NEW APPROACHES TO THE DEVELOPMENT OF PEPTOID VACCINES

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DEDICATION

In loving memory of my grandmothers

Frances Desmond

(1922-2008)

and

Emilia T. Winski

(1918-2012)

ACKNOWLEDGEMENTS

If you want to walk fast, walk alone. If you want to walk far, walk together.

African proverb

I thank, first and foremost, my mentor, Dr. Ellen Vitetta, for the opportunity to pursue a project that brought together immunology, peptoid chemistry, and virology, and was therefore decidedly outside the box of my previous experience in cancer molecular biology. This kind of bold, interdisciplinary science was one of the main attractions of UT Southwestern for me as a student, and I benefitted greatly from your experience as I navigated the unknown.

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Tubbs, Dr. Jizhou Yan, Dr. Yuqing Yang, Dr. Ya-Xiong Chen, Ms. Jennie Hildebrandt, Dr. Xinjiang Wu, and Dr. Brian Keith, all that you taught me I used in this work. Thank you for choosing me and for your patience when I was starting in the lab as an undergraduate!

To Mom and Dad, who sacrificed so much for my education, please accept your daughter's humble thanks. If I ever have enough money, I will buy you a house down the shore, but don't hold your breath. To the rest of my beloved family, Sarah, Uncle Ed, Aunt Jane, Grandpop, Aunt Ginny, Uncle Carl, Stephen, Megan, Aunt Mary, Uncle George, Michelle, Austin, Bobby, Uncle Daryl, Aunt Colleen, Shaun, Jason, Amy, Uncle Tony, Kelly Miller, Karen, the new ones Paul, Sophia, Paul Anthony, and Josh, and so many more, I feel your support and love down here in Texas.

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Lastly, to all my teachers in the humanities, don’t worry. I’m still working on my novels.

NEW APPROACHES TO THE DEVELOPMENT OF PEPTOID VACCINES

by

ANGELA DESMOND

DISSERTATION

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The ideal prophylactic vaccine against a toxin or pathogen should elicit the production of broadly protective antibodies against conserved epitopes. However, the epitopes that elicit these antibodies are often not immunodominant and even when they are, characterizing and synthesizing them can be difficult, particularly if they are conformational. The long-term goal of this work was to develop prophylactic vaccines that elicit such antibodies without epitope characterization. To develop such a vaccine platform, it was hypothesized that screening large one-bead-one-compound libraries of synthetic compounds with monoclonal antibodies that have already been shown to be broadly protective against a toxin or pathogen would allow the identification of mimetic B cell epitopes. For this platform, peptoids were chosen to construct one-bead-one-compound

libraries. Peptoids are *N*-oligosubstituted glycines that resemble peptides but bear their side groups on backbone nitrogens instead of carbons. This renders them protease resistant and enormously diverse, since they are not restricted to the twenty standard amino acids. Furthermore, previous work had demonstrated that a monoclonal antibody could be used to screen libraries of peptoids. Moreover, while peptoids themselves were not immunogenic, the attachment of peptoids to carrier proteins using a linker elicited antibodies against the peptoid/linker. Such T-cell dependent antigens elicited high-affinity, class-switched antibodies. The goal of this dissertation research was to continue optimizing the magnetic and color-based assays by which peptoid vaccine candidates could be identified and to screen libraries with neutralizing monoclonal antibodies against West Nile virus and murine norovirus type 1. In addition, the immunogenicity of peptoids was further examined by designing a peptoid-carrier, using it to immunize rabbits, and demonstrating that anti-peptoid antibodies could be affinity-purified from the resulting antisera. This antibody was then used in further optimization of the magnetic screening assay to ensure that future screens will efficiently and specifically identify the best vaccine candidates.

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PRIOR PUBLICATIONS

Case A*, **Desmond A***, Yi KJ, Chakravarty P, Smallshaw JE, Collins A, Dye K, Mapes K, Ruback SL, Yang J, Tran H, Connolly MD, Zuckermann RN, Chirayil S, Luebke KJ, Astle JM, Reddy MM, Kodadek T, Vitetta ES. A novel platform to generate peptoid-based mimetic vaccines. J Immunol. 2013 May;190(Meeting Abstract Supplement):178.6. *A. Case and A. Desmond are co-first authors.

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La P, Schnepp RW, Petersen CD, Silva AC, Hua X. Tumor suppressor menin regulates expression of insulin-like growth factor binding protein 2. Endocrinology. 2004 Jul;145(7):3443-50. Epub 2004 Mar 24. PMID: 15044367. Acknowledged.

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LIST OF DEFINITIONS

-mer	polymer with number of units preceding the hyphen
°C	degrees Celsius
4-MP	4-methylpiperidine
A	L-alanine
A6.2	1A6.2.1, a monoclonal antibody against murine norovirus type 1
ACN	acetonitrile
Ahex	6-aminohexanoic acid
AIDS	acquired immune deficiency syndrome
Ala	L-alanine
ARC	Animal Resources Center
Asp	L-aspartic acid
BAA	bromoacetic acid
BCG	Bacillus of Calmette and Guerin (a strain of *Mycobacterium bovis*)
BHK	baby hamster kidney cells
bnMAb	broadly neutralizing monoclonal antibody
BOC	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
C	carboxy terminus
CAA	chloroacetic acid
CHCA	α-cyano-4-hydroxycinnamic acid
CFA	complete Freund’s adjuvant
CIC	Cancer Immunobiology Center

cm	centimeter
CNBr	cyanogen bromide
cpm	counts per minute
Cys	cysteine
Cys•HCl•H_2O	cysteine hydrochloride monohydrate
D	L-aspartic acid
Dasp	D-aspartic acid
DCE	1,2-dichloroethane
Dgln	D-glutamine
DI	deionized
DIC	1,3-diisopropylcarbodiimide
Dlys	D-lysine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dtyr	D-tyrosine
E	L-glutamic acid
EAE	experimental autoimmune encephalomyelitis
EDT	ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E protein	envelope protein (here, of West Nile virus)
FBS	fetal bovine serum

Fc	fragment constant
Fig.	figure
$FLAG^+$	the peptide N-DYKDDDDK-C, using the single letter amino acid code
$FLAG^-$	the peptide N-DAKDDDDK-C, using the single letter amino acid code
FMOC	9-fluorenylmethyloxycarbonyl
FT	flow-through
g	gram
G	glycine
Gly	glycine
Gly-OtBu	glycine *tert*-butyl ester
Gly-OtBu•HCl	glycine *tert*-butyl ester hydrochloride
h	hour
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCl	hydrochloric acid
HCV	hepatitis C virus
Hib	*Haemophilus influenzae* type B
HIV	human immunodeficiency virus
HMWM	high molecular weight marker
HOBt	1-hydroxybenzotriazole monohydrate
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
hTGFα	human transforming growth factor alpha

I	L-isoleucine
IACUC	Institutional Animal Care & Use Committee
Ig	immunoglobulin
IL-1β	interleukin-1 beta
IRF	interferon regulatory factor
K	L-lysine
kD	kilodalton
KLH	keyhole limpet hemocyanin
L	liter; or L-leucine
LMWM	low molecular weight marker
Lys	L-lysine
M	Molar
MAb	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization
MDA5	melanoma differentiation-associated protein five
MERS-coV	Middle Eastern respiratory syndrome-coronavirus
Met	methionine
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mmol	millimole

MNV-1	murine norovirus type 1
MOG	myelin oligodendrocyte glycoprotein
mol	mole
MS	mass spectrometry
MSTP	Medical Scientist Training Program
MS/MS	tandem mass spectrometry
MW	molecular weight
m/z	mass-to-charge ratio
N	amino terminus; or L-asparagine
N_2	nitrogen gas
Na_2HPO_4	sodium phosphate dibasic
Na_2SO_4	sodium sulfate
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate monobasic
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NaOH	sodium hydroxide
NIH	National Institutes of Health
NK	natural killer cells
nm	nanometer
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
nM	nanomolar
NMO	neuromyelitis optica

nmol	nanomole
NMM	N-methylmorpholine
NMP	*N*-methyl-2-pyrrolidinone
OBOC	one-bead-one-compound
OtBu	*tert*-butyl ester (*tert*-butyl group protecting a carboxylic acid)
OVA	ovalbumin
P domain	protruding domain of the norovirus capsid protein
P particle	An assembly of 24 copies of the protruding domain of the norovirus capsid protein formed when the protruding domain is expressed in cells
PAb	polyclonal antibody
PBS	phosphate buffered saline
PBST	PBS-Tween 20; a solution of 0.01% (v/v) Tween 20 in phosphate buffered saline
peptomers	peptide-peptoid hybrid molecules
PES	polyethersulfone
PGDs	Protein G Dynabeads
prM	premembrane protein, here of West Nile virus
PTFE	polytetrafluoroethylene
PVP	polyvinylpyrrolidone
Q	L-glutamine
R	L-arginine
R group	side chain of a peptoid or peptide monomer

R5	pentamer peptoid used to immunize rabbits (rabbit 5-mer)
RAMIg	rabbit polyclonal antibody against mouse immunoglobulin (rabbit anti-mouse Ig) used as an irrelevant control for RAR5
RAR5	rabbit polyclonal antibody against R5 peptoid (rabbit anti-R5)
RC	heptamer peptoid used as an irrelevant control for R5 peptoid (rabbit control)
RIA	radioimmunoassay
RIG-I	retinoic acid-inducible gene 1
RNA	ribonucleic acid
RP-HPLC	reverse phase-high performance liquid chromatography
rpm	revolutions per minute
RT	room temperature
S	L-serine
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
SPR	surface plasmon resonance
STAT1	signal transducer and activator of transcription 1
T	L-threonine
Tab2	a monoclonal antibody raised against human transforming growth factor alpha
TB	tuberculosis
tBu	*tert*-butyl protecting group

TCEP	tris(2-carboxyethyl)phosphine
TentaGel	amino-functionalized macrobeads, composed of polystyrene and polyethylene glycol
TFA	trifluoroacetic acid
THF	tetrahydrofuran
THP	tetrahydropyran
TIS	triisopropyl silane
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TOF/TOF	time-of-flight/time-of-flight
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Tris	tris(hydroxymethyl)aminomethane
Trityl	triphenylmethyl
Trt	triphenylmethyl
Tween 20	polyoxyethylenesorbitan monolaurate
Tyr	L-tyrosine
UTSW	University of Texas Southwestern Medical Center
UV	ultraviolet
v/v	volume/volume
VLP	virus-like particle; specifically, VLPs are formed spontaneously when the norovirus capsid protein is expressed in cells
W	L-tryptophan
WNV	West Nile virus

w/v	weight/volume
X	times
x *g*	times gravity
Y	L-tyrosine
μg	microgram
μL	microliter
μm	micrometer
μM	micromolar

ADDENDUM

List of Additional Figures

List of Additional Definitions

ΔA	change in absorbance
DTT	dithiothreitol
R5A	R5 peptoid, version A (see Fig. A.1B, page lvi, Fig. 2.7, page 110, Fig. 2.8, page 112)
R5B	R5 peptoid, version B (see Fig. A.1C, page lvi)
RAR5A	Polyclonal antibody rabbit anti-R5A peptoid
SPDP	*N*-succinimidyl 3-(2-pyridyldithio)-propionate
SPDP-BSA	SPDP-modified BSA

Introduction to Addendum

Within a month of successfully defending my doctoral dissertation in Immunology on July 26, 2013, my colleagues brought to my attention a pair of errors that impacted on the sections of my thesis that address the immunization of rabbits to generate the first affinity-purified anti-peptoid antibody. These errors were discovered when this work was continued in Dr. Vitetta's laboratory and samples that I had not previously tested were evaluated. Following the discovery of these errors, Dr. Vitetta and I agreed that the best way to handle the completion of my thesis was to describe the errors and their impact on the conclusions of the work. It should be noted these errors themselves should not negate the key findings of my thesis regarding the affinity purification of anti-peptoid antibodies. The errors and the manner in which they are now addressed are summarized here, with more detailed Methods, Results, and Discussion sections appearing below (pages xliv-lx, mirroring the overall organization of this dissertation):

1. To perform additional studies examining the immunogenicity of peptoids and to generate the first affinity-purified antibody against a peptoid, we planned to immunize rabbits with a 5-mer peptoid (peptoid R5) conjugated to a carrier protein and adsorbed to alum. Drs. Smallshaw, Case, Yi, Vitetta and I designed the 5-mer peptoid shown in Fig. 2.7 (page 110) and Fig. 2.8 (page 112), and this peptoid was designated R5. Drs. Case, Yi, and I then attempted the first synthesis of this peptoid. Subsequent syntheses were carried out by Drs. Case and Yi, who found that this peptoid was difficult to synthesize and sought a solution. After the peptoid group met, we agreed that the best solution was to alter the order of the monomers in the 5-mer peptoid. Both the original sequence (here designated **R5A**) and the modified sequence (here designated **R5B**) are

shown in Fig. A.1 (page lvi). Following the first immunization with R5A, R5B was then used for several subsequent immunizations. Unfortunately, and incorrectly, we continued to refer to the modified 5-mer peptoid sequence simply as the “R5 peptoid” rather than “R5B”. Furthermore, the structure of R5 peptoid as shown in team documents (332) did not reflect the change in the sequence. When I began to spearhead the rabbit immunization studies just prior to the final immunizations, I returned to the documents I had used when Drs. Case, Yi, and I first attempted to synthesize the 5-mer peptoid. Consequently, I synthesized R5A for the final immunizations, as well as for all my subsequent experiments for affinity purification of anti-R5 peptoid antibodies and optimization of on-bead screening. When Drs. Vitetta, Pop, and Smallshaw, along with Mr. Ruback, continued the work on this portion of the project after I left the lab, the discrepancy between R5A and R5B in team documents was discovered. The immunization scheme, originally shown in Fig. 2.12 (page 119), has now been revised as Fig. A.2 (page lvii) to show when R5A and R5B were used, and the implications are discussed below.

2. In the rabbit immunization studies, we had planned to carry out all immunizations with the 5-mer peptoid conjugated to the maleimide-activated carrier protein keyhole limpet hemocyanin (KLH). We then planned to use peptoids conjugated to another carrier protein, bovine serum albumin (BSA), in enzyme-linked immunosorbent assays (ELISAs) to detect rabbit antibodies against the immunizing peptoid and against the linker connecting the peptoid to the maleimide-activated carrier protein, as shown in Fig. 3.1 (page 166). However, when Dr. Pop examined the rabbit sera from all time points by ELISA, the data

clearly demonstrated that serum from Day 155 (after the third immunization on Day 83; Figs. 2.12 and A.2) contained high levels of anti-BSA antibody (Figs. A.4C and D, page lix), suggesting that the rabbits had been immunized with peptoid-BSA instead of peptoid-KLH during the third immunization. This mostly likely occurred because the vials containing the two conjugates were erroneously interchanged by my colleague. Re-examination of records indicated that the same batch of 5-mer peptoid conjugate that was used for the third immunization was also used for the fourth immunization of rabbit 12D on Day 155. The methods and data from Dr. Pop's experiments are presented and the implications are discussed below.

While the realization that these errors occurred, and that they were not discovered by me personally, was disappointing, fortunately, they in no way affected the subsequent affinity purification of anti-R5A peptoid antibodies since the affinity column and all experiments thereafter used peptoid R5A. However, the use of two different, albeit related, peptoids for immunization would help account for the low amount of anti-R5A antibody recovered from the rabbit sera, which had been difficult to explain. I am therefore grateful to my colleagues whose work has brought these insights to light, and for the opportunity to provide a more accurate account of what transpired in my dissertation research by including this addendum. Again, more detailed revisions are described below (pages xliv-lx).

More recently, additional experiments performed by members of the Vitetta laboratory using the anti-peptoid R5A antibody have raised questions regarding the efficiency of conjugation of peptoids to carrier proteins. This work is ongoing, and as such, all conclusions based on it are preliminary. Therefore, I will not present any of the methods

or data from those experiments or discuss this work further in this addendum beyond a few statements here focusing on the implications of this recent work for my thesis. I include again here Figure 3.1 as it appears later in this dissertation (pages 166-167) to facilitate this discussion.

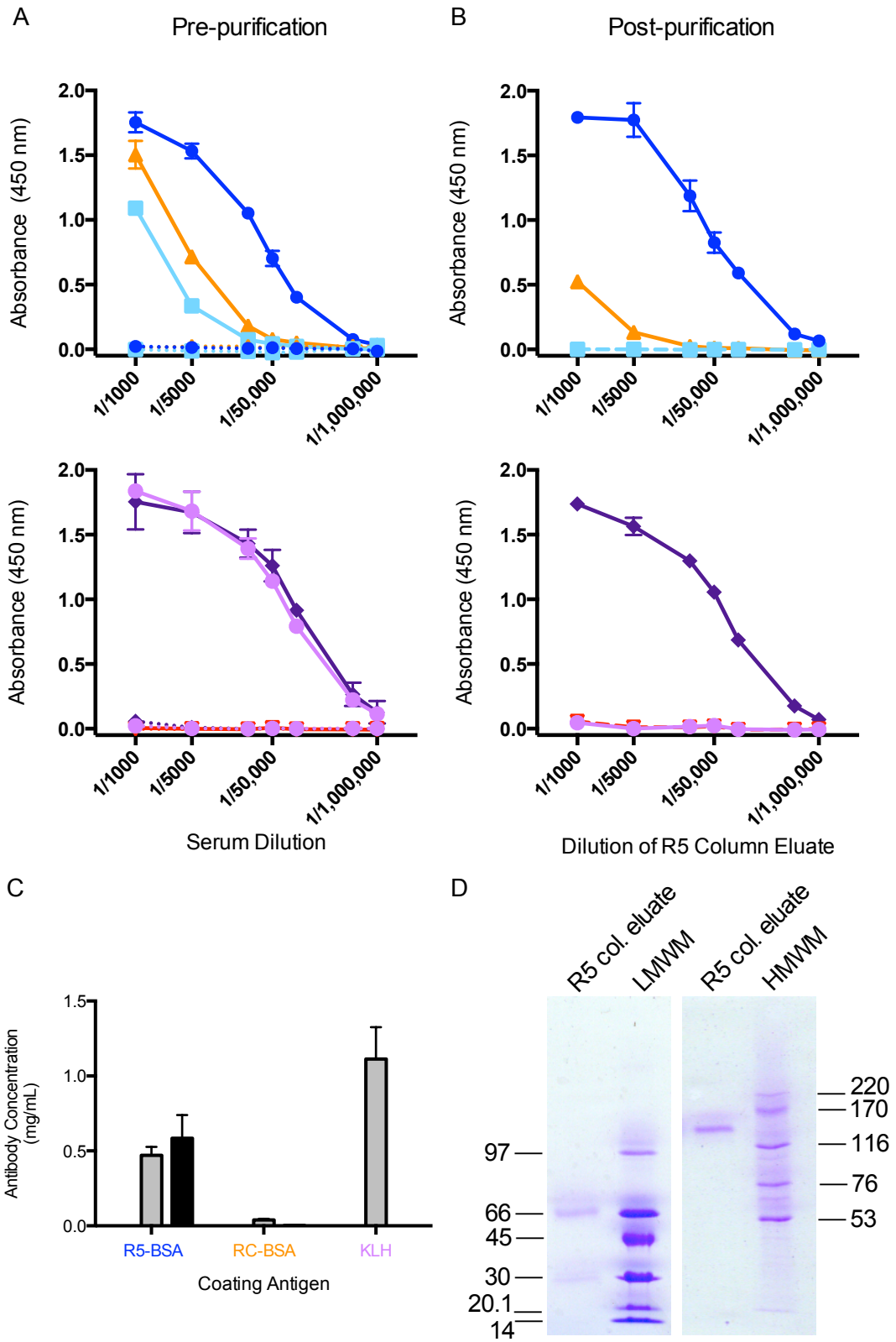

A
Pre-purification
B
Post-purification
Absorbance (450 nm)
2.0
1.5
1.0
0.5
0.0
1/1000
1/5000
1/50,000
1/1,000,000
Serum Dilution
Dilution of R5 Column Eluate
C
Antibody Concentration (mg/mL)
1.5
1.0
0.5
0.0
R5-BSA
RC-BSA
KLH
Coating Antigen
D
R5 col. eluate
LMWM
R5 col. eluate
HMWM
97
66
45
30
20.1
14
220
170
116
76
53

