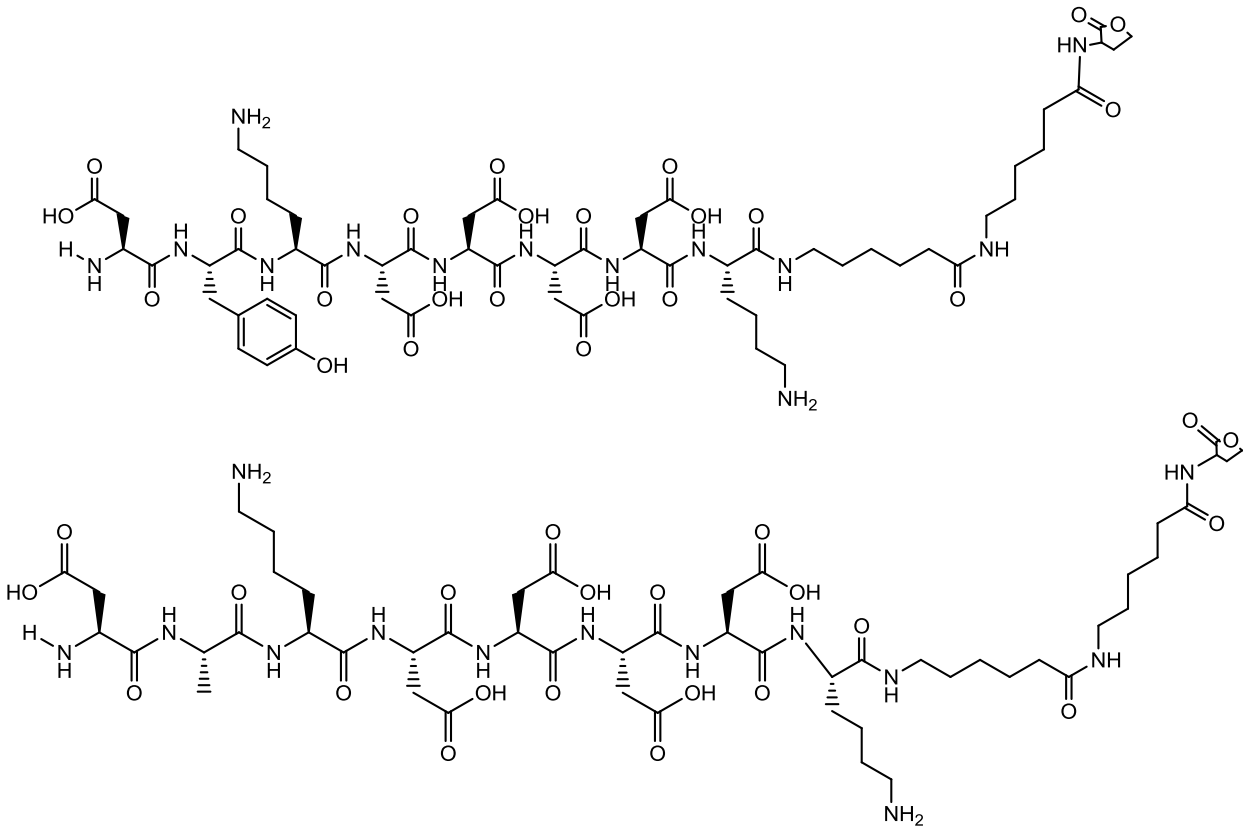


Figure 8: FLAG^{+} (top) and FLAG^{-} (bottom) peptide structures with M-A-A linker following cleavage from Tentagel resin. [Peptide sequences are displayed N'-C'].

Peptoid Synthesis

General Protocol

Peptoids were synthesized on Tentagel or Rink resin and syntheses were performed on a custom-made automated synthesizer at the Molecular Foundry (Lawrence Berkeley National Laboratory, Berkeley, CA) or on an Aapptec Titan 357 synthesizer (Louisville, KY) at UTSW. All syntheses began with the addition of a cleavable linker (**Table 3)** added by DIC - mediated peptide couplings of the linker amino acids. Briefly, following the processes described in *Automated Synthesizer General Protocol,* resin was loaded onto the synthesizer by adding 100 mg of resin to each reaction vessel. The resin was then prepared for synthesis (swollen and/or deprotected as described above in the *Resin and Linker* section). For each linker amino acid coupling, the amino acid was dissolved to 0.4 M in 0.4 M HOBt in NMP

and loaded onto the synthesizer. The synthesizer then delivered 20 mL/mM-of-resin of each amino acid solution along with 2.84 mL/mM-of-resin of DIC (50% in DMF) to each reaction vessel, and the solution and resin were incubated for 1 h at 35 °C followed by five washes with DMF. Two rounds of Fmoc de-protection with 40 mL/mM-of-resin of 20% 4-methyl piperidine in DMF and five DMF washes, 40 mL/mM-of-resin, followed each amino acid coupling cycle as described in *Automated Synthesizer General Protocol*. Peptoid synthesis was carried out in each reaction vessel according to the submonomer addition method [37]. Seventeen milliliters per mM of resin of 0.6 M BAA in DMF and 2.84 mL/mM-of-resin 50% DIC:DMF (9 mM and 18 mM respectively) were added to the resin by the synthesizer and mixed for 19 min by N_2 bubbling at 35°C during the acylation step of the submonomer cycle. After 5 DMF washes (described in detail in *Automated Synthesizer General Protocol)*, the resin-bound bromine was then displaced with 1 mL/100 mg-of-resin of the submonomer (**Table 4)** ("displacement"), which was delivered automatically by the synthesizer to each reaction vessel. Library submonomer amines were prepared as 1 M solutions in DMF except for tyramine (Sigma Aldrich St. Louis, MO), aminomethylcyclohexane (Sigma Aldrich, St. Louis, MO), and 2-(1-cyclohexenylethylamine) (Sigma Aldrich, St. Louis, MO) which were prepared as 1 M in dimethyl sulfoxide (DMSO; Sigma Aldrich St. Louis, MO), NMP, and NMP, respectively. This displacement reaction was carried out for 60 min at 35°C with periodic N_2 bubbling. Following displacement, the resin was washed five times with DMF. This process of acylation and displacement was repeated until the desired peptoid chain length was reached. Then, five final washes with DMF were carried out.

Peptoid Library Synthesis

Peptoid libraries were created using the protocol above either by fully automated solid-phase "split and pool" synthesis [49] on the synthesizer or partial automated solid-phase split and pool synthesis carried out by a combination of the synthesizer and manual synthesis steps. Each method yielded a one-bead, one-compound

(OBOC) library, meaning that each resin bead was coated with multiple copies of a single sequence. The linker amino acids and the first peptoid submonomer did not require splitting and pooling ("split and pool"). However, following the first peptoid submonomer addition, the resin from each reaction vessel was collected and combined into a common mixing vessel (Adams and Chittenden Glass, Berkeley, CA, or Chemglass, Vineland, NJ) for split and pool (**Figure 4;** and described in detail in the next section). Following split and pool, the resin was then redistributed into the reaction vessels either by the synthesizer (fully automated split and pool – as in Library 1) as an isopycnic slurry in 65% DCE in DMF, or by hand, ("manual split and pool", see *Manual Split and Pool* below, all other libraries) by dividing the resin evenly by weight and adding it to the synthesizer prior to the next peptoid residue coupling. Peptoid couplings continued as described above in *Peptoid Synthesis General Protocol*. Following the final peptoid residue coupling, the resin was not combined. Instead, the resin remained in the individual reaction vessels, with the identity of the final amine known. Upon completion of the synthesis, dry resin was removed from the synthesizer using a disposable spatula (VWR, West Chester, PA) and collected into its own 5 mL fritted syringe (Applied Separations, Allentown, PA). Residual resin in the reaction vessels was suspended in ~ 2 mL DMF by pipetting, removed from the reaction vessels with a transfer pipette and added to the corresponding dry resin. All resin was then washed as described below in *Manual Split and Pool*, but with a 5 min vacuum dry.

Manual Split and Pool

After the addition of the linker amino acids and the first submonomer addition, dried resin was removed from the synthesizer using a disposable spatula and collected into a manual synthesis vessel (Adams and Chittenden Glass, Berkeley, CA or Chemglass, Vineland, NJ). Remaining resin was suspended in ~ 2 mL DMF by pipetting, removed from the reaction vessels with a transfer pipette, and added to the pooled resin. The manual synthesis vessel was fitted with a two way stopcock which allowed for a line carrying N_2 and a port from which solvent was drained. Two

hundred milliliters of DMF was added, forcing all of the resin to the vessel bottom. The DMF was drained by opening the stopcock. Two hundred milliliters of DCE (65% in DMF) was then added, forming an isopycnic slurry, and the resin was mixed by N_2 bubbling for 5 min before draining the solvent. This process was repeated once more. Then, 300 mL DCM was added and the resin was mixed again for 5 min by N_2 bubbling. The DCM was drained, collected, and re-poured over the resin twice before the resin was dried for 1 h under vacuum in a vacuum flask (Chemglass, Vineland, NJ). Dried resin was collected using a disposable spatula and weighed on a scale (Mettler Toledo, Columbus, OH). The resin was divided equally by weight according to the number of reaction vessels and added back to the automated synthesizer to resume synthesis. The next submonomer was added in a two step acylation and displacement process before this split and pool procedure was repeated for each remaining submonomer.

Library Specifications

Three peptoid libraries were made using the procedure described above. Library 2 A-C and Library 5 were the only libraries made without the use of an automated synthesizer and are described below. Library specifications for all libraries are summarized in **Table 4** including identity and number of submonomers used, sequence length, linker amino acids, and amount of resin (in g). All libraries were synthesized on Tentagel resin. Libraries were designed to cover chemical space by including at least one submonomer representative of each major chemical characteristic (**Figure 9**) including but not limited to size, charge, polarity, hydrophobicity, and aromaticity.

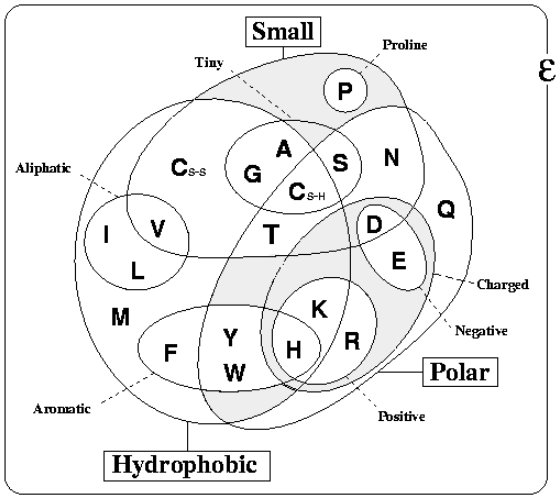
Table 4: Specifications of five peptoid libraries

	Library Number						
Submonomers	**1**	**2A**	**2B**	**2C**	**3**	**4**	**5**
1-(3-aminopropyl)-2-pyrrolidinone* (Npy)	X				X		
4-(2-aminoethyl) benzene sulfonamide* (Nbs)	X				X		
1-aminoindane[†] (Nai)	X						X
Tyramine* (Nty)	X	X		X	X	X	X
Free-based glycine tert-butyl ester* hydrochloride* (Nas)	X	X	X	X	X	X	X
benzylamine* (Nbn)	X	X	X	X	X	X	X
N-Boc-1,4,diaminobutane[‡] (Nlys)	X	X	X	X	X	X	X
Isobutylamine** (Nle)	X	X	X	X		X	
2,2-diphenylethylamine* (Ndi)	X	X	X	X		X	X
THP-protected 2-aminoethanol[×] (Net)	X	X	X	X	X		
Cyclobutylamine* (Ncy)		X	X	X			X
3-methoxypropylamine*(Nmp)		X	X	X			X
Tetrahydrofufurylamine (racemic) * (Nth)		X	X	X			
3,4-dimethyoxybenzylamine* (Ndm)		X	X	X			X
4-aminomethyl-boc-piperidine* (Nam)		X	X	X			
Piperonylamine[¥] (Npi)		X	X	X		X	
4-(aminomethyl)pyridine* (Nap)		X		X			X
Histamine* (Nhi)		X		X			
Tryptamine* (Ntry)		X		X		X	X
Furfurylamine[¥] (Nffa)			X				
2-(1-cyclohexenyl)ethylamine* (Nch)			X				X
R (+) α-methylbenzylamine* (Nmba)			X				
Boc-ethylenediamine* (Ned)					X		
Butylamine* (Nbu)					X		
3-butoxypropylamine*(Nbp)					X		
Allylamine[¥] (Nal)		X	X	X	X	X	
2-thiophenethylamine* (Nth)					X		
2,4-chlorophenylethylamine* (Nchl)					X		
Veratrylamine* (Nver)					X		
Aminomethylcyclohexane* (Namc)					X		
Propargylamine* (Nprg)							X
Exo-2-aminonorbornane* (Nexo)							X
4-phenylbenzylamine* (Nphe)							X
4-(aminomethyl)benzonitrile* (Namb)							X
2-(aminomethyl)-5-methylpyrazine[†](Namm)							X
2-aminoethanol (O-t-butyl protected) [€] (Net)						X	X
Length (# monomers)	5	6	6	5	10	10	8
# Available Monomers for Library Synthesis	10	17	16	17	15	10	18
Resin Amount (g)	3	2.5	2.5	3.5	4	5	21.6
Linker	M-A-A	M-E	M-Me	M-E	M-A-A	M-A-A	M-Me

* Sigma Aldrich St. Louis, MO; [‡]Fluka, St. Louis, MO; [†]Tokyo Chemical Industry Co, Portland, OR; [×]Synthesized at the Molecular Foundry, Berkeley, CA; **Alfa Aezar, Ward Hill, MA; [¥]Acros Organics, Morris Plains, NJ; [€]CSPS Pharmaceuticals, San Diego, CA.

Libraries 2 and 5 were synthesized by manual synthesis on Tentagel resin. Pooled resin was loaded into a single fritted glass reaction vessel (Chemglass, Vineland, NJ) and swollen at room temperature (RT) overnight by adding 40 mL of DMF. The DMF was then drained through the stopcock and Fmoc deprotection was performed before and after the methionine addition with two additions of 20% (v/v) piperidine (Sigma Aldrich, St. Louis, MO) in DMF, each for 15 min at RT while shaking on a MaxQ 2000 (Fisher Scientific, Pittsburgh, PA) at 180 rotations per minute (rpm). Each deprotection was followed by six washes with 25 mL DMF. The DMF was drained and 15 mL Fmoc-methionine was dissolved to 0.2 M in 0.2 molar 2-(1H-Benzotriazole-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate (HBTU; AnaSpec, Freemont, CA) and HOBt in DMF with 0.4 M N-methylmorpholine (NMM; Sigma Aldrich, St. Louis, MO) in a 50 mL conical vial, and then added to the fritted glass vessel by pipetteting. The methionine solution was shaken for 1 h at RT followed by six washes with DMF. Linker amines (O-t-butyl-ethanolamine for sub-libraries 2A an 2C, and methylamine for sub-library 2B and Library 5) were added to the reaction vessels according to the submonomer addition method [37] by first adding 25 mL of 0.4 M CAA (10 mmol) in anhydrous DMF (Sigma Aldrich, St. Louis, MO) and 6 mL of 2 M DIC (2 mmol) in anhydrous DMF to the resin within the reaction vessel. The vessel was shaken for 10 min at 37°C on the shaker and then drained through the vessel stopcock. After washing the resin with DMF six times, the resin-bound chloride was displaced with the linker amino acids or amines by adding 20 mL of a 2.0 M O-t-Bu-ethanolamine (CSPS Pharmaceuticals, San Diego, CA) or 2.0 M methylamine solution in anhydrous NMP into each reaction vessel. This displacement reaction was carried out for 1 h while shaking at 37°C. Following displacement the resin was washed six times with DMF. The resin was then distributed evenly by volume in transfer solvent into peptide synthesis reaction vessels (Chemglass, Vineland, NJ) and the acylation and displacement process was begun for the peptoid chain by adding the first peptoid submonomer. The process was repeated until the desired peptoid chain length was reached. Then, six final washes with DMF were carried out. In between each peptoid submonomer addition, the resin was combined into a large fritted glass reaction vessel, suspended in

anhydrous DMF, mixed well by N_2 bubbling and then split into the seventeen reaction vessels. After the addition of the final residue, the beads were combined again for Library 2 and but left undivided for Library 5 as described above and quality controlled (QC'ed) (see *Quality Control* below).



Hydrophobicity	Structure	Charge	Size	Aromatic
Ethanolamine (philic)	1-3 Aminopropyl-2-pyrrolidinone (cyclic)	1,4 Diamonobutane (+)	Piperonylamine (large)	2,2 Diphenylethylamine
Isobutylamine (phobic)	Allylamine (double bond)	Ethylenediamine (+)	Propylamine (small)	Benzylamine
Isopentylamine (phobic)	Propylamine (linear)	Glycine (-)		Tryptamine

Figure 9: Library design and coverage of chemical space. Taken from Betts *et al* [102], the top panel depicts "chemical space", showing both the interconnected characteristics of an amino acid and the amino acids under each classification. Similarly, in designing peptoid libraries, one goal is to include a representative member of each major chemical characteristic. In the bottom panel are examples of submonomers chosen for peptoid Libraries 1 through 5 are listed under the characteristic they represent.

Control Sequence Synthesis

Control sequences containing the M-A-A linker followed by each submonomer (i.e. M-A-A-SM1-SM2-SM3-SM4-SM5-SM6-SM7-SM8-SM9-SM10, where SM represents a submonomer) were synthesized simultaneously with both Library 3 and 4. After deprotecting and cleaving these sequences (see below-*Deprotection, Cleavage, Quality Control*), the molecular weight (MW) of the test compounds was determined

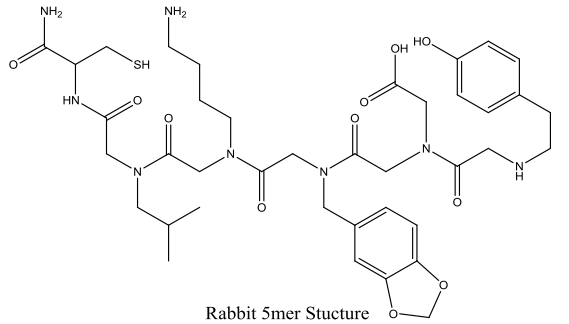
by Matrix Assisted Laser Desorption/Ionization (MALDI) time of flight (TOF) mass spectroscopy (MS) indicating that each addition cycle succeeded, and each submonomer was identified by MALDI TOF tandem MS (MS/MS), indicating that each submonomer chosen could incorporate well under the synthesis conditions.

Generating a Peptoid/Anti-Peptoid Antibody Pair: Overview and Peptoid Sequence Synthesis

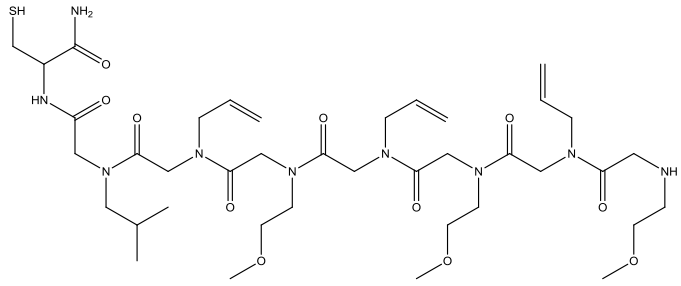
Two peptoids were synthesized on both Tentagel and Rink resin (with appropriate linkers- see **Table 3**) for use in on-bead screens and rabbit immunizations. Following synthesis on Rink resin, the 5 monomer (R5) and 7 monomer control sequence (RC) (**Table 5, Figure 10)** were cleaved from the resin (see *Deprotection and Cleavage, Rink Resin, General Protocol* and *Deprotection and Cleavage, Tentagel Resin, General Protocol*), checked for quality by MALDI MS and MS/MS (see below *Deprotection and Cleavage, Rink Resin*, *Quality Control; Deprotection and Cleavage, Tentagel Resin, Quality Control; MALDI)*, purified by reverse phase (RP)-high performance liquid chromatography (HPLC) and lyophilized (see *High Performance Liquid Chromatography and Lyophilization)*. The lyophilized peptoid was conjugated to carrier protein, keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) and adsorbed to alum (see below-*Conjugation of Peptoids and Peptides to Carrier Proteins and Aluminum Hydroxide Adsorption*) for injection (see *Immunization, Rabbits)* in the case of the R5 or stored for *in vitro* assays in the case of the RC. After immunization and boosting, the rabbits were bled and sera were prepared and purified (see *Immunizations* and *Affinity Purification)*. These sera were used to screen on-bead R5 or RC peptoids.

Table 5: Sequences of the R5 and RC peptoids

R5: cys[1]-Nle[2]-Nas[3]-Npi[4]-Nlys[5]-Nty[6]
RC: cys[1]-Nle[2]-Nall[7]-Nme[8]-Nall[7]-Nme[8]-Nall[7]-Nme[8]
[1]Fmoc-Cysteine(Trt)-OH; [2] isobutylamine, [3] free-based tert-butyl ester hydrochloride; [4] piperonylamine; [5] N-Boc-1,4-diaminobutane; [6] tyramine; [7] allylamine; [8] 2- methoxyethylamine



Rabbit 5mer Stucture



Rabbit 7mer Structure

Figure 10: R5 (top) and RC (bottom) peptoid structures for use in rabbit immunization and/or testing. Depicted here are the R5 and RC sequences post deprotection and cleavage from the resin used for rabbit immunizations and *in vitro* testing. R5 was synthesized, cleaved, purified by HPLC and lyophilized. It was then conjugated to KLH, adsorbed to alum, and injected into two rabbits to elicit rabbit anti-R5 peptoid polyclonal antibodies. RC was used as a negative control for R5 *in vitro* assays. [Peptoid sequences are displayed C'-N'].

E. Deprotection and Cleavage

Tentagel Resin

Tentagel bound sequences to be used for assays (on-bead screening) were deprotected (see below) to remove the submonomer protecting groups, but not cleaved from the bead. The bead-bound sequences were then washed in 50:50 acetonitrile (ACN; VWR, West Chester, PA) and H_2O and used in screening. A schematic depicting the overall procedure for both Rink and Tentagel resin is depicted in **Figure 11**.

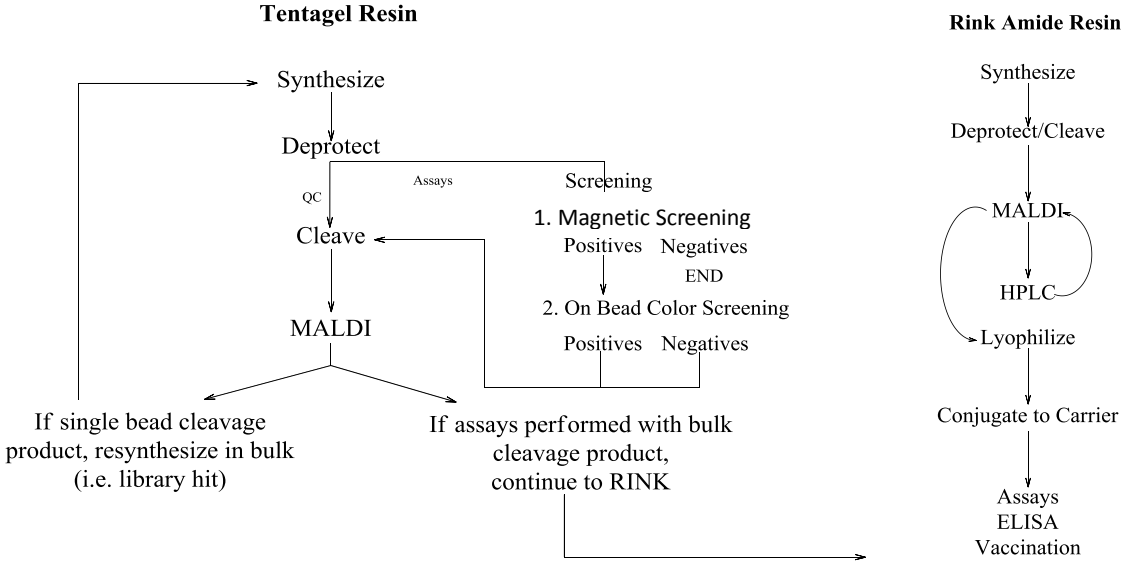


Figure 11: Peptoid processing from on-bead synthesis to bulk scale up. On-bead sequences were synthesized, deprotected with TFA solution, and the majority used immediately in assays ("assays" arrow above). A few beads were QC'ed by cyanogen bromide (CNBr) cleavage and MALDI analysis (QC arrow). If the QC results indicated a poorly synthesized sequence or library, the beads were not used. If QC indicated successful synthesis, the beads were used in magnetic screening and on-bead color screening. The "hits" (MAb bound beads) were then cleaved and analyzed by MALDI MS and MS/MS to determine their sequence. If the bead was from a library screen, it was re-synthesized on Tentagel resin in bulk and validated by cleavage and MS, MS/MS and used in large-scale testing (magnetic screening or color screen in bulk). If the sequence was still a candidate for immunization following this testing, it was synthesized on Rink resin in preparation for blocking ELISAs and immunizations.

Deprotection Protocol

For Tentagel resin, protecting groups were removed in a process separate from that which cleaves the sequence from the resin. Resin was loaded into fritted syringes and 1.5 mL/100 mg-of-resin of 94% trifluoroacetic acid (TFA; Fisher Scientific, Pittsburgh, PA), 2.5% 1,2-ethanedithiol (EDT; Fluka, St. Louis, MO), 2.5% H_2O, and 1% triisopropylsilane (TIS; Sigma Aldrich ,St. Louis, MO) was added and shaken for 2 h to deprotected the Tentagel bound sequences. The resin was then washed three times in ACN: H2O as described in *Library Synthesis* and used either in on-bead assays (screening) or were cleaved from the resin and analyzed for quality control.

Cleavage Protocol

Tentagel bound sequences were cleaved by adding 2 mg/mL cyanogen bromide (CNBr; Sigma Aldrich, St. Louis, MO) in 50% ACN containing 0.25 N hydrochloric acid (HCl; Fisher Scientific, Pittsburgh, PA) to resin in a fritted syringe. The CNBr solution was removed either by SpeedVac concentrator (Eppendorf Vacufuge plus Vacuum Concentrator, Eppendorf North America, Hauppauge, NY) for 2 h or until dry, at 45˚C or, after an 18 h incubation by evaporation into air overnight in a chemical hood. One microliter of cleaved product was mixed with 1 uL of prepared matrix and spotted onto a MALDI plate. Prepared matrix was made by creating 5 milligrams per milliliter (mg/mL) α-hydroxycinnamic acid (CHCA; Sigma Aldrich, St. Louis, MO) solution by adding 50:50 ACN: H_2O + 0.1% TFA to the CHCA in a MCF tube. Samples were analyzed using MALDI MS and MS/MS. If the sample was dried completely, it was first re-suspended in ACN: H_2O before mixing with prepared matrix.

Quality Control (QC)

For library quality control, following de-protection and before cleavage, five to ten Tentagel bound beads from each library submonomer pool were isolated for QC. These beads were isolated one bead per tube and cleaved as described

immediately above. One microliter of prepared matrix was mixed with 1 uL cleaved sample and plated for MS analysis as described. Libraries were evaluated for fidelity according to the requirements outlined by Figliozzi *et al* [36]. To QC bulk syntheses on Tentagel resin (i.e. $FLAG^+$ peptide), ten to fifty beads were deprotected and cleaved in bulk in a single microcentrifuge (MCF) tube (USA Scientific, Orlando, CA). Cleavage products were plated and analyzed by MALDI MS and MS/MS.

Rink Amide Resin

General Protocol

In the case of the Rink resin, the groups protecting the side chain monomers (protecting groups) were removed after synthesis at the same time as sequence cleavage from the resin by adding 1.5 mL/100 mg-of-resin of TFA/EDT/H_2O/TIS (94:2.5:2.5:1 by volume) to on-bead sequences in fritted reaction vessels while shaking for 2 h on a reciprocating orbital incubator/shaker (ROSI1000; Thermolyne, Fisher Scientific, Pittsburgh, PA). The solution was expelled into a 15 mL conical vial (Falcon, BD Biosciences, San Jose, CA) and argon or N_2 was introduced to the tube and delivered until all TFA was evaporated. The resulting cleavage product was dissolved in ACN and H_2O until the solution was clear and purified by RP-HPLC on a C18 column and lyophilized to dryness in preparation for conjugation to KLH. For more information on purification by HPLC, see *High Performance Liquid Chromatography and Lyophilization* below.