Figure 3.1. Purification of anti-R5 peptoid antibodies. (reprinted from pages 166-167) **A.** Rabbit 12D pre-immunization serum (*dotted lines*; Day -8 in Fig. 2.12, page 119) and post-immunization serum (*solid lines*; pooled from Days 356, 370, and 397) were analyzed by ELISA for the presence of serum antibodies. Plates were coated with 10 μg/mL of the following antigens: R5 peptoid conjugated to a carrier protein irrelevant to the original immunization (R5-BSA; ●); an irrelevant peptoid conjugated to BSA (RC-BSA; ▲); BSA alone (■); the immunogen, R5-KLH (◆); KLH alone (●); and an irrelevant protein, OVA (▼). Following blocking, dilutions of sera were applied and bound antibody was detected using an HRP-conjugated goat anti-rabbit IgG secondary antibody and the substrate TMB. The reaction was stopped with 2 M sulfuric acid and the absorbance at 450 nm was recorded for each well. The normalized absorbance averaged from triplicate wells is shown from one representative experiment of three performed. Error bars represent mean normalized absorbance ± standard deviation. **B.** To affinity purify antibodies against R5 peptoid, as described in Section 2.III.B.2 (page 85), rabbit 12D post-immunization serum was passed over a KLH-sepharose column to remove anti-KLH antibodies. The final flow-through from this column was then passed over an R5-SulfoLink column, which allowed the presentation of R5 peptoid on the column resin without the maleimide linker used to conjugate R5 peptoid to KLH for immunization. Anti-R5 peptoid antibodies were eluted from the column, dialyzed into PBS, then concentrated using ammonium sulfate precipitation and centrifugal concentrator devices. The concentrated R5 column eluate was analyzed by ELISA simultaneously with the sera in panel A. Data shown are from one representative experiment of three performed. Error bars represent mean normalized absorbance ± standard deviation. **C.** Quantification of anti-R5 peptoid, anti-linker, and anti-KLH antibody concentrations in pre-immunization serum (*white bars*) and post-immunization serum (*gray bars*) from panel A, and R5 column eluate (*black bars*) from panel B, using a standard curve included in those ELISAs as described in Section 2.III.B.4 (page 94). Since the R5-BSA conjugate used to determine the concentration of anti-R5 peptoid still contained the maleimide linker used to conjugate R5 to KLH for immunization, the antibody concentration against an irrelevant peptoid conjugated to BSA (RC-BSA) was calculated to indicate the concentration of anti-linker antibodies. Data shown represent the average of three experiments; error bars represent mean plus standard deviation. **D.** Concentrated R5 column eluate was analyzed by SDS-PAGE using a PhastSystem to assess the purity of anti-R5 antibodies. Under reducing conditions [left-hand panel, showing the low molecular weight marker (LMWM) in the second lane], heavy and light chains were visible in the R5 column eluate, whereas non-reducing conditions [right-hand panel, showing the high molecular weight marker (HMWM) in the second lane] suggested that the R5 column eluate contained intact IgG.

At this point, the Vitetta laboratory's recent experiments suggest that peptoid R5A and several other peptoids do not conjugate well to the maleimide-activated carrier proteins used in my dissertation research and in other portions of the peptoid vaccine project. These experiments also suggest that peptoid RC (Figs. 2.9 and 2.10, pages 115-117), the peptoid I

used in my dissertation research as an irrelevant control, may conjugate well to maleimide-activated carrier proteins, making it relatively unique among the admittedly small number of peptoid-maleimide-activated carrier protein conjugates tested thus far. To explain these findings, the current hypothesis is that the chemistry of conjugating *peptoids* to maleimide-activated carrier proteins may differ in as yet uncharacterized ways from that of conjugating *peptides* to maleimide-activated carrier proteins, for which the manufacturer's protocols are designed. We hypothesize that some of this difference in conjugation chemistry may be accounted for by peptoid primary, secondary, or tertiary structure, the study of which is only beginning. More specifically, if we apply this hypothesis to peptoids R5A and RC, the relatively simple structure of peptoid RC (Figs. 2.9 and 2.10) may allow more efficient conjugation than other, more structurally complex peptoids such as peptoid R5A.

If peptoid R5A indeed failed to conjugate well to maleimide-activated carrier proteins and/or peptoid RC conjugated with better efficiency, these findings would have several important consequences for my dissertation research:

1. The rabbits used to generate the anti-R5A peptoid antibodies may have been immunized with less R5-carrier protein conjugate than planned. This could help account for the low concentration of anti-R5A antibodies purified from the rabbit serum.
2. The rabbits may have been immunized with maleimide-activated carrier proteins that contained unconjugated maleimide linker (the structure of which can be seen in Fig. A.1A, page lvi), which itself may be immunogenic.
3. Purification of anti-R5A antibodies from immune rabbit serum was performed using a SulfoLink column (see Section 2.III.B.2, page 86 for the method used and Fig. 2.8D, page 113 for an abbreviated diagram of the R5A SulfoLink affinity

column structure based on the manufacturer's depiction). Despite structural differences between the maleimide linker used for immunization and the SulfoLink linker used for purification, this purification may not have purified anti-R5A antibodies away from all antibodies against the maleimide linker. As presented in Fig. 3.1 and concluded in my dissertation, the inclusion of peptoid RC-maleimide-activated carrier protein conjugates as an irrelevant peptoid control would seem to detect residual anti-linker antibodies in the product of anti-R5A antibody purification. However, if peptoid RC conjugates to maleimide-activated carrier proteins well, free maleimide sites may not have been available on these peptoid-carrier protein conjugates to detect residual anti-linker antibodies. Admittedly, the inclusion of maleimide-activated carrier proteins in my experiments as a control might have detected anti-maleimide linker antibodies, but these were not included based in part on my hypothesis (untested by me) that free maleimides might react with other ELISA reagents such as the secondary antibody and confound interpretation.

4. Any residual anti-maleimide linker antibodies in the product of anti-R5A antibody purification could potentially cross-react when applied to other conjugates of maleimide-activated carrier proteins if their conjugation is also inefficient.

Importantly, even if these recent findings prove true, several major conclusions of my dissertation remain intact, in that rabbit immunization with peptoid-carriers generated anti-peptoid antibodies, and that these antibodies could discriminate between a peptoid immunogen, R5A, and an irrelevant peptoid, RC, in on-bead assays where no conjugation was required. Again, while the efficiency of conjugation was discussed and explored

experimentally to some extent by me as well as others during the time my dissertation research was conducted (unpublished data), we were unable at that time to address the issue as thoroughly as we would have liked. The attempt to generate an affinity-purified antibody against a known peptoid, as described in my dissertation, was intended to give us a peptoid/antibody pair that could help us answer questions about conjugation as well as other questions arising in the work to develop peptoid-based vaccines and in other peptoid applications. If the anti-peptoid R5A antibody generated in my dissertation research does indeed cross-react with other conjugates of peptoids and maleimide-activated carrier proteins, this antibody unfortunately may not be as pure as I thought at the time when I wrote and defended my dissertation. However, this peptoid/antibody pair is still fulfilling its larger goal, allowing the Vitetta laboratory to conduct experiments previously not possible and bringing to light an issue that may have broad implications for peptoid vaccines and other peptoid applications. If conjugation of peptoids to maleimide-activated carrier proteins is indeed inefficient, methods for conjugating peptoids to carrier proteins for use in vaccines and other applications may need to be revisited. The amount of peptoid conjugated to carrier protein needed to elicit a protective immune response is presently unknown and may be variable; therefore, even inefficient conjugation may be enough to provide protection. In any case, further studies are needed and the peptoid/antibody pair described in my dissertation may continue to facilitate such studies, albeit, somewhat ironically, not in the manner originally intended.

On the whole, the question of peptoid conjugation efficiency is emblematic of many of the difficulties I encountered in the course of my dissertation research while working as part of the Vitetta lab peptoid vaccine team to adapt methods designed for peptides to be used with peptoids. Therefore, I look forward to assisting my colleagues on the question of

conjugation efficiency and other issues that emerge as they continue the peptoid vaccine development work, in the spirit of science being a process that presents us with constant challenges as we try to expand the frontiers of what is known and make a positive impact on human health.

Dr. Vitetta has agreed that these corrections and explanations, supplemented by the more detailed explanation below, should render this thesis acceptable for the awarding of my degree. My dissertation committee has also agreed.

Revisions to Methods

As described previously in this Addendum, the portion of this dissertation research that involved the immunization of rabbits with 5-mer peptoids conjugated to carrier proteins was confounded by two errors. In the first error, the peptoid sequence designated in this dissertation as R5 peptoid (Figs. 2.7 and 2.8, pages 110-114) was modified by intentionally interchanging the positions of two monomers, the aspartic acid-like monomer and the lysine-like monomer (shown in green in Fig. A.1, page lvi) after the first immunization (Fig. 2.12, page 119, revised as Fig. A.2, page lvii), due to concerns over difficulty with synthesis. However, miscommunication resulted in designating both versions as “R5 peptoid,” and I synthesized what I now refer to as R5A for the final rabbit immunizations (Day 326 in Fig. A.2), rather than the modified version, R5B. Additionally, we had planned to immunize rabbits with R5-KLH and detect anti-R5 peptoid antibodies using plated R5-BSA. A second error occurred when the rabbits were immunized with peptoid conjugated to BSA as suggested by experiments performed by Dr. Pop and noted in Fig. A.2, the revised version of Fig. 2.12.

The implications of these errors for the Methods section of my dissertation include the following:

1. Section 2.1.C (pages 53-63), describing the preparation of single peptoids and peptides, can be taken to describe the synthesis of R5A.
2. Conjugation of peptoids to maleimide-activated carrier proteins, described in Section 2.1.D (page 63), may have been inefficient as suggested by recent experiments in the Vitetta lab. Therefore, the rabbits may have been immunized with less peptoid than intended and with unreacted (free) maleimide groups on carrier proteins. Since the same conjugation method was used to generate peptoid-carrier protein conjugates for ELISAs (for example, those described in Section 2.III.A.4, page 82 and Section 2.III.B.4, page 94), this inefficiency may have impacted ELISA results as well.
3. After the final immunization with R5A but prior to the exsanguination of the rabbits (Fig. 2.12, page 119 and Fig. A.2, page lvii), Dr. Case and I tested the rabbit sera by ELISA to determine whether adequate amounts of anti-R5 peptoid antibody were being made, as described in Section 2.III.A.4 (page 82). These ELISA plates, prepared by Dr. Case, were coated with conjugates of R5B rather than R5A. Although I hypothesize that some antibodies against R5A likely cross-react with R5B, this complicates any quantitative interpretation of anti-R5 antibody titers from those experiments. Quantitation is additionally complicated by the second error, immunization of the rabbits with R5B-BSA (Fig. A.2, page lvii), when we had planned to immunize only with R5-KLH and to detect anti-R5 peptoid antibodies using plated R5-BSA.

4. Boosting the rabbits with R5A-KLH on Day 326 likely produced lower concentrations of anti-R5B antibodies in the final rabbit sera than if we had boosted with conjugates of R5B.
5. By the time we performed the ELISAs described in Section 2.III.A.6 (page 84) to assess antibody concentrations in rabbit sera pooled from the bleeds collected after the final immunizations, we had begun preparing ELISA plates using R5A. We also used R5A for all subsequent steps in the affinity purification of the polyclonal antibody (PAb) rabbit anti-R5 peptoid (RAR5), as well as in experiments using this antibody to optimize the magnetic screening assay for on-bead peptoids. Therefore, Methods sections 2.III.A.6 through 2.V (page 84 through the end of the Methods chapter) are unaffected by these two errors, except to note that RAR5 is an antibody purified using peptoid R5A (therefore it can be designated "RAR5A") and that on-bead R5A was used in the optimization experiments.

Since these errors were brought to light in part by experiments performed by Dr. Pop as shown in Fig. A.4 (page lix), I provide a summary of the methods he used here.

Conjugation of peptides and peptoids to carrier proteins via the *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) crosslinker

Conjugation was performed according to the manufacturer's instructions (360). As an example, the conjugation of peptoid R5A to SPDP-modified BSA (SPDP-BSA) is shown in Fig. A.3 (page lviii). Briefly, the SPDP reagent (#21857, Pierce Biotechnology, Rockford, IL) was equilibrated to room temperature (RT). Immediately before use, it was dissolved in 320 microliters (μL) dimethylsulfoxide (DMSO) to give a 20 millimolar (mM) solution. Twenty-five

microliters of this SPDP solution were then added to 3 milligrams (mg) BSA (#A7030, Sigma-Aldrich, St. Louis, MO) that had been dissolved in 1 milliliter (mL) of phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA) [100 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA (#S311-500, Fisher Scientific), 0.02% (weight/volume) (w/v) sodium azide, pH 7.5]. This mixture was then incubated for 30 minutes (min) at RT. To remove undesired contaminants such as unreacted SPDP reagent from the desired SPDP-BSA, a Zeba spin desalting column (Pierce) was then equilibrated with 20 mL PBS-EDTA prior to the addition of the SPDP-BSA solution. Following desalting, the level of SPDP modification was then assessed by detecting the level of free pyridine-2-thione released from the modified protein by reducing the disulfide bond (Fig. A.3B). First, 100 μL of the desalted SPDP-BSA were diluted to 1 mL using PBS. The absorbance at 343 nanometers (nm) in this sample was then measured using an ultraviolet (UV) spectrophotometer (DU 730, Beckman Coulter, Brea, CA) and compared to that of triplicate samples of PBS-EDTA alone. To reduce the disulfide bond and release pyridine-2-thione, 10 μL of 15 mg/mL dithiothreitol (DTT; #D0632, Sigma-Aldrich) were then added to the 1 mL SPDP-BSA and mixed. After 15 min, the absorbance at 343 nm was again measured. The change in absorbance (ΔA) was then calculated between the non-reduced (pre-DTT addition) and reduced (post-DTT addition) samples. The molar ratio of SPDP to BSA was then calculated using the following formula: (ΔA/8080) x molecular weight of BSA/concentration of BSA in mg/mL), where 8080 is the extinction coefficient for pyridine-2-thione at 343 nm [8.08×10^3 $Molar^{-1}centimeters^{-1}$ ($M^{-1}cm^{-1}$)] and 6.6×10^7 milligrams/mole (mg/mol) is the molecular weight of BSA.

To conjugate the desired peptide [$FLAG^+$-cysteine, prepared by Dr. Haydn Ball at the Protein Chemistry Technology Core at the University of Texas Southwestern Medical Center

(UTSW)] or peptoid [R5A-cysteine, synthesized by Ms. Kelly Dye and Ms. Kelly Mapes and purified by reverse phase high performance liquid chromatography (RP-HPLC) by Ms. Mapes] to SPDP-BSA (Fig. A.3C), 2 mg peptide or peptoid (~27-30 moles peptide or peptoid excess per mole of BSA) were added to the SPDP-BSA solution. The reaction was incubated overnight at RT and the resulting conjugates were then stored at 4 degrees Celsius (°C) until further use.

ELISAs to detect rabbit serum antibody levels against KLH, the peptoid R5A, and BSA

Ninety-six-well plates (#9018, Corning, Corning, NY) were coated with 10-15 micrograms/milliliter (μg/mL) solutions of the following proteins or conjugates in PBS at 50 μL per well: KLH (#H7017, Sigma-Aldrich); R5A-SPDP-BSA; $FLAG^{+}$-SPDP-BSA; or BSA alone (#A8022, Sigma-Aldrich). Following coating for 2 hours (h) at RT or overnight at 4°C, plates were washed four times by removing the contents and adding 250 μL PBS to each well. After the final wash, the contents were again removed and the plates were dried by blotting the plates on paper towels. The plates were then blocked with 150 μL Starting Block (#37538, Pierce) per well for 1 h at RT or overnight at 4°C, followed by washing as previously. Sera to be used as the primary antibody were then thawed and centrifuged at 14,000 times gravity (x *g*) for 10 min at 4°C to pellet immune complexes. The supernatant was transferred to a new tube, from which dilutions of sera in Dilution Buffer [1% Starting Block in 0.01% (volume/volume; v/v) Tween 20 in PBS (PBST)] were then prepared (1/50, 1/100, 1/500, 1/1000, 1/5000, 1/10,000, 1/50,000, and 0). Standard curves using affinity-purified PAb rabbit anti-KLH prepared in-house, RAR5A, and mouse monoclonal antibody (MAb) anti-FLAG (#F3165, Sigma-Aldrich) were also prepared in Dilution Buffer (0.5, 0.1,

0.05, 0.01, 0.005, 0.001, 0.0005, and 0 μg/mL). These primary antibodies were added to duplicate wells on the appropriate plates at 50 μL per well and incubated for 1 h at RT with gentle rocking. The plates were then washed four times with PBST and dried as described above. Secondary antibody solutions of horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (#111-035-144, Jackson ImmunoResearch, West Grove, PA) and HRP-conjugated goat anti-mouse IgG (#115-035-164, Jackson ImmunoResearch) were then prepared at 1:20,000 and 1:10,000, respectively, in Dilution Buffer and 50 μL per well were applied to the appropriate wells on each plate. The secondary antibodies were incubated for 1 h at RT with gentle rocking. Meanwhile, 3,3',5,5'-tetramethylbenzidine (TMB; #34028, Pierce) was equilibrated to RT. Following washing as described after the primary antibody incubation, TMB was applied to the plates at 50 μL per well and the color change was observed for approximately 6-8 min before the reaction was stopped with a solution of 2 M sulfuric acid in ultrapure water. The absorbance of each well when light at a wavelength of 450 nm was applied was immediately quantified using a microplate reader (THERMOmax, Molecular Devices, Sunnyvale, CA) with SOFTMax software (version #2.32, Molecular Devices). Background absorbance when the primary antibody solution at 0 μg/mL was applied to the plate was subtracted from the absorbance for each well, the normalized absorbance from duplicate wells was averaged, and the results were displayed as average normalized absorbance versus log dilution or concentration using GraphPad Prism (Version 6.0a for OS X, GraphPad Software Inc., La Jolla, CA). Error bars representing the standard deviation of the normalized absorbance were calculated using Prism.

Revisions to Results

The previously described errors in the composition of the immunogens administered to the rabbits complicate the interpretation of the results presented in Section 3.IV (page 138) and shown in Fig. 3.1 (page 166-167, reprinted on page xxxix-xl) as follows:

1. In the ELISAs performed to generate Fig. 3.1A, immune rabbit sera prior to affinity purification were titrated onto plated R5A-BSA, BSA, RC-BSA, KLH, R5A-KLH, and ovalbumin (OVA). At the time, we thought that BSA was a carrier protein that was irrelevant to the immunizations. However, in light of the results from experiments by Dr. Pop (shown in Fig. A.4, page lix and described further below), BSA was not an irrelevant carrier protein. Therefore, in Fig. 3.1A, the signal from serum titrated onto plated BSA alone can be interpreted as the detection of anti-BSA antibodies. Due to the inclusion of the irrelevant control peptoid RC in this experiment, the difference in signal between serum titrated onto plated RC-BSA versus R5A-BSA may be interpreted as the detection of anti-R5A antibodies. Similarly, the difference between the signal from serum titrated onto plated BSA alone versus RC-BSA may be interpreted as the detection of anti-linker antibodies. However, both of these observations may be complicated by the possible limitations in peptoid conjugation efficiency described above. The difference in signal between serum titrated onto plated RC-BSA versus R5A-BSA and onto BSA versus RC-BSA may also include anti-maleimide linker antibodies. However, given that affinity-purified anti-R5A peptoid antibodies could discriminate between peptoid R5A and peptoid RC in on-bead assays where no conjugation was required, it is likely that anti-R5A antibodies were elicited during immunization and detected in this assay.

2. The peptoid R5A was used for affinity purification of anti-R5 antibodies, and the ELISA plates in Fig. 3.1 were coated with R5A conjugates. With this consistency in using R5A rather than R5B, the experiments in which post-purification rabbit serum was analyzed were poised to detect anti-R5A antibodies. Signal from titrated rabbit serum plated onto R5A-BSA remained, while signal from the same serum titrated onto BSA alone and KLH alone was no longer present, suggesting the anti-R5A antibodies were successfully purified away from anti-carrier antibodies. Additionally, the signal from titrated rabbit serum plated onto RC-BSA was no longer present, suggesting the anti-R5A antibodies were successfully purified away from anti-linker and anti-BSA antibodies. However, this interpretation could be complicated by limitations of peptoid conjugation efficiency. The signal from serum plated onto R5-BSA could include anti-maleimide linker antibodies as well as anti-R5A antibodies if conjugation of peptoid R5 to BSA was inefficient and free maleimide groups were present on conjugates of R5A for both immunization and in this experiment, while conjugation of peptoid RC to BSA for this experiment proceeded efficiently and did not leave any free maleimide groups.
3. Since the affinity column used for purification of anti-5-mer peptoid antibodies was made with R5A peptoid, and given that affinity-purified anti-R5A peptoid antibodies could discriminate between peptoid R5A and peptoid RC in on-bead assays where no conjugation was required, what was designated PAb rabbit anti-R5 peptoid or RAR5 in this dissertation is more accurately designated PAb rabbit anti-R5A peptoid or RAR5A. However, based on recent experiments suggesting

problems with peptoid conjugation efficiency, RAR5A may still contain anti-maleimide linker antibodies.

4. The peptoid/antibody pair, R5A peptoid and RAR5A, was used in subsequent experiments optimizing on-bead magnetic screening for peptoid libraries, the findings from which are described in Section 3.IV.C (page 142) and shown in Tables 3.16-3.18 (pages 168-170). The interpretation of those results should not be impacted by the errors in rabbit immunization, highlighting the utility of screening for candidate peptoids using an antibody that has been affinity purified, rather than using serum. Despite any concerns that RAR5A may still contain anti-maleimide linker antibodies, these on-bead experiments, which do not rely on conjugation, demonstrate that RAR5A contains antibodies that can discriminate between R5A and RC. However, if RAR5A does contain anti-linker antibodies, these may have contributed to the isolation of on-bead peptoids from Peptoid Library 1 in addition to on-bead R5A in the magnetic screening experiments described in Section 2.IV.B (page 97), the results of which are described in Section 3.IV.C (page 142) and shown in Table 3.17 (page 169).

The results of the experiments conducted by Dr. Pop to identify the errors in the rabbit immunization can now be described. Sera from rabbit 12D (the same rabbit from whose sera RAR5A had been purified) were analyzed by ELISA to detect antibodies against plated KLH, R5A-SPDP-BSA (which used a different linker for conjugation than the maleimide-based linker used for immunization), BSA alone, and $FLAG^{+}$-SPDP-BSA, (included as an irrelevant control for R5A-SPDP-BSA and because we had no anti-BSA antibody to include as a standard curve on the BSA only plates, but did have MAb anti-

FLAG). The results of these experiments are shown in Fig. A.4 (page lix). As expected from the results shown in Fig. 3.1 (page 166, reprinted on page xxxix), pre-immunization serum from Day -8 (Fig. 2.12, page 119 and Fig. A.2, page lvii) did not appear to contain antibodies against any of the coating proteins tested (Fig. A.4A-D, right- and left-hand panels). Again as expected, anti-KLH (Fig. A.4A) and anti-R5A (Fig. A.4B) antibodies were detected in serum from Day 13 (following the first immunization) and in sera from all subsequent days tested. However, beginning with post-immunization 3 serum from Day 155, anti-FLAG^{+}-SPDP-BSA (Fig. A.4C) and anti-BSA (Fig. A.4D) antibodies were detected, strongly suggesting that the BSA conjugate of the 5-mer peptoid was used to immunize the rabbits on Day 83 (Fig. 2.12 and Fig. A.2). Re-examination of records indicated that the same batch of 5-mer peptoid conjugate that was used for immunization on Day 83 was also used for immunization on Day 155, suggesting the BSA conjugate was also used for the fourth immunization of rabbit 12D (Fig. 2.12 and Fig. A.2). Therefore, BSA was not a carrier protein irrelevant to the immunogens used, and the experiments in which it was used as an irrelevant carrier were confounded as described above.

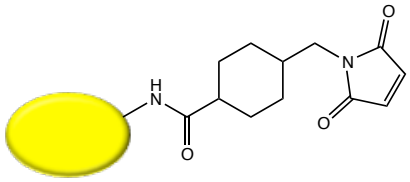
Discussion

This dissertation describes several phases of work in carried out in the early stages of the development of peptoid-based vaccines. Once our peptoid team had optimized magnetic and color screening assays using the FLAG peptide system in the first phase of the project, I began screening Peptoid Library 1 with neutralizing MAbs against West Nile virus (WNV) and murine norovirus-1 (MNV-1) as part of the second phase. The results and implications of that work, in which potential vaccine candidates were identified for MNV-1, remain unaffected by the errors in rabbit immunization that more clearly impacted the

detailed interpretation, but not the major conclusions, of the rabbit peptoid immunogenicity studies. The goal of that portion of my dissertation research was to extend previous studies of peptoid immunogenicity suggesting that peptoids are haptens. The results of this work still show that immunization with 5-mer peptoids conjugated to carrier proteins and adsorbed to alum elicited antibodies specific for at least one of the immunizing peptoids (R5A), and that anti-carrier and anti-linker antibodies were also elicited. The anti-R5A antibodies were purified away from anti-carrier antibodies, and experiments conducted during my dissertation research suggested that the anti-R5A antibodies were purified away from anti-linker antibodies as well. However, experiments by the Vitetta laboratory conducted after I wrote and defended my dissertation suggested that inefficient conjugation of some peptoids, including R5A but not RC, may have allowed unreacted maleimide groups to persist through rabbit immunization and associated ELISAs, complicating the detection of anti-maleimide linker antibodies in the ELISAs presented in my dissertation. Since the RAR5A antibody and peptoid R5A were integral to the experiments conducted to identify this potential inefficiency in conjugation, this peptoid/antibody pair has still proven useful in furthering the development of peptoid vaccines, as was a goal of my dissertation research. Additionally, RAR5A was able to discriminate between peptoid R5A and peptoid RC in on-bead assays in which no conjugation was used, highlighting the utility of using screening methods to identify peptoid vaccine candidates that eliminate potential confounding linker epitopes used to generate screening antibodies. Furthermore, the R5A/RAR5A peptoid/antibody pair could still discriminate between R5A and a library of on-bead 5-mer peptoids composed of some of the same monomers used in R5A, allowing optimization of on-bead magnetic screening assays beyond what had been possible prior to the generation of this peptoid/antibody pair. Thus, in the process of generating RAR5A and using it in subsequent experiments, we have

continued to make discoveries that may lead to peptoid vaccines, albeit not strictly in the anticipated manner, and R5A and RAR5A may continue to be useful in this regard.

A



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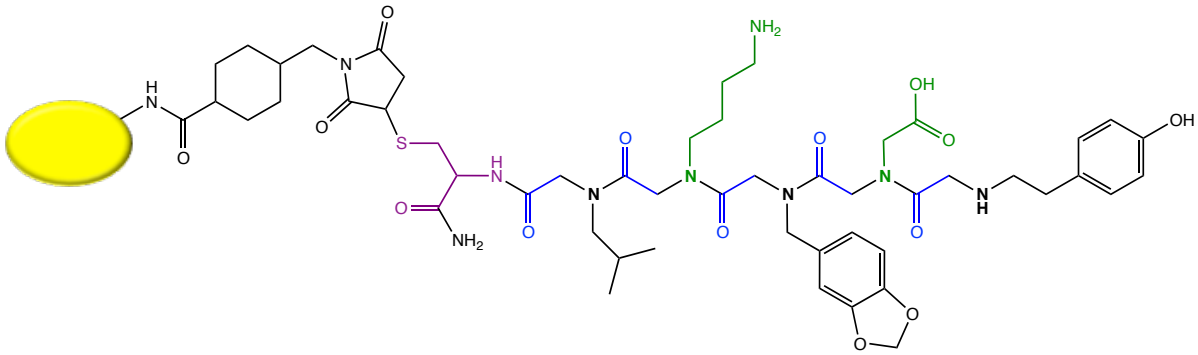


C

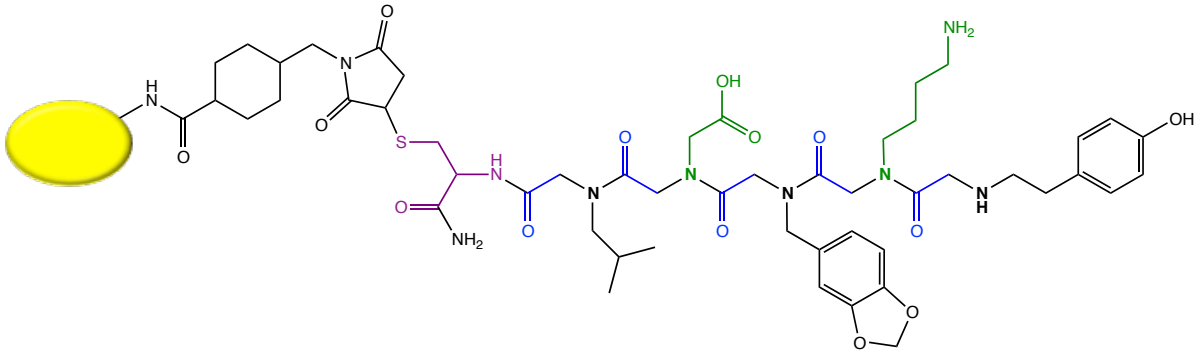


Figure A.1. Two versions of R5 peptoid (R5A and R5B) conjugated to carrier proteins for rabbit immunization (compare with Figs. 2.7 and 2.8, pages 110-114).
A. Unconjugated maleimide-activated carrier protein, with the carrier protein represented by the yellow oval. **B.** R5A conjugated to a maleimide-activated carrier protein. R5A was incubated with a maleimide-activated carrier protein (KLH) before passage over a desalting column to remove contaminants (Section 2.I.D.2, page 63). The side chains of the peptoid residues that were interchanged to generate peptoid R5B are shown in green. **C.** R5B conjugated to a maleimide-activated carrier protein (KLH or BSA).

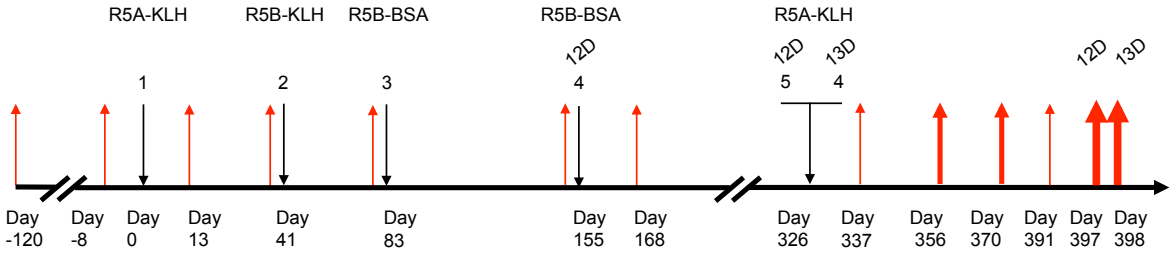


Figure A.2 (revised Fig. 2.12, page 119). Immunization of rabbits with R5 peptoids conjugated to carrier proteins and adsorbed to alum to produce affinity-purified rabbit PAb anti-R5A peptoid.

A timeline of rabbit bleeds and immunizations with R5 peptoids conjugated to carrier proteins and adsorbed to alum is shown. Two rabbits, designated 12D and 13D, were bled (red arrows) on Day -120 and Day -8 prior to receiving any immunizations. Sera from these bleeds, prepared by allowing the blood to clot overnight at 4°C followed by centrifugation and removal of the supernatant as the serum component, were designated pre-immunization sera. The rabbits were then immunized on the days shown (black arrows) with 250-500 μg of the indicated peptoid-carrier adsorbed to alum. Based on experiments conducted by Dr. Pop after my defense of this dissertation, the 5-mer peptoid BSA conjugate was inadvertently used by my colleague for immunization on Day 83, rather than the KLH conjugate. Records also indicated that the same batch of peptoid R5 conjugate was used for immunization on Day 155. Further re-examination of records indicated that R5A was used on Days 0 and 326, while R5B was used on Days 41, 83, and 155. Rabbit 12D received a total of five immunizations (as indicated above the black arrows), while rabbit 13D received four. Throughout the process of immunization, test bleeds (thinnest red arrows) of approximately 5 mL were collected (Days 13, 41, 83, 155, 168, and 337). Sera prepared from later test bleeds were used in ELISAs that attempted to monitor the anti-R5 peptoid and anti-KLH antibody concentrations. However, the quantification from these experiments, performed to determine when the rabbits had produced adequate antibody to warrant exsanguination, was confounded by the errors in peptoid immunogen composition. Nevertheless, two production bleeds of approximately 30 mL (Days 356 and 370, thicker red arrows), one additional test bleed on Day 391, and exsanguination bleeds (thickest red arrows) on Day 397 (rabbit 12D) and 398 (rabbit 13D) were collected. Sera from the two production bleeds and exsanguination were prepared, pooled for each rabbit, and designated post-immunization sera. Approximately 60 mL of post-immunization sera were obtained for rabbit 12D and 40 mL for rabbit 13D.

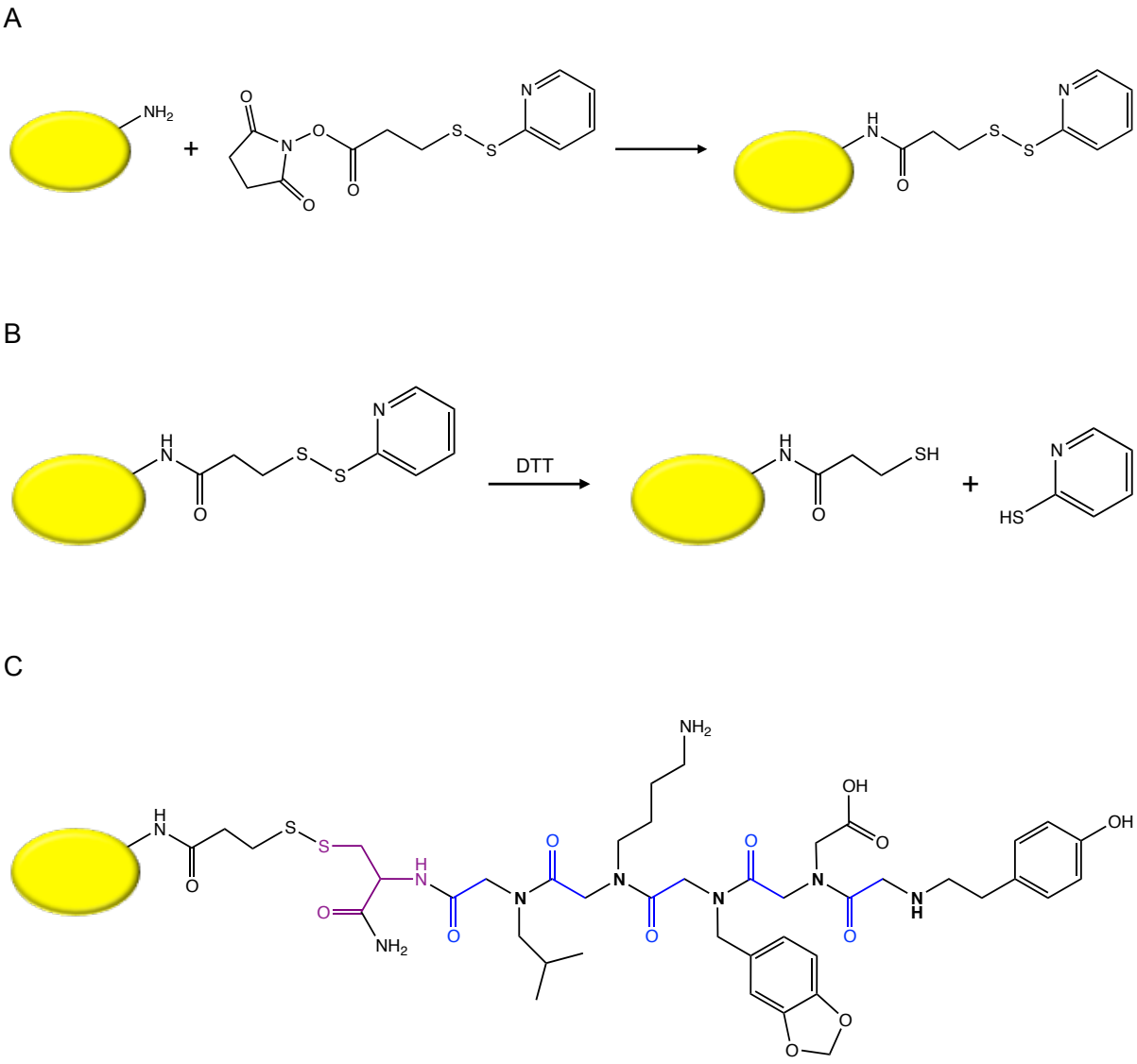


Figure A.3. Conjugation of peptides and peptoids to carrier proteins via the SPDP crosslinker.
A. The carrier protein BSA, represented by the yellow oval, was modified by SPDP at free amines to give SPDP-BSA. **B.** The reducing agent DTT was used to reduce the disulfide bond in SPDP-BSA, releasing pyridine-2-thione. Quantification of this reaction using UV spectrophotometry allowed quantification of the level of BSA modification with SPDP. **C.** Addition of a peptide or peptoid containing a free sulfhydryl (in the side chain of a cysteine residue, synthesized as described in the Methods section of this addendum) to SPDP-BSA produced the desired conjugate. Here, peptoid R5A conjugated to SPDP-BSA (R5A-SPDP-BSA) is shown as an example. While only one copy of the peptoid is shown conjugated to BSA, multivalent conjugation likely occurs.