Quality Control (QC)

All synthesis products were checked for quality by MALDI MS and MS/MS (see next section). Peptoids synthesized on Rink resin and deprotected and cleaved by TFA solution were checked for quality by mixing 1 uL cleaved product dissolved in ACN: H_2O with 1 uL prepared matrix. The matrix and sample were then mixed by pipetting and spotted onto a MALDI plate (Applied Biosystems, Carlsbad, CA) by pipetting 1 uL onto the plate.

F. Matrix Assisted Laser Desorption/Ionization (MALDI)

All MALDI/TOF MS and tandem MS (MS/MS) analyses were performed on an ABI 4800 MALDI/TOF MS (Applied Biosystems, Carlsbad, CA) in the reflector positive mode. Samples were prepared as described above by mixing 1 uL cleavage product with 1 uL prepared matrix. One microliter was spotted onto the MALDI plate by pipette for analysis. Sequence assignments were made by examining the mass difference between two adjacent peaks.

G. High Performance Liquid Chromatography and Lyophilization

Synthesized products cleaved from Rink resin were dissolved in ACN followed by the addition of H_2O such that the product precipitated out of solution. ACN was then added drop-wise to dissolve the product to a point just before precipitation. Samples were filtered using 0.45 micrometer polytetrafluoroethylene (PTFE) filter placed on a 1 mL syringe (BD Bioscience, San Jose, CA). Semi-preparative RP-HPLC was performed at the Molecular Foundry (Varian Prepstar; Agilent Technologies, Santa Clara, CA) using a C18 column (Varian Dynamics Omnisphere 5C18; Agilent Technologies, Santa Clara, CA) (250 millimeter (mm) x 21.44 mm inner-diameter (id)) or at UTSW (Hitachi Elite LaChrome; Hitachi, Peoria, IL) using a C18 column (Grace C18 Column; W.R. Grace and Co., Augusta, GA) (250 mm x 10 mm id, 5 micrometer (um) particle size). A linear elution gradient of 3–95% B over 60 min was used at a flow rate 2.5 mL/min at UTSW or 5 mL/min at the Molecular Foundry, Berkeley, CA (Solvent A: H_2O:0.1% TFA, Solvent B: ACN/0.1% TFA). Fractions were collected into glass tubes by hand or by using a FoxyJr (Teledyne ISCO, Lincoln NE) or Varian ProStar 701 (Agilent Technologies, Santa Clara, CA) fraction collector. Each fraction was analyzed using MALDI-TOF MS and MS/MS as described, and dried by lyophilization (Thermo Fisher Scientific/Thermo Savant, Pittsburgh, PA). The yield was determined by weighing the lyophilized product.

H. Conjugation of Peptoids or Peptides to Carrier Proteins and Aluminum Hydroxide Adsorption

After lyophilization, peptoids and peptides were conjugated to maleimide activated KLH (mcKLH) or maleimide activated bovine serum albumin (mcBSA) using kits from Pierce (Rockford, IL). Briefly, the activated carrier protein was dissolved to 2 mg/mL in ultrapure H_2O while the cysteine-containing peptoid or peptide was dissolved to 2 mg/mL in conjugation buffer [0.083 M sodium phosphate, 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.9 M NaCl, 0.1 M sorbitol, 0.02% sodium azide (NaN_3)]. The two were then mixed together by pipetting and incubated for 2 h at RT without shaking. After 2 h, the un-conjugated material was isolated from the conjugate by centrifugation over a Zeba spin column (Pierce, Rockford, IL). The column was prepared by centrifuging the column at 1,000 times the force of gravity (*g)* for 2 min into a 15 mL conical vial to remove the storage solution, followed by washing with 1 mL phosphate buffered saline (PBS; From 10x stock: 175.3 g NaCl, 21 g disodium hydrogen phosphate (Na_2HPO_4; Fisher Scientific, Pittsburgh, PA) 6.4 g sodium bicarbonate (NaH_2PO_4; Fisher Scientific, Pittsburgh, PA), brought up to 2 L in deionized H_2O), added by pipette, and centrifuged (ISC BioExpress, Kaysville, UT) for 2 min at 1,000 x *g* while collecting the PBS into a conical vial. Both the wash and centrifugation were repeated four times. Finally, the sample was loaded onto the Zeba spin column and centrifuged for 2 min at 1,000 x *g*. The sample was collected into a 15 mL conical vial and the optical density measured on an ultraviolet (UV) spectrophotometer at wavelength (λ) of 280 (OD_{280}) using a Tecan Spectafluor Plus plate reader (Maennedorf, Switzerland). The data was analyzed with Magellan 2 software (Tecan).

Samples were adsorbed to alum (Alhydrogel; Accurate Chemical and Scientific Corporation, Westbury, New York) by sterilizing the conjugates using a syringe filter (Millipore, Billerica, MA) attached to a 1 mL syringe, and then preparing 2 mg/mL conjugate and 2 mg/mL alum. The conjugate and alum were mixed together in a sterile MCF tube (sterilized by autoclave; Getinge USA, Rochester, NY) at 1:1 (v/v) and incubated for 20 min at 4˚C while inverting on a rotisserie. Sample adsorption to alum was confirmed via OD_{280} by centrifuging both alum alone and the sample

adsorbed to alum at 18,000 x *g* for 2 min, setting the OD_{280} equal to zero on the alum supernatant (blanking), and then reading the supernatant of the adsorbed sample. If the OD_{280} was close to zero, all samples were assumed to be adsorbed to the alum.

I. Immunizations

Mouse Immunizations

Female Swiss Webster mice were purchased from Taconic (Taconic Farms, Germantown, NY). All mice were housed in groups of four in sterilized cages with filter tops in accordance with institutional Animal Care Policy. Mice were allowed to acclimate in the facility for at least seven days prior to handling and then ear notched and weighed. Mice were weighed twice weekly throughout the vaccination schedule. All protocols were approved by the University of Texas Southwestern Medical Center at Dallas Institutional Protocol Review Committee (IACUC). The mice were approximately eight weeks of age at the time of the first pre-bleed and vaccination, described below. Following vaccination, the mice were monitored for signs of ill health as determined by alopecia and a loss of > 25% initial body weight. Before each vaccination, 50-100 uL blood was collected by first placing the mouse under sedation then collecting blood via retro-orbital bleed for serum using capillary tubes (Fisher Scientific, Pittsburgh, PA). The blood was then transferred from the capillary tubes to MCF tubes and sera was prepared by allowing the blood to clot during an overnight incubation at 4˚C, followed by centrifugation at 1,000 x *g* for 10 min at 4˚C. Sera were pipetted off with a micropipette and placed in a new MCF tube. This process of centrifugation was repeated once. The resulting sera were transferred to a new MCF tube by pipette before being stored at -20˚C until ELISAs were performed. Mice were injected intramuscularly (IM) in the calf muscle with 25-50 ug $FLAG^+$ peptide conjugated to KLH (FLAG peptide/KLH) or peptoid/KLH adsorbed to alum at 1 mg/mL final concentration. Immunizations were administered at four weeks intervals for eight weeks and were accompanied by a test bleed (again by retro-orbital bleeding) before each immunization (see **Figure 12A)**. Future work will

titer the mouse sera by ELISA to ensure anti-FLAG titers have returned to baseline. Following this return to baseline, potential FLAG mimetic peptoids will be conjugated to carrier protein and injected into the mice following the same protocol outlined for FLAG immunizations. Sera will be evaluated by ELISA for antibodies that cross react between the immunizing peptoid and FLAG peptide.

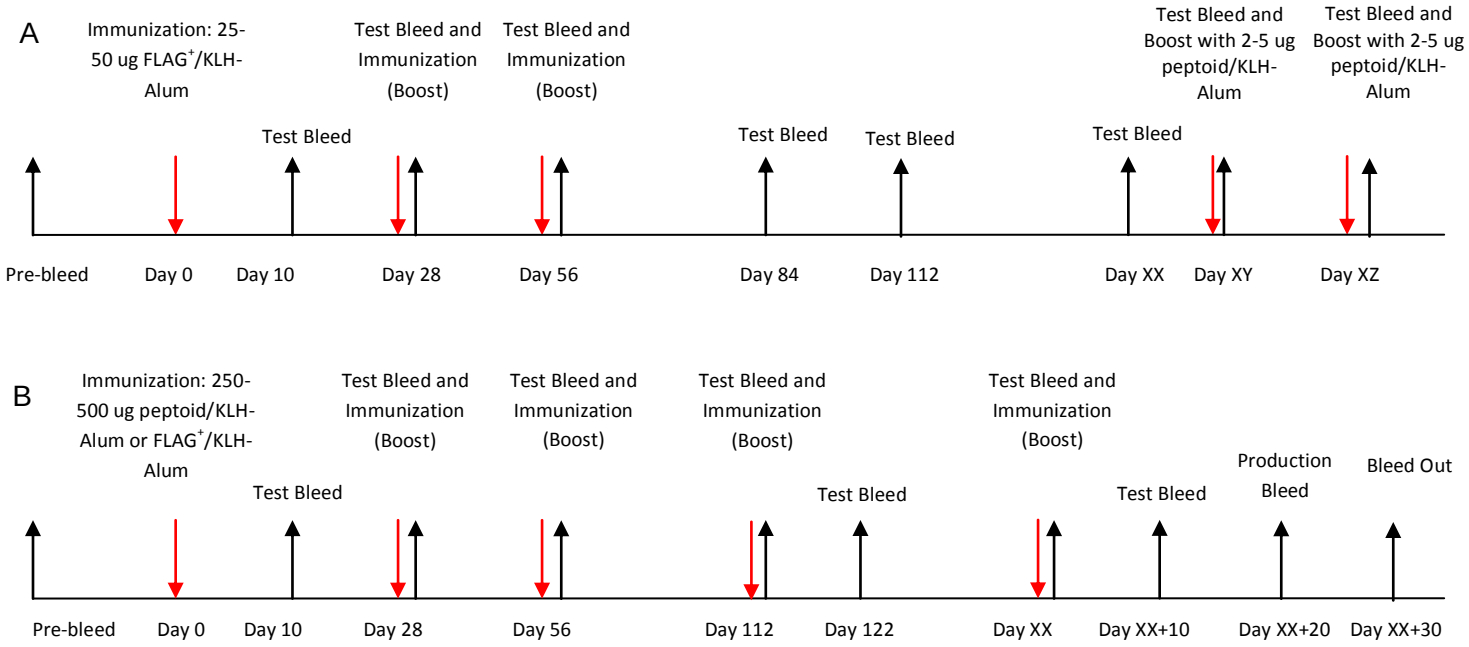


Figure 12: Mouse and rabbit immunization protocols. Depicted above are the mouse (A) and rabbit (B) immunization protocols. Upward pointing arrows indicated bleeds while downward pointing arrows indicate injections. Below each protocol timeline is the time, in days, of each event.

Rabbit Immunizations

Four female New Zealand White (NZW) rabbits, 3-4 months of age, were purchased from Myrtle's Rabbitry (Thompson Station, TN). Two rabbits were used per immunogen ($FLAG^+$ peptide/KLH or R5/KLH). Rabbits were housed in cages in accordance with the institutional animal care policy and were allowed to acclimate in the facility prior to handling. Rabbits were then ear tattooed. The health of the rabbits was monitored throughout the study by the Animal Resource Center (ARC) personnel and Cancer Immunobiology Center personnel. All protocols were

approved by IACUC. Following immunization, the rabbits were monitored for signs of ill health as determined loss of appetite or thirst.

Before the first immunization, the rabbits were pre-bled by central ear artery catheterization. The blood was allowed to clot and the sera was then processed as described above and tested in an ELISA. The sera from each rabbit were stored separately in a labeled MCF tube. Rabbits were immunized with 250-500 ug peptoid/KLH or $FLAG^+$ peptide/KLH each adsorbed to 1 mg/mL final concentration of alum. Rabbits were injected IM in each hind leg. A test bleed was performed 14 days following the first injection by central ear artery catheterization. Test bleeds and immunizations were repeated at four week intervals (**see Figure 12B**) until high titers (as determined by ELISA), were obtained. Rabbits will be bled again ten days after high titers are observed, every ten days for twenty days. Finally, the rabbits will be exsanguinated by cardiac puncture under sedation by an ARC technician.

J. Affinity Purification

$FLAG^+$ peptide columns were prepared using a SulfoLink Immobilization kit for peptides (Pierce, Rockford, IL). Briefly, 2 mg cys-$FLAG^+$ peptide was dissolved in coupling buffer (50 mM tris-(hydroxymethyl) aminomethane (Tris), 5 mM EDTA-Na, pH 8.5) reduced with 0.1 mL 25 mM tris(2-carboxyethyl) phosphine (TCEP), and incubated for 30 min at RT on a rotisserie. The Sulfolink column resin was suspended by turning the column end over end by hand and the column was then centrifuged for 1 min at 1,000 x *g* and this process repeated. Two milliliters coupling buffer was then added to the column. Two milliliters of coupling buffer containing $FLAG^+$ peptide was then added to the column and mixed end over end for 15 min on a rotisserie. The column was then kept upright for 30 min at RT without mixing. The column was then placed in a 15 mL conical vial and centrifuged at 1,000 x *g* to collect unbound peptide. The column was washed three times with 2 mL of wash solution (1.0 M NaCl and 0.05% NaN_3) which was pipetted onto the column and removed by centrifugation at 1,000 x *g* for 1 min into a 15 mL conical vial. Finally, the column was washed twice with coupling buffer which was removed by

centrifugation, and the wash was repeated once. Nonspecific binding was blocked by adding 15.8 mg L-Cysteine HCl to 2 mL coupling buffer and applying this solution to the column. The column was inverted end over end on a rotisserie at RT for 15 min at RT followed by 30 min incubation at RT without mixing. The column was equilibrated with 5 mL wash buffer (1.0 M NaCl and 0.05% NaN_3) and 2 mL or less of sera was added. Zero point two milliliters wash buffer was added and the samples were incubated 60 min at RT on a rocker to allow sera to bind. The column was centrifuged at 1,000 x *g*, washed with wash buffer and centrifuged again. To wash the column, 2 mL wash buffer was added and the column was centrifuged for 1 min at 1,000 x *g.* This was repeated fourteen times. Each time, the flow through was collected in a 15 mL conical vial. Finally, the protein was eluted with 2 mL 0.1 M Glycine HCl (pH 2.8) and collected into a tube containing 100 uL of 1M Tris HCl at pH 8.5. The protein was eluted a total of four times, and each time collected into a 15 mL conical vial. The eluate was labeled PAb anti-FLAG followed by the number of the elution pass that yielded the antibodies (i.e. PAb anti-FLAG eluate 1). The eluate was also tested in on-bead screens. Columns of cysteine-R5 peptoid were also prepared following this protocol. These elutions were also labeled according to the eluate number that resulted in the antibodies. PAb anti-R5 (eluate 1) ("anti-R5") was used for the majority of the experiments in this dissertation unless otherwise indicated. Two independent purifications of rabbit sera were performed and the flow through and eluate tested by ELISA. The third independent purifications and subsequent ELISAs are ongoing.

K. Radiolabeling and Trichloroacetic acid (TCA) Assay

Mouse anti-FLAG MAb was radiolabeled as described previously [103]. Briefly, 50 uL antibody at 1 mg/mL was pipetted into to an Iodo-Gen 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Iodogen; Sigma Aldrich, St. Louis, MO)-coated borosilicate 12 x 75 mm glass tube (Fisher Scientific, Pittsburgh, PA) and the tube placed on ice. Five to 10 uL radiolabeled iodine ($Na^{125}I$; Perkin Elmer, Waltham, MA) (stock: 17 Ci/mg) were added to the tube and incubated for 20 min on ice in a radioactive hood.

Sephadex G25 spin columns (Amersham Biosciences, Piscataway, NJ) were prepared by removing the top and bottom closure, centrifuging (Beckman Coulter Microfuge (Brea, CA) at 1,000 x *g* for 2 min and collecting the storage buffer into a MCF. Then, BSA was added and the column centrifuged, and all flow through was collected into a 15 mL conical vial. Lastly, the columns were washed by adding 1 mL PBS and then centrifuging to collect the PBS into a MCF tube. The radiolabeled sample was loaded into a prepared G25 spin column to remove free $Na^{125}I$, and centrifuged for 2 min at 1,000 x *g*. Samples were then collected into a MCF tube and diluted in 150 uL of 5 mg/mL BSA and 595 uL PBS. The incorporation of ^{125}I into the protein was quantified using a trichloroacetic acid (Fisher Scientific, Pittsburgh, PA) assay (TCA) and radioactivity in counts per minute (CPM) was determined by counting samples in a 1470 Automated Gamma Counter (Perkin Elmer, Waltham, MA). The specific radioactivity of the labeled protein was in the range 4-10 x 10^6 CPM/ug with < 10 % free $Na^{125}I$ as determined in the TCA and according to the equation:

$$\frac{\text{Average CPM of TCA precipitable protein sample}}{\text{Average CPM of iodinated sample}} \times 100$$

TCA assays were performed in a chemical hood designated for radioactive materials use by adding 10 uL of radiolabeled protein to each of four 12 x 75 mm glass tubes. Five milliliters of 10% TCA solution [10 g TCA (Sigma Aldrich, St. Louis, MO) in 100 mL MilliQ H_2O (purified and deionized water)] and 50 uL of 500 mM sodium iodide (NaI; Sigma Aldrich, St. Louis, MO) (7.59 g NaI in 100 mL MilliQ H_2O) were added into two of the 12 x 75 mm glass tubes (referred to as the precipitable protein samples) and allowed to incubate for 10 min at RT. During the incubation, two Millipore (Billerica, MA) HA filters were placed in MilliQ H_2O and allowed to soak for the duration of the incubation. After 10 min, forceps were used to place the filters into a filter holder (custom made) and one tube of the precipitable protein samples was poured over the filter. The filter was washed with 5 mL of 10% TCA a total of three times followed by two washes with 5 mL of 70% ethyl alcohol (Midwest Grain Products, Atchison, KS). The filter was then removed with forceps and placed into the bottom of a 12 x 75 mm glass tube. This process was repeated for the second

filter and precipitable protein sample and all four samples (two precipitable protein samples, two radiolabeled protein only samples) were read in the gamma counter using a 15 sec count. The percent precipitable protein was calculated using the equation above.

ASSAYS

L. On-Bead Screening

Statistics

All experiments described in this section were repeated three times (with the exception of library screens, which were only performed once). All screening optimization experiments were performed using duplicate tubes for each data point. The data shown represent the average of the three experiments and are represented as the mean plus or minus (+/-) the standard deviation (S.D). A student t-test (2–tailed, unpaired) was used to evaluate significance at a level of $p < 0.05$.

Antibodies and Control Proteins

The mouse anti-FLAG MAb M2 was purchased from Sigma Aldrich, St. Louis, MO and was used as part of the "FLAG system" consisting of the $FLAG^+$ peptide, the $FLAG^-$ peptide and the anti-FLAG. Anti-FLAG was also used to screen all peptoid libraries for peptoid mimetics of FLAG peptide. RFT5, a MAb mouse immunoglobulin 1 (IgG1) anti-CD25 [104], was used as an isotype-matched control MAb, and was prepared in-house. BSA was also used either as a non-immunoglobulin (non-Ig) control or an irrelevant control protein as needed. Affinity purified antibodies from mouse and rabbit sera were prepared in house as described below in *Immunizations and Affinity Purifications* and used to screen pure populations of the on-bead R5, on-bead R5 sequence spiked into on-bead RC, and on-bead R5 sequence spiked into an on-bead peptoid library aliquot.

General Magnetic Screening Protocol

On-bead screens were performed either in fritted syringes (up to 1 mL bead volume) or MCF tubes (no more than 250 uL bead volume). All libraries except Library 2, which was screened as aliquots of Library 2A, 2B, or 2C, were screened in aliquots of ~ 30,000 beads, divided according to their final submonomer. On-bead compounds were pipetted into the vessel, washed three times with PBST (PBS + 0.01% polyoxyethylenesorbitan monolaurate (Tween 20; Sigma Aldrich, St. Louis, MO)) by drawing up 5 mL PBST into the syringe, shaking by hand for 30 sec, and expelling the PBST. If a MCF tube was used, 1.5 mL PBST was added by pipetting, the tube vortexed (Fisher Scientific, Pittsburgh, PA) for 10 sec, and centrifuged at 2,000 x *g* and the PBST removed by pipetting off the supernatant. The screening MAb was added at twice the bead volume at 10 ug/mL and the mixture incubated on the rotisserie (Fisher Scientific, Pittsburgh, PA) for 1 h at RT. Unbound MAb was expelled from the syringe or pipetted out of the MCF tube after centrifugation and the beads were washed three times with PBST as described above. PGDs in PBST at a volume equal to twice the bead volume were added at a 1:10 dilution in PBST and the sample incubated on a rotisserie for 60 min at RT. Beads in solution were then transferred to a MCF tube if not already in one and placed on a magnet for 1 min or until the solution clarified (indicating that all PGDs were held to the side of the tube by the magnet). The magnet with samples loaded onto it, was then turned upside down multiple times to allow the samples to traverse the magnet before turning the magnet right side up. Peptoids or peptides not bound by PGDs (the negatives) formed a pellet at the bottom of the tube and were removed by pipetting. At least 250 uL of solution was left behind each time liquid was removed so as to not disturb the PGD bound by the magnet. Two hundred fifty microliters PBST were then added to the tube and the wash step was repeated twice by mixing the solution by pipetting, placing it on the magnet, allowing the solution to clarify, and removing the negative pellet. The remaining “hits” (beads bound by both MAbs and PGDs) were held on the inside wall of the tube by the magnet, and washed with PBST by adding PBST to the hit pellet, shaking the tube by hand until the sample was cloudy (indicating that the sample well mixed) and then the tube was placed on the magnet

until the solution clarified. PBST was removed by pipetting and the beads were counted by eye under a light microscope (10 x magnification) (Nikon Diaphot; Nikon, Melville, NY). To count by eye, all beads in PBST were pipetted into a Petri dish (VWR, West Chester, PA) and the dish with beads placed under a light microscope at 10x magnification. By moving the plate in a coordinated fashion, the number of beads was counted (referred to as "counted or counting" in the remainder of this section). These beads were then screened in an on-bead color screening assay (described below) for secondary validation.

Screening Optimization using the FLAG System

The optimal concentration of the anti-FLAG used in screening on-bead peptides was determined by adding 105 uL anti-FLAG (^{125}I labeled and unlabeled) or RFT5 (control antibody) (^{125}I labeled and unlabeled) to 1.6 uL bead volume of on-bead FLAG^{+} peptides or FLAG^{-} peptides in duplicate MCF tubes at 0-10 microgram per milliliter (ug/mL) in PBST. The MAb and peptide beads were incubated for 1 h at RT on a rotisserie to allow the labeled and unlabelled MAb to bind. After 1 h, the tubes were centrifuged at 2,000 x *g* and the supernatant removed by pipetting. The beads were washed three times by adding 200 uL PBST to each tube, placing the MCF tube on the vortex (Fisher Scientific, Pittsburgh, PA) to mix the beads in solution, and centrifuging at 2,000 x *g*. The supernatant was removed and retained by pipetting and the wash repeated twice. Each wash was transferred into a 12 x 75 mm borosilicate glass tube and the radioactivity counted in a gamma counter. The pellet of peptide beads was then re-suspended in 100 uL PBST, pipetted into a glass tube, and counted in a gamma counter. The resulting CPM were then converted to micrograms antibody bound per concentration in a TCA assay as described above and the binding of the MAb for the peptide beads was calculated based on the CPM of ^{125}I MAb bound and the ratio of labeled vs. unlabeled MAb added to the beads.

The optimized PGD concentration for use in the proof-of-principle screening platform was identified by adding 25 uL of 20 ug/mL radiolabeled protein (anti-FLAG, RFT5, or BSA) to 25 uL of washed PGDs (0-4.8 ug/uL) in a MCF tube, and incubated for 30

min at RT on a rotisserie. To wash and prepare stock PGDs, the PGDs are removed from the stock tube and placed on the magnet for 1 min. Storage solution was removed by pipetting and 1 mL PBS was added by pipetting. The sample was mixed well by shaking by hand for 10 sec and then replaced on the magnet. The PBS was removed and this process repeated twice more. Finally, the PGDs were brought to the desired final concentration by adding PBST by pipette. After allowing the PGDs to bind the constant fragment (Fc) portion of the antibody, the tubes were then placed on a magnet, the solution was allowed to clarify and the supernatant was removed by pipetting. The pellet was then washed six times with PBST, each time shaking the sample by hand, and then placing the tube on the magnet, allowing the solution to clarify, and removing the supernatant into separate tubes by pipetting. The pellet was then re-suspended in 150 uL PBST and the pellet, washes, supernatant and the empty tubes were counted in a gamma counter. The antibody retained was calculated by converting CPM into micrograms antibody bound using the radioactivity of each radiolabeled protein.

The effect that temporal order of reagent addition on the number of positive beads retained was determined by comparing two protocols. In the first protocol, 25 uL PGDs were pre-incubated with 25 uL MAb at 2, 5, 10, 20 or 50 ug/mL on the rotisserie for 30 min at RT. The MAb/PGD conjugates were then placed on the magnet, the supernatant removed, and the conjugates were washed three times with PBST. PBST was then added to make a stock dilution of the MAb/PGD conjugates. Four more serial dilutions were made from this stock and these five dilutions of MAb/PGD conjugates were added to 20 $FLAG^+$ or 20 $FLAG^-$ peptide beads in a MCF tube and incubated for 30 min at RT on the rotisserie. After 30 min, the samples were placed on the magnet and positive and negative bead isolation was carried out as described above. Briefly, the samples were placed on the magnet, the non-binders and supernatant removed, and the binders washed and counted under the microscope. In the second protocol, screening proceeded as described above. Briefly, 20 $FLAG^+$ or $FLAG^-$ peptide beads were added to MCF tubes and washed. Twenty microliters of screening MAb at 10 ug/mL were added to the beads, incubated for 1 h at RT on the rotisserie, and then washed. Twenty five microliters

of prepared PGDs were then added to the samples; the samples were incubated for 1 h at RT on the rotisserie, and then placed on a magnet. Positives were isolated from negatives and counted. The number of beads retained from the two protocols was compared.

Screening Validation using the FLAG System

Peptide "Spiking" Experiments

Spiking experiments were performed by adding three or five $FLAG^+$ peptide beads into 250 uL $FLAG^-$ beads and the mixture screened using anti-FLAG as described above in *On-Bead Screening, General Screening Protocol*. Beads retained by the MAb/PGDs on the magnet were counted under a microscope as described immediately above. Positive beads were washed in ACN: H_2O and isolated to MCF tubes (one bead per tube) using a P2 pipette set at 2 uL or less. These beads were then cleaved with CNBr as described above in *Tentagel Resin Cleavage* and the sequences were determined using MALDI-TOF MS and MS/MS.

Peptide/Peptoid "Spiking" Experiments

Spiking experiments were repeated using a peptoid library aliquot instead of $FLAG^-$ beads by adding 1, 3 or 5 $FLAG^+$ peptide beads into 250 uL Library 1 aliquot (refer to **Table 4** for a list of Library 1 monomers) and the mixture screened as described above. Beads retained by the magnet were counted under a microscope. Positive beads were washed in ACN: H_2O and isolated in MCF tubes (one bead per tube) as described above. These beads were then cleaved with CNBr as described above and the sequences were determined using MALDI-TOF MS and MS/MS.

"Preclearing": Removing Nonspecific Peptoid Sequences

Since peptoids might bind to sites on the MAb other than the antigen binding site (*e.g*. ligands for the Fc of the antibody), we tested how many beads would be bound

by a species and isotype-matched control antibody (RFT5). The hits were removed and the library was then screened with anti-FLAG. To this end, two Library 1 peptoid aliquots were spiked with $FLAG^+$ peptide beads and screened with 500 uL of 10 ug/mL RFT5, 500 uL of a 1:10 PGD dilution in PBST, and then the sample was placed on the magnet. Any positive beads were removed by pipetting. The remaining beads were washed three times in PBST and screened with 500 uL of 10 ug/mL anti-FLAG in PBS and peptide or peptoid-antibody complexes were isolated with PGDs and a magnet and counted. The peptides and peptoids retained were quantified under a microscope as described above then cleaved from the beads using CNBr as described in *Tentagel Resin Cleavage* and sequenced using MALDI-TOF MS and MS/MS.

Screening Optimization using the R5 peptoid system

To determine if screening conditions determined with the FLAG system were sufficient for the retention of R5 peptoids, four aliquots of 20 R5 beads and one aliquot of RC peptoid beads were made. Affinity purified rabbit anti-R5 sera in PBS were added at a volume equal to twice the bead volume of 5.88 ug/mL anti-R5 (eluate 1) were added to three R5 aliquots and one RC aliquot. Ten ug/mL RFT5 were added to the final R5 aliquot. The remainder of the screening protocol proceeded as described above. Briefly, samples were incubated on the rotisserie for 1 h at RT. Samples were then washed and twice the bead volume 1:10 PGD dilution in PBST was added and the samples incubated. Samples were then placed on the magnet, and the positive beads were counted.

Screening Validation Using the R5 peptoid system

Peptoid "Spiking" Experiments

Spiking experiments were performed by adding three or five R5 peptoid beads to 250 uL of RC peptoid beads and the mixture screened with antibodies from affinity purified rabbit anti-R5 sera (PAb anti-R5 (eluate 1); "anti-R5"; described above in

Affinity Purification). Beads retained by the magnet were counted under a microscope as described above. Retained beads were washed in ACN: H_2O and isolated to MCF tubes (one bead per tube). These beads were then cleaved with CNBr as described and the sequences were determined using MALDI-TOF MS and MS/MS. These spiking experiments were then repeated in a peptoid library aliquot by adding three or five R5 peptoid beads to aliquots of 250 uL bead volume of Library 2C, screening was repeated using 10 ug/mL anti-R5 and PGDs and the positive beads retained were quantified.

M. Library Screening

Peptoid libraries were deprotected and washed as described above, then screened to remove any peptoids bound to non-antigen binding sites on the antibody ("pre-clearing"). To do this, Libraries 1, 3, and 5 were screened in aliquots of 250 uL bead volume each, according to their terminal monomer, Library 2 was not subdivided by terminal monomer, using 500 uL RFT5 and 500 uL of PGDs at a 1:10 dilution in PBST and by placing the samples on the magnet. After these peptoids bound by RFT5 and PGDs were removed, the negatives were washed three times, and the screen was repeated with 500 uL MAb mouse anti-FLAG at 10 ug/mL and 500 uL of PGDs at a 1:10 dilution in PBST and MAb/PGD bound peptoid beads were isolated on the magnet and counted. These hits were then washed with PBST and validated using a secondary validation assay described in the next section.

N. Secondary Validation and Sequencing of Antibody Bound Peptoid Beads

FLAG color-screening was validated in two on-bead assays. First, the optimized concentration of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was optimized by transferring 20 $FLAG^+$ peptide beads into six MCF tubes by pipetting. Twice the bead volume of 10 ug/mL anti-FLAG in PBST was added to the beads in five of the tubes for 1 h at RT while rotating on a rotisserie. The remaining tube was incubated with twice the bead volume of 10 ug/mL RFT5. The beads were then

washed three times by adding 1 mL PBST by pipetting and vortexing the beads in solution, centrifuging at 2,000 x *g* and removing the supernatant with a pipette. The beads were then incubated with twice the bead volume of 0, 1/10,000-1/1,000 dilution in PBS of HRP-conjugated goat anti-mouse IgG secondary antibody (BD Pharmingen, San Diego, CA) and incubated on a rotisserie for 1 h at RT. After 1 h the beads were washed with PBS again as described. The beads were transferred to a 96 well plate, one well per tube, by micropipette and one drop 3, 3′, 5, 5′-tetramethylbenzidine (Ultra-TMB; Pierce, Rockford, IL) was added to the beads. When color change was observed, the reaction was stopped by pipetting twice the bead volume of 2 M sulfuric acid (H_2SO_4; Sigma Aldrich, St. Louis, MO) onto the plate and the OD_{450} of the samples in each well of the plate were measured on a plate reader.

The primary antibody concentration was optimized by transferring 20 $FLAG^+$ peptide beads into seven MCF tubes by pipetting. Twice the bead volume of 0, 0.00016 -1 ug/mL anti-FLAG in PBST was added to the beads in six of the seven aliquots for 1 h at RT while rotating on a rotisserie. The remaining aliquot was incubated with twice the bead volume of 10 ug/mL RFT5. The beads were then washed three times by adding 1 mL PBST by pipetting and vortexing the beads in solution, centrifuging at 2,000 x *g* and removing the supernatant with a pipette. The beads were then incubated with twice the bead volume of 1:500 dilution in PBS of HRP-conjugated goat anti-mouse IgG secondary antibody (BD Pharmingen, San Diego, CA) and incubated on a rotisserie for 1 h at RT. After 1 h the beads were washed with PBS again as described. The beads were transferred to a 96 well plate, one well per tube, by micropipette and one drop Ultra-TMB was added to the beads. When color change was observed, the reaction was stopped by pipetting twice the bead volume of 2 M H_2SO_4 onto the plate and the OD_{450} of the samples in each well of the plate were measured on a plate reader.

R5 color-screening was validated in two on-bead assays. First, the optimized concentration of HRP-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA) was optimized by transferring 20 R5 beads into eight MCF tubes

by pipetting. Twice the bead volume of 10 ug/mL anti-R5 in PBST was added to the beads in seven of the tubes for 1 h at RT while rotating on a rotisserie. The remaining tube was incubated with twice the bead volume of 10 ug/mL RFT5. The beads were then washed three times by adding 1 mL PBST by pipetting and vortexing the beads in solution, centrifuging at 2,000 x *g* and removing the supernatant with a pipette. The beads were then incubated with twice the bead volume of 0, 1/100,000-1/1,000 dilution in PBS of HRP-conjugated goat anti-rabbit IgG secondary antibody and incubated on a rotisserie for 1 h at RT. After 1 h the beads were washed with PBS again as described. The beads were transferred to a 96 well plate, one well per tube, by micropipette and one drop Ultra-TMB was added to the beads. When color change was observed, the reaction was stopped by pipetting twice the bead volume of 2 M H_2SO_4 onto the plate and the OD_{450} of the samples in each well of the plate were measured on a plate reader.

The primary antibody concentration was optimized by transferring 20 R5 beads into seven MCF tubes by pipetting. 20 RC beads were transferred into a MCF tube by pipetting. Twice the bead volume of 0, 0.02 – 1 ug/mL PAb anti-R5 (eluate 1) in PBST was added to the beads in six of the seven R5 aliquots and the RC aliquot for 1 h at RT while rotating on a rotisserie. The remaining aliquot was incubated with twice the bead volume of 10ug/mL RFT5. The beads were then washed three times by adding 1 mL PBST by pipetting and vortexing the beads in solution, centrifuging at 2,000 x *g* and removing the supernatant with a pipette. The beads were then incubated with twice the bead volume of 1:10,000 dilution in PBS of horseradish HRP-conjugated goat anti-rabbit IgG secondary antibody and incubated on a rotisserie for 1 h at RT. After 1 h the beads were washed with PBS again as described. The beads were transferred to a 96 well plate, one well per tube, by micropipette and one drop Ultra-TMB was added to the beads. When color change was observed, the reaction was stopped by pipetting twice the bead volume of 2 M H_2SO_4 onto the plate and the OD_{450} of the samples in each well of the plate were measured on a plate reader.

Any peptoid sequences bound by MAb/PGDs (hits) in a primary screen with anti-FLAG were subjected to on-bead color screening as a secondary validation for positivity. Each hit bead was transferred to a MCF by pipetting and 10 - 50 uL of 10 ug/mL anti-FLAG or anti-R5 in PBST was added to the bead for 1 h at RT while rotating on a rotisserie. The bead was then washed three times by adding 1 mL PBST by pipetting and vortexing the bead in solution, centrifuging at 2,000 x *g* and removing the supernatant with a pipette. The bead was then incubated with a 1:500 dilution in PBS of HRP-conjugated goat anti-mouse IgG secondary antibody (BD Pharmingen, San Diego, CA) or 1:10,000 HRP–conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA) at a volume equal to that of the screening antibody and incubated on a rotisserie for 1 h at RT. After 1 h the bead was washed with PBS again as described, one drop Ultra-TMB was added to the bead and the bead then viewed under a microscope. If a color change occurred (from translucent to blue, ~ 0-10 min), the bead was placed in a drop of 0.1 M glycine (Sigma Aldrich, St. Louis, MO)- HCl (Fisher Scientific, Pittsburgh, PA) at pH 2.8 in a Petri dish to remove the TMB, and the tube was placed on a vortex for 30 sec. This glycine wash was repeated twice more and the residual glycine neutralized with 1 mL PBST, added by pipette. The positive beads were washed three times in PBST, isolated one bead per tube, cleaved as described in *Tentagel Resin Cleavage* and the sequences were determined using MALDI-TOF MS and MS/MS.

O. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates (BD Bioscience, San Jose, CA) were coated with 50 uL of 10 ug/mL coating protein in PBS by pipetting the solution into each well using a multichannel pipette (Eppendorf, Eppendorf North America, Hauppauge, NY). Plates were covered with parafilm (VWR, West Chester, PA) and incubated at 4˚C overnight or for 1 hr at RT while shaking on a Bellco Rocker Platform (referred to as “shaking, shaker or shaken” in the remainder of this section). Plates were then washed twice in PBS by delivering PBS with a multichannel pipette and aspirating the liquid with an aspirator comb (Fisher, Pittsburgh, PA) connected to a vacuum flask and vacuum source (referred to as “washing, wash or washed” in the remainder of this section).

One hundred microliters starting block (proprietary formula containing 0.05% Tween 20; Pierce, Rockford, IL) was added by multichannel pipette and the plates were covered and incubated for 20 min on a shaker. Plates were again washed twice with PBS. Then, 50 uL MAbs or PAbs were added to each well in triplicate using a pipette (Mettler Toledo, Columbus, OH). The plates were covered with parafilm and incubated for 1 hr at RT while shaking. Plates were washed three times with 200 uL PBST. Secondary antibody (HRP–conjugated goat anti-mouse IgG or HRP–conjugated goat anti-rabbit IgG) was diluted to 1:2500 in dilution buffer (PBS + 0.01% Tween 20 + 1% starting block) and 50 uL added to the plates by multichannel pipette. The plates were again covered with parafilm and incubated for 1 h at RT while shaking. The plates were again washed in wash buffer and 50 uL TMB solution was added to each well by multichannel pipette. Plates were shaken in TMB for 5-20 min until blue color was observed by eye. The reaction was stopped by pipetting 50 uL 2 M H_2SO_4 onto the plate and the OD_{450} of the samples in each well of the plate were measured.

Sandwich ELISAs were performed by first coating the plates with 50 uL of affinity purified rabbit anti-KLH (produced in house) delivered by multichannel pipette followed by washing three times with PBS. Twenty five microliters FLAG/KLH, or KLH at 0, 0.01 – 10 ug/mL were then added to the plate by pipetting and allowed to incubate for 1 h at RT while shaking. The remaining ELISA protocol proceeded as described above.

Blocking ELISAs were performed as described above with slight modifications. Twenty five microliters anti-FLAG or RFT5 at 0.1 mg/mL were pre-incubated with 25 uL blocking protein (FLAG/BSA, KLH, or BSA) at 0, 0.025 - 250 M fold blocking protein concentration in a MCF tube at RT for 15 min followed by centrifugation at 18,000 x *g* for 10 min. The supernatant was removed by pipetting, leaving behind any pellet, and added to the FLAG/KLH coated plate at serial dilutions. RFT5 was included as a negative control antibody. Following this primary antibody addition step, the remaining ELISA protocol proceeded as described.

All ELISAs were repeated three times each, with triplicate wells per sample per plate.

III. RESULTS

Results Overview

We designed a platform in which peptoid libraries were screened with MAbs. MAb-bound peptoid sequences were retained with PGDs and further tested using two validation assays designed to confirm binding by the MAb and to evaluate the relative affinity of the MAb for the peptoid sequence, as well as to confirm binding of the sequence by the MAb's binding site.

This platform was evaluated in three phases. In Phase I, the magnetic screening and validation assays were optimized using the FLAG peptide system. In Phase II we continued optimizing the magnetic screening and validation assays using a peptoid/anti-peptoid pair. Finally, in Phase III, peptoid libraries were screened with anti-FLAG to identify potential FLAG peptide mimetic peptoids. Secondary validation assays were used to confirm binding and measure the relative binding affinity of the anti-FLAG to the potential B cell epitope mimetic (peptoid). Accordingly, these three phases are described in the three chapters below:

Chapter 1: Platform Development and Optimization Using a Peptide/Anti-Peptide MAb pair

Chapter 2: Platform Optimization Using a Peptoid/Anti-Peptoid Antibody Pair

Chapter 3: Application of the Optimized Screening Platform: Identifying Potential Peptoid Mimetics from Peptoid Libraries using a MAb.

The completion of these three phases resulted in an optimized platform from which potential mimetic peptoids could be identified with sensitivity, specificity, and reproducibility. The platform developed in this dissertation is now being applied to identify potential peptoid mimetics of HIV, HCV and WNV epitopes, which will be developed as vaccine candidates.

CHAPTER 1: PLATFORM DEVELOPMENT AND OPTIMIZATION USING A PEPTIDE/ANTI-PEPTIDE MAB CONTROL PAIR

A. Objective and Overview

Without a peptoid/anti-peptoid antibody pair to optimize our platform, a peptide/MAb pair consisting of on-bead $FLAG^+$ and $FLAG^-$ peptide and anti-FLAG, was chosen to establish and optimize on-bead magnetic screening. The selection of the peptide/MAb pair was based on the commercial availability of anti-FLAG and the ability to compare the binding of the MAb to $FLAG^+$ peptide versus $FLAG^-$ peptide.

The $FLAG^+$ and $FLAG^-$ peptides were synthesized as described in the Materials and Methods and validated by MALDI MS and MS/MS. After the synthesis and purity of the peptides was determined, they were used in the following manner:

- The FLAG system (anti-FLAG, $FLAG^+$ peptide, $FLAG^-$ (control) peptide) was tested by ELISA to verify that anti-FLAG bound to the $FLAG^+$ peptide and not to the $FLAG^-$ peptide (control) as described by Slootstra *et al* [100] (data not shown).

- Magnetic screening conditions were established and optimized by determining i) the binding capacity of anti-FLAG to on-bead $FLAG^+$ and $FLAG^-$ control peptides, ii) the binding capacity of PGDs to anti-FLAG, and iii) the effect that the order of reagent addition had on the retention of the beads.

- The optimized magnetic screening conditions established above were validated in two assays. In the first assay, a small number of $FLAG^+$ peptide beads were added to an aliquot of $FLAG^-$ (control) peptide beads and the magnetic screening protocol was applied to retain these added ("spiked") beads. In the second assay, $FLAG^+$ peptide beads were added to a peptoid library aliquot to more closely approximate screening peptoid libraries and the magnetic screening platform was applied to retain these spiked beads.

- An experiment was performed to determine whether peptoid libraries should be pre-cleared of peptoids that bound to other portions of the antibody not involved in antigen binding using a species and isotype-matched control MAb.

- Two assays were developed to serve as secondary validations of retained peptoids. In the first assay, anti-FLAG and goat anti-mouse-HPR were added to beads retained and washed in library screening. Beads that were positive in this "color-screening" assay turned blue. The second assay determined whether sequences retained in the magnetic screen were bound by the binding site of the MAb and with what relative affinity ("blocking ELISAs").

The completion of this first phase of work resulted in an optimized platform to screen on-bead peptoid libraries with MAbs and two validation assays to verify that peptoids retained during the magnetic screening mimicked the native antigen for which the MAb was specific.

B. Results

i. Platform Development: Optimization of on-bead magnetic screening using the FLAG system

a. Affinity of anti-FLAG for FLAG peptides as determined by ELISA

Before beginning work on the screening and platform development, an ELISA was performed to ensure that anti-FLAG bound $FLAG^+$ peptide and not the $FLAG^-$ control peptide as described by Slootstra *et al* [100]. Cysteine (cys)-$FLAG^+$ peptide and cys-$FLAG^-$ peptide were synthesized on Rink resin using the automated synthesizer and were deprotected and cleaved as described in Materials and Methods. The peptide MWs were analyzed by MALDI MS and the peptide sequences were analyzed by MALDI MS/MS both before and after HPLC purification. Both $FLAG^+$ and $FLAG^-$ peptides were successfully synthesized and purified before use (data not shown). Cysteine was included to facilitate attachment of the peptide to a carrier protein after cleavage from the resin. Attachment to the carrier was necessary because small peptides do not bind well to ELISA plates. Conjugation of the peptide

to the carrier protein enabled coating of the peptide conjugate onto the plate and created a multivalent display of the peptide for the MAb to bind. Carrier protein conjugates were used to coat ELISA plates. Control plates were coated with the carrier protein. Binding of anti-FLAG to $FLAG^+$ and $FLAG^-$ control peptide was measured. RFT5 was included as an isotype (IgG) and species (murine) specific antibody control. Anti-FLAG bound cys-$FLAG^+$ peptide but neither cys-$FLAG^-$ peptide nor carrier protein. RFT5 bound neither $FLAG^+$ nor $FLAG^-$ peptide on carrier protein. The saturating concentration of anti-FLAG for $FLAG^+$ peptide on BSA (carrier protein) was 0.05 ug/mL.

This standard ELISA confirmed the binding of anti-FLAG to FLAG peptides as described by Slootstra *et al* [100] and determined the saturating concentration of anti-FLAG for $FLAG^+$ peptide in a plate ELISA. This saturating concentration was used as a guide when designing downstream on-bead experiments

b. Affinity of anti-FLAG for on-bead FLAG peptides

To determine whether anti-FLAG would recognize bead bound M-A-A-$FLAG^+$ peptide, and if so, how much antibody was bound by a single $FLAG^+$ peptide bead, $FLAG^+$ peptide or $FLAG^-$ peptide beads were incubated with ^{125}I labeled and unlabeled anti-FLAG or RFT5. The antibody bound to on-bead peptide was measured using a gamma counter and CPM were converted to total micrograms of antibody bound by dividing by the specific radioactivity of each antibody. RFT5 was included as an isotype and species specific negative control. As shown in **Figure 13**, the saturating concentration of anti-FLAG for on-bead $FLAG^+$ peptide was 1 ug/mL in this on-bead assay. Anti-FLAG did not bind to on-bead $FLAG^-$ peptide nor did RFT5 bind to on-bead $FLAG^+$ peptide.

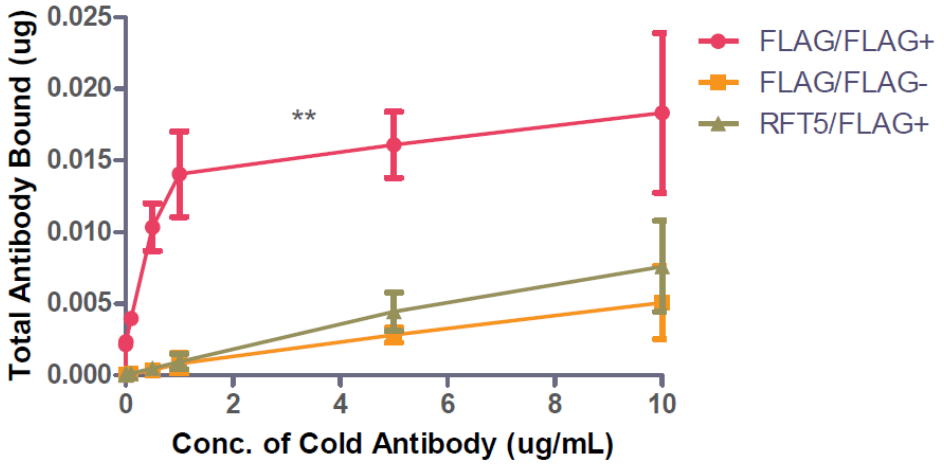


Figure 13: Binding of anti-FLAG to on-bead FLAG$^+$ and FLAG$^-$ (control) peptides. One point six microliters of on-bead FLAG$^+$ peptide beads or FLAG$^-$ peptide beads were incubated with 100 μL (0-10 μg/ mL) of ^{125}I labeled anti-FLAG or RFT5 for 1 h. The supernatant was removed and the beads were washed with PBST. The peptide bound antibody was determined by placing the bead pellet, suspended in PBST, in a glass tube and placing the tube in a gamma counter. CPM were converted to micrograms of antibody bound by the peptide by dividing by the specific radioactivity (CPM/ug) of each protein and by multiplying by the ratio of unlabeled to labeled antibody added to each sample. The signal to noise ratio of FLAG$^+$/FLAG$^-$ was 14.7. This experiment was repeated three times, using duplicate tubes for each data point. The data shown represent the average of the three experiments and are represented as the mean +/- the S.D. A student t test (2–tailed, unpaired) was performed yielding a *p* value <0.05 (**) for binding of anti-FLAG binding to FLAG$^+$ peptide vs. FLAG$^-$ peptide, and p value < 0.05 for anti-FLAG binding to FLAG$^+$ peptide vs. RFT5 binding to FLAG$^+$ peptide.

Anti-FLAG recognized both plate bound FLAG$^+$ peptide in the ELISA above and on-bead peptide presented on an M-A-A linker in the on bead assay. On-bead FLAG$^+$ peptide was saturated with anti-FLAG at 1 ug/mL. A concentration tenfold higher than the saturating concentration (10 ug/mL) was adopted as the screening concentration for the magnetic screen.

c. Affinity of Protein G Dynabeads for anti-FLAG

To determine the amount of PGDs needed to bind 10 ug/mL anti-FLAG, titrated concentrations of PGDs were incubated with ^{125}I labeled anti-FLAG, RFT5, or BSA, a protein that would not be expected to be bound by PGDs. The PGD bound radiolabeled antibody was counted in a gamma counter and the CPM were converted to total protein bound by dividing by the specific radioactivity determined for each labeled protein. RFT5 and BSA were included as positive and negative controls respectively. As shown in **Figure 14**, at 2 uL of stock PGD volume, the PGD binding of anti-FLAG was saturated, corresponding to a binding capacity of 0.134 micrograms of antibody per microliter of stock PGDs (0.134 ug/uL). Two microliters of stock PGDs corresponded to a 1:12.5 dilution. PGDs did not bind BSA, the "non-Ig" control, but did bind RFT5 because RFT5 has an Fc portion antibody.

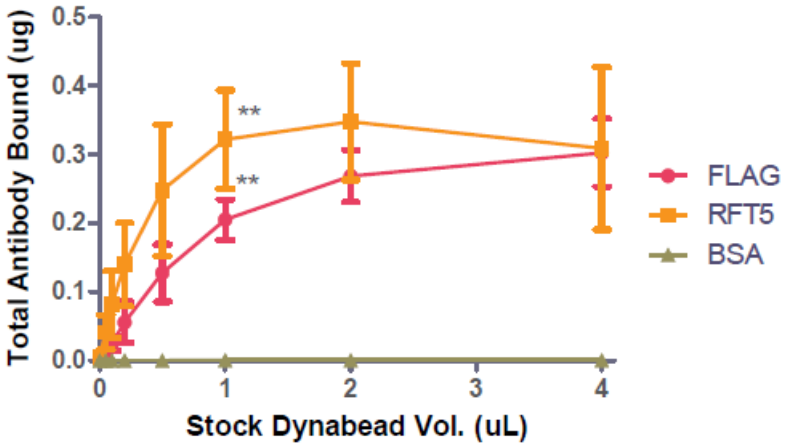


Figure 14: Binding of anti-FLAG by PGDs. Twenty five μL of 20 μg/ml ^{125}I labeled anti-FLAG and isotype-matched control MAb, RFT5, or BSA were incubated with 25 μL of titrated PGDs in PBST (0-4.8 μg/μL) while rotating on a rotisserie at RT. After 30 min, unbound protein was removed; the PGDs were washed six times with PBST, the pellet transferred to a glass tube, and counted in the gamma counter. CPM were converted into total protein bound by dividing by the specific radioactivity of each protein. This experiment was repeated three times, with duplicate tubes for each data point. Data are displayed as the average of the three experiments and presented as mean +/- S.D. A 2-tailed, unpaired student t- test was performed and yielded a *p* value < 0.05 (**) for anti-FLAG vs. BSA and a p value <0.05 for RFT5 vs. BSA.

With regard to PGDs, 0.134 ug/uL, or a 1:12.5 dilution of stock PGDs was required to bind 10 ug/mL of anti-FLAG. Therefore, a 1:10 dilution was adopted as the dilution of PGDs necessary to retain and isolate on-bead sequences saturated by antibody from a library screen. This experiment also confirmed that saturation of a PGD with antibody did not prevent their binding to the magnet.

d. Retention of on-bead sequences and the effect of order of reagent addition to retention of on-bead sequences

Having determined the binding capacity of PGDs for anti-FLAG, we next determined whether the strength of interaction between the two was sufficient to retain a positive bead on the wall of a tube using a magnet. To do this, the conditions determined above were used in combination. Two versions of this assay were compared to determine which order of reagent addition resulted in the highest retention of $FLAG^+$ peptide beads. In the first version, anti-FLAG was incubated with PGDs before adding the MAb/PGD complex to $FLAG^+$ or control ($FLAG^-$) peptide beads. These data were plotted as the percentage of $FLAG^+$ beads retained vs. the concentration of the MAb (ug/mL). In version two, $FLAG^+$ or control ($FLAG^-$) peptide beads were incubated with anti-FLAG followed by the addition of PGDs. Each sample was placed on the magnet and the number of peptide beads retained was quantified. RFT5 was included as the negative control antibody. These data were plotted as the percent of $FLAG^+$ peptide beads retained vs. increasing concentration of PGDs (reflected in **Figure 15** as the theoretical volume of stock (30 mg/mL) PGDs present in each tube). Version 2 (right hand panel), with sequential addition of anti-FLAG and PGDs was the preferred option since 100% vs. 35% of $FLAG^+$ peptide beads (10 ug/mL) were retained. In both versions of this assay, both anti-FLAG added to the on-bead control peptide or RFT5 added to the on-bead $FLAG^+$ peptide resulted in no bead retention.

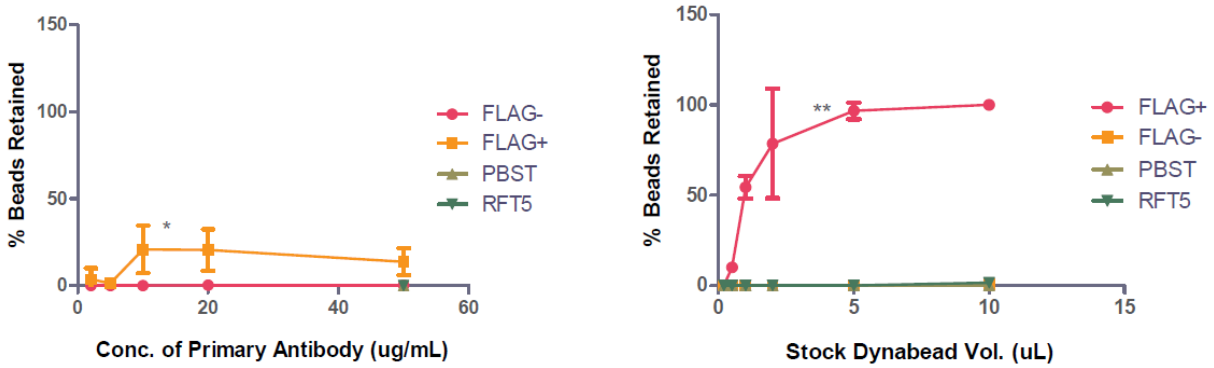


Figure 15: Effect of order of reagent addition on on-bead sequence retention. The left panel shows the percentage of beads retained by the pre-incubated MAb/PGD conjugate versus concentration of the MAb. The right panel shows the percentage of beads retained by the sequential incubation of anti-FLAG with on-bead peptide followed by PGD addition versus volume of stock dynabeads per tube. Sequential addition retained 100% of the beads as compared to 35% with preincubation of MAb/PGDs. These experiments were repeated three times. Data are displayed as the average of the three experiments for the pre-incubation and the average of two experiments for sequential incubation and presented as mean +/- S.D. A 2-tailed, unpaired student t-test was performed and yielded a *p* value < 0.10 (*) for anti-FLAG binding to $FLAG^+$ beads vs. $FLAG^-$ beads for the left panel and a *p* value < 0.05 (**) for anti-FLAG binding to $FLAG^+$ beads vs. RFT5 and $FLAG^-$ for the right panel.