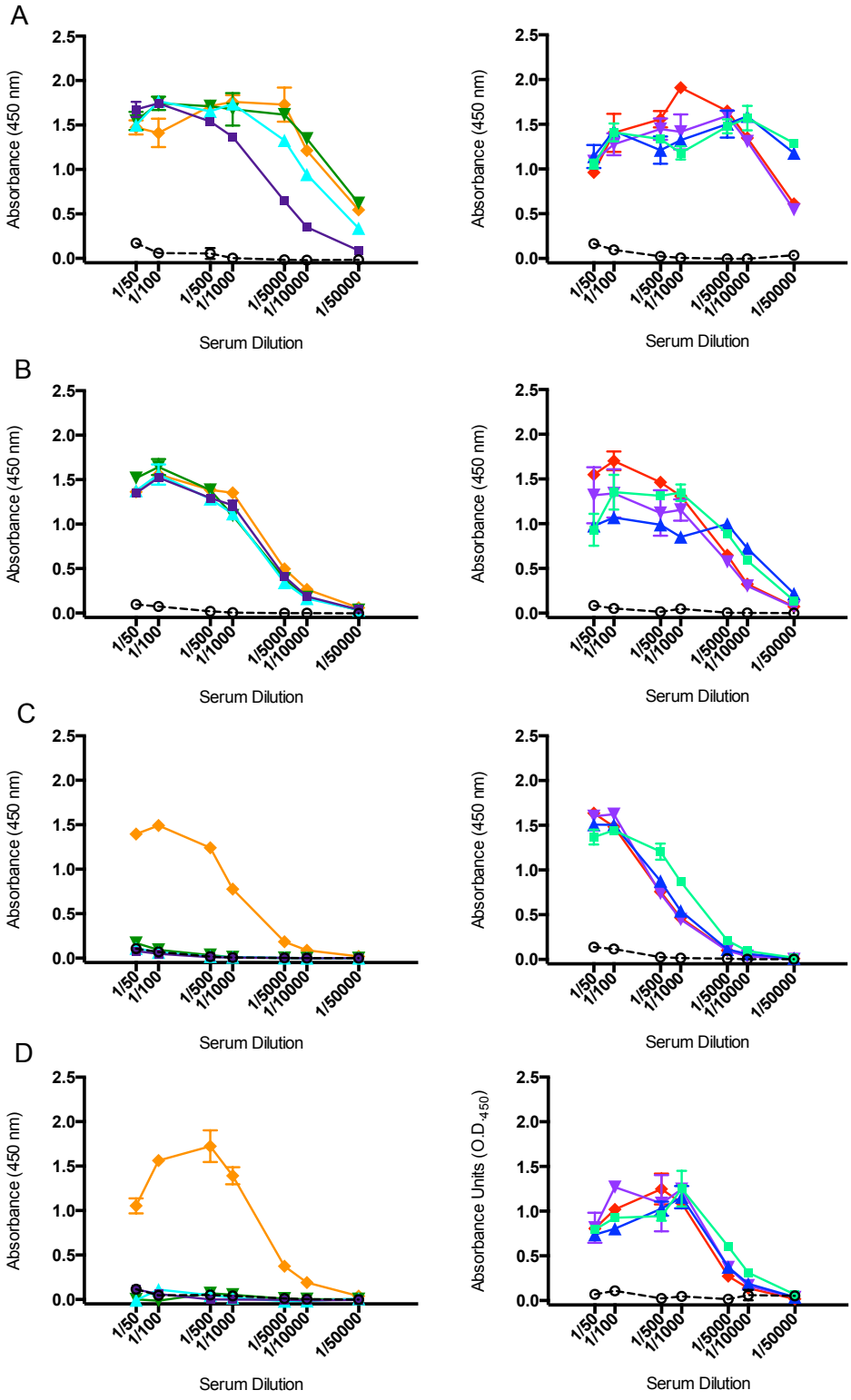

A
B
C
D
Absorbance (450 nm)
Absorbance Units (O.D.450)
2.5
2.0
1.5
1.0
0.5
0.0
1/50
1/100
1/500
1/1000
1/5000
1/10000
1/50000
Serum Dilution

Figure A.4. ELISAs used to detect serum levels of rabbit antibodies against KLH, the peptoid R5A, and BSA, indicating that the BSA conjugate of the 5-mer peptoid was used to immunize the rabbits on Day 83.
Rabbit 12D pre-immunization serum (*black dotted lines*; Day -8 in Figs. 2.12, page 119 and A.2, page lvii) and post-immunization sera (*solid lines*) were analyzed by ELISA for the presence of serum antibodies. Plates were coated with 10-15 μg/mL of **A.** KLH, **B.** R5-SPDP-BSA, **C.** $FLAG^{+}$-SPDP-BSA, or **D.** BSA alone. Following blocking, dilutions of post-immunization 1 serum from Day 13 (■); post-immunization 1 serum from Day 41 (▲), post-immunization 2 serum from Day 83 (▼), post-immunization 3 serum from Day 155 (◆), post-immunization 4 serum from Day 168 (■), post-immunization 4 serum from Day 337 (▲), post-immunization 5 pooled serum from Days 356, 370, and 397 as used in Fig. 3.1A (page 166, reprinted on page xxxix) (▼), and post-immunization 5 serum from exsanguination only on Day 397 (◆) were applied and bound antibody was detected using an HRP-conjugated goat anti-rabbit IgG secondary antibody and the substrate TMB. The reaction was stopped with 2 M sulfuric acid and the absorbance at 450 nm was recorded for each well. The normalized absorbance averaged from duplicate wells is shown. Error bars represent mean normalized absorbance ± standard deviation.

Additional Reference

360. SPDP crosslinking. Rockford, IL: Pierce Biotechnology. p. 1-4.

CHAPTER ONE

Introduction

1.I. Perspective

The success of vaccination as a public health intervention is well recognized, and evident in my own life. To date I have never encountered anyone with the measles, and routine smallpox vaccination ended in the United States almost a decade before I was born (see Table 1.1, page 29). In contrast, my mentor, who is only 1.5 generations older than I am, contracted measles, mumps, and more, and received the smallpox vaccination. Fortunately, she was one of the 400,000 children enrolled in the clinical trial of the Salk polio vaccine (something she considers to be her major achievement in immunology), and she escaped polio. In comparing our two generations, it becomes apparent why lack of personal experience with vaccine-preventable diseases might contribute to misconceptions about their potential severity, and lead to resistance toward vaccination in some segments of the population (1). Listening to someone who lived through these diseases and comparing this to the current public questioning about the need for vaccination is a reminder to me that if I want to develop prophylactic vaccines, and therefore propose this critical medical intervention to healthy people, then I have a responsibility to ensure that vaccines are as safe as possible and founded on sound principles in immunology. I also have the responsibility to convey scientifically founded risks to the public. On a small scale, my work in this area has already begun. When family and friends outside of science and medicine learned of the topic of my dissertation research, they often followed up with questions about the vaccines that they or someone close to them might receive. Meanwhile, major news stories during the time in which I have worked on this dissertation project, such as the H1N1

swine flu (2) and the results of the RV144 Thai human immunodeficiency virus (HIV) vaccine trial (3) in 2009, the upswing in cases of West Nile virus (WNV) in Dallas (4) in 2012 (which brought an influx of new members to the support group I attended in conjunction with this dissertation project), and most recently, the emergence of the Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV) (5), highlighted the need for technologies that can produce vaccines for emerging, resurging, and established pathogens. Thus, the long-term goal of this dissertation research, to develop new vaccines, is one that excited me as a future physician-scientist interested in immunology and public health. However, it also made me aware that once vaccinations eliminate a disease, other issues concerning the use of these vaccines for future generations can come into play.

The method that the Vitetta laboratory proposed to develop these new vaccines was based on a very simple concept in immunology: the hapten-carrier effect. It was hypothesized that if protease-insensitive mimetics of protective B cell epitopes on pathogens (haptens) could be identified and attached to proteolytically susceptible carrier proteins, then these hapten-carrier conjugates would elicit robust T cell-dependent immunoglobulin G (IgG) antibody responses against the mimetic hapten, and these antibodies would cross-react with the critical native epitope on the pathogen. In order to identify these mimetic B cell epitopes, members of the Vitetta lab would screen large libraries of synthetic compounds with monoclonal antibodies (MAbs) that were already available and known to be protective. To accomplish these goals, a team of immunologists, virologists, and chemists who would work together was needed, and when I joined the Vitetta lab as a graduate student in the fall of 2008, I began my training to become an immunologist as a part of this team. Once the team was assembled, the technology had to be developed, tested, and validated. This dissertation focused on that mission. In this introduction, I will call attention to some general

concepts from the fields of immunology, virology, and chemistry that are relevant to this work, and devote more focused sections to points at which these fields intersect. I will address the attributes of currently approved vaccines, what is presently understood about the requirements for protection, and lastly, the rationale behind the choice of exploring peptoids as our platform for making these entirely new mimetic vaccines. Should this platform work, it would be applicable to the generation of new vaccines for a wide variety of pathogens, toxins, and even cancers.

1.II. The attributes of current vaccines demonstrate the requirements for a good immunogen and illustrate the opportunity to improve vaccine development strategies

Vaccination has been named one of the ten greatest public health achievements of the 20th century in the United States (6), and of the first decade of the 21st century in the both the United States and the world (7, 8), because of its ability to prevent devastating diseases in large populations at a relatively low financial cost. Table 1.1 (page 29) shows the diseases preventable using vaccines currently licensed in the United States, the year of discovery of the first vaccine or licensure, the types of vaccines currently licensed, and some notes about the indications for administering these vaccines. For 2001, it was estimated that seven of these childhood vaccines (the combined diphtheria and tetanus toxoids and acellular pertussis, the combined tetanus and diphtheria toxoids, the Hib conjugate, the inactivated poliovirus, the combined measles, mumps, and rubella, the hepatitis B, and the varicella vaccines) prevented 14.3 million cases of disease and 33,564 deaths from a hypothetical birth cohort of about 4 million United States children (9). In addition to these health benefits, this was estimated to save $9.9 billion in direct costs (those associated with the care of the patients who would have contracted these diseases)

and $43.4 billion in societal costs (*e.g.*, lost productivity) (9). One decade later, a revised estimate was produced that included 20 million cases of disease and 42,000 deaths prevented, while approximately $14 billion in direct costs and $69 billion in societal costs were saved (7). Furthermore, vaccination for smallpox was so successful that it was declared eradicated by the World Health Organization in 1980 (10), and it is hoped that polio will become the second eradicated pathogen (11). Of course, eradication may not be possible for all pathogens, particularly those such as WNV, an early focus of my dissertation research, that have a reservoir outside of humans. Nevertheless, it is an inspiring goal, in large part because it suggests that vaccination was applied successfully in the developing world, which, nonwithstanding a rise in the proportion of total disease burden from noncommunicable diseases, still bears a disproportionate burden from communicable diseases (12).

Despite all that these vaccines have achieved, there are many diseases for which no viable prevention and/or treatment options have yet been discovered, or for which the current prevention or treatment is less than ideal (for example, in its schedule, side effects, lack of efficacy, or cost). Examples of some pathogens that cause such disease are shown in Table 1.3 (page 32), and two of these will be discussed in more detail shortly. However, even the currently approved vaccines, especially when considering administration to certain populations, have particular drawbacks that highlight areas in which new vaccination strategies would be beneficial. As shown in Table 1.1 (page 29), the currently approved vaccines fall into several categories: live attenuated; inactivated; and subunit, or portions of pathogens derived from the pathogen itself or made in the laboratory, including recombinant proteins, polysaccharides, and conjugates of polysaccharide B cell epitopes to larger proteins, which can provide T cell epitopes. The advantages and disadvantages of each are

outlined in Table 1.2 (page 30). Live, attenuated vaccines, in which a pathogen is weakened in some way, such as serial passage through human and non-human cells to acquire mutations [*e.g.*, the varicella vaccine (13)], or in which a less virulent but related pathogen is used (*e.g.*, the smallpox and BCG vaccines), are infectious, and thus have the advantage of eliciting a robust immune response. Since these live pathogens may be transmitted from person to person, even vaccination of one person could make a larger contribution to herd immunity, in which a large vaccinated population prevents the spread of a pathogen to the unvaccinated individuals. However, the infectious nature of these vaccines is also a contraindication for those in which vaccination would be most desirable; for example, the measles, mumps, and rubella vaccine should not be given to patients with severe immunodeficiency (14). To avoid these contraindications, as well as the risk of reversion to virulence from such vaccines, pathogens such as hepatitis A virus have been inactivated using, for example, formalin (15). However, this move toward safety comes with the cost of reduced immunogenicity, and adjuvants and booster immunizations are often required (15). Finally, vaccines composed of pathogen subunits, including recombinant proteins, polysaccharides alone, or polysaccharides conjugated to proteins, suffer from a lack of immunogenicity like inactivated vaccines, but provide both an advantage and another disadvantage essential to the goals of this dissertation research. Unlike the live, attenuated and the inactivated vaccines, which are composed of whole pathogens, these vaccines begin to narrow the scope of the pathogen components presented to the immune system, which may focus the immune response toward protective epitopes. However, a barrier to developing conjugate, protein, or polysaccharide vaccines is that the epitope(s) that elicit a protective immune response must be identified prior to making the vaccine. The same is true for two types of vaccines that have been tested but not yet been approved for human

clinical use, but are included optimistically in Table 1.2, synthetic peptide vaccines and vaccines composed of deoxyribonucleic acid (DNA) that encodes pathogen proteins of interest.

Regardless of the advantages and disadvantages of each of these types of vaccines, for nearly all currently approved prophylactic vaccines, the development of an antibody response correlates with protection (16). This point is critical for understanding the rationale that underpins the attempt to develop new vaccines in the Vitetta laboratory, which is explored further in the next sections. Although we hope to use this platform for novel vaccine development to overcome some of the shortcomings presented here for the currently approved vaccines, eliciting protective antibodies is still the goal.

1.III. Studies of the immune response to pathogens for which no vaccine is available provide insights into the requirements for vaccine development

1.III.A. The role of broadly protective antibodies in the proposed vaccine development strategy

Since currently approved prophylactic vaccines induce protection by the production of protective antibodies, the Vitetta lab proposes to create new vaccines that will induce the 'right' antibodies, *i.e.*, those that are protective and that bind to epitopes conserved across many strains of a pathogen or toxin. This is an important point since both natural infection and vaccination induce polyclonal responses, and not all the antibodies that are made are protective. In many cases the critical epitopes are not immunodominant, meaning antibodies that do not protect predominate. Indeed, this proposed vaccine development platform should avoid the production of antibodies against those portions of a pathogen or toxin that will not be useful in protection. In addition, the goal of this vaccine platform is to elicit antibodies that

are protective against epitopes that are so critical for pathogen function that they do not undergo frequent mutation, and thus the epitopes are shared by many strains of the pathogen in question. Such antibodies are termed *broadly protective*. The method proposed to develop these vaccines is briefly explained in Figure 1.1 (page 31). To identify vaccine candidates that can elicit broadly protective antibodies, MAbs that are **already known** to be broadly protective themselves are first obtained from our collaborators. Some of these MAbs recognize rare, non-immunodominant epitopes that would not normally be robust immunogens, or immunogens that would not stimulate a protective immune response by administering an intact pathogen or protein derived from it. These MAbs are then used to screen libraries of synthetic molecules termed peptoids, which will be addressed below. It is hypothesized that if these libraries are diverse enough, they will contain peptoids that mimic the shape of the epitope on the pathogen or toxin to which the screening MAb binds, even though the chemical building blocks of the mimetic are quite different. By applying various detection agents, complexes of the screening MAb and bound peptoids can be recognized and the identity of the peptoids of interest determined. The peptoids can then be re-synthesized in larger quantities and many copies will be attached to carrier proteins for immunization. Both copy number and mimicry should result in a robust T cell-dependent response that is class switched and of high affinity. When used as vaccines, successful candidates will elicit antibodies that cross-react with the protective epitopes on the native antigen that the screening MAb recognized, and hence provide protection against the pathogen or toxin in question.

The search for the broadly protective antibodies that are key components of this vaccine development platform has intensified in recent years, particularly with regard to HIV and influenza (17), for which universal vaccines would be very desirable. While

administering such antibodies to patients as prophylaxis or therapy could be attempted, this would have to be performed repeatedly and is extremely costly. It is therefore impractical for large populations. The more prudent use of broadly protective antibodies is to apply them to epitope selection. Table 1.4 (page 33) shows the MAbs against the toxin and pathogens from Table 1.3 (page 32) that have been acquired by the Vitetta lab and that, as described in the references noted, have been shown to exhibit neutralizing activity. Although an antibody does not need to neutralize *in vitro* in order to be protective, the antibodies that the lab has acquired are neutralizing and can therefore be considered broadly neutralizing MAbs (bnMAbs). The precise methods by which these MAbs were produced or identified varied, but involved immunizing mice or screening sera from many infected human patients, then isolating B cells, fusing them with immortalized cells to produce hybridomas, and screening the products of these cells to determine which clones produced neutralizing MAbs. Furthermore, although one of the potential strengths of the peptoid vaccine platform is that the epitopes on the native antigen need not be known, for the MAbs listed, the native antigens are indeed known and are listed in Table 1.4. In many cases, more specific structural information about the epitopes has been obtained.

At the start of my dissertation research, I was responsible for the WNV antibodies, while another graduate student, Ms. Angela Collins, and a postdoctoral fellow, Dr. Kate Yi, would work with the HCV and HIV antibodies, respectively. Meanwhile, a third graduate student, now Dr. Allison Case, would spearhead the use of a model system unrelated to a pathogen in proof-of-principle experiments. Due to the expertise in the Vitetta lab in developing a ricin vaccine, Dr. Joan Smallshaw and Mr. Stephen Ruback would pursue this avenue. Over the course of my dissertation research, we acquired the MNV-1 MAb, and this also became part of my dissertation research. While these pathogens and toxin were our

first targets for vaccine development, we hope to acquire broadly protective MAbs against other pathogens in the future. The laboratory is in the process of obtaining MAbs against both herpes simplex virus and influenza.

To explore the role of protective antibodies in the context of the immune response to the pathogens the peptoid vaccine project has targeted, I will now focus on the two viruses in Table 1.3 for which I attempted in this dissertation research to identify peptoid vaccine candidates: WNV and MNV-1.

1.III.B. WNV

As listed in Table 1.3 (page 32), WNV is a member of the *Flaviviridae* family of enveloped virsuses with single-stranded, positive-sense ribonucleic acid (RNA) genomes. Other members of this family include dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis, tick-borne encephalitis, Murray Valley encephalitis, and Kunjin viruses. West Nile virus cycles between mosquitoes and birds, while humans are usually infected by mosquitoes as incidental, dead-end hosts (18). In the United States, human cases occur as summer-fall epidemics in relation to mosquito exposure (19). Additionally, infection through transplanted organs (20), blood transfusions (21-24), occupational (laboratory) exposure (25), or transmission from mother to infant through breast milk can occur (26, 27). Once infected, the severity of the clinical manifestations in humans varies. Most people infected remain asymptomatic (approximately 70-80%), while about 20-30% develop WNV fever, characterized by a flu-like illness, and less than 1% develop the most severe form of WNV infection, WNV neuroinvasive disease (28). This neuroinvasive disease can take several forms, which may coexist: meningitis, causing fever and headache; encephalitis, causing fever, altered mental status, seizures, and/or focal neurological deficits; or poliomyelitis,

causing acute flaccid paralysis even without previous signs and symptoms such as fever (28). Elderly and immunocompromised patients are more likely to experience severe WNV disease (29-33). While complete recovery is possible, WNV neuroinvasive disease can leave behind fatigue and neurological deficits. In about 10% of cases, WNV neuroinvasive disease is fatal (34).

Since WNV was first isolated in 1937 (35), outbreaks have occurred in Africa, the Middle East, and Europe (36, 37) and in 1999, WNV arrived in New York City (38), from which it spread rapidly across the United States (39). After a decline in cases in the latter part of the past decade, in 2012 WNV reemerged, particularly in Texas (39). Different strains of WNV have caused the various outbreaks of WNV around the world and are divided into several lineages, although most isolates fall into lineage 1 or 2 (40, 41). An ideal vaccine would thus need to cover multiple strains of the virus.