The conditions determined above were sufficient to isolate a bead using a magnet. Furthermore, sequential addition of anti-FLAG and PGDs greatly enhanced bead retention. This was adopted as the standard protocol for magnetic screening. Since peptoid sequences did not bind to the PGDs, blocking buffer was not considered necessary.

ii. Magnetic Screening

a. Retention of $FLAG^+$ peptide beads "spiked" into on-bead control ($FLAG^-$) peptide beads

Ultimately, this magnetic screen would be used to isolate vaccine candidates form peptoid libraries. To further refine the screening conditions in order to begin approximating conditions under which this magnetic screening assay could be applied to retain a presumably small number of mimetic peptoids from a large peptoid library, $FLAG^+$ peptide beads were spiked into increasingly complex on-bead sequences. Also, the potential existed that the presence of other non-$FLAG^+$ peptide sequences could adversely affect the amount of antibody available to the $FLAG^+$ peptide beads, and thus retention of the beads would be decreased. Furthermore, we needed to determine whether the magnetic screening conditions were sufficiently specific to distinguish $FLAG^+$ peptides from control ($FLAG^-$) peptides when the two were mixed together. To answer these questions, three or five $FLAG^+$ peptide beads were spiked into ~20,000 control ($FLAG^-$) beads and the mixtures were subjected to the magnetic screening assay described above. **Table 6** summarizes the total number of the retained beads for the three experimental repeats, the number of the retained beads that were successfully analyzed by MALDI MS, and the number of $FLAG^+$ and non-$FLAG^+$ sequences identified.

Many factors affect the success of MALDI MS including the efficiency of cleavage of the compound from the bead, the interaction between the compound and the matrix, the amount of compound spotted on the plate, and the ionization pattern for each sample as it was ionized and analyzed. When 5 $FLAG^+$ peptide beads were spiked into 20,000 $FLAG^-$ control beads (in triplicate), 24 were retained. All 24 beads were successfully analyzed by MALDI MS and 12 of the 24 beads analyzed by MALDI MS were identified as $FLAG^+$ peptide. In other words, 12/15 (80%) $FLAG^+$ peptide beads originally introduced into the three $FLAG^-$ peptide bead aliquots were retained. Six beads successfully analyzed by MALDI MS were not $FLAG^+$ peptides. When 3 beads were spiked into 20,000 $FLAG^-$ beads, 10 of 9 were retained, 9 of which were successfully analyzed by MALDI MS, and 8 of which were $FLAG^+$ peptide. The remaining sequence was $FLAG^-$ peptide (1230 Da).

The results of these experiments indicated that the magnetic screening platform was **sensitive** (beads were retained from samples as dilute as 3 beads in 20,000),

specific (88%+ FLAG$^+$ beads were retained), and **reproducible** in the retention of FLAG$^+$ peptide sequences.

Table 6: The retention of known numbers of FLAG$^+$ peptide beads "spiked" into an excess of FLAG$^-$ (control) peptide beads

Ratio of FLAG$^+$: FLAG$^-$ Beads	Beads Retained from the Spike: Beads Added	Successfully Analyzed by MALDI	Confirmed as FLAG$^+$	Confirmed as Non-FLAG$^+$
5/20,000 [3]	24/15	24/24	12/15 (80%)	6 x 1230 Da (FLAG$^-$)
3/20,000 [3]	10/9	9/10	8/9 (88%)	1 x 1230 Da (FLAG$^-$)

Either 3 or 5 FLAG$^+$ peptide beads were spiked into ~ 20,000 FLAG$^-$ beads and the mixture was screened with 10 ug/mL anti-FLAG and 1:10 dilution of PGDs and the hits isolated on a magnet and counted under a microscope. Each spiking experiment was repeated three times as indicated in column one. Column two summarizes the number of beads retained. Column three is the number of beads with sequences successfully analyzed by MALDI MS. Columns four and five summarize the number of beads which were successfully analyzed in MALDI found to be FLAG$^+$ peptide or non-FLAG$^+$ sequences (peptoid sequences).

b. Retention of FLAG$^+$ peptide beads "spiked" into peptoid library aliquots

To further increase the complexity of the on-bead sequences into which FLAG$^+$ peptide beads were added and to evaluate the affect of peptoid beads on the magnetic screening the assays above were repeated by spiking either 1, 3 or 5 FLAG$^+$ peptide beads into aliquots of Library 1, reasoning that this complex mixture would more closely approximate the application of this magnetic screening assay to the identification and retention of small numbers of mimetic peptoids from a large library of peptoid beads. We also determined how many, if any, potential peptoid mimetics would be identified. The experiment was performed as described above, but instead of FLAG$^-$ beads, FLAG$^+$ peptide beads were spiked into peptoid library aliquots of approximately 30,000 beads (Library 1). This aliquot size was chosen

because 30,000 beads was a reasonable number of beads for a MCF tube, the tube for which our magnet was designed. **Table 7** summarizes the data. The results of these experiments indicated that the magnetic screening platform was **sensitive** (beads were retained from samples as dilute as 1 beads in 30,000), **specific** (100% $FLAG^+$ beads were retained from spikes of 3:30,000 beads), and **reproducible** in the retention of $FLAG^+$ peptide sequences from $FLAG^-$ peptide. Furthermore, the presence of peptoid beads did not adversely affect the retention of $FLAG^+$ peptide beads. Peptoid sequences were not bound directly by the PGDs, indicating that blocking buffer was not necessary to inhibit non-specific binding.

Table 7: The retention of known number of $FLAG^+$ beads "spiked" into a peptoid library aliquot

Ratio of $FLAG^+$: Peptoid Aliquot Beads	Beads Retained from the Spike: Beads Added	Successfully analyzed by MALDI MS	Confirmed as $FLAG^+$	Confirmed as Non $FLAG^+$: (peptoid MW)
5/30,000 [3]	20/15	15/20	10/15 (67%)	5/20: 1167, 1205, 1115, 1007, 1126
3/30,000 [3]	21/9	20/21	9/9 (100%)	11/20: 1122, 1014, 1239, 1391, 877, 1077, 1375, 834, 1008, 1127, 1106
1/30,000 [3]	9/3	9/9	2/3 (67%)	7/9: 919, 1145, 1143, 1081, 1374, 1258

5, 3, or 1 $FLAG^+$ peptide beads were spiked into a peptoid library aliquot (30,000 beads) and the aliquot was screened with 10 ug/mL anti-FLAG and 1:10 dilution of PGDs and the hits isolated on a magnet. The beads retained were counted under the microscope. Each spiking experiment was repeated three times as indicated in column one. Column two summarizes the number of beads retained from the spike. Column three is the number of beads with sequences successfully analyzed by MALDI MS. Columns four and five summarize the number of beads which were successfully analyzed in MALDI and found to be $FLAG^+$ peptide or non-$FLAG^+$ sequences (peptoid sequences). Also shown in column five is the molecular weight of the non-$FLAG^+$ peptide sequences which will be evaluated as potential mimetic sequences.

Table 8 shows the sequences of the peptoid beads retained from spikes into three aliquots of Library 1. **Figure 16** below shows the MALDI MS spectra of three beads retained from these spiking experiments. When the sequences were cleaved from beads and analyzed, their MW was determined to be 1322 Da by MALDI MS, indicating that they were M-A-A-FLAG$^+$ peptides.

Twenty six of the thirty beads retained came from a single Library 1 aliquot whose terminal monomer was tyramine. Some consensus did appear among the sequences; tyramine and 1,4 diaminobutane together accounted for 50% of the monomers. In this project, all but one subsequent libraries included tyramine and all included 1, 4 diaminobutane.

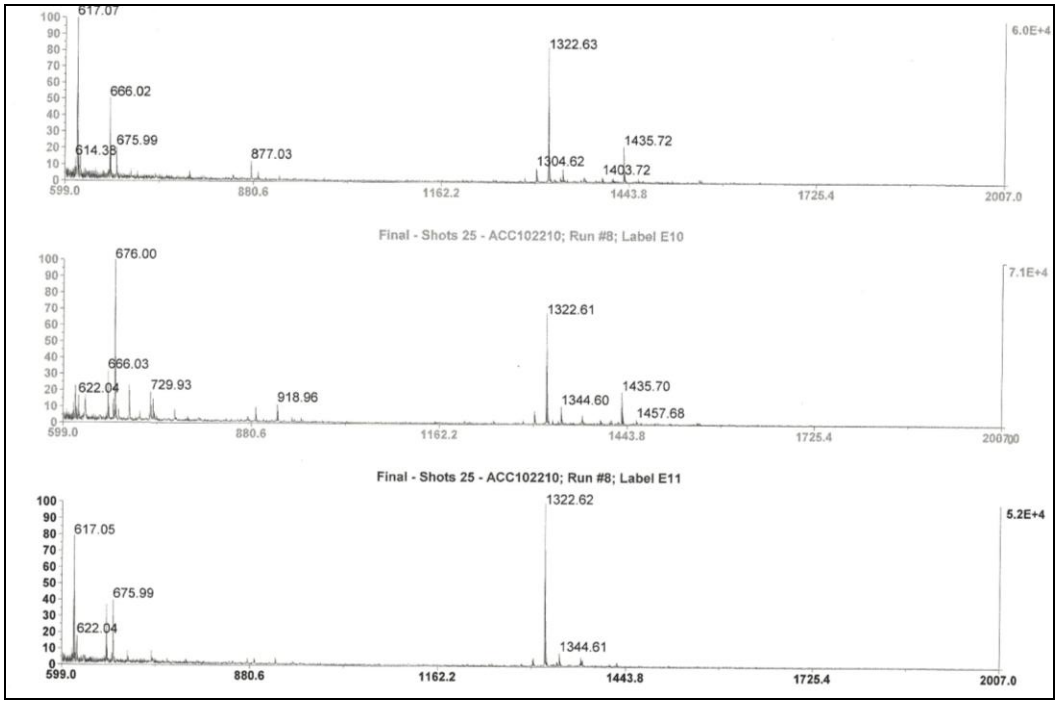


Figure 16: MALDI MS spectra of three beads retained in a "spike" of three FLAG$^+$ peptide beads into 30,000 peptoid beads. 1322 m/z was seen for all three samples and corresponds to the MW of M-A-A-FLAG$^+$ peptide (1322 Da).

Table 8: Sequences of the peptoids retained from spiking FLAG$^+$ beads into a peptoid library aliquot

Terminal Monomer	Hits	Sequences					MW
Benzylamine	3	Nbn	Ndi	Ndi	Net	Nty	1227
		Nbn	Nly	Net	Nly	Npy	1014
		Nbn	Nly	Npy	Nly	Nly	1041
Ethanolamine	1	Net	Nty	Net	Nbs	Nly	1075
Tyramine	26	Net	Nty	Nly	Nbs	Ndi	1211
		Nty	Nty	Nbs	Net	Nbn	1205
		Nty	Nly	Nty	Nly	Nty	1115
		Net	Nbs	Ndi	Net		1007
		Nty	Nly	Nly	Nly	Ndi	1126
		Nty	Npy	Ndi	Nai	Nty	1274
		Nty	Nbs	Nbs	Ndi	Nbs	1462
		Nty	Npy	Ndi	Nly		1052
		Nty	Nty	Nbs	Net	Npy/Nbn	1205
		Nty	Nly	Nty	Nly	Nty	1115
		Nty	Nly	Npy	Ndi	Npy	1167
		Ndi	Npy	X	Nbs	Nbn	1374
		Nty	Nbn	Nas	Npy		949
		Nty	Nbn	Nly	Nty	Nly	1085
		Nty	Nbn	Nly	Net	Nly	1066
		Nty	Nty	Nbn	Nbs	Ndi	1306
		Nty	Nai	Nly	Nly	Nbs	1174
		Nty	Nbs	Nbs	Nly	Nly	1241
		Nty	Nty	Nly	Nly		1115
		Nty	Nbs	Nly	Nbs	X	1112
		Nty	Nbs	Net	Nly	Nbn	1121
		Nty	Nas	Nas	Net	Npy	1018
		Nbn	Nly	Nly	Nbn	Nly	1005
		Nty	Nly	Nty	Nly	Nty	1115
		Nty	Ndi	Nbn	Npy	Net	1172
		Nty	Nly	Nly	Ndi	Nly	1126

Three peptoid library aliquots were spiked with FLAG$^+$ peptide beads and magnetically screened. In addition to the retention of the FLAG$^+$ peptide beads spiked into the aliquots, peptoid sequences were retained. These peptoid sequences are listed in addition to the terminal monomer associated with that aliquot and the sequence MW in Daltons. Sequences are color coded (each monomer was assigned a unique color as shown above)

to help identify consensus sequences/monomers. **Table 4** gives the full submonomer name for the monomer abbreviations used here.

c. "Preclearing" peptoid library aliquots: Removing peptoid sequences that bind to other portions of the MAb

Isolating peptoid sequences in addition to $FLAG^+$ peptide during the spiking experiments suggested the possibility that peptoids bound to sites on the screening antibody other than at the antigen binding site might be retained during screening. The need to remove peptoids which might bind to other sites on the antibody (i.e. hinge, Fc) was evaluated by spiking two peptoid aliquots from Library 1 with $FLAG^+$ peptides and then screening with an isotype matched MAb, RFT5 ("pre-clearing") before screening with MAb anti-FLAG. Any peptoids bound by RFT5 were removed and the aliquots were then re-screened until no peptoid beads were retained with the preclearing antibody, RFT5. As shown in **Table 9**, five beads were retained in the first round of pre-clearing in two aliquots. No peptoid beads were retained from the second round of "pre-clearing" screen. The spiked aliquots were then screened using with anti-FLAG. 90% of the original $FLAG^+$ peptide beads added in to the aliquot was retained. In the second aliquot, 35 of 36 beads were retained but only 64% were confirmed to be $FLAG^+$.

Based on these results, pre-clearing did not adversely affect the isolation of $FLAG^+$ peptide beads. Library 1 did not have a significant number of non specific binders and would not require pre-clearing. The need for preclearing was evaluated on a library by library basis since each peptoid library had the potential to contain non-specific binders.

Table 9: Evaluating the need to remove peptoids bound to sites outside the MAb's binding site

	Number of FLAG$^+$ Peptide Beads:			% Beads
Peptoid library aliquot number	**Spiked into the aliquot**	**Retained during "pre-clearing" (RFT5)**	**Retained during "screening" (anti-FLAG)**	**Retained in "screening" and verified as FLAG$^+$**
Aliquot 1	32	5/32	35/32	29/32 (90%)
Aliquot 2	36	0/36	35/36	23/36 (64%)

Two peptoid library aliquots were washed three times with PBST. FLAG$^+$ peptide beads were added into each aliquot, (Aliquot 1: Experiment 1: 10 beads added, Experiment 2: 9 beads added, Experiment 3: 13 beads. Aliquot 1 total: 32 beads total. Aliquot 2: Experiment 1: 9 beads, Experiment 2: 10 beads, Experiment 3: 17 beads. Aliquot 2 total: 36 beads) and the aliquots then screened with RFT5 and PGDs. The beads were placed on the magnet and beads were counted under a microscope. The number of beads retained is indicated in the third column. These "preclearing" beads were removed and the remaining beads were then washed and screened with 10 ug/mL anti-FLAG and PGDs. The number of beads retained in anti-FLAG/PGD screens is indicated in column four. The last column indicates the number of beads retained by screening with anti-FLAG/PGDs that were FLAG$^+$ as determined by MALDI MS. This experiment was repeated three times per aliquot.

iii. Screening Validation: Development of secondary validation assays

a. "Color screening"

No further testing could be done on the original peptoid sequences retained in the spiking experiments because the sequences were cleaved from the bead in preparation for MALDI MS analysis. Sequence cleavage, sequencing, and re-synthesis were labor and material intensive. In addition, our goal was to develop a method to validate the positivity of a bead retained from a library screen in a secondary assay before cleavage, synthesis and scale up. Therefore, we designed an assay referred to as "color screening" which could be applied to individual beads immediately after they were isolated from a peptoid library and before cleavage from the beads. This intermediate assay would reduce the time, labor, and materials required to assess any false positives identified in the magnetic screening. Specifically, color screening determined whether peptoids retained by the magnetic screen were indeed bound to anti-FLAG determined by adding an enzyme-coupled secondary antibody and a substrate that would change color to the antibody bound

sequences. This validation assay eliminated any beads that represented false positives in the magnetic screening step.

For color screening, anti-FLAG was added to the retained sequences followed by HRP-conjugated goat anti-mouse IgG. TMB was then added and the color change of the on-bead sequence was determined by eye. Before applying this color screening assay to potential mimetic sequences retained during screening, the conditions were optimized in a two step experiment.

To optimize the secondary antibody dilution, titrated dilutions of HRP-conjugated goat anti-mouse IgG were added to twenty $FLAG^+$ peptide beads that had been incubated with 10 ug/mL anti-FLAG for 1 h and washed. The absorbance was measured in a plate reader and plotted vs. the dilution of the secondary antibody. The data are presented in the left panel of **Figure 17**. RFT5 was included as a negative control and did not bind to $FLAG^+$ peptide as indicated by baseline absorbance. Absorbance was maximized when 1:500 dilution of HRP-conjugated goat anti-mouse IgG was added to the $FLAG^+$ peptide bound by anti-FLAG and accordingly this dilution was adopted as the secondary antibody dilution for color screening.

The concentration of anti-FLAG to add to individual peptoid sequences was optimized by adding known amounts of the MAb to 20 $FLAG^+$ peptide beads and incubating for 1 h. Maximum absorbance, correlating to the saturation of $FLAG^+$ peptides with antibody, was observed at 0.10 ug/mL for 20 $FLAG^+$ peptide beads.

Color screening was determined to be sufficiently sensitive and specific to be used for validating peptide beads retained from a screen. A 1:500 dilution of secondary antibody would be used along with 10 ug/mL of primary antibody, which was chosen to be saturating and to parallel the magnetic screening conditions. The $FLAG^+$ peptide sequences turned blue in under 1 min and the color change was intense enough to be observed by eye.

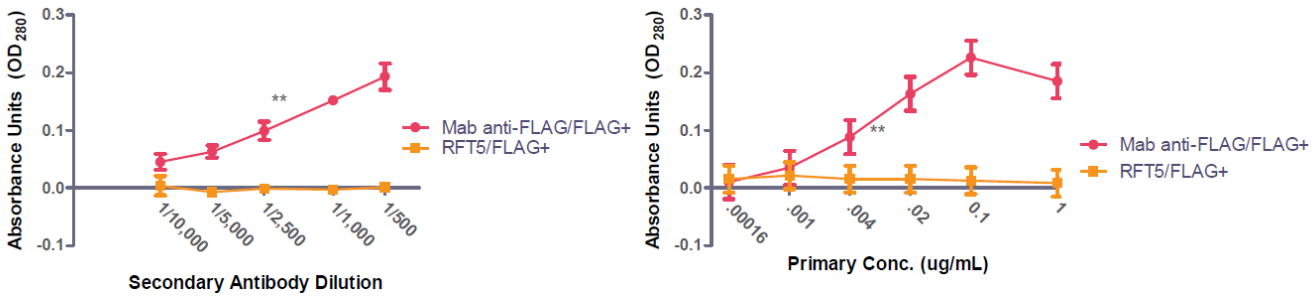


Figure 17: Color screen optimization with the FLAG system. On the left are the results of the optimization of the secondary antibody dilution performed by adding titrated dilutions of HRP-conjugated goat anti-mouse IgG to 20 $FLAG^+$ peptide beads which had been pre-incubated with 10 ug/mL anti-FLAG or RFT5 and the absorbance of the bead bound antibody was measured on a plate reader. On the right, the primary antibody concentration was optimized by adding titered concentrations of antibody to 20 $FLAG^+$ beads followed by a 1:500 dilution of HRP-conjugated goat anti-mouse IgG as described in Materials and Methods. These data are plotted in semi-log scale. These experiments were repeated three times each. Data are displayed as the average of the three experiments and presented as mean +/- S.D. A 2-tailed, unpaired student t-test was performed and yielded a *p* value < 0.05 (**) for anti-FLAG binding to $FLAG^+$ beads vs. RFT5 binding to $FLAG^+$ beads for both the left and right panel.

When color screening was applied to nineteen of the thirty peptoid beads retained in the previous spiking experiments, none changed color indicating that none of these sequences were retained specifically in the magnetic screening assay. These results were in stark contrast to the immediate and intense color change observed for $FLAG^+$ peptide. What was yet to be determined was whether the lack of color change was due to a lack of mimicry of the retained peptoids to $FLAG^+$ peptide, to low affinity binding, or whether the conditions sufficient for $FLAG^+$ peptide to change color differed from those for peptoid mimetics. An assay was necessary which would determine whether the sequences identified were bound by the antibody binding site and with what relative affinity. Furthermore, a peptoid/anti-peptoid antibody pair was needed to determine the magnetic and color screening conditions appropriate for peptoids. Both are described in the following sections.

b. Blocking ELISA

Having developed an assay to reduce the number of sequences identified in the magnetic screening (indicating sequences bound by anti-FLAG and PGDs) to those positive during both magnetic screening and a secondary assay (color screening), we then hoped to reduce the number of peptoids further to only those that best mimicked the native antigen. We designed an assay to determine whether retained sequences were binding in the antigen binding site of the antibody, and if so, which sequences bound with the highest relative affinity. This assay was referred to as a "blocking ELISA", reasoning that peptoids on carrier protein that block binding of the MAb to the native antigen must bind to the antigen binding site of the MAb.

To establish reference blocking conditions, an ELISA plate was coated with $FLAG^+$ peptide/BSA and control proteins. Anti-FLAG was incubated with FLAG/BSA, KLH or BSA. RFT5 incubated with FLAG/BSA was used as an isotype matched antibody control. As shown in **Figure 18**, RFT5 did not bind to $FLAG^+$ peptide/BSA. BSA and KLH were used as negative control blocking proteins and neither blocked binding of the MAb to FLAG/BSA. FLAG/BSA blocked the binding of anti-FLAG to FLAG/BSA. KLH and BSA did not. Fold excess molar concentration was defined as the ratio of the molar concentration of the blocking protein to the molar concentration of the antibody added. A 1.98 fold excess Molar concentration of FLAG/BSA was required to block 50% of anti-FLAG binding of to FLAG/BSA.

This blocking ELISA would indicate which peptoids were bound by the binding site of the MAb. A 1.98 fold excess Molar protein concentration was necessary for soluble $FLAG^+$ peptide on carrier to block the binding of anti-FLAG to plate bound $FLAG^+$ peptide on carrier protein. These data served as a reference against which future potential B cell epitope mimetic peptoids would be compared.

Because none of the thirty sequences color-screened positive we concluded that none of these sequences were potential mimetic sequences. Therefore no blocking ELISAs were performed.

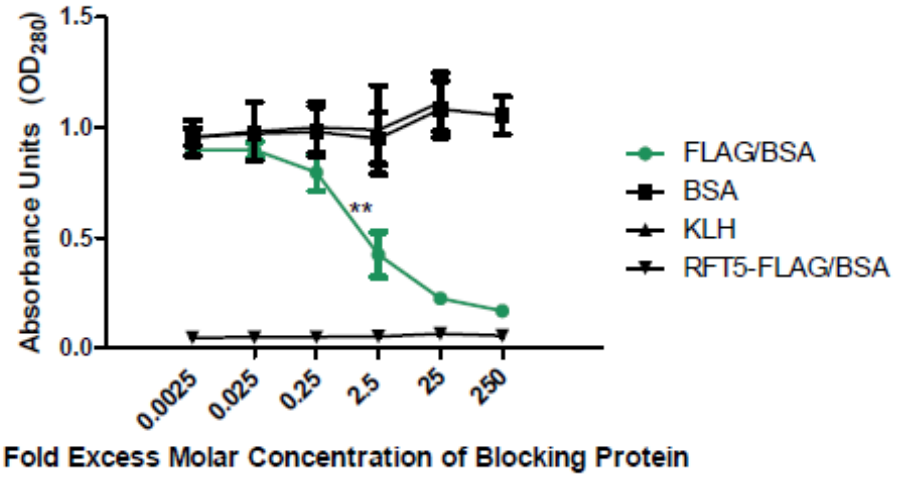


Figure 18: Fold excess Molar concentration of soluble FLAG peptide on carrier protein (BSA) needed to block binding of anti-FLAG to plate-bound FLAG peptide/BSA. Twenty five uL of 0.1 ug/mL anti-FLAG or RFT5 was pre-incubated with FLAG peptide/BSA or control proteins and the supernatant was plated in triplicate on the ELISA plate. The absorbance of plate bound antibody was read with a plate reader. This experiment was repeated three times with triplicate wells. Data are displayed as the average of the three experiments and presented as mean +/- S.D. A 2-tailed, unpaired student t-test was performed and yielded a p value < 0.05 (**) for blocking of anti-FLAG binding to $FLAG^{+}$/BSA by FLAG/BSA vs. with KLH.

C. Summary

The completion of this first phase of work resulted in the generation of a magnetic screening platform that was **sensitive, specific** and **reproducible** as applied to the retention of peptides from a peptoid library using a MAb.

1. The **magnetic screen** was sensitive, specific, and reproducible in retaining $FLAG^{+}$ peptide beads spiked into $FLAG^{-}$ peptide beads and from a peptoid library.

2. Non-specific binding was not observed in these experiments such that a blocking buffer was not included during library screening or color screening.

3. Sequential addition of the screening reagents resulted in 100% bead retention as compared to 35% with pre-incubated anti-FLAG and PGDs. Sequential addition was adopted for the remained of this platform.

4. **Secondary assays** were developed and optimized so that they could be used downstream to validate the mimicry of potential peptoid hits retained from the optimized screen. With regard to the FLAG system, these secondary assays confirmed the $FLAG^{+}$ peptide as a sequence that was bound specifically by the binding site of the anti-FLAG.

Next, this platform would be further optimized for application to the identification of peptoid vaccine candidates from a peptoid library.

CHAPTER 2: PLATFORM OPTIMIZATION USING A PEPTOID/ANTI-PEPTOID ANTIBODY CONTROL PAIR

A. Objective and Overview

To continue optimizing this platform for the identification of potential peptoid mimetics, a peptoid/anti-peptoid antibody pair was created by immunizing rabbits with the R5 peptoid on carrier protein and adsorbed to alum.

- The R5 and RC peptoids were synthesized on Rink resin and Tentagel resin and the quality of the synthesis was analyzed by MALDI MS. All peptoids were > 95% pure before use (data not shown).
- Two NZW rabbits were bled before the first immunization. The rabbits were immunized and boosted several times with the R5 peptoid on carrier protein adsorbed to alum before they were exsanguinated. Serum was prepared from blood taken before each immunization and ten days after each boost starting with boost four (test-bleeds).
- Rabbit anti-R5 sera were tested by ELISA to determine whether anti-R5 antibodies were induced, and if so, PAb anti-R5 titers were determined.
- Rabbit anti-R5 sera were affinity purified on a column of R5 peptoid attached to Sulfolink column resin and the ELISAs described above were repeated using the column flow through and eluate.
- The magnetic screen created in Phase 1 was refined by the application of the affinity purified antibodies from the rabbit anti-R5 sera (PAb) to the:
    - R5 beads alone to ensure that the R5 sequence was recognized by the antibodies “on-bead” and using a different linker than the one used for immunization.
    - R5 peptoid beads spiked into RC peptoid beads or a peptoid library and the retention of the R5 peptoid beads by the affinity purified antibodies from rabbit anti-R5 sera was evaluated.

- Secondary validation assays created in Phase I were then re-applied using the peptoid control pair and anti-R5 and the need for platform changes was assessed.

The completion of the second phase of optimization resulted in a screening platform that was **sensitive, specific** and **reproducible** as demonstrated by the successful identification of not only peptide ligands but peptoid ligands from a peptoid library using a PAb. Also, two secondary validation assays were optimized for application to peptoids.

B. Results

i. Rabbit immunizations and measuring rabbit anti-R5 sera titers by ELISA

Following the optimization of the magnetic screening platform with a peptide/anti-peptide pair, a peptoid/anti-peptoid antibody pair was created and the platform optimization repeated.

A five monomer long peptoid (R5) was chosen for immunization because peptoids of this length had been successfully synthesized and purified in our laboratory previously. Although pilot studies in our laboratory indicated that six monomer peptoids were immunogenic, and certainly peptoids have been shown to be immunogenic [71] [72], no formal study has been published regarding the use of peptoids as epitopes, so our decision to create a five monomer peptoid for immunization was an educated guess based on our previous experience. A seven monomer peptoid was chosen as the control peptoid (RC) because our original research plan involved the use of two immunizing peptoids; the first peptoid was five monomers long and the second peptoid was planned to be ten monomers. A seven monomer control peptoid, we hypothesized, would be a reasonable control for both.