Natural, or, in the case of susceptible laboratory animals such as mice, experimental infection with WNV has shown that the immune response to this virus involves a plethora of molecules, pathways, and cells that might be expected to combat viral infection, and has suggested the limited treatments that have been applied. Of the topics addressed in this introduction, this is one of the most extensively studied and has been frequently reviewed (31, 33, 42-47). Implicated in innate immune recognition of infection and anti-WNV function are Toll-like receptor (TLR) signaling pathways, including TLR3 and TLR7 and the adaptor protein MyD88 (48-53), signaling pathways including retinoic acid-inducible gene I (RIG-I) and related receptors such as melanoma differentiation-associated gene 5 (MDA5) (54-58), the inflammasome and pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) (59-63), and both type I and type II interferon (57, 64-78), with involvement from Langerhans and dendritic cells (79-81), neutrophils (82), macrophages (83), and natural killer (NK) cells

(84, 85). Gamma-delta T cells have also been shown to play a role (86-90). Adaptive immune responses have been found to include the cytotoxic activity of $CD8^+$ T cells (91-99), particularly in viral clearance (94, 96, 97), acting through various mechanisms, including perforin (97, 100), Fas ligand (95), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (96). Antibody production by B cells, aided by $CD4^+$ helper T cells, has also been shown to be critical for WNV protection (101-104). Both polyclonal antibodies (PAbs) and MAbs have been shown to be protective when used as prophylaxis (105-109) or treatment (107, 108, 110, 111). Taken together, these findings have led to attempts to treat WNV with interferon alpha and intravenous immunoglobulin G (IVIG) (112, 113). However, in the absence of more specific or widely used treatment, currently, the best way to combat WNV is through surveillance monitoring of horses, birds, mosquitoes, and other animals, as well as vector control measures and public education to avoid mosquito exposure.

Given this lack of specific treatment, many and varied attempts have been made to develop vaccines against WNV. Several have been approved for animals and some clinical trials have been undertaken using formulations related to these veterinary vaccines, as well as for additional vaccine candidates. The strategies used in each of the vaccines that have progressed to clinical trials is largely reflective both of the types of vaccines addressed in Section 1.II (page 3) and of many other published reports. Two live, attenuated vaccines have been tested in clinical trials. One, called ChimeriVax-WN02, is a chimeric vaccine using a yellow fever virus 17D strain backbone to express the WNV E protein and another WNV structural protein, the precursor transmembrane (prM) protein (114, 115). The other is a chimeric vaccine using a dengue virus type 4 backbone to express WNV E and prM proteins (116-119). The former is related to a similar vaccine for horses (120, 121); additional mutations were introduced into that virus for use in the clinical trials. The second

type of WNV vaccine to progress to a clinical trial, which was conducted in Belgium, is an inactivated vaccine produced in PER.C6 cells, which are derived from the human retina (122-124). This vaccine is also related to veterinary vaccines for horses and geese, but unfortunately the company producing this vaccine for humans halted their WNV vaccine program to focus on other areas (125). The third type of vaccine to enter clinical trials is a recombinant protein vaccine composed of a truncated WNV E protein (WNV-80E, containing 80% of the E protein amino acids) (126-130). In accord with the lower immunogenicity of subunit vaccines, in this trial, three immunizations were given and a comparison between administration with and without the adjuvant alum was made (131). The fourth type of vaccine to progress to clinical trials is a DNA vaccine encoding the E and prM proteins (122-124). Again, because of the lower immunogenicity of these types of vaccines, three immunizations were given. This vaccine is also an approved vaccine for horses (132). Notably, the E protein, which is known to be a target for neutralizing antibodies (133), is included in all the vaccines listed here that have progressed to clinical trials, and is the target of the neutralizing MAbs in hand listed in Table 1.4 (page 33). Moreover, in the published reports from the clinical trials of these vaccine candidates, or in prior testing in animals, the development of an antibody response, and often, a neutralizing antibody response, was studied as a correlate of protection. This supports the goal of the Vitetta lab peptoid-based vaccine project, which posits that having protective antibodies in the blood and tissue fluids prior to infection is the best approach for protection. Clearly many other cells and pathways are involved once infection has occurred, but successful vaccines against many pathogens need only elicit the right antibodies.

1.III.C. MNV-1

As shown in Table 1.3 (page 32), MNV-1 is a member of the family *Caliciviridae*, genus *Norovirus*, a group of non-enveloped, positive-sense single-stranded RNA viruses. The first was isolated in 1968 in Norwalk, Ohio (134), giving rise to a former name for this group, the Norwalk-like viruses. For many years since this discovery, the study of noroviruses was hampered by the inability to culture them in cells, and by the lack of an animal model smaller than pigs (135, 136). However, in 2003, MNV-1 was isolated from mice (137). This virus could be cultured in cells and, as a natural pathogen of mice, was suited for study in the mouse model. A neutralizing MAb that had been raised against MNV-1 was obtained from one of the original investigators who identified MNV-1, Dr. Christiane Wobus, and was used in this dissertation research.

The noroviruses have been classified into five genogroups (138). Members of genogroups I, II, and IV infect humans, while genogroup III infects cows, and additional members of genogroup II infect pigs. Genogroup V infects mice, and includes MNV-1 as well as additional variants (139, 140). In humans, new strains of norovirus emerge every few years; currently, the most common strains fall within the GII.4 cluster (141-144). These viruses cause epidemics of gastroenteritis, with symptoms colloquially referred to as the "stomach flu" (despite having no relation to influenza). Generally, the infection is self-limited and symptoms of nausea, vomiting, and diarrhea resolve in several days (145). While one might question why a vaccine would be needed for a disease that is self-limiting in many people, the rationale results from several factors. The first relates to the fact that norovirus is responsible for 21 million cases of gastroenteritis per year in the United States (146). The virus is easily spread (147) through fecal-oral transmission *via* person-to-person contact or contamination of food or water (148). Therefore, the virus causes outbreaks of disease

where large numbers of people are in close contact, such as cruise ships (149, 150) or United States naval vessels (151). Outbreaks also occur within such institutions as schools (152), nursing homes (153-156), and hospitals (157, 158), which include many people with developing, waning, or compromised immunity. Accordingly, the outcomes of norovirus infection tend to be poorer in these populations, including hospitalization and death (153, 155, 158-161), which may be more pronounced for children in developing countries (160). Finally, costs such as missed work and the efforts involved to stop the spread of the virus can result in significant economic losses (162). Therefore, a vaccine against norovirus would be desirable due to the incidence, the patient populations most affected, and the economics of the disease caused.

The immune response against norovirus, studied following natural infection as well as introduction into mice and pigs, is known to involve the innate immune system, including dendritic cells and macrophages (163-166) and components of signaling pathways and cytokines such as MDA5 (167), nucleotide-binding oligomerization domain-like receptors [NLRs; (168)], signal transducer and activator of transcription 1 [STAT1; (137, 169)], interferon (170-173), and interferon regulatory factors [IRFs; (173, 174)]. Later, the T cells of the adaptive immune system play a role (175, 176). However, viral clearance is eventually accomplished by the production of antibodies (177), and antibody has been shown to correlate with protection against norovirus (178). Furthermore, passive transfer of antibody, but not $CD4^+$ or $CD8^+$ T cells, was found to protect severe combined immunodeficient (SCID) mice from MNV infection (179). Regarding the many strains of norovirus, the antibody response that develops after infection may be effective against the homologous virus, but the development of an effective response against heterologous virus is less clear (151, 180-182). Additionally, the immune response in mice against re-challenge with the

homologous virus at the mucosal surface was not found to be effective at preventing infection when the primary infectious dose was increased, while the peripheral immune response was more effective in reducing viral loads than that of control mice infected for the first time (183). The authors suggested that this may indicate the inappropriate induction of tolerance by high doses of the virus in the context of a tolerogenic mucosal environment, or the failure of infected mucosal antigen-presenting cells to stimulate the memory cells appropriately (183). Taken together, these findings regarding the inconsistent ability of previous infection to protect against re-infection, coupled with the emergence of new strains of norovirus every few years, suggest a need for vaccines that are effective against many strains of norovirus.

To this end, many investigators have made MAbs against the norovirus capsid and mapped their epitopes (184, 185). Importantly for vaccine development, some conserved epitopes have been identified in the capsid protein (144, 186-191). For this reason, and because live, attenuated and inactivated vaccines cannot be made for human norovirus in the absence of the ability to culture it in cells, the capsid protein has been used in the most common types of vaccines against norovirus to be studied. These vaccines are composed of virus-like particles (VLPs), or assemblies of the capsid protein that form spontaneously when this protein is expressed in cells. They can be thought of as shell-like versions of the virus that lack the nucleic acid required to be infectious. Since the VLPs take the form of icosahedrons, like the intact virus would, the VLPs display capsid protein epitopes in the native conformation, but are not infectious. To produce the VLPs for vaccination, recombinant DNA encoding the norovirus capsid protein is expressed in one of several culture systems. These have included insect cells (192-199), yeast cells (200), baby hamster kidney (BHK) cells (in which the capsid protein is expressed from a Venezuelan

equine encephalitis virus backbone) (201), and BSRT7 cells (a cell line related to BHK cells, in which a vesicular stomatitis virus backbone has been used to express the capsid protein) (202). Additionally, VLPs have been produced in plants, with the idea that eating plants containing VLPs could provide a useful means of administering the vaccine, especially in developing countries (198, 203-207). Clinical trials have been reported for VLPs expressed and purified from insect cells and administered either by oral (193) or intranasal routes (208). The oral vaccine was found to induce a serum IgG response, but no challenge was performed, while the intranasal vaccine was found to be protective against viral challenge. A related strategy, studied in mice, involved the intranasal administration of a recombinant adenovirus expressing the capsid protein, either alone (209) or with booster immunizations of VLPs prepared from insect cells (210). Alternatively, vaccines have been formulated using only the P domain of the capsid protein (199, 211-215). When this domain is expressed in *Escherichia coli* (*E. coli*) cells, it spontaneously assembles, with the help of a cysteine (Cys)-containing peptide to promote intermolecular disulfide bonding, into structures called P particles. These P particles are composed of 24 P domains that, as 12 dimers, form an octahedron, and like the VLPs, these particles are not infectious (214). Therefore, as described in Section 1.III.B (page 9) for WNV, the major constituents of the vaccines that have been attempted for norovirus are the target of the neutralizing MAb (Table 1.4, page 33) with which I sought to identify vaccine candidates in this dissertation research.

1.III.D. Summary

The strategy to develop new vaccines employed in this dissertation is to use MAbs that are known to be broadly protective, and which may also be neutralizing, to identify

vaccine candidates that mimic conserved epitopes on toxins or pathogens. In the case of both WNV and MNV-1, which were targeted for vaccine development in this dissertation, no vaccine has yet been approved, although attempts to develop vaccines have been made and antibody has been shown to be protective. These attempts at vaccine development have included whole proteins or pathogens, which may elicit many antibodies against immunodominant epitopes. However, these antibodies may not be broadly protective, since epitopes that are important for cross-protection may be rare, obscured, or otherwise not immunodominant. One of the major strengths of the novel vaccine platform explored in this dissertation research is that a small mimetic structure selected by a protective antibody should focus the antibody response on those epitopes that will elicit broadly protective antibodies. The molecules used as these small, mimetic structures, termed peptoids, will now be discussed.

1.IV. Peptoids are peptide-like molecules that can be synthesized and screened as diverse, combinatorial libraries for biomedical applications, including vaccine development

1.IV.A. Comparison between peptoids and peptides

Peptoids were first synthesized and described as potential pharmaceutical agents by Simon et al. (216). Peptoids are oligo-*N*-substituted glycines that resemble peptides. However, while the side group (R group) of a peptide monomer is attached to a chiral carbon atom, the R group of a peptoid monomer is attached to the nitrogen of its amide group (Fig. 1.2, page 34). This difference is responsible for conferring critical advantages in the manipulation and application of peptoids versus peptides:

i. Unlike peptides, peptoids are resistant to proteolysis (216-218);

ii. The chemistry required to synthesize peptoids was greatly simplified with the development of the submonomer method by Zuckermann et al. [(219); Fig. 1.3, page 35]. This was made possible because peptoids lack the chirality of peptides where the side group is attached. Using this method, individual peptoid monomers need not be synthesized, as was performed in the original description of peptoids (216). Rather, this method potentially allows over 1000 commercially available primary amines to be used as submonomers to form peptoid side groups, and indeed, many amines have been used successfully (220). Thus, the diversity of structures far exceeds that of peptides built from the 20 standard amino acids; and

iii. Peptoids are capable of assuming a greater variety of conformational states than peptides, due to the loss of the intramolecular hydrogen bonds and differences in steric hindrance (216). The relationship between peptoid sequences and their resulting three-dimensional structure is an area of active research, but it appears that cis/trans isomerization of the peptoid amide bond is common. Furthermore, the secondary structure of peptoids can include helices, turns, and loops, and steric constraints, hydrogen bonding, and hydrophobic interactions may play a role in determining their conformations (221).

Despite these important differences, peptoids share certain characteristics with peptides and these similarities have resulted in the ability to synthesize individual compounds and to construct large, one-bead-one-compound (OBOC) libraries of both peptides and peptoids. Solid phase synthesis (on resin, or small beads), widely used for peptides, has been commonly used for individual peptoids since they were initially described (216), as it has been for the construction of OBOC libraries. In these libraries, a single bead contains many copies of the same peptoid. However, use of the “split and pool” method,

developed for peptides (222-224) and adapted for peptoids (225), allows many different peptoid sequences to be synthesized on different beads. This allows a large library to be made efficiently (Fig.1.4, page 37). While the sequence of the peptoid on each individual bead is not known during synthesis, peptoids, like peptides, can be sequenced *via* Edman degradation (226), or peptoids can be cleaved from the resin by using a linker that was included by the manufacturer (for certain types of resin), or by adding a linker during synthesis (227-229). The cleaved peptoids can then be sequenced by tandem mass spectrometry (MS/MS) (230-232). A hybrid method called partial Edman degradation, which employs Edman degradation and mass spectrometry, has also been developed (229). Finally, peptoid chemistry has advanced to incorporate monomers of different structures and modified backbones (233-240), as well as macrocyclization, or formation of cyclic peptoid chains (241-243), further broadening the diversity of peptoids.

1.IV.B. Use of peptoids in biological applications

This ability to synthesize and sequence diverse single peptoids and peptoid libraries using the techniques described above, combined with other methods, has prompted investigators to explore peptoids, peptide-peptoid hybrids [termed peptomers (244)], or peptoids conjugated to moieties such as lipids for a variety of biological uses:

i. anti-microbial activity (245-255);
ii. anti-neoplastic activity (236, 256-258);
iii. binding to RNA (259, 260);
iv. binding to transcriptional co-activators (261-264);
v. binding to autoreactive T cells (265);
vi. transfecting DNA into cells (266-269);

vii. sequestering bacterial toxins (254);

viii. inhibiting enzymes (270-272);

ix. inhibiting the proteasome 19s regulatory particle (273-275);

x. inhibiting protein-protein interactions (276, 277);

xi. inhibiting costimulation of T cells through CD28 (278);

xii. inhibiting receptors with neurological or endocrine functions (216, 279-284);

xiii. diagnosing or treating neurodegenerative diseases (285-289); and

xiv. mimicking proteins such as collagen (290) or lung surfactants (291-293).

Most relevant to this dissertation research, however, are several studies in which the interactions of peptoids with antibodies and sera have been examined. These provide some illustrative vignettes regarding approaches to screening peptoid libraries.

1.IV.C. Previous studies of peptoid interactions with antibodies and sera

The first of these was a study by Heine et al. (294), in which the MAb Tab2, raised against human transforming growth factor alpha (hTGFα), a mitogenic peptide composed of 50 amino acids and containing three disulfide bonds (295), was used to screen a library of peptoids and peptomers. This library was designed to be six monomers in length and was composed of 40 different monomers, of which 35 were peptoid monomers and 5 were amino acids. This would produce a library with the theoretical size of 40^6, or approximately 4×10^9 compounds. However, because this library was synthesized on a cellulose array using the SPOT method (296), this large number would not fit on the array. Therefore, 8000 compounds were chosen at random and synthesized using an automated SPOT synthesizer. Following blocking and washing, the Tab2 MAb was added to the cellulose array, the excess was washed away, and a secondary antibody conjugated to horseradish

peroxidase (HRP) was applied. Addition of an appropriate substrate allowed quantification of the reaction using an imaging device. Since the compounds had been synthesized at particular addresses on the array, the sequences at resulting dark spots could be determined from the automated synthesizer program used, and two of these positive sequences, as well as two sequences that were negative in screening, were resynthesized on resin. The binding affinity of the lead compounds for Tab2 was determined by surface plasmon resonance (SPR) in comparison with the negative control compounds, an irrelevant, subtype-matched control MAb, and a positive control compound, the native heptamer peptide epitope for Tab2. While the inclusion of these controls is admirable and the selection of monomers and synthesis of the library were diligently optimized, the authors of this study devoted comparatively less attention to the optimization of their library screen in this report. This may be because members of this group had previously published many studies using the SPOT method, some including Tab2 [for example, a study by Reineke et al. (297)]. However, to strengthen the results of these particular experiments, the Tab2 native epitope peptide as well as a known irrelevant peptide could be synthesized on the array as positive and negative controls. Nevertheless, these authors reported the identification of two ligands with dissociation constants in the micromolar (μM) range (2-400 μM), in comparison with the nanomolar (nM) range dissociation constant (20 nM) for the native Tab2 epitope peptide. This suggested that antibody ligands of reasonable affinity could be obtained by screening peptoid libraries with MAbs.

In a related study performed by some of the same investigators, similar techniques were used to transform, in an iterative fashion, the native Tab2 epitope peptide one residue at a time into a peptoid (298). In this case, the compound from the previous step was

synthesized on the subsequent array as a positive control. The resulting compound of interest from each stage was assessed by SPR and competition enzyme-linked immunosorbent assay (ELISA) with the native epitope. From the 1470 compounds examined, the best peptoid was found to have a dissociation constant of 200 nM. This study demonstrated that it was possible to identify peptoids to which a MAb could bind with a nanomolar-range affinity. Furthermore, neither in this study nor the previous study (294) did the side chains of the selected peptoids correspond to those in the native epitope, suggesting that the peptoids selected mimicked the three dimensional shape, or conformation, of the native peptide rather than its sequence.

Two additional studies attempting to select members of peptoid libraries that bind to antibodies deserve mention here (287, 299). The goal of the first study (287) was not to identify peptoids bound by a known antibody, but to identify biomarkers in patient serum, with the goal of developing diagnostic tests. These investigators proposed that a peptoid library could do the work of identifying antibody biomarkers without knowing the nature of the antibody or its antigen. To do this, they performed several experiments in mouse model systems prior to testing patient samples. A peptide from myelin oligodendrocyte glycoprotein (MOG), which induces experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, was first used to immunize two mice. For this immunization, the peptide was combined with complete Freund's adjuvant (CFA), and as a control, two mice were immunized with CFA alone. On day 36 following immunization, blood was collected, and sera were prepared and diluted to a standard protein concentration. The sera were then hybridized to a peptoid microarray, which was constructed with a subset of 4600 peptoids synthesized from a library in which seven different monomers were used to make octamer peptoids, giving this library a theoretical size of 7^8 or approximately 5.8×10^6 compounds.

This library “under-sampling,” or synthesizing only a fraction of all possible compounds, was similar to that used in the two studies screening with the Tab2 antibody (294, 298). Following serum hybridization, a fluorescently labeled secondary antibody was applied, or was applied directly to control spots to avoid false-positives due to direct binding to the secondary antibody. The microarrays were then examined to determine whether peptoids bound by antibodies in the sera of the mice immunized with MOG/CFA, but not by the sera from mice immunized with CFA alone, could be identified. Three such peptoids were identified, and these were found to discriminate in a blinded study between 14 additional serum samples (seven from mice immunized with MOG/CFA and seven from mice immunized with CFA alone). However, the design of this assay and subsequent, similar assays examining mice immunized with ovalbumin (OVA), or comparing patients with Alzheimer’s disease to controls and patients with other diseases, made it difficult to assess the authors’ conclusions regarding the specificity of the selected peptoids and their ability to capture diagnostically relevant antibodies from serum without further experimentation. First, the sera were diluted to a standard total protein concentration, but may have varied in their antibody concentrations. Even the use of a mouse model of systemic lupus erythematosus in comparison with EAE, and patient samples with Parkinson’s disease in comparison with Alzheimer’s disease, cannot ensure that the serum samples tested contained similar levels of antibody. Furthermore, in time-course studies, where mouse sera were obtained once a week for a month following immunization, the rise in fluorescence intensity when serum samples were applied to the microarrays did not occur until days 14-21. This is inconsistent with a primary antibody response, which generally occurs in a shorter time frame (7-10 days). Finally, although sera from immunized mice were affinity-purified using the immunogen and the flow-through was found to be depleted of binding activity for the peptoid

candidates, this did not necessarily confirm that the depleted entity was an antibody. To demonstrate this, experiments could be performed in which antibodies are purified from the sera of interest, then titrated to determine whether their binding to the candidate peptoids is significantly different from antibodies purified from the sera of controls. This would provide more evidence of potentially very exciting findings in using peptoids to develop needed diagnostic tests for diseases such as Alzheimer's.