Monomers for the R5 peptoid sequence were chosen such that as a group, they represented diverse molecular properties (polarity, hydrophobicity, size, charge, etc). Groups of monomers were chosen to include representative members of such

molecular properties are said to “cover chemical space”. In addition to covering chemical space, the monomers were chosen to facilitate purification by HPLC, since highly hydrophobic sequences can be difficult to purify by HPLC. The monomers chosen for the RC peptoid had little in common with the R5 peptoid. In addition to a common cysteine (necessary to both sequences for attachment to a carrier protein), R5 and RC shared only the first monomer, isobutylamine (**Table 5)**. The six remaining monomer positions for the RC peptoid were occupied by two alternating amines, allylamine and 2-methoxyethylamine, which were chosen such that the resulting sequence was not overly hydrophobic.

Rabbits as compared to mice were chosen for immunization because large volumes of sera could be obtained from the test bleeds and production bleeds. Large volumes of sera were critical to the development of this platform. Serum is a rich source of proteins. However, purification requires large volumes of sera to obtain, in relation, a small sample of serum derived antibody of interest. The use of rabbits yielded large volumes of anti-R5 sera from which we could obtain anti-R5 antibodies. Furthermore, with sufficient supply of sera and anti-R5 antibodies, we were able to include all the controls necessary for well characterized and well controlled serum-related assays.

Purified R5 or RC peptoids were conjugated to a carrier protein for use in ELISAs or to immunize the rabbits. After conjugating the purified peptoids to carrier and adsorbing to alum, rabbits were injected IM according to the protocol depicted in **Figure 12B**. The rabbits were bled before immunization (pre-bleed) and then immunized and boosted several times before they were exsanguinated. Blood was collected and sera prepared from the blood before each immunization and ten days post immunization (test-bleeds).

To evaluate whether the rabbits produced antibodies against R5, ELISA plates were coated with R5/BSA, BSA, KLH or FLAG/BSA. FLAG/BSA was used as a standard curve (by adding Anti-FLAG at known concentrations). The pre-bleed or test-bleed sera were added to triplicate wells at titrated serum dilutions (**Figure 19** x-axis) and the absorbance measured (y axis) using a plate reader. As shown in the left panel

of **Figure 19**, the pre-bleed sera did not contain anti-R5 antibodies nor did it contain antibodies against KLH, BSA or FLAG/BSA. The test bleed titers are depicted in the right panel of **Figure 19**. The rabbits produced antibodies against the R5 peptoid, the carrier protein KLH, and the monomers linking the R5 peptoid to KLH (as determined by binding to ovalbumin (OVA) conjugated to bovine serum albumin (OVA/BSA)) linker. BSA and OVA were included as negative controls and only baseline absorbance was seen for each.

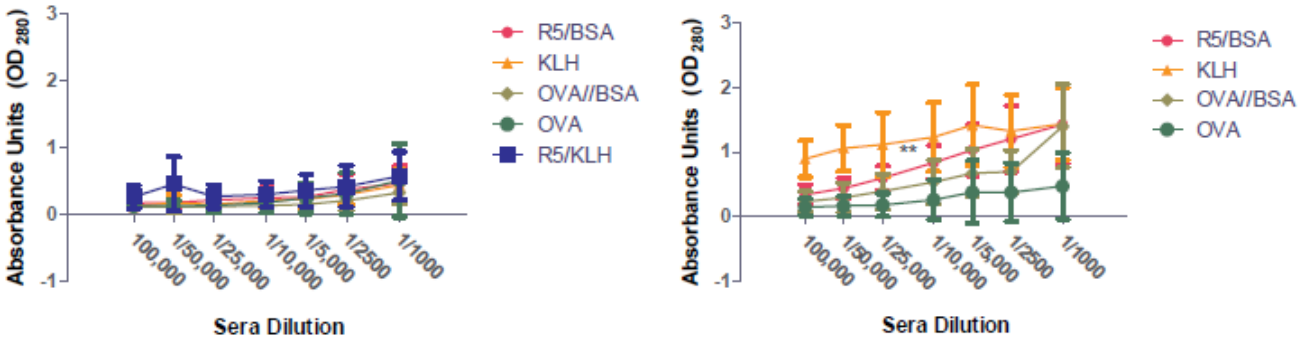


Figure 19: Rabbit anti-R5 titers: Prebleed and test bleed sera. Two NZW rabbits were injected with 250-500 ug of R5/KLH adsorbed to alum and injected IM at 4 week intervals. The titers from pre-bleed (left) sera and test bleed (right) sera show a lack of response to any protein in the pre-bleed sera and a significant anti-R5, anti-KLH, and anti-linker titers in the test bleed sera. Based on a MAb anti-FLAG + FLAG/BSA standard curve, the rabbit anti-R5 titer for the test bleed sera was between 462 - 1000 ug/mL. ELISAs were performed using triplicate wells and were repeated three times. Data are displayed as the average of the three experiments and presented as mean +/- S.D. A 2-tailed, unpaired student t-test was performed and yielded a *p* value < 0.05 (**) for test bleed sera binding to R5/BSA vs. OVA and a *p* value <0.05 (**) for all proteins vs. FLAG standard curve for the pre-bleed sera.

We concluded that the rabbits immunized with R5/KLH produced anti-R5 antibodies. Hence, the R5 peptoid was immunogenic when conjugated to a carrier protein adsorbed to alum as determined by the anti-R5 antibody titer (measured by ELISA). A five monomer peptoid was sufficient to induce an immune response when conjugated to carrier protein and adsorbed to alum.

ii. Affinity purification of rabbit anti-R5 sera

The rabbit serum could contain anti-R5, anti-linker, and anti-KLH antibodies. The next series of experiments were designed to determine which antibodies were present. We therefore affinity purified the polyclonal rabbit anti-R5 sera and isolated a pure pool of anti-R5 antibodies using an affinity column made by attaching R5 peptoid to SulfoLink linker and pouring the sera over the column multiple times. For each sera pass, the column flow through and eluate were retained. Both the flow through and eluate were evaluated in an ELISA for both the presence of anti-R5, anti-carrier protein, and anti-linker antibodies. To do so, ELISA plates were coated with R5/BSA, BSA, KLH, OVA, OVA/BSA or FLAG/BSA (included as a standard curve by adding known concentrations of anti-FLAG to the FLAG/BSA). The column flow through and column eluate were added to the plate. The results of the column purified antibody titers as determined by ELISA are shown in **Figure 20**. Overall, the eluate (left panel) from the column had significant levels of antibody against R5 peptoid/BSA and the carrier protein KLH, but not against BSA or FLAG/BSA. The lack of binding to FLAG/BSA indicated that we had successfully removed anti-linker antibodies. At a 1/100 dilution, the anti-R5 antibody titer was high while both the anti-KLH and anti-linker antibody titer was baseline. At this dilution, column flow through bound FLAG/BSA, KLH, OVA/BSA and BSA. Of note in **Figure 20** below, the student t test was performed for all six dilutions of anti-R5 binding to R5/BSA vs. KLH. This *p* value was 0.15. However, this analysis includes points above saturation; analysis of the same data at the 1/100 dilution reveals a p value <0.05 for anti-R5 binding to R5/BSA vs. KLH.

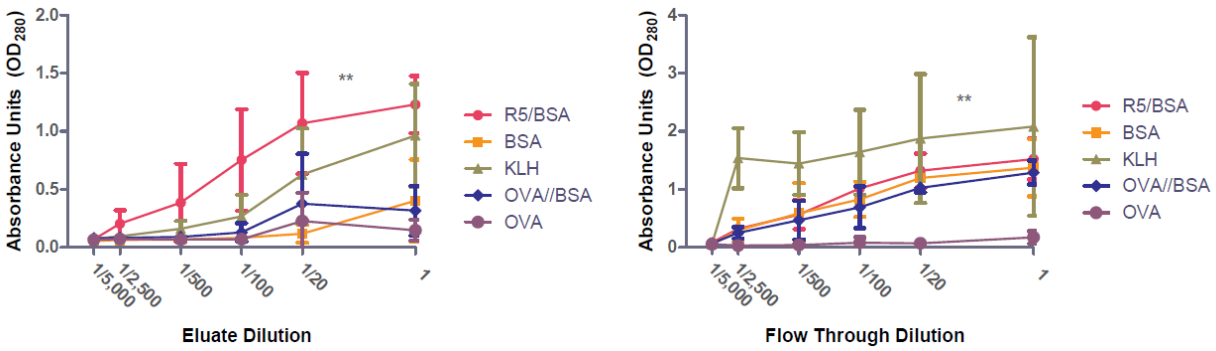


Figure 20: Column purification ELISAs: Anti-R5 titers in the column flow through vs. the column eluate. Two NZW rabbits were injected with 250-500 ug of R5/KLH at 1 month intervals. The test bleed sera were column purified over an R5-Sulfolink column. By comparing the eluate (left) from the flow through (right), the diminished anti-KLH and anti-linker antibody titers are evident, indicating that the column successfully purified the sera. Data are displayed as the average of the two experiments and presented as mean +/- S.D. A 2-tailed, unpaired student t-test was performed and yielded a *p* value < 0.05 (**) for eluate binding to R5/BSA vs. OVA and a *p* value < 0.05 flow through binding to R5/BSA vs. OVA. The *p* value for eluate binding to R5/BSA vs. KLH was *p* < 0.15 as compared to flow through binding to R5/BSA vs. KLH, *p* value = 0.44.

We concluded that affinity purification removed anti-linker and anti-carrier protein antibodies from the eluate as compared to the column flow through at a 1/100 dilution of eluate. Thus we were able to purify antibodies specific for the R5 peptoid with a range of antibody affinities, since antibodies were eluted from each of several passes of elution buffer. Antibodies that eluted after the first pass of elution buffer are of lower affinity than those that elute later. All experiments were performed with the first eluate (lower affinity antibodies) to determine the limits of affinity of the platform. Future work will repeat these assays with the higher affinity elutions.

iii. Primary validation of optimized magnetic screening conditions

a. Preliminary validation of anti-R5 binding to on-bead R5 peptoid

To determine whether the polyclonal anti-R5 antibodies (PAb) would recognize the R5 peptoid synthesized on an M-A-A linker and attached to a bead (each event potentially altering the conformation adopted by R5), four aliquots of 20 R5 beads and 1 aliquot of 20 RC beads were made. Five point eight ug/mL of anti-R5 (eluate 1) were added followed by PGDs and the number of bead retained was quantified. 10 ug/mL RFT5 was added to the remaining R5 aliquot. As shown in Table **10**, anti-R5 antibodies recognized the R5 sequence i) on-bead and ii) following the M-A-A linker.

The synthesis of the peptoid following the M-A-A linker and presented on a bead did not alter the binding of the antibodies to the sequence to the extent that binding was abrogated. Anti-R5 did not bind the RC beads, indicating that the affinity purified antibodies (even eluate 1) were specific for R5. Between 85%-95% of the R5 beads were retained in each of three repeats. Finally, RFT5 did not bind R5 beads.

Table 10: The retention of known numbers of R5 peptoid beads with column purified anti-R5 sera

	Number of peptoid beads per tube	Beads retained with eluate
Experiment 1	20	17/20 (85%)
Experiment 2	20	18/20 (90%)
Experiment 3	20	19/20 (95%)
RC beads/ Eluate	20	0/20 (0%)
R5 beads / RFT5	20	0/20 (0%)

Twenty on-bead R5 peptoid sequences were added to four tubes and 20 on-bead RC tubes added to one tube. The beads were screened with anti-R5 (eluate1) or RFT5 and the number of magnet bound beads was quantified. Column one indicates the experiment number, column two indicates the number of beads added to each tube, and the final column indicates the number of beads retained by the screening antibody and PGDs. Between 85-95% of the beads were retained indicating that anti-R5 recognized the on-bead peptoid sequence and prepared with an irrelevant linker.

b. Retention of on-bead R5 peptoid beads "spiked" into on-bead RC peptoid beads

To begin to approximate the retention of peptoid B cell epitopes from a peptoid library, 1 or 3 R5 peptoid beads were spiked into an aliquot of RC peptoids beads and the spiking experiments described in Phase I were repeated. **Table 11** summarizes the sum of the beads retained in the three experimental repeats per spiking condition. Also summarized are the number of beads retained from the spike, the number of beads that were successfully analyzed by MALDI MS, the number of beads confirmed as R5, and the number of beads confirmed as non-R5.

When 5 R5 peptoid beads were spiked into 20,000 RC peptoid beads in triplicate experiments, 22 beads were retained in total. The number 14 appears instead of 15 as the number of R5 beads added to the three aliquots because one R5 bead was left behind in the tube during the addition of the R5 beads into the aliquots. Of the 22 beads retained, 20 could be analyzed by MALDI MS. Of these 20, only 14 of which could be R5, 13 (93%) of the original 14 beads were retained. One of the twenty beads was confirmed as RC. When three R5 peptoid beads were spiked into 20,000 RC beads in triplicate experiments, 11 beads total were retained. Nine of these were successfully analyzed by MALDI MS and 9/9 (100%) were R5.

Table 11: The retention of known numbers of R5 peptoid beads spiked into an excess of RC peptoid beads

R5: RC Beads	Number of Beads Retained: Beads Added	Successfully Analyzed by MALDI MS	Confirmed as R5	Confirmed as Non-R5
5/20,000 [3]	22/14	20/22	13/14 (93%)	1/7
3/20,000 [3]	11/9	9/11	9/9 (100%)	0/11

Either 3 or 5 R5 beads were spiked into ~ 20,000 RC beads and the mixture screened with 10 ug/mL anti-R5 and 1:10 dilution of PGDs and the hits isolated on a magnet and counted under a microscope. This experiment was repeated in triplicate and the data for the number of beads recovered in the screen, the number of these retained beads that were able to be analyzed with MALDI MS, and the number of these MALDI MS analyzed beads that were R5 vs. non-R5 are summarized in columns two through five.

The results of these experiments indicated that the magnetic screening platform was **sensitive** (beads were retained from samples as dilute as 3 beads in 20,000), **specific** (100%+ R5 beads were retained vs. 0.01% RC beads), and **reproducible** in the retention of R5 peptoid sequences from RC peptoid sequences using PAb anti-R5 (eluate 1).

The magnetic screen developed in Phase I was sufficient to distinguish between R5 and RC on-bead sequences, and isolated 3 out of 20,000 beads 100 % of the time. As compared to MAbs, PAbs did not prevent isolation of the R5 sequences, leading us to conclude that the affinity and clonality of the screening antibody did not negatively affect the isolation of R5 peptoid in contrast to the retention of $FLAG^{+}$ peptide. PGDs were able to bind the PAbs and together the PGD/PAb complex could be successfully isolated on the magnet.

A noteworthy observation made during these experiments was the difficulty with which the R5 sequences were analyzed by MALDI MS. The R5 beads retained from the spike were very densely covered with PGDs. Their coverage with PGDs was so extensive that positive beads could be easily identified by eye under the microscope. When these positive beads were analyzed with MALDI MS, despite the benefit of foreknowledge of their MW and the location of their MALDI MS peak, difficulty in identifying the sequences was encountered. MALDI MS conditions were finally identified that permitted analysis of these samples, but the spectrum peak intensities were drastically reduced (MALDI MS spectra intensities on the order of hundreds instead of thousands). We hypothesized that the density of the PGDs and anti-R5 on the beads or the binding affinity of the antibodies was too high such that access of the cleavage cocktail to the peptoid sequences was occluded, resulting in poor spectrum intensity. In response to this difficulty, and based on the success others [55] we used 0.1 M glycine (pH 2.8) to remove antibody from the on-bead-peptoids before cleavage and sequencing the peptoids. This antibody was not reused since low pH can denauture antibodies. The peptoids were not affected by the low pH buffer.

c. Retention of on-bead R5 peptoid beads "spiked" into an on-bead peptoid library

To more closely approximate the retention of peptoid B cell epitopes from a peptoid library, R5 peptoid beads were next spiked into a peptoid library aliquot. However, before doing so, the chosen aliquot from Library 2C was screened with PAb anti-R5 to remove any R5 sequences that might be present on the library naturally. To clarify, Library 2C was created using the same monomers found in the R5 peptoid. It was possible for Library 2C to contain native-R5 (R5 sequence(s) synthesized over the course of library synthesis).

To do so, Library 2C was screened with 10 ug/mL PAb anti-R5 (eluate 1) and 1:10 dilution of PGDs. Fifty eight beads were retained in magnetic screening, but only three (0.0004%) strongly positive beads were observed by eye under the microscope. The remaining sequences were retained because of microscopic cracks, non-specific binding, or were retained specifically but with low affinity. Ongoing work is being performed to screen these fifty eight sequences with other column eluates (containing higher affinity antibodies) followed by cleavage and MALDI MS and MSMS analysis to identify the sequence and identity of the monomers making up these peptoids.

These results indicated that Library 2C did in fact contain potential R5 or R5 mimetics. A hit rate of 0.0004% was observed for this "sub-library", or a library made using monomers known to be important for antibody binding.

To return to the spiking experiments, devoid of any native-R5 or R5-related sequences, Library aliquot 2C was then divided evenly into six aliquots. Into each aliquot, either 1 or 3 R5 beads were added. These aliquots were magnetically screened with 10 ug/mL anti-R5 (eluate 1) and 1:10 dilution of PGDs. **Table 12** summarizes the sum of the beads retained per spiking condition, the number of beads successfully analyzed by MALDI MS, the number of R5 beads retained as confirmed by MALDI MS, and finally the number of non-R5 beads retained. When 3 R5 peptoid beads were spiked into 30,000 peptoid library beads, 10 beads total were retained. Two of these beads were lost when preparing the beads for cleavage.

Of the remaining 8 beads, 7 were successfully analyzed by MALDI MS. Of these 7, only 3 out of the theoretical 9 R5 peptoid beads were retained as determined by MALDI MS analysis. Four non-R5 beads were retained and will be analyzed in future work.

Table 12: The retention of known numbers of R5 peptoid beads spiked into a Library 2C aliquot

R5: Peptoid Library Aliquot Beads	Number of Beads Retained: Beads Added	Successfully Analyzed by MALDI MS	Confirmed as R5	Confirmed as non-R5 peptoids
3/30,000 [3]	10/9	7/8*	3/9 (33%)	4/8
1/30,000 [3]	5/3	4/5	3/3 (100%)	1

3 or 1 R5 beads were spiked into a peptoid library aliquot (~30,000 beads) and the aliquots were screened with 10 ug/mL anti-R5 and 1:10 dilution of PGDs and the hits isolated using the magnet. Retained beads were counted under the microscope. Each spiking experiment was repeated three times as indicated in column one. Column two shows the number of beads retained out of the number added to the aliquots. Column three is the fraction of beads retained which were analyzed successfully by MALDI MS. The asterisk indicates that although 10 beads were retained, two were lost. Therefore, only eight beads were present for MALDI MS, seven of which were successfully analyzed. Column 4 is the number of beads (and percentage) of these that were R5. The last column is the fraction of retained beads that were analyzed by MALDI MS and MS/MS, but were not R5.

Finally, when 1/30,000 R5 beads was screened, 5 beads total were retained. Four of these were successfully analyzed by MALDI MS. Three beads (100%) were confirmed as R5 by MALDI MS and one peptoid sequence was identified by MALDI MS.

The results of these experiments indicated that the platform was **sensitive** (beads were retained from samples as dilute as 1:30,000), **specific** (100% of R5 beads were retained vs. 0% non-R5) and **reproducible** for the experiments in which 1 R5

bead was introduced into each 2C peptoid aliquot. However, for experiments adding three R5 beads to each aliquot, only three (33%) out of eight R5 beads were retained. As observed in the case of spiking R5 beads into RC beads as described above, difficulty was experienced when MALDI MS analysis was performed on these beads (despite the use of glycine to the beads to remove antibody before sequencing).

Perhaps the concentration of the screening antibody must be increased when antigen-related libraries are screened. To be more specific, because Library 2C was made with the same monomers as R5, perhaps the incidence of mimetics was higher than for naïve libraries. If so, the increased number of positive sequences could reduce the antibody available to the three R5 beads in each tube, thereby preventing their retention. In contrast, because there were fewer R5 beads in the spikes of 1:30,000 perhaps sufficient antibodies was present to retain these beads. Furthermore, perhaps other column eluates (affinity purification eluates 2, 3, etc.) would bind and isolate these beads.

iv. Secondary validation assays

Both the color screening assay and the blocking ELISA created and optimized in Phase I were repeated using the R5 peptoid and anti-R5 to establish optimized conditions for the application of this platform to the isolation of mimetic peptoids and to obtain reference data against which data for potential peptoid mimetics would be compared.

a. Color screening validation using the R5 peptoid control

To investigate whether the color screening assay conditions developed in Phase I were optimized to validate *peptoids* using a PAb or if significant changes to the conditions would be necessary to apply color-screening to the R5/anti-R5 pair (and to potential mimetic peptoids later on) titrated dilutions of HRP-conjugated goat anti-mouse IgG were added to 20 R5 peptoid beads that had been pre-incubated with

anti-R5 and then washed. RFT5 and RC beads were included as negative controls. As shown in **Figure 21** (left panel), absorbance was maximized when 1:5,000 dilution of HRP-conjugated goat anti-rabbit IgG was added to the R5/PAb conjugates. RFT5 did not bind to R5 peptoid as indicated by baseline absorbance. 1: 10,000 dilution was adopted as the optimized secondary dilution in all further peptoid color screening assays. The concentration of primary antibody (PAb anti-R5) (right panel) was optimized by adding known concentrations of the anti-R5 to 20 R5 peptoid beads or 20 RC peptoid beads and incubating for 1 h. Maximum absorbance, correlating to saturation of the R5 peptoid beads with anti-R5, was observed at 0.04 ug/mL anti-R5 for 20 R5 peptoid beads. A concentration of 0.1 ug/mL was chosen to be in excess of saturation and was adopted as the anti-R5 concentration for color screening.

These experiments demonstrated that color screening was both sensitive and specific for use with both the peptide control pair and the peptoid control pair. As compared to the color screening data of the FLAG system, the R5 peptoid beads bound by anti-R5 and HRP-conjugated goat anti-rabbit IgG changed color as quickly and as intensely as the $FLAG^+$ peptide beads bound by a MAb. Because of this, the peptoid beads validated by color-screening in Phase I which did not color change were probably not FLAG peptide mimetics because this experiment clearly demonstrates that peptoid and peptides alike color change if they are either B cell mimetics of, or are the native antigens against which the antibody was raised.

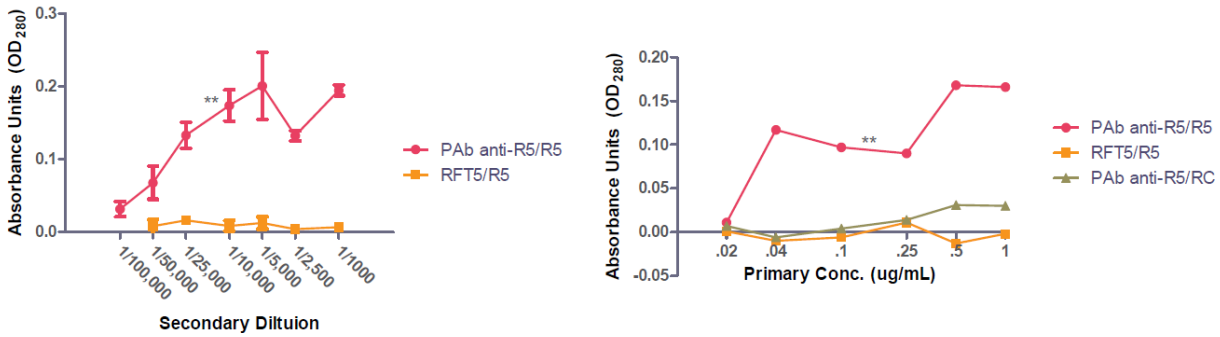


Figure 21: Color screen optimization using the peptoid control pair. On the left, the optimization of the secondary antibody dilution (HRP-conjugated goat anti-rabbit IgG) performed by adding titrated dilutions of HRP-conjugated goat anti-rabbit IgG antibody to 20 R5 peptoid beads which had been incubated with 10 ug/mL anti-R5 or RFT5 and the absorbance of the bead bound antibody was measured on a plate reader. On the right, the primary antibody concentration was optimized by adding known concentrations of antibody to 20 R5 peptoid beads, followed by a 1:10,000 dilution of the appropriate species specific secondary antibody (HPR conjugated) rabbit. A 1:10,000 dilution was adopted as the optimal secondary antibody concentration and 0.1 ug/mL was chosen as the concentration of anti-R5 concentration. These data are plotted on semi-log scale. Secondary dilution optimization experiments were repeated three times each. Data are displayed as the average of the three experiments and presented as mean +/- S.D. Primary antibody concentration experiments are on-going. One of two repeats is shown. A 2-tailed, unpaired student t-test was performed and yielded a p value < 0.05 (**) for PAb anti-R5 binding to R5 beads vs. RFT5 binding to R5 beads for both the left and right panel.

a. Color screening beads retained from library aliquot 2C

In preparation for spiking experiments, library aliquot 2C was screened with anti-R5 antibodies to identify native R5 sequences or R5-related sequences. Fifty eight sequences were color screened by adding anti-R5 and HRP-conjugated goat anti-rabbit IgG to the beads. One sequence changed color with intensity that was equal to that of the five R5 beads that were included as a color-change-positive-control indicating that equal amounts of anti-R5 were bound to both the potential mimetic sequence and the R5 sequence. **Table 13** shows the sequence of the only color-screen positive sequence resulting from the screening of Library aliquot 2C.

Table 13: Color-screen-positive peptoid sequence retained by screening Library 2C with PAb anti-R5

Peptoid Sequence (MW)	Color Screen Intensity with respect to R5 peptoid
Met- Meth- Nall- Nty- Nam- Nam- Ncy (896 Da)	Equal to R5 control beads

Fifty eight were validated in color screening and one was positive in color screening. This table displays the sequences of color-screen positive sequence as well as the intensity of the color change as compared to R5. For a list of the three or four letter abbreviations and their corresponding monomers, see Table 4. Each sequence was color-screened once.

b. Blocking ELISA optimization using the R5 peptoid control

The fold excess molar concentration of soluble peptoid/carrier needed to block binding of PAb anti-R5 to plate bound peptoid/carrier was determined using the R5 peptoid system. ELISA plates were coated with R5/BSA. Anti-R5 antibodies were incubated with R5/BSA, KLH or BSA and the conjugates were centrifuged. BSA and KLH were used as negative control binding proteins. RFT5 was included as an antibody control. As shown in **Figure 22**, RFT5 did not bind R5/BSA. R5/BSA blocked the binding of anti-R5 to R5/BSA as compared to KLH alone or BSA alone which did not. A 3.46 fold excess Molar concentration of blocking protein was needed to block binding of the anti-R5 to plate-bound peptoid/BSA.

R5 peptoid conjugated to carrier protein blocked binding of anti-R5 to plate bound R5/BSA. These data were referenced for further peptoid blocking experiments. The fold excess Molar concentration necessary to block 50% binding of anti-R5 to R5/BSA was 3.46.

Having successfully screened a peptoid library using anti-R5 in the magnetic screen and having retained peptoid sequences which were positive in color screening as potential R5 or R5 mimetics from a sub-library, a vaccine candidate isolation platform has been demonstrated which isolated potential mimetic peptoids from a peptoid library.

All experiments described in this chapter were first performed with the FLAG peptide system (data not shown) to create and optimize the protocols, column chemistry and spiking conditions before using the anti-R5 antibodies with R5 peptoid in each assay.

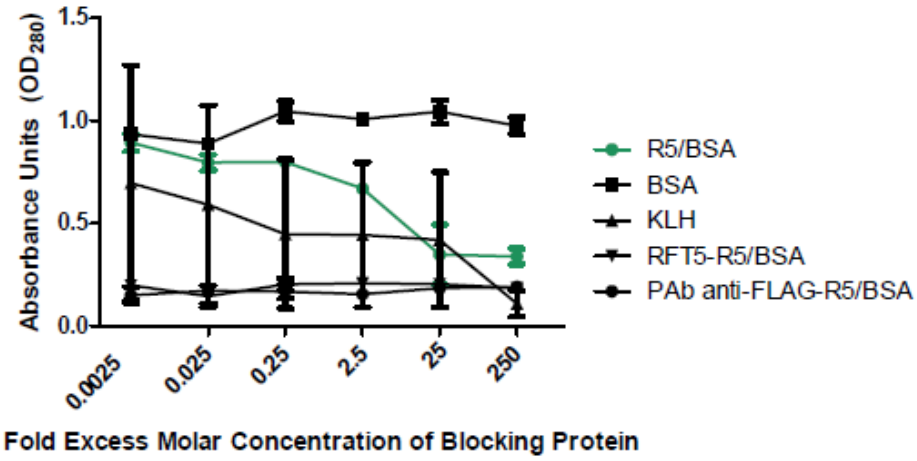


Figure 22: Fold excess Molar concentration of soluble R5/BSA needed to block binding of PAb anti-R5 to plate bound R5/BSA. Twenty five uL of 0.1 ug/mL eluate (PAb anti-R5) or RFT5 was pre-incubated with FLAG peptide on carrier protein or control proteins and the supernatant was plated in triplicate on the ELISA plate. The absorbance of plate bound antibody was read with a plate reader. This experiment was repeated three times with triplicate wells. Data are displayed as the average of the three experiments and presented as the mean +/- S.D. A 2-tailed, unpaired student t-test was performed and yielded a p value < 0.05 (**) for anti-R5 binding to R5 /BSA blocked with R5/BSA vs. blocking with KLH.