In their subsequent study (299), this group focused on a disease, neuromyelitis optica (NMO), in which high levels of serum antibodies had been found against the protein aquaporin 4. This would seem to remove a level of uncertainty compared to their previous study, in which both the antigen and the antibodies for Alzheimer's disease were uncharacterized. In this system, peptoid beads were first screened with control serum using fluorescent quantum dots as a detection reagent, washed, then screened with serum from patients with NMO. Unlike their previous study, this patient serum was first submitted to a test for the presence of anti-aquaporin 4 antibodies *via* their ability to cause complement-dependent cytotoxicity. Here, NMO patient sera were applied to transfected cells expressing aquaporin 4, and to control cells that did not express this protein. Cell killing, as measured by the release of proteases, was then assessed in comparison with cell lysis by the detergent Triton X-100. However, the definition of what constituted a positive or negative result in this assay was unclear from this report, and the authors speculate that antibody levels may still differ widely among patient samples.

Having identified candidate peptoids in their on-bead screens, they then submitted these compounds to another round of screening on microarrays. Since the native antigen for their serum antibodies of interest was known to be aquaporin 4, they applied to their arrayed peptoid candidates a MAb against anti-aquaporin 4 and an irrelevant control MAb as

controls. While these are elegant controls, to strengthen these experiments, the purified native antigen, aquaporin 4, which was available to the authors for inclusion in their affinity purification experiments, could be included on the arrays if technically feasible. Furthermore, titrations of sera and the control MAbs could be performed in this array-based screening. Even if aquaporin 4 cannot be included on the arrays, the anti-aquaporin 4 MAb and purified aquaporin 4 antigen could be used as a standard curve in an ELISA or similar experiment to determine the concentration of anti-aquaporin antibodies in patient serum before application to the microarrays. In the absence of such experiments, the results of the additional assays performed by these investigators (for example, depleting sera over an aquaporin 4 affinity column), as well as their conclusions that peptoid ligands of anti-aquaporin 4 antibodies have been identified, are difficult to interpret.

While some of this detail may seem tedious to sort through, the strengths and shortcomings of these studies had a direct impact on how our peptoid team approached the goal of developing peptoid-based vaccines. Taken together, these four studies by Heine et al. (294), Hoffmann et al. (298), Reddy et al. (287), and Raveendra et al. (299) suggested that the published work to identify peptoids that bind to antibodies had great promise. However, as will become apparent in the remainder of this dissertation, even when PAbs or MAbs were used in peptoid screening assays instead of sera, the identification of specific peptoid ligands could be challenging without including positive and negative controls in the assays performed and optimizing the concentration of the screening reagents. This acute attention to antibody specificity is an area in which we felt our team of immunologists could make a strong contribution to the developing field of biological applications for peptoids. To work toward our goal of using peptoids in vaccines, we were fortunate to team up with expert peptoid chemists whose thinking and expertise was essential to the implementation

of our immunologic approach. We became official Users of the Molecular Foundry, a facility sponsored by the Department of Energy at Lawrence Berkeley National Laboratory. This allowed us to learn the techniques of peptoid design and chemistry under the tutelage of Dr. Ronald Zuckermann, one of the original inventors of peptoids (216), and his staff, including Mr. Michael Connolly and Ms. Helen Tran. Altogether, three trips from Dallas to Berkeley, each for approximately 2.5 weeks, were made; I participated in two of these. We have maintained our status as Users, which will allow peptoid team members to return to the Foundry if needed. Additionally, Mr. Connolly made a trip to the Vitetta lab to help us establish our peptoid synthesis lab, and the chemists at the Foundry frequently advise us by email or phone when needed. I have also had the benefit of attending two of the biennial Peptoid Summits at the Foundry, in August 2010 and August 2012, to interact with many of the chemists who authored the studies of peptoids described in this dissertation.

Lastly, two studies relating to the interaction of peptoids with antibodies need to be discussed here. The first study was carried out prior to my joining the laboratory and was initiated by Drs. Kodadek and Vitetta for the purposes of testing the immunogenicity of peptoids. Astle et al. (300) examined the immunogenicity of free peptoids in comparison with peptoids attached to carriers in mice. As predicted, free peptoids behaved as haptens and were not immunogenic. In contrast, once attached to carrier proteins *via* a linker, antibodies against the peptoid/linker were elicited. However, the assay used could not distinguish between anti-peptoid antibodies and anti-linker antibodies. In a second set of experiments, a pool of seven peptoids was used for immunization either with or without conjugation to a carrier. In the assay used to analyze the resulting sera, no irrelevant peptoid control was used. While these reports were encouraging, further experiments were needed to complete this important work of exploring peptoids as haptens.

Finally, with regard to the use of peptoids as vaccines, a number of years ago, a group attempted to develop a vaccine by screening peptoid libraries with a MAb against *Neisseria meningitidis* serogroup B (301, 302). Although this strain causes significant disease, particularly in children (303), no vaccine had been developed because it was thought that self-tolerance prevented an effective immune response against the epitopes of the polysaccharide capsule that would otherwise elicit protection. Accordingly, the vaccine for meningococcal meningitis listed in Table 1.1 (page 29) does not include the serogroup B polysaccharide. Therefore, these investigators wanted to identify antigen mimetics that would avoid self-tolerance and elicit a protective response. To that end, they developed a panel of MAbs that could bind to the capsular polysaccharide of *N. meningitis* but were not autoreactive, and used these MAbs to screen libraries, including peptoids. Their initial screens were of phage libraries, including novel phage libraries in which the displayed peptides adopted secondary structure. Nevertheless, in describing their preliminary assessment of their lead compounds in the last pages of their publication, they mentioned that all of their leads had come from screening peptoid libraries. Unfortunately, these peptoid candidates did not lead to development of a vaccine, and their work to identify peptoid vaccine candidates apparently did not continue.

1.IV.D. Summary

Peptoids are peptide-like molecules that overcome some of the limitations of peptides, including protease sensitivity and limited diversity. Nevertheless, peptoids retain some features of peptides that allow them to be synthesized with relative ease as individual sequences and in the context of large OBOC libraries. This has enabled the use of peptoids in a variety of biological applications. In particular, several studies screening large libraries

to identify peptoids that bind to antibodies have been performed, but often, further experimentation was needed to reinforce the conclusions drawn. Additionally, further studies to assess whether peptoids are haptens, eliciting specific antibody when attached to carriers, needed to be performed. With the benefit of a supportive team and guided by the chemistry expertise of Dr. Ronald Zuckermann at the Molecular Foundry and the immunology expertise of my mentor, I undertook the dissertation research described in the upcoming chapters, continuing the search for peptoid vaccine candidates and the study of peptoid immunogenicity.

Table 1.1. Current vaccines.*

Disease or major disease(s) caused	Causative agent	Year use of a vaccine was first published	Year a vaccine was first licensed in the United States	Type(s) of vaccine currently licensed for use in the United States	Current notes regarding administration in the United States
Smallpox	variola virus	1798	-	live (vaccinia virus)	routine vaccination ended in 1971
Rabies	rabies virus	1885	-	inactivated	generally, post-exposure; pre-exposure for laboratory workers, others at risk (*e.g.*, spelunkers, veterinarians and staff, animal-control and wildlife officers)
Typhoid	*Salmonella typhi*	1896	-	live; polysaccharide	laboratory workers, travelers, close exposure to a carrier (> 6 years old for live; > 2 years old for polysaccharide)
Plague	*Yersinia pestis*	1897	-	vaccine approved but not available	-
Diphtheria	*Corynebacterium diphtheriae*	1923	-	toxoid	recommended childhood immunization
Pertussis	*Bordetella pertussis*	1926	-	acellular (purified culture fluids)	recommended childhood immunization
Tetanus	*Clostridium tetani*	1927	-	toxoid	recommended childhood immunization
Tuberulosis (TB)	*Mycobacterium tuberculosis (MTB)*	1927	-	live Bacillus of Calmette and Guerin (BCG) strain of *Mycobacterium bovi*s	individuals who have not been previously infected with MTB at high risk for exposure, including TB-exposed tuberculin skin test-negative infants and children, and TB-exposed health care workers in high risk settings
Influenza	influenza virus	-	1945	live, attenuated; inactivated	annual; routine childhood immunization at 6 months (inactivated) or > 2 years of age (live, attenuated)
Yellow fever	yellow fever virus	-	1953	live, attenuated	laboratory workers, travelers to or living in an endemic area (> 9 months old)
Poliomyelitis	poliovirus	-	1955	inactivated	recommended childhood immunization
Measles	measles virus	-	1963	live, attenuated	recommended childhood immunization
Mumps	mumps virus	-	1967	live, attenuated	recommended childhood immunization
Rubella	rubella virus	-	1969	live, attenuated	recommended childhood immunization
Anthrax	*Bacillus anthracis*	-	1970	cell-free filtrate from cultures of an avirulent, nonencapsulated strain	military and potentially exposed workers (*e.g.* laboratory workers, handlers of relevant animal products, veterinarians in high incidence areas)
Meningitis	*Neisseria meningitidis*	-	1975	polysaccharide conjugate	recommended adolescent immunization; special schedules for high-risk children
Pneumonia	*Streptococcus pneumoniae*	-	1977	polysaccharide; polysaccharide conjugate	conjugate is a recommended childhood immunization
Adenovirus	Adenovrus	-	1980	live	military personnel
Hepatitis B	Hepatitis B virus	-	1981	recombinant (produced in yeast cells)	recommended childhood immunization
Meningitis, pneumonia, epiglottitis	*Haemophilus influenzae* type b (Hib)	-	1985	polysaccharide conjugate	recommended childhood immunization
Japanese encephalitis	Japanese encephalitis virus	-	1992	inactivated	≥ 17 years old at risk for exposure (living in or traveling to epidemic and endemic areas, including military; laboratory workers)
Hepatitis A	Hepatitis A virus	-	1995	inactivated	recommended childhood immunization
Varicella (chickenpox)	varicella-zoster virus	-	1995	live, attenuated	recommended childhood immunization
Rotavirus	rotavirus	-	1998	live reassortant (express proteins from human- and bovine-infecting viruses)	recommended childhood immunization
Herpes zoster (shingles)	varicella-zoster virus	-	2006	live, attenuated	≥ 50 years old
Cervical cancer (bivalent, quadrivalent vaccines), vaginal, vulvar, and anal cancers, genital warts (quadrivalent vaccine)	human papillomavirus (HPV)	-	2006	recombinant (quadrivalent produced in yeast cells, bivalent produced in insect cells)	9-26 years old (quadrivalent); females 9-25 years old (bivalent)

*Adapted from Impact of vaccines universally recommended for children--United States, 1990-1998 (304).

Table 1.2. Advantages and disadvantages of current vaccines.

Type of vaccine	Examples of commonly used vaccines	Advantages	Disadvantages
Live, attenuated (mutated pathogen, pathogen of non-human cells)	influenza; measles, mumps, and rubella; varicella	very immunogenic	dangerous for immuno-compromised
		could contribute to herd immunity	risk of reversion
Killed/inactivated	influenza; polio (Salk); Hepatitis A	less dangerous for immuno-compromised	less immunogenic than live, attenuated, therefore requiring adjuvants, boosters
		No risk of reversion	
Subunit: polysaccharide, conjugate, recombinant protein also: synthetic peptide, DNA	pneumococcal; meningococcal; Hib; Hepatitis B; HPV	privileges protective epitopes	must know protective epitopes
		less dangerous for immuno-compromised	less immunogenic
		No risk of reversion	

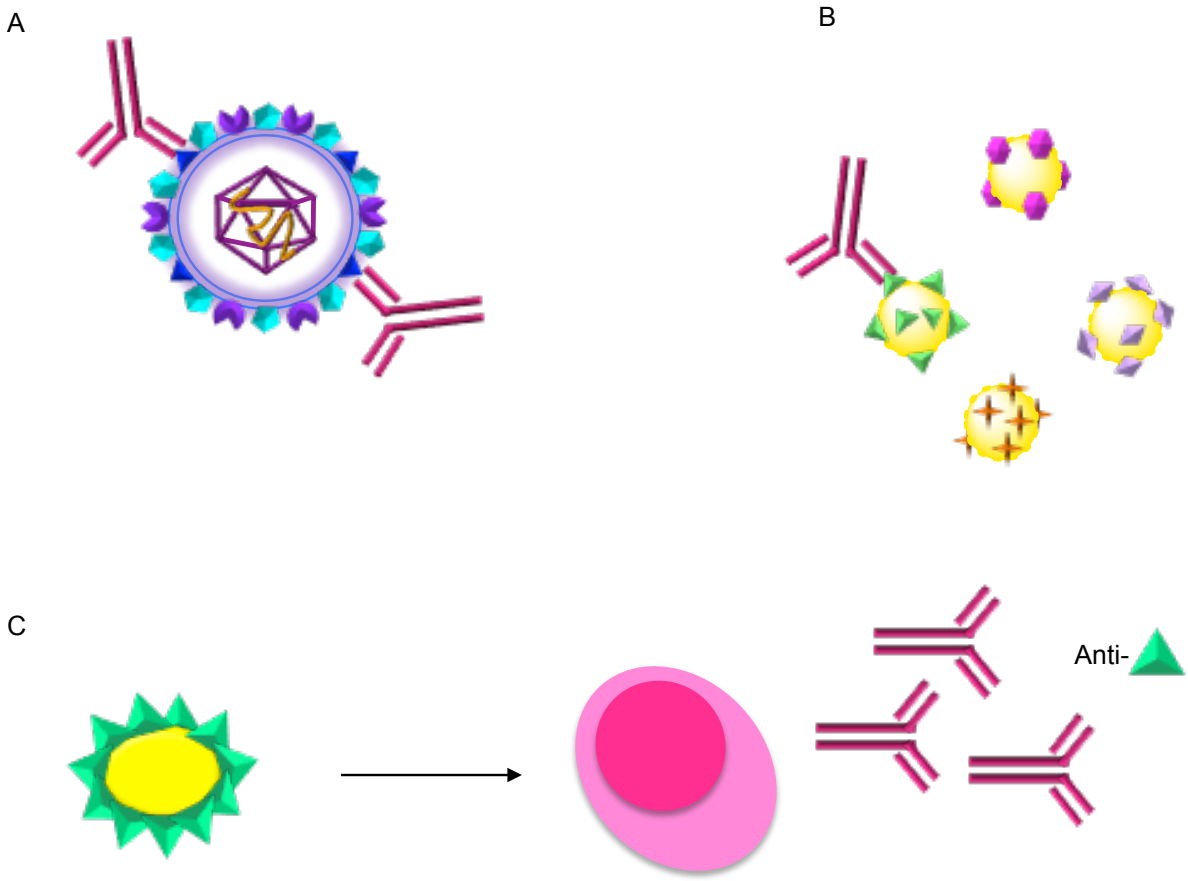


Figure 1.1. Strategy for the development of peptoid-based vaccines.
A. Collaborators have identified broadly protective MAbs against toxins or, as shown, pathogens such as viruses. The blue triangle on the virus represents the epitope of the broadly protective MAb. **B.** These broadly protective MAbs can be used to identify on-bead peptoids (represented by the colored shapes on yellow circles) that bind to the antigen binding sites of the MAb and mimic the shape of the native epitope on the toxin or pathogen. The mimietic peptoid is represented by the green triangle. **C.** For use in vaccines, the peptoid B cell epitopes (green triangles) selected by broadly protective MAbs will be conjugated to a carrier protein (yellow oval), which should provide T cell epitopes so that T cell help can contribute to the development of high-affinity IgG antibodies. Conjugating peptoid vaccine candidates to carrier proteins will also provide a multivalent display of the peptoid to facilitate B cell receptor cross-linking and antibody production. Immunization with these peptoid-carrier conjugates should generate antibodies against the peptoid that cross-react with the native antigen on the toxin or pathogen and are broadly protective.

Table 1.3. Toxin and pathogens to be targeted first in the development of peptoid-based vaccines.

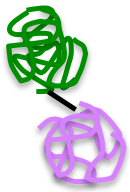
Toxin or pathogen	Ricin toxin	Murine norovirus-1 (MNV-1)	West Nile virus (WNV)	Hepatitis C virus (HCV)	Human immunodeficiency virus (HIV)
Description	derived from castor beans (*Ricinus communis*)	*Caliciviridae*, genus *Norovirus*	*Flaviviridae*		*Retroviridae*
					
Human disease	symptoms of intoxication depend on route of exposure (inhalation, ingestion, injection)	epidemic gastroenteritis	epidemic flu-like illness, meningitis/ encephalitis	chronic infection, leading to cirrhosis, hepatocellular carcinoma	acute infection, acquired immune deficiency syndrome (AIDS)

Table 1.4. Broadly protective antibodies received from collaborators with which to identify peptoid B cell epitopes for use in vaccines.

Toxin or pathogen	Neutralizing Mab	Corresponding recombinant protein antigen	Species	Isotype	Collaborator	Selected references
Ricin toxin	A18	Ricin toxin A chain	mouse	IgG2a	Seth Pincus (Lousiana State University)	(305)
	GD12			IgG1	Nicholas Mantis (Wadsworth Center)	(306)
	R70			IgG1	D. C. Wright, United States Army Medical Research Institute of Infectious Diseases/ATCC	(307)
Murine norovirus-1 (MNV-1)	1A6.2.1	Protruding (P) domain of capsid protein	mouse	IgG2a	Christiane Wobus (University of Michigan)	(308-311)
West Nile virus (WNV)	E16	Envelope (E) protein	mouse	IgG2b	Michael Diamond (Washington University in St. Louis)	(133, 312-316)
	E53			IgG2a		
Hepatitis C virus (HCV)	J6E2.36	Envelope 2 (E2) protein	mouse	IgG1	Michael Diamond (Washington University in St. Louis)	(317, 318)
	H77E2.16			IgG1		
	H77E2.39			IgG1		
	H77E2.56			IgG1		
	CBH-2		human	IgG1	Steven Foung (Stanford University)	(319-321)
	CBH-5			IgG1		
Human immunodeficiency virus (HIV)	b12	gp120 subunit of Envelope (Env) protein	human	IgG1	Dennis Burton (The Scripps Research Institute)	(322-326)
	PG9	Trimeric gp120 expressed on a transfected cell line		IgG1		(327-330)
	PG16			IgG1		

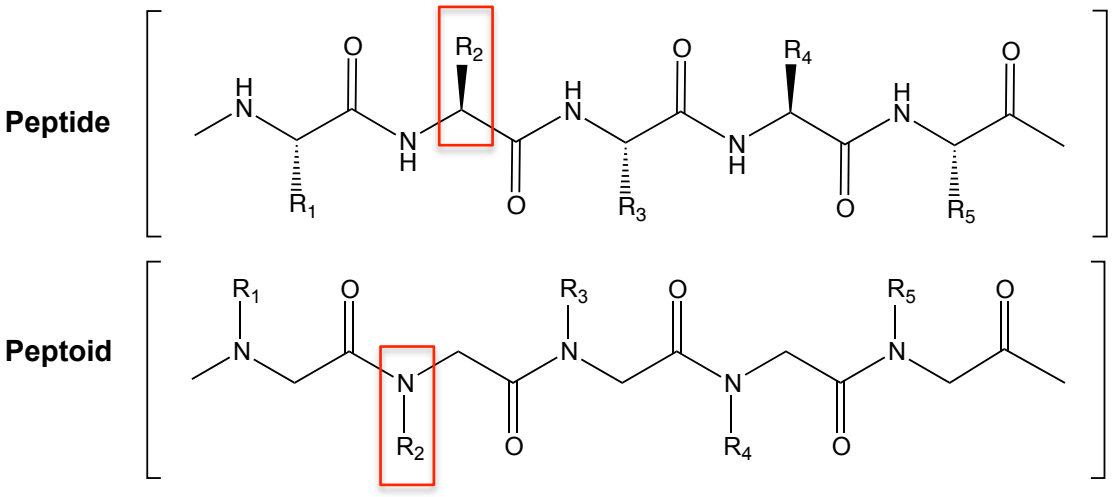


Figure 1.2. Comparison of peptide and peptoid structure.
Peptoids have similar backbone structure to peptides, but bear their R groups on their backbone nitrogen atoms. Figure adapted from Simon et al. (216).