C. Summary

Phase II of platform optimization resulted in the demonstration of a **sensitive, specific** and **reproducible** magnetic screening platform which identified peptoid sequences from a peptoid library using a PAb. Furthermore, screening a sub-library with PAb anti-R5 (eluate 1) revealed 1 potential R5 mimetic peptoid as determined by color-screening.

The successful identification of a peptoid mimetic from a sub-library with PAb anti-R5 was very encouraging in evaluating the potential of the platform for future success of naïve library screens. Blocking ELISAS will be carried out to confirm that these sequences were indeed mimetics of R5.

Color screening and blocking ELISA conditions were optimized for the application of these assays to peptoid validation and revealed that each assay was robust for peptides and peptoids.

CHAPTER 3: DEMONSTRATION OF THE OPTIMIZED SCREENING PLATFORM: IDENTIFYING POTENTIAL PEPTOID MIMETICS FROM PEPTOID LIBRARIES USING A MAB

A. Objective and Overview

We aimed to demonstrate the successful creation of a proof of principle platform for the discovery of potential vaccine candidates. To do so, the optimized magnetic screen was then used to screen five peptoid libraries with anti-FLAG and PGDs. The two validation assays developed and optimized in the first two phases were applied to any retained sequences. Each library was:

- Analyzed by MALDI MS to determine the quality of the sequences in each library before use (data not shown).
- Pre-cleared with RFT5 and any retained sequences were removed and retained.
- Screened with anti-FLAG/PGDs with the magnetic screen and any sequences retained were:
    - Quantified
    - Color screened and analyzed by MALDI MS and MS/MS (data not shown)
    - Ongoing work will evaluate these peptoids in a blocking ELISA

B. Results

All peptoid libraries were checked for the fidelity of their synthesis as described in Materials and Methods. All libraries but one, Library 4, had at last 90% sequence fidelity as determined by MALDI MS and MS/MS before use (data not shown). Library 4 was excluded from screening because of poor sequence fidelity and synthesis. Each peptoid library was screened in aliquots of ~30,000 beads at a time

in MCF tubes using the optimized magnetic screen developed in the first two phases of this work.

Each library was pre-cleared of potential non-specific peptoids by screening with RFT5 before the addition of the screening antibody.

Library 1 synthesis was performed at the Molecular Foundry under their guidance. The rationale behind the first library was to learn the basics of library synthesis and screening. To accomplish this goal, Library 1 was simple and redundant. It was simple because of its relatively short length (5 monomers) and the selection of monomers that were incorporated. A five monomer library was a choice reasonable for a first time synthesis. The chosen monomers covered chemical space, but ease of incorporation strongly influenced these monomer choices.

Library 2 was a mixture of three small libraries of 5mer/6mers whose monomers were each chosen from the same set of eighteen monomers, but in different combinations (see **Table 4**). By maintaining sequence length and increasing the number of monomers used, comparisons were drawn between the effect of diversity on hit rate and sequence affinity.

Library 3 was designed to increase library diversity and, correspondingly, the potential to identify potential B cell epitope mimetics. Library 3 was also synthesized at the Molecular Foundry. The resulting library was ten monomers and made using two alternating blocks of eight monomers each. Glycine (Nas, **Table 4**) was present in each monomer block, resulting in fifteen unique monomers. This library, nicknamed the U10 library for undersampled ten-mer, was our first attempt at increasing diversity at the cost of redundancy. The decision to synthesize the library in two blocks of eight monomers each was made because randomization of sixteen monomers at ten positions would yield a diversity of one trillion sequences (to be synthesized on two million beads). This library would be too undersampled and important monomer or monomer pairs might not appear from only two million sequences.

Library 4 was designed to complement a ten monomer sequence intended for use in rabbit immunizations. When we originally designed the rabbit project, our goals were to generate anti-peptoid antibodies and to begin to understand the importance of peptoid length on immunogenicity. Instead, the ten monomer sequence became a lesson in monomer choice, difficult chemistries, and overcoming difficult purifications. A poorly chosen tyramine was included in the library, which is prone to branching and side reactions. These deleterious effects can be attenuated using chloroacetic acid. However, synthesizing an entire library with chloroacetic acid was suboptimal for other monomers. We decided to synthesize the rabbit ten monomer library with bromoacetic acid resulting in an unsuccessful library. Figliozzi *et al* [36] described a "good library" as one whose beads were covered with sequences of the expected MW, at least 70%+ of the beads cleaved and analyzed by MALDI MS were successful and sequenceable, and, upon cleavage, the mass recovery of any individual bead was 50% of the sequences on the bead. Library 4 did not meet these requirements and was not included in library screening.

As we gained experience, we attempted to increase the library size (in grams of resin), length (number of monomers in the chain) and monomer diversity. Our latest library contained sequences that were eight monomers long and was made using eighteen monomers. **Table 14** below summarizes the size (in number of beads) and monomer diversity of each library.

Each of the four libraries were divided between five and eleven portions for distribution to other group members according to the number of neutralizing antibodies each member had in hand. **Table 14** below summarizes the division of each library. The size of each library screened in this work can be found by dividing the grams of resin used by the division factor.

Table 14: Libraries 1 through 5: Library size and diversity as compared to hit rate

Item	Library 1	Library 2	Library 3	Library 5
Beads in library	1.5×10^6	4.4×10^6	2.08×10^6	11.2×10^6
Theoretical Sequences	10^5= 100,000	42×10^6	1×10^9	11×10^9
Divided into parts:	5	6	11	11
Beads screened in this work	300,000 (triplicates of 100,000 sequences)	733,333	189,090	1×10^6
Magnetic screen hit rate	0.01% (30/300,000)	0.0023% (17/733,000)	0.007% (13/189,000)	0.0058% (26/444,444)
Color screen hit rate	0	0.0001%	0.001%	0.0005%

i. Magnetic screening and validation: Use of the novel platform to identify B cell epitope mimetics of FLAG$^+$ peptide from a peptoid library using a MAb

a. Magnetic Screening

Preclearing

All four libraries were precleared in aliquots of 30,000 beads using two times the bead volume of 10 ug/mL RFT5 and a 1:10 dilution of PGDs. Row three in **Table 15** summarizes the number of preclearing hits per library. Library 1 resulted in seven preclearing hits, while Library 5 had over three hundred.

We conclude that a correlation existed between the number of preclearing hits and the diversity of the library.

Screening

To identify potential peptoid mimetics of FLAG$^+$ peptide and to demonstrate the success of a platform by which vaccine candidates can be identified by the application of magnetic screening using a MAb of interest, Libraries 1 through 5

were screened with twice the bead volume of 10 ug/mL anti-FLAG followed by twice the bead volume of 1:10 dilution of PGDs. Retained beads were quantified by counting by eye and those data are presented in row four of **Table 15** summarizing the data for each library and the antibody applied. Again, Library 1 had thirty hits while Library 5 had 26.

Furthermore, there was a significant difference in the number of preclearing hits retained per library and the number of potential FLAG peptide mimetic sequences retained. While the number of preclearing hits rose exponentially with the diversity of the library, the magnetic screening hits, while trending with diversity, did not show large increases.

Table 15: Library screening results: Number of peptoid sequences retained during pre-clearing and screening

Screening Antibody	**Library**				
	1	**2**	**3**	4	**5**
RFT5	7	24	162	N/A	>300
Anti-FLAG	30	22	13	N/A	26
Sequences color screened (sequences positive)	19 (0)	22 (1)	13 (2)	N/A	26 (7)

Peptoid Libraries 1-3 and 5 were screened with 10 ug/mL of either RFT5 and PGDs or anti-FLAG and PGDs as described in the General Screening Protocol of the Materials and Methods section. Summarized here is the number of peptoid beads isolated per antibody per library. Each library was screened in aliquots of ~30,000 beads. Aliquots were screened one time per antibody.

b. Secondary Validation

To determine the positivity of each retained sequence in a secondary assay, each retained peptoid sequence was color screened using twice the bead volume of anti-FLAG and HRP-conjugated goat anti-mouse conjugated secondary antibody. $FLAG^+$ peptide beads screened with anti-FLAG were included as screening controls

and for reference of the intensity of the color change. The last row of **Table 15** shows the number of color-screening hits per library. Ten color-screen sequences total were retained from the four libraries screened. **Table 16** shows the sequences of these ten beads that were color-screen-positive and for reference, the FLAG$^+$ peptide sequence.

Table 16: Color-screen-positive peptoid sequences retained in library screening

Library	Sequence	Color Screening Intensity
FLAG reference	D-Y-K-D-D-D-D-K	Reference
2	1) Met-Eth-(Nap-Nty)-Npi	+
3	1) M-Ahex-Ahex-Npy-Nbs-Nas-Nas-Npy-Namc 2) M-Ahex-Ahex-Npy-Nas-Nbp-Nbu-X	+
5	1) M-Me-X-Nexo-Ntry-Nmp-Nas-Nprg-Nty 2) M-Me-Nbn-Nty-Ntry-Nmp-Ncy-Ntry OR, M-Me-Ndi-Nty-Nphe-Nas-Nphe-Ntry 3) M-Me-Nty-(Nch-Nas)-Namm-Nty-Nlys-Ntyr OR, M-Me-Nty-(Nexo-Nmp)-Namm-Nty-Nlys-Ntry 4) M-Me-Nexo-Nas-Nas-Nai-Nlys-Nlys-Nlys-Nexo OR, M-Me-Namm-Net- Nas-Nai-Nlys-Nlys-Nlys-Nexo OR, M-Me-Nch-Net- Nas-Nai-Nlys-Nlys-Nlys-Nexo OR, M-Me-Net-Nch-Nas-X-Nlys-Nlys-Nlys-Nexo 5) M-Me-Nch-Nlys-Nexo-Nlys-Nty-Nch-Nexo 6) M-Me-Ncy-Ncy-Nch-Nai-Nlys-Nexo-Nexo OR, M-Me-Ncy-Ncy-Ndi-Nbn-Nap-Net-Nexo 7) M-Me-Ndi-Nch-Ntry-Nch-Nlys-Nexo OR, M-Me-Ndi- (Nch-Nmp)-Ntry-Nch-Nlys-Nexo	+

Sequences retained were color-screened as described in Materials and Methods. Ten ug/mL anti-FLAG were added to the beads followed by HRP-conjugated goat anti-mouse IgG and the change in color from translucent to blue was monitored by eye. Each sequence was color-screened once before re-synthesis. The FLAG peptide sequence is shown in line one of the table for reference. For Library 5, either the sequence of the hit peptoid is shown or, if the sequence of monomers could not be determined, possible alternate sequences are shown yielding more than seven sequences for the seven retained beads.

As discussed previously, thirty magnetic-screen-positive sequences were retained from Library 1. Of these, twenty six came from a single aliquot. Table 17 below shows the monomer frequencies for Library 1 hits as well as the monomer nature. The most important monomers to antibody binding were tyramine (25% of the monomers) and 1,4 diaminobutane (25%). Neither tyramine nor 1,4 diaminobutane are negatively charged. In comparison, FLAG peptide (DYKDDDDK) is negatively charged and polar due to the predominance of aspartic acid.

Table 17: Monomer frequency in hit sequences from Library 1

Frequency (%)	Monomer	Abbrev.	Nature
25.69%	Tyramine	Nty	hydrophilic, cyclic, positive charge
25.00%	1,4-Diaminobutane	Nly	positive charge
11.11%	4-(2-Aminoethyl)benzenesulfonamide	Nbs	large, aromatic
9.03%	Ethanolamine	Net	hydrophilic
9.03%	2,2-Diphenylethylamine	Ndi	cyclic
9.03%	Benzylamine	Nbn	cyclic
7.64%	1,3- Aminopropyl-2-pyrrolidinone	Npy	heterocycle
2.08%	Glycine	Nas	negative charge
1.39%	1-Aminoindane	Nai	Large, aromatic
0.00%	Isobutylamine	Nle	hydrophobic, branched

When Library 2 was pre-cleared and screened, twenty four and twenty two sequences were retained respectively (**Table 15**). Of the twenty two anti-FLAG positive sequences, one was positive in color screening. However, perhaps this behavior is unique to FLAG peptide. The need for and the effect of stripping on color screening needs to be evaluated in future work.

Three monomers appeared most important to antibody binding in Library 3. These monomers are summarized in **Table 18**. Five of thirteen monomers were retained ending with 1,4-diaminobutane, two of thirteen sequences were retained with

aminomethylcyclopropane, and two of thirteen were retained ending in ethanolamine. 1,4-diaminobutane was a “consensus” monomer in Library 1 hits, accounting for 25% of the hit monomers. Ethanolamine was also important (10% of the monomers, one of the five terminal monomers (out of ten) that yielded hits) in Library 1. Library 3 was the first use of aminomethylcyclopropane.

Finally, Library 5 yielded twenty six magnetic screening hits and seven color screening hits. Three monomers were most important to binding in Library 5 (as determined by their frequency): 1,4 diaminobutane was again at the top of the list accounting for 22% of the monomers encountered, followed by exo-2-aminonorbornane accounting for 14% and 2-(1-cyclohexenyl)ethylamine accounting for 13%.

Ongoing work will determine which of the retained peptoid sequences were bound by the binding site of the screening MAb. ELISA plates will be coated with $FLAG^+$ peptide/BSA and each peptoid sequence on BSA. Anti-FLAG will be incubated with the peptoid sequences/BSA, BSA, KLH or $FLAG^+$ peptide. RFT5 will be included as an isotype matched control antibody and was incubated with $FLAG^+$ peptide/BSA. This data will be compared to the binding affinity of anti-FLAG to $FLAG^+$ peptide/BSA.

The blocking ELISA will indicate which peptoids are likely bound by the binding site of the MAb as demonstrated by the inhibition of anti-FLAG binding to $FLAG^+$ peptide/BSA. Such peptoids are likely candidates to mimic $FLAG^+$ peptide. Future work will test these color-screen-positive sequences in blocking ELISAs, and any positive sequences will be conjugated to a carrier protein, adsorbed to alum and injected into animals; the induction of antibodies which are cross reactive to $FLAG^+$ peptide will be determined.

Table 18: Color-screen-positive consensus monomers for Libraries 3, 5 and overall

Library 3 Consensus Monomers		
Monomer	**Instances**	**Frequency**
Nas	9	11.84%
Nlys	6	7.89%
Npy	6	7.89%
Nchl	6	7.89%
Nbs	4	5.26%
Nbp	3	3.95%
Nal	3	3.95%
Nchl	2	2.63%
Namc	2	2.63%
Nbu	2	2.63%
Nth	2	2.63%
Nver	1	1.32%

Library 5 Consensus Monomers		
Monomer	**Instances**	**Frequency**
Nlys	17	22.37%
Nexo	11	14.47%
Nch	10	13.16%
Ntry	7	9.21%
Nas	7	9.21%
Nty	6	7.89%
Ncy	5	6.58%
Ndi	4	5.26%
Nmp	3	3.95%
Net	3	3.95%
Nbn	2	2.63%
Nphe	2	2.63%
Namm	2	2.63%
Nai	2	2.63%
Npy	1	1.32%
Nap	1	1.32%

Consensus Monomers of Libraries 2, 3, and 5		
Monomer	**Instances**	**Frequency**
Nlys	17	17.53%
Nexo	11	11.34%
Nch	10	10.31%
Nty	7	7.22%
Ntry	7	7.22%
Nas	7	7.22%
Ncy	5	5.15%
Ndi	4	4.12%
Npy	3	3.09%
Nas	3	3.09%
Nmp	3	3.09%
Net	3	3.09%
Nbn	2	2.06%
Nphe	2	2.06%
Namm	2	2.06%
Nai	2	2.06%
Nap	1	1.03%
Npi	1	1.03%
Nbs	1	1.03%
Namc	1	1.03%
Nbp	1	1.03%
Nbu	1	1.03%
Unknown	1	1.03%
Npy	1	1.03%
Nap	1	1.03%

Summary

This work demonstrated the retention of 91 potential $FLAG^+$ peptide mimetics from five peptoid libraries using a MAb. Each of these candidates was evaluated for binding by anti-FLAG by color screening and ten potential $FLAG^+$ peptide mimetic peptoids were identified. The data in this third and final phase of this research demonstrated the successful creation of a novel platform to generate synthetic vaccine candidates (peptoids) by applying magnetic screening with a MAb of interest. Any resulting sequences can be validated for their candidacy for use in immunization by using the two assays developed here. This platform has laid the ground work for the discovery of novel vaccine candidates. This work revealed that library size and diversity correlated with both the preclearing and magnetic screening hit rate. In reviewing the color-screening results, we identified the lower limit for library diversity, retaining zero hits from Library 1, while the upper limit of library diversity has yet to be determined.

IV. DISCUSSION

A. Objectives and Major Findings

Vaccination remains the optimal means of preventing infectious disease. Currently there are more than sixteen vaccines approved for use in the American population [8]. Vaccines induce antibodies that confer protective immunity against the pathogen in question *before* exposure [1, 2]. Although protective, many of these approved vaccines have significant drawbacks [9].

The long-term goal of this project was to develop a vaccine candidate discovery platform that could overcome some of the drawbacks of traditional vaccine development and identify vaccine candidates for any pathogen against which a broadly neutralizing MAb already exists or can be made. Specifically, our goal was to discover vaccine candidates without foreknowledge of the conserved and/or neutralizing epitopes that would induce broadly neutralizing antibodies.

As described previously, to accomplish this goal a platform was developed utilizing neutralizing MAbs to bind B cell epitope mimetics in a peptoid library. These sequences were isolated by PGDs and the sequences validated and characterized.

We began platform optimization using a FLAG peptide/anti-peptide control pair. We then created a peptoid anti-peptoid antibody pair which involved immunizing rabbits with a five monomer peptoid. The platform optimization was repeated using this peptoid/anti-peptoid antibody pair. The remainder of this discussion consists of the results and discussion of the results of our findings using each pair and concludes with the application of this platform to identify potential peptoid B cell epitope mimetics of FLAG peptide.

The major accomplishments to emerge from this study were 1) the creation of an optimized magnetic screening platform for the isolation of peptide B cell epitopes from an on-bead library, 2) a magnetic screening platform optimized for the isolation of peptoid B cell epitopes from a peptoid library, and 3) the identification of potential peptoid B cell epitope mimetics of FLAG peptide from a peptoid library using a MAb. This platform displayed sensitivity, specificity, and reproducibility for retaining

peptide and peptoid B cell epitopes from libraries. All parameters were tested with appropriate negative and positive controls (something missing from most other studies with these platforms).

B. The creation of an optimized magnetic screening platform for isolation of peptide B cell epitopes from an on-bead library

The FLAG/anti-FLAG system

In the absence of a peptoid/anti-peptoid antibody pair, we began optimizing the magnetic screening conditions with the FLAG peptide system. FLAG peptide was chosen for use in this platform based on the work of Slootstra *et al* [100] who described three peptides, $FLAG^{+}$, $FLAG^{-}$ and FLAG50, bound by anti-FLAG with varying avidities (binding, no binding, 50% binding).

First, to confirm Slootstra *et al*'s [100] findings, we performed an on-bead ELISA by incubating on-bead $FLAG^{+}$, $FLAG^{-}$ and FLAG50 peptides with increasing concentrations of anti-FLAG (M2). For FLAG positive and negative peptide, our results were consistent with those of Slootstra *et al* [100]. However, FLAG50 peptide was bound by only 10% of the MAb bound to FLAG positive peptide. The use of the FLAG positive and FLAG negative peptide pair allowed us to optimize the platform screening conditions in the presence of a negative control peptide. While this negative control was critical in establishing screening conditions to identify B cell epitope mimetics with sensitivity and specificity, the platform optimization could have been more robust if FLAG50 peptide had been bound by anti-FLAG with 50% avidity. In designing a screening platform, there is interplay between screening stringency and yield [73, 79, 105]. If conditions are too stringent, potential lead sequences bound with low to medium affinity are lost and even some medium-high affinity ligands are lost as only the fittest binders are selected, resulting in low yield of hit compounds. On the other hand, lack of stringency increases the likelihood of sequences being retained because of nonspecific binding and increases the time, money, and resources needed to work through all of the resulting sequences. Smith *et al* [79] describes these two antithetical conditions as the "greedy" vs. "non-greedy"

approach. The “greedy” approach eliminates the possibility of identifying groups of monomers, which in the right sequence, could lead to high affinity binding. The “non-greedy” approach is to relax the stringency of selection such that many sequences are selected. FLAG50 would have represented a medium affinity ligand. With such a ligand, we could have tested the lower limit of ligand-affinity retained by screening with “greedy” vs. “non-greedy” conditions, and modified our platform accordingly.

The screening conditions for this platform were determined in three on-bead assays using anti-FLAG with $FLAG^+$ and $FLAG^-$ peptides. To optimize the primary antibody concentration, on-bead FLAG peptides were incubated with increasing MAb concentrations. An antibody concentration tenfold higher than the saturating concentration (10 ug/mL) was selected for on-bead magnetic screening. By working under ten-fold supersaturating conditions (“non-greedy” conditions) we increased the likelihood of retaining ligands bound specifically by the MAb but with low affinity. Screening under supersaturating conditions was possible because under these conditions, binding to $FLAG^-$ peptide remained at baseline levels.

In the second on-bead optimization assay, the optimal concentration of PGDs to retain the screening MAb was determined. We identified the optimal concentration (corresponding to a 1:12.5 dilution) to retain MAbs and adopted this concentration (a 1:10 dilution was adopted).

The third on-bead optimization assay revealed that sequential incubation of the on-bead $FLAG^+$ peptide beads with the MAb followed by incubation with PGDs greatly improved bead retention. Perhaps steric hindrance affected the ability of multivalent PGD/MAb complexes to bind to multivalent on-bead peptides.

To refine these screening conditions to those sufficient for the retention of a presumably small number of sequences from a large library, the magnetic screening conditions optimized above were used to retain $FLAG^+$ peptide beads spiked into 20,000 $FLAG^-$ peptide beads. The results of these spiking experiments led us to conclude that the magnetic screen was sensitive, specific and reproducible in isolating *peptide* epitopes from a simple mixture ($FLAG^+$ peptide in $FLAG^-$ peptide).

Furthermore, the presence of $FLAG^-$ peptide beads did not appear to adversely affect the retention of $FLAG^+$ peptide beads.

To further optimize the screening conditions, to more closely approximate the retention of peptoid sequences from a peptoid library, and to determine whether the diversity of the peptoid aliquot impacted the screening conditions necessary to retain B cell epitope mimetics, $FLAG^+$ peptide beads were spiked into peptoid library aliquots (Library 1) and the magnetic screening was repeated. We hypothesized that the increasing diversity of the peptoid library aliquot as compared to $FLAG^-$ peptide would require an increased concentration of anti-FLAG to retain $FLAG^+$ peptide beads.

The results of these experiments indicated that the magnetic screen was sensitive, specific and reproducible for the retention of peptide epitopes from a more complex mixture (a peptoid library aliquot) as compared to $FLAG^-$ peptide, but this data disproved the hypothesis that library diversity negatively affected the retention of B cell epitopes.

In addition to the retention of the $FLAG^+$ peptide beads from the aliquot, thirty peptoid sequences were retained (a hit rate of 0.01%). Either these thirty sequences were potential B cell epitope mimetics or they were retained non-specifically. A case existed for each possibility.

As compared to the typical hit rates reported for the identification of a B cell epitope from synthetic and biological peptide libraries, the hit rate reported here was two orders of magnitude higher, but was still within the range of hit rates reported for the identification of B cell epitopes from a naive library. Two examples are discussed in detail below.

One of the first instances of screening an on-bead library with a MAb was done by Lam *et al* [49][1]. Two million pentapeptides were screened with MAb anti-β-

[1] For an excellent, comprehensive review of on-bead screening methods and applications of on-bead libraries to biology, see the review Lam, KS., A new type of synthetic peptide library for identifying ligand-binding activity. Nature, 1991.**354** (6348): p. 82-84.

endorphin. The theoretical diversity of a library of pentamers constructed with nineteen of the twenty naturally occurring L-amino acids is 2.5 x 10^6 sequences. Therefore, the library used by Lam *et al* [49] was slightly undersampled. However, as described in this work, the number of beads containing any individual sequence will follow a Poisson distribution, so a library many times larger than 2.5 x 10^6 beads would be required to fully cover the library. Cysteine was excluded for simplicity as cysteines can form disulfide bonds. Lam *et al* [49] had a peptide for which MAb anti-β-endorphin bound with high affinity (Ki= 17.5 nM). Six peptides were retained (a hit rate of 0.0003%). One of these sequences was bound with an affinity almost equal to the native epitope. This peptide varied from the native peptide by only one terminal amino acid. Unfortunately, negative controls were not included in this work, or the author excluded their discussion in this brief letter to Nature. The hit rate of our spiking experiments was 1 FLAG peptide bead in 30,000 peptoid beads (0.003%). Lam ***et al's*** [49] hit rate is an order of magnitude lower than ours. Although untested, we believe that our platform is capable of hit rates similar to Lam *et al* [49]. Our limitation is not the sensitivity or selectivity of the platform but instead the number of beads that can be screened in a microcentrifuge tube. Microcentrifuge tubes are used to retain PGD bound beads on a magnet. Lam *et al* [49] made no mention of blocking non-specific binding, but our results appeared unaffected by non-specific binding.

Phage display is most commonly used to display antibody fragments, which are screened with potential epitopes. However, inspired by Geysen ***et al's*** [21] work displaying peptides on pins, phage libraries displaying peptide epitopes instead of antibodies were created. In work by Prezzi *et al* [91] 2 x 10^{11} phage clones displaying nonamer peptides were screened using antibodies from HCV infected patients to identify mimetic epitopes HCV. Of these clones, 770,000 (a hit rate of 0.00035%) were selected by the serum antibodies in the first round of screening. Normal sera were used as negative controls. After amplification and further rounds of screening, first with sera from two HCV infected patients, and then using sera from eighty three infected patients, three peptides were retained which bound antibodies from 37%+ of the sera samples and not the control. Although considered

specific by the authors because of positive reactivity with anti-HCV serum antibodies, partial sequence consensus, and antibody-epitope binding inhibition, true mimicry of HCV epitopes by these retained clones is yet to be determined because of several oversights including the lack of appropriate negative screening controls and the non- affinity purification of sera (using only anti-human Fc), to isolate HCV specific antibodies. (There are many other non-anti-HCV antibodies in sera from uninfected individuals).

Although the size of the phage library was orders of magnitude larger than the on-bead screening libraries, biological factors limiting effective library size including the transformation efficiency of E. coli, and limits on enrichment, amplification, and screening efficiency close this size gap in part. Surprisingly, the hit rate of Lam *et al's* [49] work described above is equal to the hit rate in the work involving phage display. Both works involved the binding of peptide antigen by a MAb.

As compared to these works, our hit rate was two orders of magnitude greater. Although within the range of reported hit rates for similar works, the hit rate here indicated that perhaps these results could be attributed to non-specific binding.

Alternatively, these beads could have been retained non-specifically. Based on previous work, we expected non-specific binding to affect our screens. Certainly in phage display and on-bead peptide screens, measures are taken to reduce nonspecific binding including "blocking" nonspecific binding by introducing gelatin [39], E. coli lysate [55], or by varying the pH and detergent content of the screening solution. Sources of nonspecific binding can include plastic plates or beads, capturing reagents (streptavidin, protein G secondary antibody), blocking agents (BSA, milk) and "selection related target unrelated sequences" [106].

Secondary validation assays were designed to determine whether sequences in the magnetic screen were retained specifically or nonspecifically. However, three observations were made during these experiments, one of which shed light on whether these retained peptoids were B cell epitope mimetics or non-specific binders, even before these sequences were validated with secondary assays.

First, sequences which were not fully covered in PGDs were included as potential positive sequences in these initial spiking experiments. We realized that microscopic cracks in the resin led to a low level of PGD binding at the site of the crack. Beads bound by PGD because of cracks distinguish themselves from potential hits by the density of the PGD coverage. Sequences retained as potential mimetics were fully covered with PGDs. In contrast, PGDs bound to beads with cracks exhibited a slim band of PGDs bound to the crack while the remainder of the bead remained uncoated. In addition, cracked beads were identifiable by jagged edges. Although distinguishable from potential hits, we modified our screening protocol to exclude actions that would apply strong mechanical forces to the beads resulting in microscopic cracks; these included suspending the on-bead sequences with a magnetic stir bar. Furthermore, Chen *et al* [107] noticed the formation of cracks in the resin when resin beads were swollen in aqueous buffer immediately following organic solvent washes. By implementing a gradient wash which gradually brought the resin from organic to aqueous solutions, the authors observed a reduction in the amount of cracks observed. If reducing the mechanical forces applied to the resin does not eliminate cracks in the resin, we will also incorporate a gradient wash, as these cracks can cause problems during screening and sequencing.

Next, we observed that not all on-bead sequences were successfully analyzed by MALDI MS and MS/MS. Even though cleavage was successful for a majority of the $FLAG^+$ peptide beads (indicating sufficient cleavage solution incubation), and despite use of an appropriate matrix for MALDI MS and MS/MS analysis of FLAG peptide, there remained beads with sequences that could not be determined. As described by Figliozzi *et al* [45], MALDI has limitations in deconvoluting some sequences (peptoids and peptides alike). Firstly, MALDI MS and MS/MS commonly result in two sets of ionization patterns (b and y) which can make the spectra difficult to interpret. Furthermore, peak signal intensity depends on ionizability of each component of the sequence, which can be dramatically different. To account for the potential difficulty in sequencing some retained beads, the peptoid libraries were subdivided by terminal monomer following synthesis to aid in deconvoluting the

peptoid sequences by MALDI MS and MS/MS. By doing this, the identity of at least one monomer (the terminal monomer) was known.

Finally, truncated peptoid sequences, even in libraries with sequence quality in excesses of 95% were observed. An algorithm designed by the Zuckermann lab can be used to calculate the presence of truncated sequences as a function of the synthesis efficiency. For 99% efficiency, a 5mer library will consist of 5% 4mers, and a 10mer library will consist of 10% 9mers. With a decrease of only 5% efficiency, 95% efficient synthesis of a 10mer consists of 60% 10mers, 30% 9mers, 8% of 8mers and 1% 7mers. We have taken and continue to take steps to increase synthesis efficiency including making changes to our resin handling procedures and closely monitoring the status of the reagents used for synthesis.

Of the three observations made during Phase I experiments, none were novel; instead all have been previously described in detail in the literature. Although not novel, these observations impacted the interpretation of our findings in Phase I. The impact of the inclusion of cracked beads as hits on hit rate, non-specific binding, and on the conclusions we made regarding the impact of library diversity on screening conditions are discussed below.

Hit Rate. Regarding the inclusion of beads with visible cracks or that were impartially covered by PGDs in the "hits" for Library 1, the manifestation of this decision was a hit rate two orders of magnitude higher than that reported in the literature for similar works. In addition to the impartial coverage of these beads by PGDs, two additional observations added strength to the likelihood that these beads were retained because of resin cracks. First, triplicate sequences were designed into Library 1 to create a redundant, well characterized and well controlled starter-library. Hence, of the 300,000 beads screened in Library 1, only 100,000 unique sequences were represented. Therefore, any true mimetic sequences should have been identified in triplicate. An examination of the hit sequences from Library 1 revealed no triplicate sequences were retained. Next, none of the beads retained in magnetic screening were positive in color-screening. The color-screening assay was validated in a series of two experiments that demonstrated that color-screening was

a robust secondary assay to determine potential B cell epitope mimetics. Furthermore, color-screening reliably distinguished between $FLAG^+$ and $FLAG^-$ sequences. The fact that none of these sequences was positive in color screening was strong evidence that they were retained non-specifically.

Non Specific Binding. If a majority of the sequences retained for Library 1 were due to cracks in the resin, the remainder were bound non-specifically. Therefore, a low level of non-specific binding was observed for Library 1. Although the need for blocking was evaluated on a library to library basis, the results from Phase I indicated that blocking buffer was not necessary for this platform.

Impact of Library Diversity on Screening Conditions. The fact that no true mimetic sequences were identified from Library 1 raises another point. The results of spiking $FLAG^+$ peptide beads into a Library 1 aliquot led us to conclude that the diversity of the peptoid library (or the mixture being screened) did not affect the screening conditions (more specifically the amount of antibody needed to retain potential B cell epitopes). However, in light of the findings that no B cell epitope mimetics were retained from Library 1 means that Library 1 was effectually inert with respect to anti-FLAG binding. Based on this result, the hypothesis of the impact of mixture diversity on the screening conditions remained untested.

Secondary validation assays using the FLAG peptide system

The FLAG system was used to develop two secondary assays, color screening and blocking ELISAs, with which to validate the sequences obtained from a library screen. Other groups [39, 59, 86, 108-110] have used techniques similar to the color screening assay we employed by implementing fluorescent identification of bead bound sequences (for a review of on-bead screening, see Lam *et al* [39]*)*, and competition/inhibition ELISAs are standard immunological assays to validate the binding of a ligand by the MAbs binding site. These color screening assays will be critical in identifying only true epitope mimetics (which will reduce the cost, time and resources required) and are particularly important when this platform is applied to

pathogens whose epitopes are unknown and for which blocking ELISAs cannot be done because of limited quantities of native antigen. However, the blocking ELISA reveals not only that the potential B cell epitope mimetic is bound by the antibody, but that it is bound by the MAb's binding site.

To develop on-bead color screening, two assays to optimize the secondary antibody concentration followed by the primary antibody concentration were performed. The anti-FLAG bound $FLAG^+$ peptide beads changed color from translucent to blue in less than one minute and with color intensity observable by eye when incubated with anti-FLAG and HRP-conjugated goat anti-mouse IgG. As compared to the conditions used to retain peptides from a library using the magnetic screen, the primary antibody color screening conditions were equivalent to those for the magnetic screening. As for the secondary reagent, although direct comparisons between the concentrations of PGDs vs. species-specific secondary antibodies conjugated to HRP cannot be made, protein G has a medium affinity for mouse IgG1 (the isotype and specie of antibodies used in these screens), while anti-mouse secondary antibodies have a high affinity for all subclasses of mouse antibody. Despite this fact, the number of color-screen positive sequences is consistently less than the number of magnetic screen positive sequences. Indeed, contrary to our findings, Lam *et al* [39] cited a higher incidence of false positives using HRP detection systems. By presupposing that each retained sequence is covered by equal primary antibody in magnetic and color-screening, equal if not more numerous sequences should be positive in color-screening. However, a difference in the amount of antibody bound to the bead between the two assays is one potential explanation for the observed reduction in hit rate number in color screening vs. magnetic screening. Perhaps the absence of glycine stripping carried out between magnetic screening and color screening resulted in no available sites for the HRP-conjugated secondary antibody to bind. Alternatively, perhaps the magnetic screening identified minor contributing sequences on the bead. While sufficient antibody bound the minor contributing sequences for the bead to be retained by PGDs, perhaps the corresponding color-change associated with antibody bound to such a small quantify of mimetic sequence might be indistinguishable by eye.

Other potential sources for false positives in magnetic screening were sequences bound (either specifically or nonspecifically) by PGDs or sequences bound because of the aforementioned cracks in the resin. Regarding false negatives, it is possible that mimetic sequences might not be isolated by magnetic screening if the density of the mimetic sequence on the surface of the resin is present at low copy numbers.

Regarding the retention of thirty beads in the magnetic screen, none of which were positive in color screening, two explanations exist. Perhaps monomers within these sequences were important for antibody binding but the *sequence* of these relevant monomers resulted in a low affinity ligand (a “non-greedy” hit). Our results indicated that this hypothesis was possible as some consensus did appear among the sequences. Tyramine and 1,4-diaminobutane together accounted for 50% of the monomers. In this project, all but one subsequent libraries included tyramine and all included 1,4-diaminobutane.

Secondly, and most likely as evidenced and discussed above, perhaps these peptoids were retained by binding non-specifically to the antibody or because of microscopic cracks in the resin.

Blocking ELISAs were used as a relative measurement of antibody affinity and to ensure that only B cell epitope mimetics bound by the MAbs binding site were selected. Soluble FLAG/BSA blocked the binding of anti-FLAG to plate bound FLAG/BSA at 1.98 fold protein concentration for 0.1 ug/mL anti-FLAG.

Because no sequences were positive in color-screening, no blocking ELISAs were performed.

C. The magnetic screening platform was optimized for the isolation of peptoid B cell epitopes from a peptoid library

Design of a peptoid/anti-peptoid antibody pair

Following the optimization of the magnetic screening platform with a *peptide*/anti-peptide pair, a peptoid/anti-peptoid antibody pair was created and the optimization of the platform was repeated.

The R5 peptoid was used for immunization and on-bead testing as a linear, non-constrained sequence. Peptoids are “floppy” because the amide bond in both the c*is* and *trans* conformations are equally favored. Although constraints can be imposed to stabilize the peptoid, both floppy and constrained peptoids have been identified as biologically relevant ligands [47]. Specifically for immunological applications, it has yet to be determined if such constraints are necessary to elicit the production of high affinity antibodies. However, it is known is that there is a correlation between the conformational stability of an epitope and the affinity of the antibodies induced. Hence, floppy or unconstrained epitopes create energy barriers (entropy factors) that are unfavorable for antibody binding. Immunization with such epitopes elicits the production of antibodies with lower binding affinity [111, 112].

There are many forces involved in antigen/antibody binding (van der Waals interactions, hydrogen bonds, salt bridges and hydrophobic forces). Because peptoids are “floppy”, they can take on a variety of conformations depending on the forces acting on them. Thus, there is the potential that a single peptoid could display several different shapes.

Techniques to induce constraints include cyclization [113], the introduction of cysteines to form disulfide bridges [105], or the addition of peptide monomers within the peptoid chain [45]. Unfortunately, cyclical sequences are difficult to sequence in tandem mass spectroscopy [114]. To overcome this difficulty, Joo *et al* [115] developed a strategy based on the one-bead-two compound (OBTC) approach developed by Lam *et al* [116] to encode a linear peptoid inside the resin and a cyclic peptoid on the surface of the resin. Kwon *et al* [44] developed a similar technique, but synthesized both the linear and cyclic structures on the surface of the bead. The cyclical sequence was used for binding assays while the linear sequence was analyzed by MALDI MS. However, in our platform, we would be unsure if the antibodies were binding to the cyclic or linear conformations.

Another technique to induce conformational constraints unique to peptoids is the inclusion of peptide monomers within the peptoid chain to create some stability. These sequences are called hybrids, and have been used by many groups [45, 117, 118].

If necessary, in later studies, we can employ the methods listed here to constrain the immunizing peptoid sequences. However, we hypothesized that the choices we made in formulating the R5 peptoid for immunization, despite the absence of constraints, should induce high affinity antibodies since it was displayed as a multivalent hapten [119], attached to a carrier protein (such that both B and T cell epitopes were present in abundance) and used to immunize the rabbits several times at one month intervals (boosting memory cells).

Affinity purification of rabbit anti-R5 sera

Sera from healthy individuals vs. individuals with infectious disease, autoimmune disease or cancer likely contain different constellations of antibodies and other proteins. There might also be large variations among normal individuals due to their different immunologic histories. Hence, the omission of key controls could easily lead to the selection of the wrong peptoids. Examined below are four such commonly omitted controls which we included in our work.

Prebleed sera. Before either rabbit received an immunization, each rabbit was bled twice. These prebleed sera served as a negative control for many of the assays in this platform. Other groups using sera to screen libraries have not obtained pre-bleed sera from the same animals.

Control proteins in the ELISA. ELISAs were used to titer the prebleed and immune sera from each rabbit. Included in these ELISAs were control proteins to measure non-specific binding, to measure the antibodies generated against the monomers linking the sequence to the carrier protein, and finally to measure the response to the carrier protein alone. Without controlling for the presence of these antibodies, anti-linker and anti-carrier antibodies could be mistaken for anti-R5 antibodies.

Furthermore, each ELISA was performed using multiple dilutions of the sera and a standard curve from which a linear equation was derived relating antibody concentration and absorbance units.

Antibody Purification. Rabbit anti-R5 sera were affinity purified on the relevant affinity columns. As summarized in the Results, affinity purification yields antigen specific proteins. In comparison to other studies utilizing subtraction screening (ligands selected by screening with sera from healthy controls are subtracted from those positive in screening with serum from individuals with the disease of interest), screening with affinity purified sera is far more specific.

An R5-Sulfolink column was chosen for affinity purification because this column had advantages over other column chemistries. For instance, the R5 peptoid was attached to the Sulfolink column using iodoacetyl-sulfhydryl chemistry, while the R5 peptoid was linked to the carrier protein for immunization by maleimide-sulfhydryl chemistry. By avoiding the use of the same attachment chemistries, anti-linker antibodies were not retained during affinity purification. Furthermore, we preferred this column because it was designed to select antibodies specific for R5. In contrast to purification by subtraction, affinity purification eliminates all other proteins and antibodies, resulting in purification of the antibodies which bind to the peptoid. Purification by depletion can lead to errors and incomplete depletions.

Validation of affinity purified antibodies. Finally, it is critical to validate the purity and activity of the affinity purified antibodies. In this study, the purified antibody that was eluted from the columns was analyzed by both gel electrophoresis and by ELISA. The former indicated that the eluate contained IgG only; the latter indicated that antibodies against the carrier protein and linker monomers had been removed from the eluate by affinity purification. By comparison, antibodies against KLH and the linker monomers were present in the column flow through.

Affinity purification with the Sulfolink-R5 column yielded antibodies from the anti-R5 sera that were i) specific for R5, ii) IgG, and iii) that recognized R5 as it was presented on the affinity column. These polyclonal antibodies had different affinities

as determined by their elution from the column. Hence, some antibodies could be eluted off the affinity column with the first pass of low pH buffer, while other antibodies required multiple passes of low pH buffer to displace the antibodies from the column. These pools of antibody were tested individually in ELISA but the first eluate was used in on-bead screens.

On-bead optimization

Confident of the purity of anti-R5 (R5 specific Ig), and having avoided the pitfalls of other studies using unpurified sera to screen libraries, we optimized the platform for the retention of peptoid B cell epitopes from a peptoid library in three on-bead assays.

The first on-bead assay tested whether affinity purified PAb anti-R5 bound bead-bound R5 peptoid. Because antibodies bind with such high specificity, even small changes in the epitope can attenuate or even abrogate antibody binding. Such changes can be induced by techniques used in screening the library. For example, linker amines preceding the sequence of interest, the scaffold on or within which the sequence is presented [120], and sequence constraints (applications of which are reviewed in [39, 121]), can impact the adopted conformation. In our platform, by using R5 on a bead with a cleavable linker; the conformation adopted by the R5 peptoid could vary significantly from the conformation used to induce the antibodies and to select them by affinity purification. However, the results of our first on- bead assay indicated that any change in conformation to R5 due to the presence of the three monomers linking R5 to the carrier protein or due to the attachment of R5 to a resin bead were insufficient to abrogate antibody binding.

Experiments in which we spiked one or three R5 peptoid beads into aliquots of RC peptoid beads revealed that the magnetic screening platform was sensitive, specific and reproducible. The presence of RC peptoid beads did not affect the retention of R5 beads. However, spikes of 3 R5 beads into Library 2C did not display these same qualities. It appeared that the amount of antibody available to bind the R5

beads was insufficient to retain all B cell epitopes as only three out of nine R5 beads were retained. A better control would have been to first spike R5 beads into an irrelevant library Based on the success of these spikes, R5 would then be spiked into a sub-monomer library and the retention of R5 beads compared between the two library types. Such an experiment will be performed in future work to determine the effect of the diversity and presumed number of hits within a library on the retention of B cell epitopes.

Screening Library 2C with anti-R5 determined that a library made from monomers used to synthesize R5 did contain potential R5 or R5-related sequences native to the library (sub-libraries). Naïve libraries are those constructed without biases for the conformational space or functional groups suspected to play a role in the binding between two proteins. Sub-libraries, in contrast, are those made from monomers known or suspected to participate in the protein-protein (or in our case antibody-peptoid) interaction. While Sidhu *et al* [90] suggest that naïve libraries of greater than one billion compounds are necessary to identify nanomolar quantities of ligands, sub-libraries of significantly smaller size and diversity can yield equally avid binders [73, 79, 80, 86, 122]. Library 2C was a sub-library with respect to R5 and was screened with anti-R5. In contrast to Library 1 which yielded no color-screen-positive hits when screened with anti-FLAG, Library 2C yielded fifty eight (0.023%) magnetic screening positive sequences and one color screen positive sequence (0.0004%). The demonstration of the retention of potential R5 mimetics from a sub-library pointed to possible future success employing naïve libraries to do the same.

In addition to demonstrating the successful execution of the platform and pointing to future success, these data also helped assess the screening conditions employed to screen naive libraries for potential B cell epitopes (Phase III). Library 2C was screened with eluate 1 from the R5 affinity column. The antibodies in eluate 1 were the lowest in affinity of all of the antibodies retained by the column (affinity increases as the number of passes required to remove the antibodies from the column). We began with these antibodies to establish the relative limits of the screening platform. Had screening with eluate 1 yielded no potential B cell epitope mimetics, we would

know that sequences that block binding of anti-R5 to R5/BSA at 3.47 fold excess molar concentration were insufficiently avid to identify sequences from a peptoid sub-library. On the contrary, the antibodies in eluate1 were effective in retaining peptoids from Library 2C.

In light of the application of this platform to peptoid libraries screened with a MAb, these data indicated a strong potential for success, as peptoid sequences were retained from the sub-library despite the low affinity of the screening antibody.

As compared to the hit rates cited above in works by Lam *et al* [49] and Prezzi *et al* [91] (0.0003% and 0.00035%) the hit rate observed in the retention of peptoid B cell epitopes from an epitope-related peptoid library (0.0004%) was on the same order of magnitude. However, to draw more relevant comparisons, the hit rate for a similar work screening peptoid libraries with sera was examined. Reddy *et al* [123] described the identification of three peptoids isolated from a microarray of 4,608 peptoid sequences using sera from mice immunized with myelin oligodendrocyte glycoprotein (MOG). Encouraged by these findings they then used a microarray of 15,000 peptoid sequences and screened with sera from patients with Alzheimer's disease. Three peptoids were identified. Therefore the hit rates were 0.065% and 0.02%. The authors concluded that these peptoids, which bound to serum antibodies from either mice immunized with MOG or sera from Alzheimer's patients but not control sera, were "disease specific peptoid sequences" or biomarkers. These hit rates were orders of magnitude higher than in any other work reviewed in this discussion, and higher than most encountered in the literature. In our view their technology was not well enough controlled to come to any conclusions. Thus, Reddy *et al* [123] assumed that the positive signal observed when peptoid arrays were incubated with sera from immunized mice but not control mice could be attributed to antibody (Ig) only. There are a myriad of serum proteins in normal serum. In addition to these normal serum proteins, pathogen related proteins can be induced that are non-Ig. This is why a control is so necessary.

To further shed doubt on their results, they presented no data to indicate that the peptoid-bound Ig was anti-MOG. In an effort to confirm that anti-MOG antibodies

were responsible for binding these peptoids, the authors affinity purified an undisclosed volume of mouse sera using a MOG peptide column. No ELISAs were presented to confirm that the anti-MOG antibody depletion was successful. Furthermore, with such a small serum sample it is difficult to imagine that there were sufficient sera to pass over an affinity column more than once.

Finally, even assuming the antibody responsible for binding the three peptoids recognized disease-specific biomarkers, no correlation between anti-MOG antibody titers and spot intensity was determined experimentally. This was a critical omission. In supplemental work the authors did correlate spot intensity to total protein concentration, but this is meaningless comparison since there are many non-antibody proteins in sera. Furthermore, the sera from only two mice immunized with MOG one time and bled at day 36 were used in these screens. This is not acceptable. One needs larger groups of mice, immunized or not and multiple time points. The titers of the sera taken at each time point must also be measured to make sure that they even exist. The authors summarize the isolated peptoids as sequences retained “at this particular serum protein concentration and these instrument settings”. Indeed, without proper dose response curves, time course assays, and positive and negative instrument controls, these results are highly questionable.

Secondary validation assay optimization

To optimize the color screening assay for the R5 system, two assays were performed. The first assay optimized the concentration of the secondary antibody for anti-R5, and the second assay optimized the concentration of anti-R5 for on-bead R5. With these optimized conditions, the R5 system exhibited a color change that was intense and rapid. The color change intensity and time were similar to those of anti-FLAG for on-bead FLAG peptide. Furthermore, anti-R5 did not cause the RC beads to undergo a color change. These results indicated that the color screening assay was equally robust for both peptides and peptoids.

Blocking ELISAs were optimized for the R5/anti-R5 peptoid system. Anti-R5 binding was blocked by 3.47 fold excess molar concentration of R5/BSA. As a reference, anti-FLAG binding to $FLAG^+$ peptide/BSA was blocked by a 1.98 fold excess molar concentration of FLAG/BSA. These results demonstrated the use of the blocking ELISA to validate both peptide and peptoid sequences.

Evaluation of retained peptoids using secondary validation assays

Of the 58 sequences retained when Library 2C was screened with anti-R5, one sequence was positive in color screening with an intensity that was equal to five R5 beads included as positive controls. Although comparisons between the R5 peptoid structure and the potential mimetic revealed that both sequences were five monomers long and both contained a tyramine, conformational mimicry will be assessed using the blocking ELISA. As reviewed by Wetzler *et al* [34], determination of conformational mimicry by other techniques is difficult as the state-of-the-art in assessing and/or predicting peptoid secondary structure is in its infancy

In summary, Phase II results demonstrated that the platform was successfully applied to the retention of peptoid B cell epitopes from a library. The nature of the ligand (peptide vs. peptoid) did not diminish the sensitivity, specificity, or reproducibility of the magnetic screening platform as determined by the results of spiking experiments. Furthermore, PAbs and MAbs were equally effective at retaining B cell epitopes.

The results of screening Library 2C with anti-R5 demonstrated the identification and retention of a potential library-derived B cell epitope mimetic. The hit rate observed for the sub-library indicated that “non-greedy” conditions would be most likely to yield a hit from naïve libraries, since a low hit rate was observed from the library 2C when screened under “greedy” conditions. Both the color-screening assay and the blocking ELISAs were equally robust for application to peptoids and peptides.

D. Demonstration of a successful platform to identify potential peptoid B cell epitope mimetics from a peptoid library using anti-FLAG

Finally, proof-of-principle library screens were performed to demonstrate the sensitivity, specificity, and reproducibility of the magnetic screening platform in retaining potential B cell epitope mimetics from a naïve peptoid library using a MAb. To do so, four peptoid libraries were screened with anti-FLAG and the two secondary validation assays created and optimized in the two prior phases were implemented to validate the resulting sequences of the magnetic screen and narrowed the number of sequences to be studied in future work.

Five libraries were created for this platform. We excluded chiral centers (which can be included in peptoids to obtain secondary structures) in all but one library. The criteria used to select monomers changed as the project progressed. Initially, monomers with robust and efficient incorporation into the peptoid sequence were included to make simple, successful libraries. As the project progressed, the diversity (as determined by the number of monomers incorporated) and the length of the library sequences were increased. These increases, while maintaining the amount of resin on which libraries were synthesized, meant that our first libraries contained multiple copies of each possible sequence while later libraries were undersampled; only a fraction of the total possible number of sequences were represented.

These peptoid libraries were first screened with RFT5 to remove sequences bound to other portions of an irrelevant IgG antibody. The number of preclearing sequences retained by RFT5 correlated with the length and diversity of each library. As the diversity and length of the libraries increased, the number of preclearing sequences retained did as well; Library 1 yielded seven preclearing hits while Library 5 yielded over three hundred. These data indicated the importance of the preclearing step in removing nonspecific sequences, particularly as library size and diversity increased.

The precleared peptoid libraries were then screened with anti-FLAG to identify potential B cell epitope mimetics of FLAG peptide. The results of these screens failed to exhibit a relationship between the library size/diversity and hit rate as

observed for sequences retained in preclearing. On the order of thirty peptoid sequences were retained for each of the four libraries screened with anti-FLAG despite the library size or diversity. However, as discussed previously, a re-examination of the hit rate of Library 1 would likely result in a much lower hit rate and fewer beads considered positive. Many Library 1 sequences were retained because of microscopic cracks in the resin bead. An examination of the retained sequences indicated that no sequence was identified in triplicate, and none of these sequences were positive in color screening.

Therefore, with confidence we could exclude the thirty hit sequences from Library 1 in determining if the diversity and/or length of the libraries were related to the number of hits retained. Upon excluding these thirty beads, a relationship between library size/diversity and hit rate was observed. Specifically, the three most diverse libraries (in increasing order) were Libraries 3, 2, and 5. In absence of the thirty hits from Library 1, the three highest hit rates (in increasing order) resulted from Library 3, 2 and 5. This correlation between library diversity and hit rate was expected based on reports in the literature [39, 74, 79, 86]. Our results corroborated the well documented importance of including sufficient diversity in any libraries to be used for screening. They also demonstrated that for this platform, a diversity of 100,000 sequences was insufficient to yield a hit in magnetic screening. Although these data determined the lower limit of diversity, we have not yet determined the upper limit of diversity (the library diversity above which the hit rates are all equal).

All together, ninety one sequences were retained from approximately 2.24 million peptoid sequences resulting in a hit rate of 0.004%.

As compared to the hit rates cited above in works by Lam *et al* [49] and Prezzi *et al* [91] (0.0003% and 0.00035%) the hit rate observed in the retention of potential FLAG mimetics from four peptoid libraries with a MAb was one order of magnitude greater. Certainly, in addition to library factors important for successful screening platforms, the conditions (specifically the stringency) under which libraries were screened strongly influenced this hit rate. For the development of this platform, we chose optimized but “non-greedy” screening conditions. That is to say, with the use

of on-bead assays, we identified optimized screening conditions for the retention of on-bead FLAG peptide; we then chose a screening concentration ten-fold higher than this optimized condition to retain potential peptoid B cell epitope mimetics. As noted by Smith *et al* [79], the ultimate goal of selection is usually to isolate sequences with high fitness, but this does not mean that stringency should be increased indefinitely since this can lead to decreasing yields. Because we employed "non-greedy" screening conditions, it was not surprising that our hit rate was higher than those reported for other platforms. By decreasing the stringency of our screens, we increased the yield.

Taken together, these results indicated that our screening platform demonstrated hallmarks of successful screening platforms. The platform utilized libraries with sufficient diversity to yield potential hits, had a hit rate that was consistent with that of other similar platforms, and the screening conditions applied to the libraries yielded manageable numbers of potential FLAG mimetics.

As for the nature of the sequences retained, while no consensus sequences were observed, 1,4-diaminobutane, exo-2-aminonorbornane, and 2-(1-cyclohexenyl)ethylamine appeared at a higher frequency than the remaining twenty four library monomers. Although it is tempting to assess the retained sequences for mimicry to FLAG peptide based on the monomers or the nature of the monomers found in the hit sequences, such analysis would not necessarily distinguish hit vs. non-hit sequences. Because we are screening for conformational mimetics, "mimotopes", the retained sequences need only represent the three dimensional shape presented by FLAG peptide as opposed to resembling the FLAG peptide sequence or characteristics associated with the FLAG peptide monomers. With this being said, it was worth noting the prevalence of only three monomers from twenty seven total monomers as a potential indication of how few critical residues there may be in determining binding of the peptoid sequence by anti-FLAG.

Regarding the nature of the retained sequences, sequences that were either equal in length or shorter than the native antigen were retained as potential mimetics by anti-FLAG. These findings further support our hypothesis that the sequences

presented for binding to the antibody need only mimic the three dimensional conformation of the native antigen. Neither the nature/identity of the monomers nor the length of the hit sequences correlated directly with the native antigen.

To return to the screening platform, these ninety one retained sequences were then tested in color-screening to narrow the number of potential mimetics to a number reasonable to continue in testing and to identify only those most likely to mimic the native antigen. Of these ninety one, only ten sequences were positive in color screening. Each of these ten sequences had equal color change times and intensities as compared to the positive control.