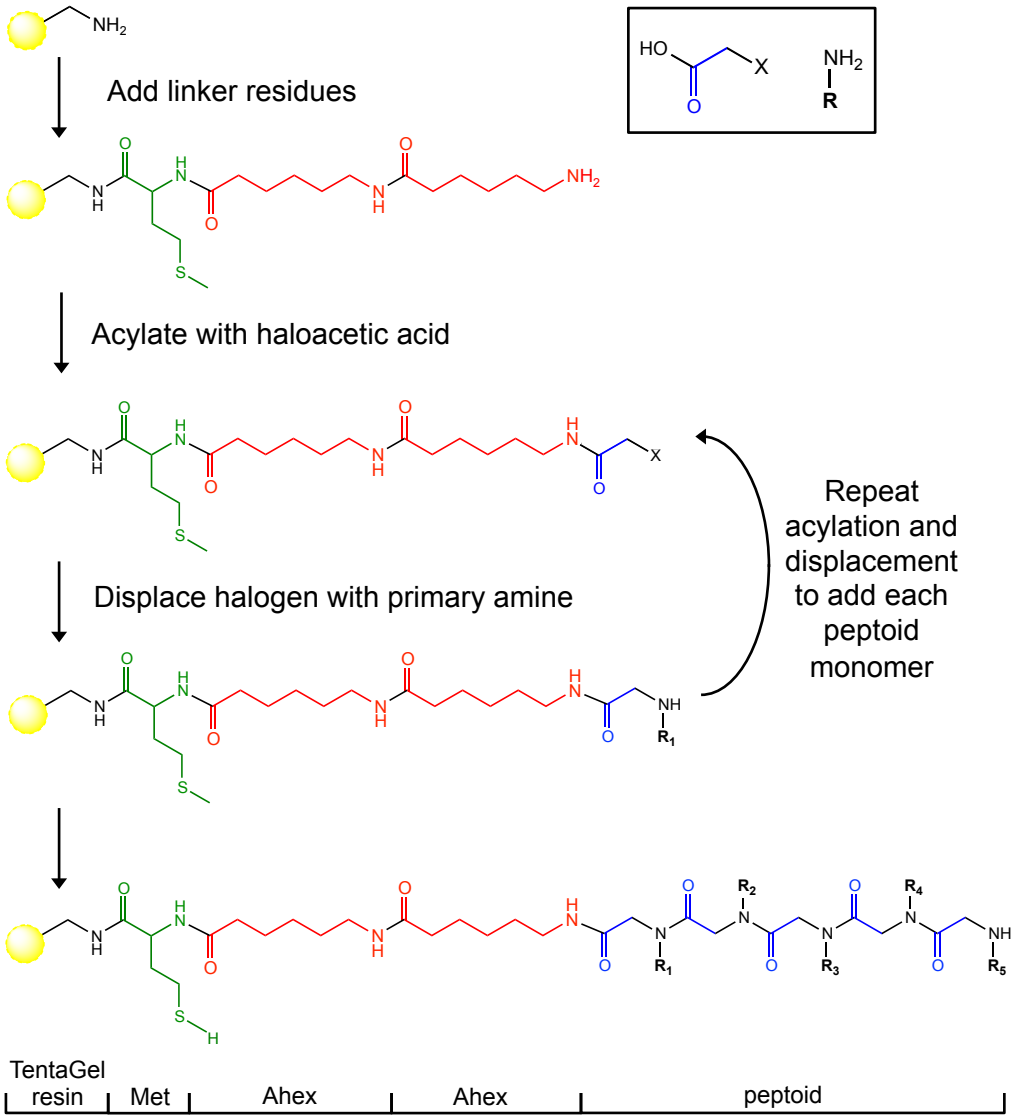


Figure 1.3. The submonomer method of peptoid synthesis.
This example shows synthesis on amino-functionalized TentaGel resin for use of peptoids in on-bead assays [see Figs. 2.1 (page 101), 2.7 (page 110), and 2.9 (page 115) and Section 2.I.C.2.b, page 56]. The cleavable linker, comprising one residue of methionine (Met; shown in green) and two residues of aminohexanoic acid (Ahex; shown in red), was synthesized on the resin first using standard 9-fluorenylmethyloxycarbonyl (FMOC) chemistry. Peptoid monomers were then added using the two-step submonomer method (219). In the first step, a haloacetic acid (inset, left-hand side) was used in an acylation reaction to provide the carbon backbone. The portion of the growing peptoid provided by the haloacetic acid is shown in blue. Bromoacetic acid (BAA) was used preferentially because it is more reactive, but the less reactive chloroacetic acid (CAA) was used to avoid undesirable side reactions

with some unprotected and/or heterocyclic peptoid side chains [(331); see Section 2.I.B.3, page 46]. In the second step, S_N2 displacement of the halogen with a primary amine (inset, right-hand side) was used to add the backbone nitrogen atom and peptoid monomer R group. The two steps of submonomer synthesis were then repeated until the desired peptoid was synthesized.

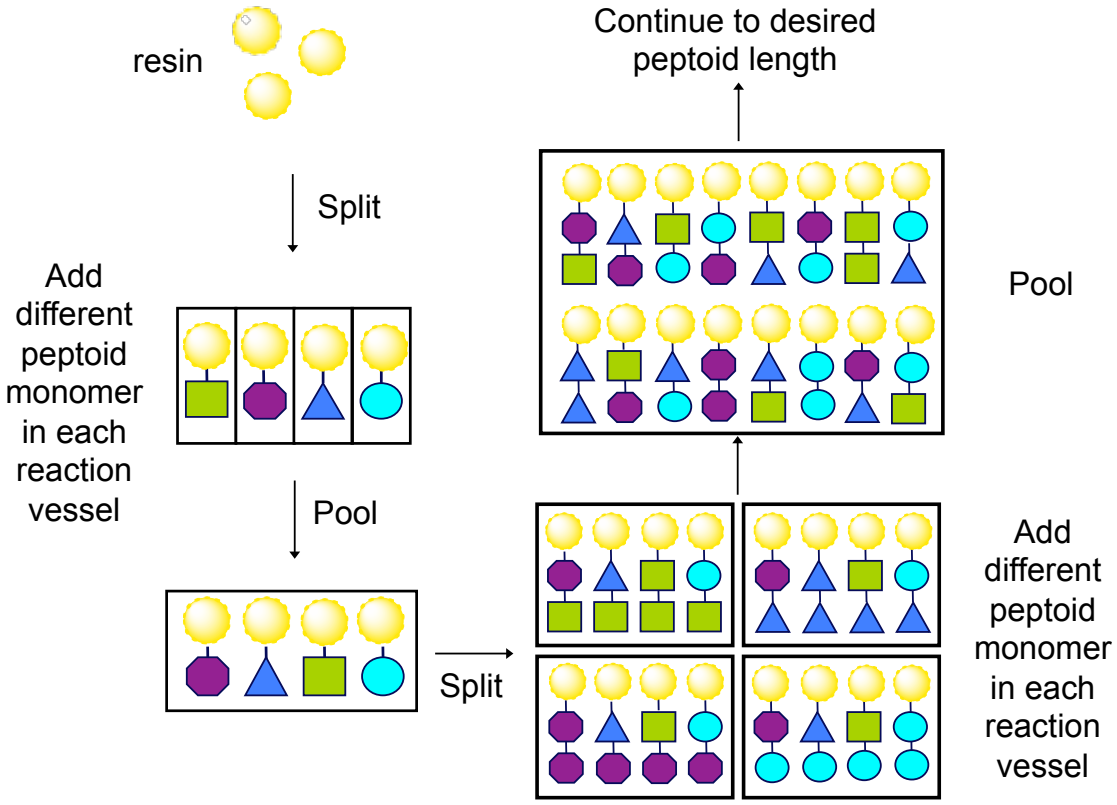


Figure 1.4. The split-pool method of peptoid library synthesis.
The split-pool method (222-224) allowed the synthesis of diverse peptoid libraries. First, the resin (yellow circles) on which the peptoid library was to be synthesized was split into as many reaction vessels as the number of peptoid monomers that were to be used at that position in the library. For example, if four peptoid monomers (represented by the green, blue, cyan, and purple shapes) were to be used at position 1 of a peptoid library, the resin would be split into four reaction vessels. One of the four peptoid monomers would then be added to the resin in each reaction vessel. Following addition of the first peptoid monomer to all vessels, the resin in each reaction vessel would then be pooled in a single, large reaction vessel and mixed. The resin would then be split randomly but evenly into the same number of reaction vessels as the number of peptoid monomers to be used at the second position in the library. One peptoid monomer would then be added to each vessel, and the split-pool method would be continued until the desired peptoid length was achieved. Libraries in hand in the Vitetta lab have ranged from 5–10 monomers in length and have used between 8–18 monomers at each library position (see Table 2.1, page 102). Notably, for some of these libraries, the resin was not pooled following the addition of the last monomer such that the last monomer in each reaction vessel was known (Table 2.1). This allowed easier sequencing of peptoid library members by MS/MS (see Sections 2.I.B.4, page 49 and 2.I.B.5, page 52). Figure adapted from Dr. Kate Yi and Ms. Angela Collins.

CHAPTER TWO

Materials and Methods

2.I. Preparation of peptoid libraries, single peptoids, and single peptides

An overview of the methods used to prepare peptoid libraries, single peptoids, and single peptides is diagrammed in Fig. 2.1 (page 101). Many of these methods have been detailed elsewhere (332) but are revised here.

2.I.A. Preparation of reagents prior to synthesis

With the exception of the two reagents described in Sections 2.I.A.1 and 2.I.A.2, reagents were used without further modification.

2.I.A.1. Protection of 2-aminoethanol with tetrahydropyran (THP)

Prior to the synthesis of Peptoid Library 1 (Table 2.1, page 102, and Fig. 2.2, page 105), 2-aminoethanol (Table 2.2, pages 103-104, and Fig. 2.2) was protected by the staff at the Molecular Foundry with THP as described (332, 333).

2.I.A.2. Preparation of glycine (Gly) *tert*-butyl ester (OtBu) free base

To use glycine as an amine in peptoid synthesis, the free base, Gly-OtBu [where OtBu indicates that the *tert*-butyl (tBu) group is protecting the side chain carboxylic acid, forming a tBu ester], was first prepared from the purchased salt, Gly-OtBu hydrochloride (HCl) (Gly-OtBu• HCl; #03072, Chem-Impex, Wood Dale, IL; see Table 2.2, pages 103-104). An appropriate mass of Gly-OtBu•HCl was weighed [*e.g.*, 15-60 grams (g)] and

dissolved in dichloromethane (DCM; #DX0835P-4, EMD Chemicals, Gibbstown, NJ, #676853, Sigma-Aldrich, St. Louis, MO, or #42006, Alfa Aesar, Ward Hill, MA) at a ratio of 15 milliliters (mL) for every 5 g of Gly-OtBu•HCl. An equimolar solution of sodium hydroxide (NaOH; #221465, Sigma-Aldrich) was then prepared in a glass bottle by dissolving the appropriate mass of NaOH in twice the volume of ultrapure water as grams of Gly-OtBu•HCl. Both solutions were added to a separatory funnel of appropriate volume (*e.g.*, 500 mL) and the solutions were mixed by gentle shaking, followed by immediate venting of the gas created. The mixing and venting was continued until no more gas was released. The funnel was then placed on a ring stand and the organic layer (bottom) was drained slowly into a flask through the stopcock of the funnel. The same volume of fresh DCM as was used initially to dissolve the Gly-OtBu•HCl was then added to the funnel and the mixing and draining was repeated. The organic (bottom) layer was collected into the same flask as previously. The process of adding DCM, mixing, and draining the organic (bottom) layer into the same flask was repeated once more for a total of three extractions. The aqueous layer left in the funnel was then poured through the top opening in the funnel into a glass bottle and retained until the end of the procedure in case of error. A second set of extractions was then performed using brine. The brine was first prepared in a glass bottle by adding sodium chloride (NaCl; #S271-3, Fisher Scientific, Pittsburgh, PA) to a volume of ultrapure water (at least as much as three times the volume of DCM used to dissolve Gly-OtBu•HCl; generally 100-500 mL). Adding NaCl was continued until as much NaCl as possible had dissolved and a layer of NaCl about 1 centimeter (cm) thick remained at the bottom of the bottle when the excess settled. The organic layer from the first set of extractions then was returned to the separatory funnel through the top opening and a volume of brine equal to that of the DCM

used to dissolve Gly-OtBu•HCl was then added. Mixing, venting, and draining of the organic (bottom) layer into a new flask was then performed. The aqueous layer remaining in the funnel was then poured through the opening at the top of the funnel into a glass bottle and retained until the end of the procedure. The organic layer was then returned to the funnel through the opening at the top and fresh brine was added. Mixing, venting, draining the organic (bottom) layer, then removing the aqueous layer were performed as previously. The organic layer was then returned to the funnel and the extraction with brine was repeated once more for a total of three extractions. To dehydrate the final organic layer, to the flask was added enough sodium sulfate (Na_2SO_4; #238597, Sigma-Aldrich) to form a layer approximately 0.5-1 cm thick. The contents of the flask were mixed by shaking and, after allowing the mixture to stand for several minutes, the supernatant was then removed with a glass serological pipet and transferred to a weighed round-bottom flask. The solvent was then removed with a rotary evaporator (Buchi, Flawil, Switzerland). Removal was determined to be complete when no more solvent was evaporating and the mass of the flask contents was equal to or less than the calculated mass of Gly-OtBu in the starting material.

2.I.B. Preparation of OBOC peptoid libraries

2.I.B.1 Overview

On-bead peptoid libraries (Table 2.1, page 102) were synthesized by solid-phase synthesis from carboxy (C) terminus to amino (N) terminus on amino-functionalized TentaGel resin composed of polystyrene and polyethylene glycol. Since this resin does not include a linker to facilitate eventual cleavage of synthesized compounds from the resin for analysis, a methionine residue was first added to allow cleavage using a cyanogen bromide (CNBr) solution. One or two additional residues, either peptoid monomers or amino acids,

were then added to act as a spacer between the resin and the desired peptoid library. Together with the methionine residue, the spacer residue(s) provided a known mass to facilitate downstream sequencing of peptoids by MS/MS. Peptoid monomers were then added to construct the variable portion of each member of the peptoid library using a split-pool technique (222-224) (Fig. 1.4, page 37): 1.) the library resin was split equally into as many vessels as peptoid monomers that were used at that position in the growing peptoid sequences; 2.) one peptoid monomer was added to the resin in each vessel; 3.) the resin from all vessels was pooled in a single large vessel; and 4.) the resin was split randomly but evenly back into separate vessels as in Step 1. This process was repeated until the monomers at the last position in the library were added, when the resin was either pooled or allowed to remain as separate aliquots according to the last monomer added. In the latter case, the last monomer added served as a known mass to facilitate sequencing by MS/MS.

2.I.B.2. Using the automated synthesizers at the Molecular Foundry: Peptoid Libraries 1 and 5

Peptoid Library 1 was designed by Mr. Michael Connolly and Dr. Ronald Zuckermann and synthesized by Dr. Allison Case and Ms. Angela Collins. Peptoid Library 5 was designed by Mr. Michael Connolly, Dr. Allison Case, and me and synthesized by Dr. Allison Case and me. High loading, amino-functionalized TentaGel resin [TentaGel Macrobeads HL MB-NH_2, #MB 160 002; particle size 140-170 micrometers (μm); resin capacity 0.4-0.6 millimoles/gram (mmol/g) or 0.86 nanomoles/bead (nmol/bead); 520,000 beads/g, Rapp Polymere GmbH, Tuebingen, Germany] was equilibrated to room temperature (RT), aliquots were weighed [ten 300 milligram (mg) aliquots for Library 1, eight 500 mg aliquots for Library 5], and the aliquots were poured into fritted glass reaction

vessels (Adams and Chittenden Scientific Glass, Berkeley, CA) on a custom-built automated synthesizer (Molecular Foundry, Lawrence Berkeley National Laboratory, Berkeley, CA) according to the split-pool technique described in Section 2.I.B.1 (page 40) and Fig. 1.4 (page 37). A disposable transfer pipet was used to deliver *N,N*-dimethylformamide (DMF; #BDH1117, VWR, West Chester, PA) to wash the resin down to the base of the reaction vessels. All subsequent steps were performed by the automated synthesizer under a blanket of nitrogen gas (N_2) with the reaction block containing the reaction vessels heated to 35 degrees Celsius (°C). Unless otherwise noted, the automated synthesizer was programmed using its attached computer to perform all steps, including delivery of solutions from glass bottles connected to its robotic arm *via* plastic tubing.

At the start of the synthesis program, the DMF used to wash the resin to the base of the reaction vessels was drained under vacuum into a waste container using the automated synthesizer's attached vacuum pump. The resin was then swollen in DMF at a ratio of 40 mL/mmol of resin capacity for 5 minutes (min) with periodic mixing by N_2 bubbling, and drained as previously.

In the first monomer addition to the resin, a methionine residue was added using 9-fluorenylmethyloxycarbonyl (FMOC) chemistry as follows. A solution of 0.4 Molar (M) methionine protected at its amino group with an FMOC group (FMOC-L-methionine, Met, #FM2400, Advanced ChemTech, Louisville, KY) was prepared by dissolving FMOC-L-methionine in 0.4 M 1-hydroxybenzotriazole monohydrate (HOBt; #CXZ010, AAPPTec, Louisville, KY) in *N*-methyl-2-pyrrolidinone (NMP; #RP8310, Advanced ChemTech) by vortex mixing and sonication (#FS-9H, Solid State Ultrasonic, Fisher Scientific). This solution was added to the synthesizer in 50 mL conical tubes [#352070, Beckton Dickinson Labware, Franklin Lakes, NJ], aspirated by the robotic arm of the synthesizer and delivered to the

resin at a ratio of 20 mL/mmol of resin capacity. A solution of 50% (volume/volume) (v/v) 1,3-diisopropylcarbodiimide (DIC; #RC8120, Advanced ChemTech) in DMF was also added to the resin at a ratio of 2.84 mL/mmol of resin capacity. The resin was mixed by periodic N_2 bubbling for 1 hour (h) before the vessels were drained and the resin was washed five times with DMF at a ratio of 40 mL/mmol of resin capacity for 1 min with periodic mixing by N_2 bubbling. The FMOC protecting group was then removed from the methionine residue by adding 20% (v/v) 4-methylpiperidine (4-MP; #M73206, Sigma-Aldrich) in DMF at a ratio of 40 mL/mmol of resin capacity. The resin was mixed by periodic N_2 bubbling for 5 min before the vessels were drained and the FMOC deprotection step was repeated with the incubation time extended to 12 min. The resin was then washed five times with DMF as previously. This constituted one amino acid addition cycle.