The resulting hit rate of the platform following color screening was 0.0004%, one order of magnitude lower than that reported for magnetic screening. As compared to the hit rates discussed in this section, the hit rate was on the same order of magnitude as those for both phage display and on-bead peptide libraries. These data indicate that color screening effectively reduced the number of potentially positive mimetics and brought the hit rate associated with this platform in line with those reported in other platforms.

Worth noting was the difficulty with which the ten-monomer sequences were analyzed by MALDI MS and MS/MS. One hypothesis is that the longer sequences could have had more protecting groups on a single sequence than their shorter counter parts. The cleavage time necessary to remove the sequence from the resin depends directly upon the number and type of protecting groups present. Perhaps longer cleavage times will eliminate this difficulty. Additionally, we can try using different matrices for MALDI MS and MSMS analysis. Other matrices might permit more productive ionizations and clearer spectra.

In summary, Phase III resulted in the successful demonstration of a peptoid based platform for the identification of potential B cell epitopes using MAbs. Magnetic screening resulted in the retention of potential B cell epitope mimetics with a hit rate one order of magnitude greater than that reported by other groups applying the same strategy to identify potential B cell epitope mimetics. There was a trend

relating the number of sequences retained to the length and diversity of the library. This pattern was observed for sequences retained in preclearing as well as in magnetic or color screening. However, the preclearing hit rate showed exponential increases in hit rate as related to library diversity, whereas the hits retained by anti-FLAG screening correlated with library diversity, but did not show large increases.

Color screening successfully reduced the number of potential positive peptoid B cell epitope mimetics from those retained in the magnetic screen (reduced from ninety one to ten sequences). This secondary validation assay will save time, money and labor in working up potential mimetic sequences for further validation.

Monomers important for antibody binding, as determined by their frequency in the retained hit sequences, were observed. However, because these sequences were potential mimotopes of FLAG peptide, no direct correlation between the monomers of the peptoid and the native peptide was expected or observed. Additionally, sequences both equal in length and shorter than FLAG peptides were retained, supporting our hypothesis that a one to one relationship was not necessary between monomer identity, monomer characteristics or sequence length.

E. CONCLUSION

In conclusion, we have created a successful platform 1) to identify peptide B cell epitopes from on-bead libraries using MAbs, and 2) to identify peptoid B cell epitopes from peptoid libraries with MAbs, and 3) to optimize this platform for the retention of potential B cell epitope mimetics from peptoid libraries. This was demonstrated using anti-FLAG and four peptoid libraries. The magnetic screening platform displayed sensitivity, specificity, and reproducibility in retaining B cell epitopes from peptide and peptoid libraries.

The resulting hit sequences were both the same length or shorter than FLAG peptide, and the nature of the monomers within the peptoid hits did not resemble the nature of the monomers in FLAG peptide, supporting the idea that the MAbs select conformational mimetics from the library.

Two secondary assays were developed with which potential B cell epitope mimetics were assessed. Color screening successfully reduced the number of potential hits by 90%.

In terms of the strategic plan presented for this work, we successfully synthesized large, on-bead combinatorial libraries, screened these libraries with MAbs to identify hits, validated the potential hits with secondary assays and sequenced the sequences that were positive in magnetic and color screening.

In applying this proof of principle screen to the retention of peptoid B cell epitope mimetics, ten potential FLAG peptide mimetics were identified.

F. FUTURE WORK

This study represents only the beginning of the creation of this novel platform. Much work lies ahead to create a sensitive and specific platform that works with antibodies with a large range of affinities.

Future work will first continue by validating the ten color-screen-positive hits by **blocking ELISA**. In addition to identifying which sequences were true FLAG peptide mimetics (as determined by binding the hypervariable regions of the MAb used for screening), the blocking ELISA results will indicate whether there is a relationship between the consensus monomers and the resulting binding avidity. The results of these blocking ELISAs will also determine any changes that might be necessary to alter the screening stringency. Any peptoid sequences that block binding of anti-FLAG to $FLAG^{+}$ peptide will be tested *in vivo* for the induction of antibodies that cross-react with FLAG peptide.

Sub-libraries will be made from consensus monomers and these libraries will be screened for high affinity binders.

Finally we are working on developing a **third secondary validation assay**. As described by Granoff *et al* [124], we are developing an on-bead blocking assay to

follow on-bead color screening. In this assay, native antigen would be coated onto a plate and the on-bead hit sequence plus the screening antibody would be added.

We will **continue our work with the R5 system**, identifying the conditions that enable sensitive retention of peptoid ligands. Furthermore, screening libraries with pools of PAb anti-R5 that vary in affinity will elucidate the relationship between antibody affinity and the sensitivity and specificity of our platform. By identifying the range of affinities within which our platform operates specifically, sensitively, and reproducibly, we will better understand the anti-pathogen antibodies that can be applied to this platform. The fifty three magnetic screen positive/color screen negative sequences will be screened with antibodies of increased affinity to assess their positivity. Ideally this work would continue with the creation of a MAb anti-R5.

In addition to screening peptoid libraries with anti-FLAG, libraries should be **screened with PAb anti-FLAG** to identify a larger set of monomers from which sub libraries can be synthesized and to determine the lower range of affinities for which a sequence can be retained by an antibody from a library screen. We have PAb anti-FLAG antibodies in hand resulting from the immunization of two rabbits with FLAG/KLH and adsorbed to alum.

Finally, peptoid libraries will be screened with **neutralizing monoclonal antibodies** against HIV, HCV and WNV with the goal of identifying peptoid mimetics of B cell epitopes with which novel, life saving vaccines can be created. Only after we have applied this platform to the identification of B cell epitope mimetics retained from screening with anti-pathogen neutralizing MAbs can we truly comment on how this platform compares to others (phage, peptide libraries). Nevertheless, this work represents a promising beginning to an alternative method for discovering B cell epitopes mimetics that can be used to make vaccines.

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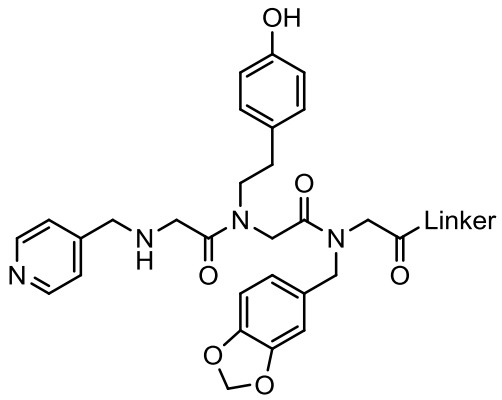
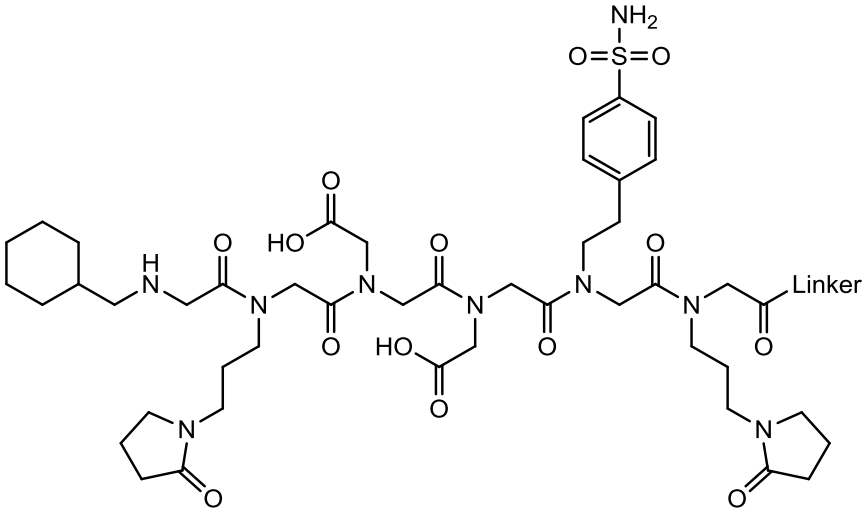
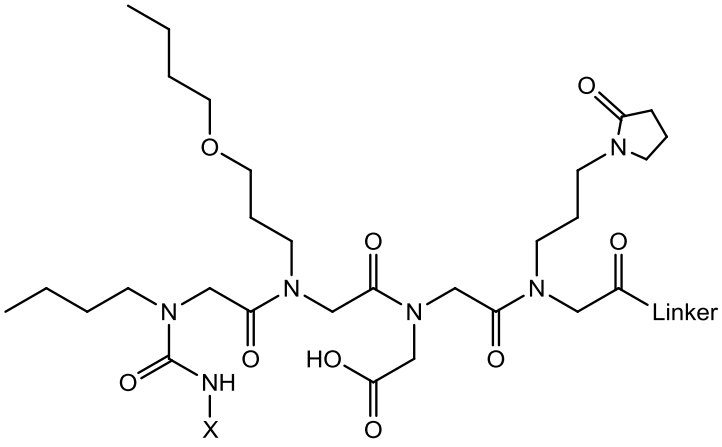
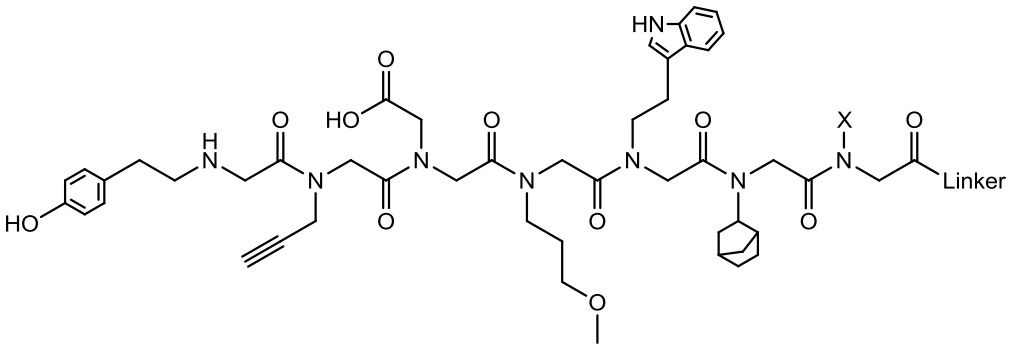
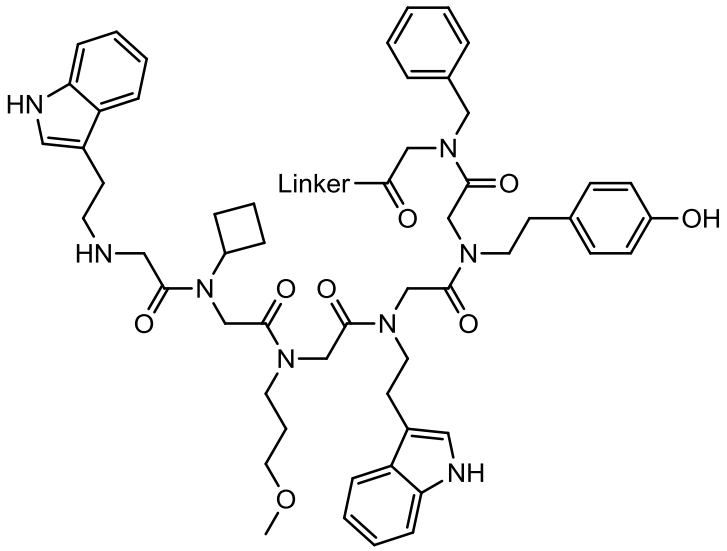
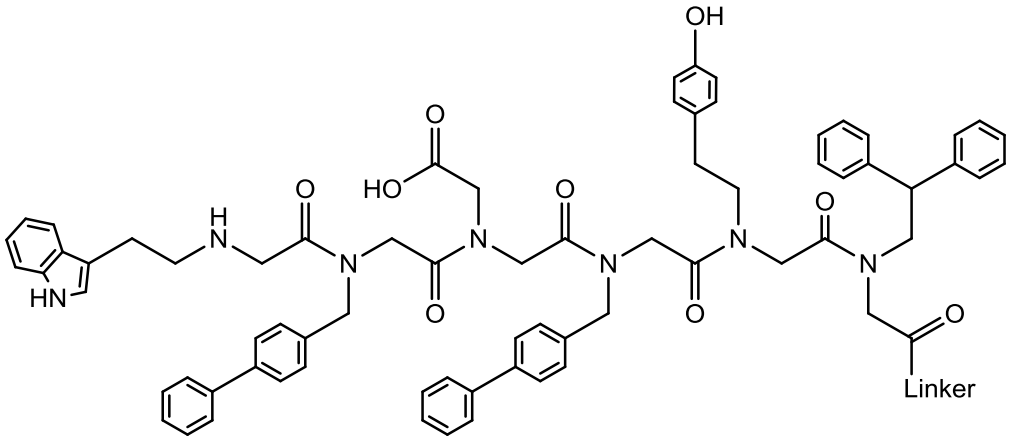
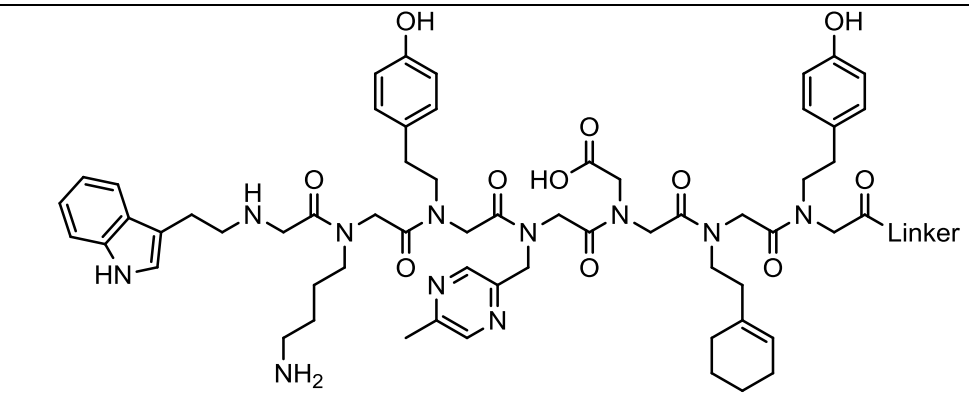
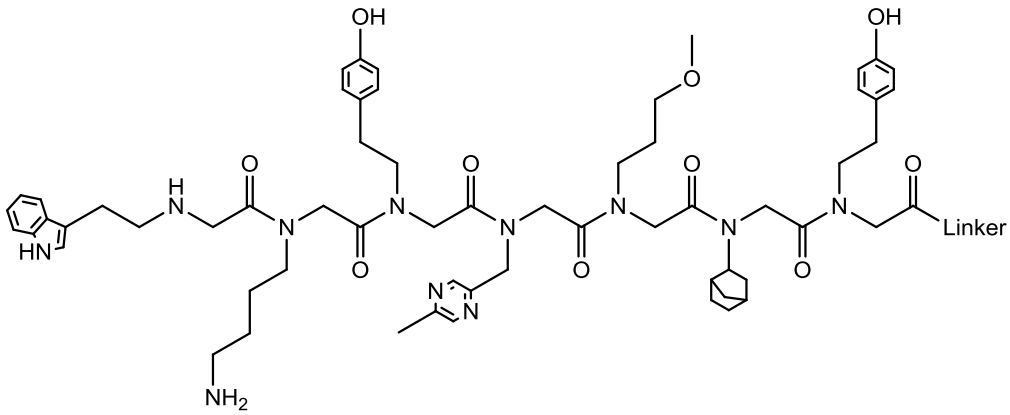
VI. APPENDIX A: Structures of Ten Potential Peptoid Mimetic Sequences

Library	Strucutre and Sequence
2	 1) (Nap-Nty)-Npi-E-M
3	 1) Namc-Npy-Nas-Nas-Nbs-Npy-A-A-M  2) X-Nbu-Nbp-Nas-Npy-A-A-M

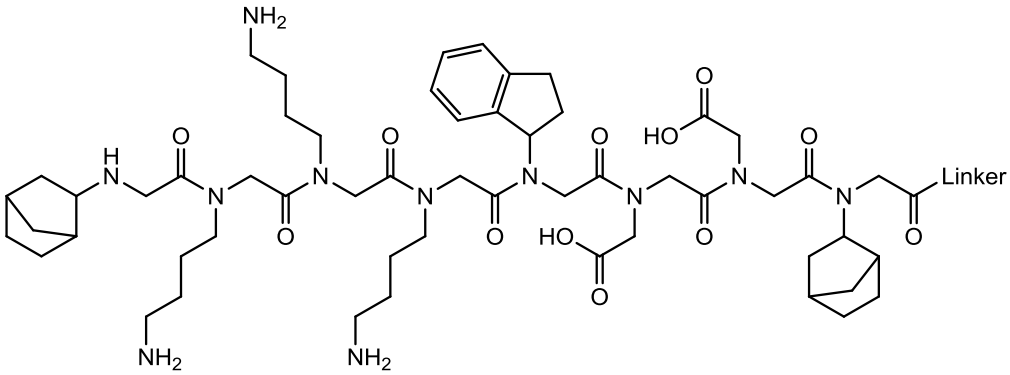
5	 1) Nty-Nprg-Nas-Nmp-Ntry-Nexo-X-Me-M  2a) Ntry-Ncy-Nmp-Ntry-Nty-Nbn-Me-M  2b) Ntry-Nphe-Nas-Nphe-Nty-Ndi-Me-M



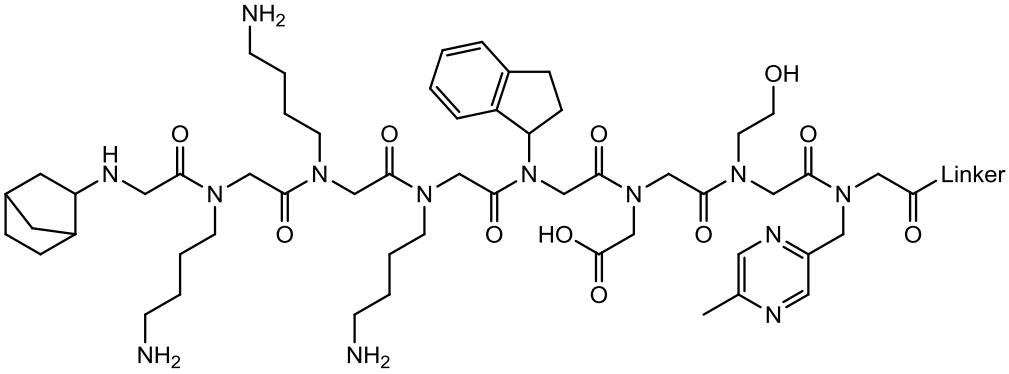
3a) Ntry-Nlys-Nty-Namm-(Nas-Nch)-Nty-Me-M



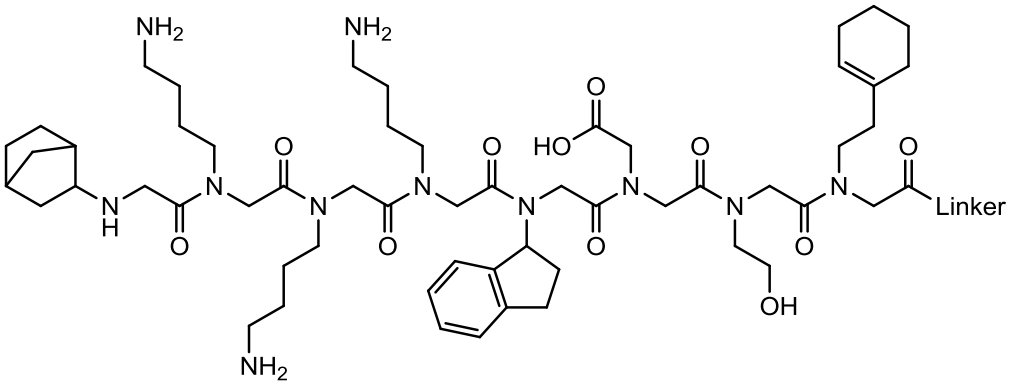
3b) Ntry-Nlys-Nty-Namm-Nmp-Nexo-Nty-Me-M



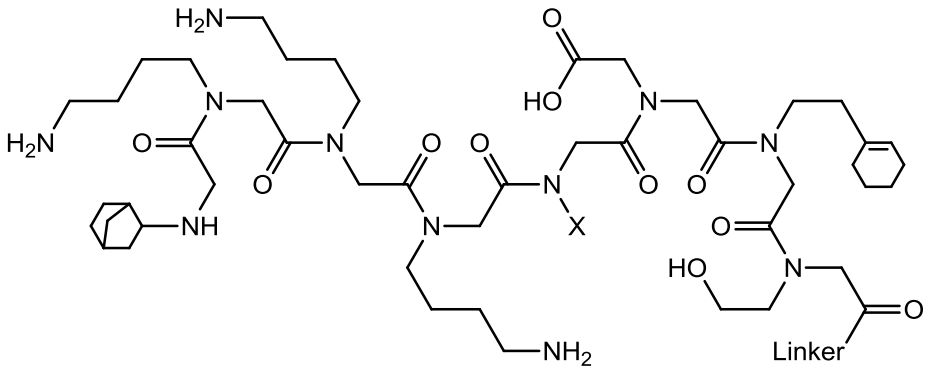
4a) Nexo-Nlys-Nlys-Nlys-Nai-Nas-Nas-Nexo-Me-M



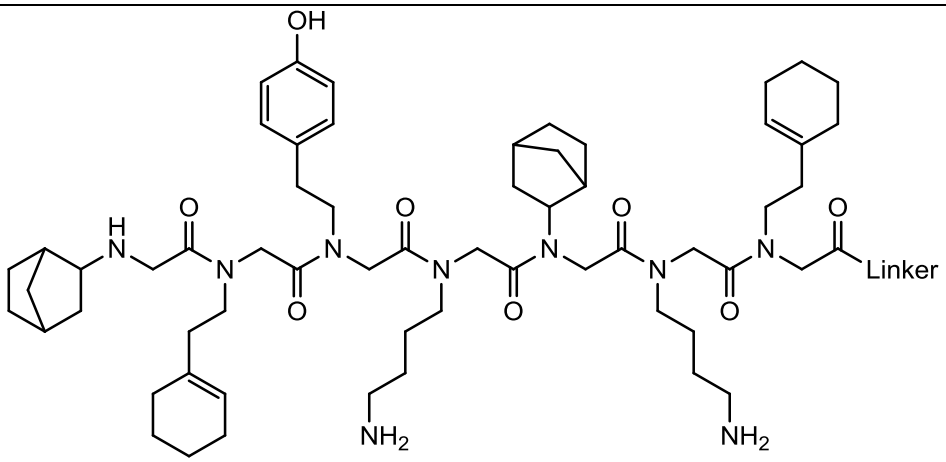
4b) Nexo-Nlys-Nlys-Nlys-Nai-Nas-Net-Namm-Me-M



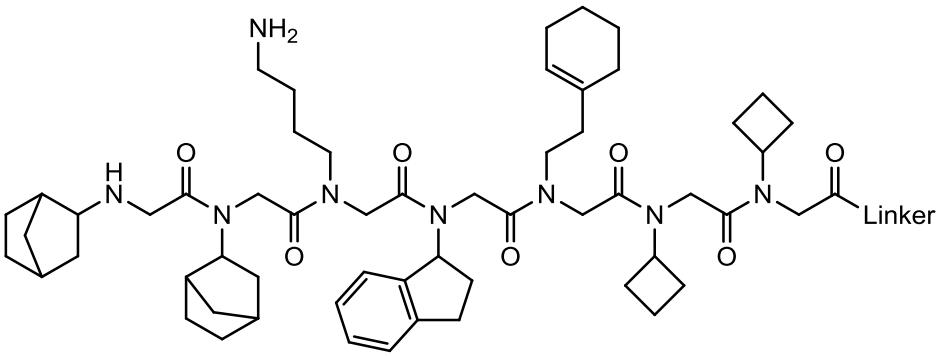
4c) Nexo-Nlys-Nlys-Nlys-Nai-Nas-Net-Nch-Me-M



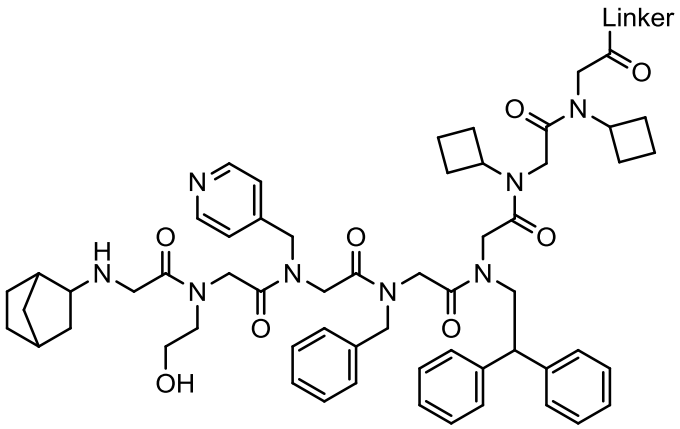
4d) Nexo-Nlys-Nlys-Nlys-X-Nas-Nch-Net-Me-M



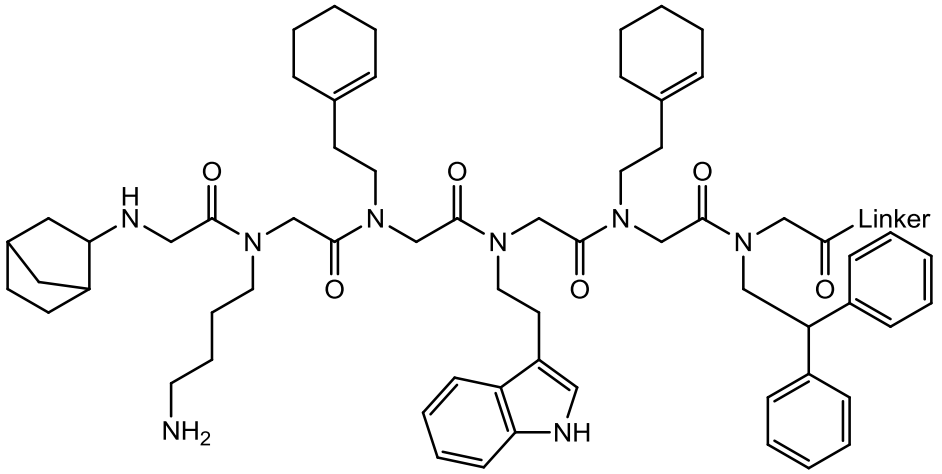
5) Nexo-Nch-Nty-Nlys-Nexo-Nlys-Nch-Me-M



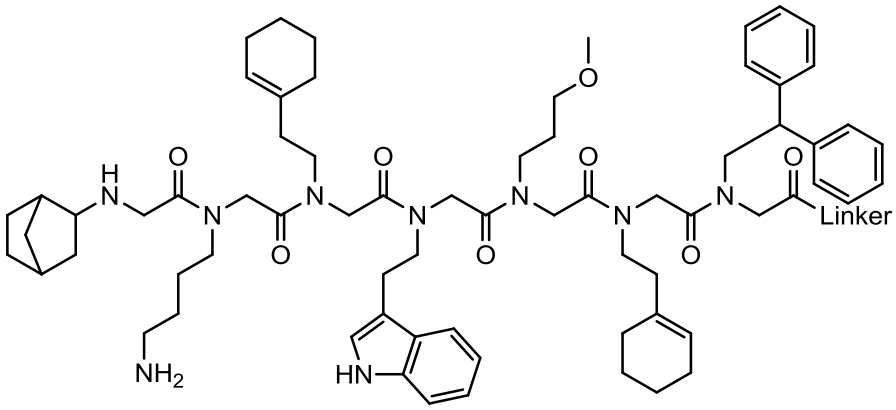
6a) Nexo-Nexo-Nlys-Nai-Nch-Ncy-Ncy-Me-M



6b) Ncy-Ncy-Ndi-Nbn-Nap-Net-Nexo



7a) Nexo-Nlys-Nch-Ntry-Nch-Ndi-Me-M



7b) Nexo-Nlys-Nch-Ntry-(Nmp-Nch)-Ndi-Me-M

Structures of potential mimetic peptoids, divided by the library of origin, are shown in the table above. These structures are associated with the sequences listed in **Table 16**. Linker amino acids and/or amines have been removed to show only the sequence and structure of the magnetic screen positive, color screen positive, potential mimetic peptoid sequences, although the linker monomers do appear in the sequence below each structure. The full names of the abbreviated linker monomers (M-E; M-A-A; M-Me) can be found in **Table 3**. More than ten structures are shown for the ten potential mimetic peptoids as some sequences could not be fully determined, so alternative structures are also displayed. Parentheses indicate that the order of the monomers inside the parenthesis is unknown. Peptoids are displayed N'-C'.