To provide a spacer between the resin and the peptoid sequence to be added, and, along with the methionine residue, provide a known mass to facilitate sequencing, two residues of FMOC-6-aminohexanoic acid (Ahex; #02490, Chem-Impex) were then added using similar amino acid addition cycles.

The first library peptoid residue was then added using the two-step submonomer synthesis method (219) (Fig. 1.3, page 35). In the first step, the peptoid monomer's carbon backbone was added to the resin in an acylation reaction. A solution of 0.6 M bromoacetic acid (BAA; #259357, Sigma-Aldrich) in DMF was added to the resin at a ratio of 17 mL/mmol of resin capacity, and a solution of 50% (v/v) DIC in DMF was added to the resin at a ratio of 2.84 mL/mmol of resin capacity. The resin was mixed for 20 min with periodic N_2 bubbling before the vessels were drained and the resin washed with DMF as previously. In the second step of submonomer synthesis, the backbone nitrogen and the side chain of the peptoid monomer were added by nucleophilic S_N2 displacement of the bromine with a

primary amine. Solutions of various primary amines (described in Table 2.2, pages 103-104 and shown in Figs. 2.2, page 105 and 2.3, page 106) were prepared at 1.0 M in DMF or, as noted in Table 2.2, in dimethylsulfoxide (DMSO; #D4540, Sigma-Aldrich or similar) or NMP in 50 mL conical tubes, added to the automated synthesizer, aspirated by its robotic arm, and delivered to the resin at a ratio of 17 mL/mmol of resin capacity in the appropriate vessels according to the split-pool technique described in Section 2.I.B.1 (page 40) and Fig. 1.4 (page 37). The resin was mixed for 1 h by periodic N_2 bubbling before the vessels were drained and the resin washed with DMF as previously. This constituted one cycle of the peptoid submonomer synthesis method.

To pool and split the resin prior to the next submonomer synthesis cycle to generate a peptoid library, the resin was pooled from each vessel, mixed, and split again as an isopycnic slurry in 65% (v/v) 1,2-dichloroethane (DCE; #E175-4, Fisher Scientific, Pittsburgh, PA) in DMF by the automated synthesizer for Peptoid Library 1, or for Peptoid Library 5, manually. In the latter case, the majority of the resin was scooped from the vessels using a disposable spatula (#80081-188, VWR) and collected into a 1 L fritted glass reaction vessel (Adams and Chittenden), which was transferred to a chemical hood and mounted on a ring stand. To wash the resin to the base of the reaction vessel, 200 mL of DMF were added, and drained under vacuum through the stopcock at the base of the reaction vessel. To mix the resin, 200 mL of 65% (v/v) DCE in DMF were added to form an isopycnic slurry and mixed for 5 min by N_2 bubbling through the second port of the two-way stopcock at the base of the vessel. The solution was then drained under vacuum. This mixing step was then repeated before 300 mL DCM were added and the resin was mixed for 5 min by N_2 bubbling. The solution was then drained, collected, and used to repeat the DCM wash step twice more. The resin was then dried under vacuum for 1 h. Meanwhile, the

residual resin remaining in the automated synthesizer reaction vessels was washed to the base of the reaction vessels with DMF delivered via a large disposable transfer pipet (#16001-196, VWR). A small transfer pipet was then used to transfer the resin into a 5 mL fritted disposable reaction vessel (Applied Separations, Allentown, PA) that was drained continuously by gravity into a glass bottle. This resin was then washed three times with DCM by filling the fritted syringe with DCM delivered by a wash bottle before being drained, dried under vacuum for 1 h, and combined with the dried resin from the glass reaction vessel. The resin was then divided into equal portions by mass and split into the appropriate reaction vessels on the automated synthesizer according to the split-pool technique described in Section 2.I.B.1 (page 40) and Fig. 1.4 (page 37). Five milliliters of DMF were added by transfer pipet to wash the resin down to the base of the reaction vessels, and the vessels were then drained by the automated synthesizer for 15 min.

This process of peptoid monomer addition followed by pooling and splitting continued until the desired peptoid length had been reached, with the omission of the resin pooling step following the last synthesis cycle. To remove the resin from the automated synthesizer at the completion of synthesis, a disposable spatula was used to collect the majority of the resin from each synthesizer reaction vessel into its own 5 mL disposable fritted reaction vessel before a disposable transfer pipet was used to deliver DMF and transfer any remaining resin into its disposable reaction vessel. The resin was then drained of DMF under vacuum. To dry the resin, DCM was added, and then drained under vacuum prior to placing the reaction vessels in 50 mL conical tubes for storage at 4°C.

2.I.B.3. Manually at the University of Texas Southwestern Medical Center (UTSW): Peptoid Libraries 2, 3, 4, and 6

Manual synthesis of peptoid libraries was performed by Dr. Sara Chirayil in consultation with me (Peptoid Libraries 2, 3, and 4) and with Dr. Allison Case (Peptoid Library 6) in a similar manner with several modifications.

First, resin was swollen in DMF by adding the total mass of resin to be used in library synthesis to one glass fritted reaction vessel (25 mL peptide synthesis reaction vessel for Libraries 2, 3, and 4, 250 mL for Library 6; Chemglass Life Sciences, Vineland, NJ), adding DMF (40 mL for Library 6), and allowing the resin to swell overnight at RT. The DMF was then drained through the reaction vessel stopcock under vacuum.

To add methionine to the resin as the first monomer, a solution of 0.2 M FMOC-L-methionine in 0.2 M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; #21001, AnaSpec, Fremont, CA) and HOBt in DMF with 0.4 M N-methylmorpholine (NMM; #M56557, Sigma-Aldrich) was prepared in a 50 mL conical tube. Fifteen milliliters of this solution (for Library 6) were added to the reaction vessel by pipetting and the reaction was mixed by orbital shaking (MaxQ 2000, Fisher Scientific) at 180 revolutions per min (rpm) for 1 h at RT. The solution was drained and the resin was washed six times with DMF (25 mL for Library 6) delivered *via* a wash bottle. The FMOC deprotection step was then performed using 20% (v/v) piperidine (#411027, Sigma-Aldrich) in DMF for 15 min at RT with orbital shaking as previously, and the resin was washed with DMF as previously. This constituted one amino acid addition cycle.

To serve as a spacer and, with the methionine residue, as a known mass, peptoid monomers as indicated in Table 2.1 (page 102; amines themselves are described in Table 2.2, pages 103-104 and shown in Figs. 2.4, page 107, 2.5, page 108, and 2.6, page 109)

were added using the two-step submonomer synthesis method as follows. In the first step, chloroacetic acid (CAA; #402923, Sigma-Aldrich) was used in an acylation reaction to add the peptoid monomer's carbon backbone to the resin. A solution of 0.4 M CAA in anhydrous DMF (#61032-0010, Acros Organics, Geel, Belgium, or #227056, Sigma-Aldrich) was prepared and, for Library 6, 25 mL (10 mmol CAA) were added to the reaction vessel by pipetting. A solution of 2.0 M DIC in anhydrous DMF was also prepared and, for Library 6, 6 mL (2 mmol DIC) were added to the reaction vessel by pipetting. The reaction was allowed to proceed for 10 min at 37°C with orbital shaking (MaxQ 4000, Thermo Scientific) before the solution was drained under vacuum and the resin washed with DMF as previously. In the second step of the submonomer synthesis method, each amine was prepared as a 2.0 M solution in anhydrous NMP (#328634, Sigma Aldrich) and, for Library 6, 20 mL were delivered to the reaction vessel by pipetting. The reaction proceeded for 1 h at 37°C with orbital shaking before the solution was drained under vacuum and the resin was washed with DMF as previously. This constituted one cycle of peptoid submonomer synthesis.

To generate a peptoid library by the split-pool technique as described in Section 2.I.B.1 (page 40) and in Fig. 1.4 (page 37), prior to the next submonomer synthesis cycle, the resin was transferred (by scooping with a spatula and using DMF delivered from a wash bottle) to a pre-weighed beaker. The resin was then distributed evenly by weight into as many additional fritted glass reaction vessels (100 mL size for Library 6) as the number of different peptoid monomers to be used at that position in the library. The first library peptoid residue was then added according to the submonomer synthesis method as described above for the addition of the spacer peptoid monomers to the pooled resin, but the volume of reagents used for the pooled resin was divided by the number of reaction vessels into

which the resin had been split. The resin was then pooled into the initial glass reaction vessel by pouring and washing using a stream of DMF delivered from a wash bottle. Approximately 25 mL of anhydrous DMF were then added, the resin was mixed by N_2 bubbling for 5-15 min, and the resin was split as previously.

This process was continued until the desired peptoid length had been reached, except in the case of Library 6, where the resin pooling step following the last peptoid synthesis cycle was omitted. To store the library overnight during synthesis, the resin was placed at 4°C. Following the completion of library synthesis, the resin was washed six times with DMF as previously before being washed 2-3 times with DCM. Libraries 2, 3, 4, and 6 were then stored in 100% ethanol (#6505-00-105-0000, Midwest Grain Products of Illinois, Pekin, IL) at 4°C in their glass reaction vessels with the caps and stopcocks closed. Libraries 2, 3, and 4 were protected from light with aluminum foil. Periodically, additional ethanol was added to replace what had evaporated. For Library 6, a sub-aliquot was removed from each reaction vessel by mixing with N_2 bubbling for screening with MAb anti-FLAG (332). The remaining resin from each reaction vessel was then removed from the glass reaction vessels for storage. Transfer pipets (Corning Samco Corp., San Fernando, CA or similar) were used to transfer the majority of the resin in each reaction vessel in 100% ethanol to its own 50 mL conical tube. A transfer pipet was then used to add DMF to the reaction vessel, aspirate the remaining resin, and transfer it to a 5 mL disposable fritted reaction vessel (#NC9160511, Fisher Scientific) attached to a needle (#305196, BD, Franklin Lakes, NJ) and allowed to drain by gravity into a vacuum flask. The remaining liquid was drained from the disposable reaction vessel under vacuum, DCM (#676853, Sigma-Aldrich, or #42006, Alfa Aesar, Ward Hill, MA) was added to the reaction vessel, and the

resin was drained and dried under vacuum. Once dry, the resin was scooped using a disposable spatula into the 50 mL conical tube containing the bulk of the resin in 100% ethanol. Additional ethanol was added to the resin and the 50 mL conical tubes of resin were stored at 4°C.

2.I.B.4. Quality control of peptoid libraries: test deprotection using trifluoroacetic acid (TFA), cleavage using CNBr, and sequence determination by MS/MS

To verify the synthesis of the desired peptoid library, small amounts of beads from each library aliquot (for libraries in which resin remained separated by last monomer, as described in Table 2.1, page 102) or from the whole, pooled library were transferred into 1.5 mL or 0.5 mL microfuge tubes using a pipet tip or spatula and subjected to test deprotection, cleavage, and sequencing by MS/MS as follows. For Library 1, verification of synthesis was performed by Dr. Allison Case and Ms. Angela Collins as described previously (332). For Libraries 2, 3, 4, and 6, verification of synthesis was performed by Dr. Sara Chirayil using a procedure similar to that described here. For Library 5, verification of synthesis was performed by Dr. Allison Case and me. Protecting groups present on amino acid and peptoid monomer side chains were first removed using a solution of 94% (v/v) TFA (#BP618, Fisher Scientific), 2.5% (v/v) ultrapure water, 1% (v/v) triisopropyl silane (TIS; #233781, Sigma-Aldrich), and 2.5% (v/v) ethanedithiol (EDT; #02390, Sigma-Aldrich). The water, TIS, and EDT in this solution serve as scavengers to prevent protecting groups from reattaching to the peptoid. Following incubation with orbital shaking for 1 h, the beads were pelleted by centrifugation and the supernatant was removed by pipetting. The beads were then washed several times in a solution of 50% (v/v) acetonitrile (ACN; #A998-4, Fisher

Scientific) in ultrapure water by adding the wash solution and briefly vortexing. The beads were pelleted by centrifugation, and the supernatant was removed by pipetting.

To cleave the library peptoids from individual beads, the beads were then transferred by pipetting to a tissue culture dish (#3002, Becton Dickinson Labware, Lincoln Park, NJ) containing 50% (v/v) ACN in ultrapure water, or additional 50% ACN in ultrapure water was added directly to the microfuge tube. The beads were visualized under a light microscope (Stereomaster, #1256212, Fisher Scientific; Diaphot, Nikon, Garden City, NY; or CK2, Olympus, Tokyo, Japan) and individual beads were transferred using a P2 pipet in a minimal volume of solvent [1 microliter (μL) or less] to 0.5 mL microfuge tubes. To cleave peptoids from the resin, a solution of 2-5 mg/mL CNBr (#C91492, Sigma-Aldrich) was prepared in 50% (v/v) ACN in 0.25 N HCl (#H613-05, Mallinckrodt, Phillipsburg, NJ or #HX0603, EM Science, Gibbstown, NJ) in ultrapure water. Twenty microliters of this solution were added to each 0.5 mL microfuge tube containing a bead, and the cleavage reaction was incubated for 3-22 h. To remove the CNBr solution, 0.5 mL tubes were opened to the air (in a chemical hood) until the solution evaporated or the solution was evaporated using a vacuum concentrator (at the Molecular Foundry, Vacufuge Concentrator, #022820109, Eppendorf North America, Hauppauge, NY) heated to 45°C.

To determine the peptoid sequence from individual beads, analysis by matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) mass spectrometry (MS) using a 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX, Foster City, CA) in reflector positive mode was performed as follows. MALDI matrix was first prepared by allowing α-cyano-4-hydroxycinnamic acid (CHCA; #476870, Sigma-Aldrich) to equilibrate to RT from storage at -20°C and several milligrams were weighed in a 0.5 mL microfuge tube

using a disposable microspatula (#80081-194, VWR) for transfer. Solutions of 0.1% (v/v) TFA in ultrapure water and 0.1% (v/v) ACN in ultrapure water were prepared and added to the CHCA by pipetting such that a solution of 10 mg/mL CHCA in 50% (v/v) ACN in ultrapure water with 0.1% (v/v) TFA resulted. The solution was mixed by vortexing for 1 min or sonication in an ultrasonic cleaner (#08895-04, Cole Parmer Instrument Co., Vernon Hills, IL) for 30 seconds, followed by centrifugation at approximately 425-2650 times gravity (x *g*) for 1 min or allowing the tube to stand upright for 5 min so that undissolved CHCA could settle. The matrix tube was then wrapped in aluminum foil and stored at 4°C. Generally, fresh matrix was prepared if the matrix was more than one week old.

If MS/MS was performed before the CNBr cleavage solution had evaporated, 1 μL of the CNBr solution containing cleaved peptoid was mixed by pipetting with 1 μL of CHCA matrix in a 0.5 mL microfuge tube. If the CNBr cleavage solution had evaporated, the cleaved peptoid remaining in the 0.5 mL microfuge tube was resuspended in 5 μL 50% (v/v) ACN in ultrapure water and 1 μL of this solution was mixed with 1 μL CHCA matrix. One microliter of the peptoid/CHCA matrix mixture was then spotted by pipetting onto an Opti-TOF 384-well insert (#1016629, AB SCIEX) and allowed to air-dry. This process was repeated for each peptoid sample to be analyzed.

To determine the mass of unknown library peptoids, an appropriate mass range was first estimated for MS as follows. The mass of the lowest mass peptoid library monomer was multiplied by the length of the library peptoid and added to the mass of the linker (cleaved methionine plus spacer) to determine the theoretical lowest mass of the desired peptoids synthesized. This process was repeated for the peptoid library monomer with the highest mass. The mass of protecting groups was also considered in case the protecting groups had

not been fully removed during the deprotection step. Finally, the mass of truncated peptoids was considered as no chemical reaction proceeds with 100% efficiency. The mass-to-charge ratio (m/z) of the cleaved peptoid was then determined using 4000 Series Explorer software (AB SCIEX). Peaks in the MS spectrum were then selected for further analysis by MS/MS. The spectra were printed directly or exported to and printed from Data Explorer software (AB SCIEX). To determine peptoid sequences, the unique mass of each peptoid monomer within a peptoid library (Table 2.2, pages 103-104 and Figs. 2.2-2.6, pages 105-109) was used to assign peptoid monomer identity to the difference calculated using a handheld calculator or Microsoft Excel between peaks in each MS/MS spectrum. The quality of peptoid library synthesis was judged to be adequate when single peaks of appropriate m/z were observed from MS spectra and when approximately 80% of the peptoids cleaved from the beads could be sequenced.

2.I.B.5. Bulk deprotection of peptoid libraries

Prior to the use of peptoid libraries or portions of peptoid libraries in screening assays (described below), protecting groups on peptoid libraries were removed. Bulk deprotection of Peptoid Library 1 was performed by Dr. Allison Case as described previously (332). For Library 3, this was performed by Dr. Kate Yi, and for sub-aliquots of Library 6, by Mr. Stephen Ruback. The remaining libraries have not been deprotected as storage in the protected state is preferred. Bulk deprotection was performed as described in Section 2.I.B.4 (page 49), with the following modifications: deprotection using TFA was carried out in 5 mL disposable reaction vessels (#99.274, Intavis, Cologne, Germany, or #NC9160511, Fisher Scientific) or 10 mL disposable reaction vessels (#NC9003437, Fisher Scientific); sub-aliquots (approximately 250 μL bead volume) from Library 6 were washed to remove

the storage solution with 6 mL DMF three times by allowing the DMF to stand for 2 min, followed by drainage under vacuum for 2 min, then with 6 mL DCM three times by allowing the DCM to stand for 2 min and draining it under vacuum for 2, 5, and 30 min to dry the resin; and following incubation with the TFA solution, the solution was expelled from the reaction vessels using the included plungers, and the resin was then washed with DMF and dried with DCM. To ensure that several screening antibodies could be used to screen fresh peptoid library resin, the dried resin from each aliquot from Library 1 was split into 5 sub-aliquots (see Table 2.1, page 102) and stored in 1.5 mL microfuge tubes at 4°C before use. To verify that deprotection of side groups occurred correctly, the library was again subjected to quality control as described in Section 2.I.B.4 (page 49), beginning with the distribution of single beads to 0.5 mL microfuge tubes for CNBr cleavage, before further use.

2.I.C. Preparation of single peptides and peptoids

2.I.C.1. Overview

In the absence of a peptoid and an antibody raised against it with which to optimize assays for peptoid vaccine development, FLAG peptide (334) and a commercially available mouse monoclonal antibody (MAb) against it (clone M2, #F3165 or #F1804, Sigma-Aldrich) (335) were chosen for initial experiments, as described previously (332), by Mr. Michael Connolly and Dr. Ronald Zuckerman with Dr. Allison Case and Ms. Angela Collins, the first members of the Vitetta lab to go to the Molecular Foundry. During this trip, an alanine replacement peptide to which MAb anti-FLAG did not bind (336) was also adopted as a negative control. To indicate this difference in binding to MAb anti-FLAG, FLAG peptide (from N terminus to C terminus, where D is L-aspartic acid, Y is L-tyrosine, and K is L-lysine: N-DYKDDDDK-C) was designated **FLAG$^+$ peptide** and the L-alanine replacement peptide

(N-DAKDDDDK-C) was designated **FLAG$^-$ peptide**. To refine the screening assays used in peptoid vaccine development, a peptoid was designed by the peptoid team, myself included, and was used to generate the first affinity-purified anti-peptoid antibody. This peptoid was five monomers in length (Figs. 2.7, page 110 and 2.8, page 112) and was designated **rabbit 5-mer** or **R5 peptoid** to indicate that the peptoid would be used to immunize rabbits with the goal of generating antibodies. An additional peptoid was also designed to serve as a negative control and was designated **rabbit control** or **RC peptoid** (Figs. 2.9, page 115 and 2.10, page 116). The first portion of this study has been described previously (332); further details appear in Section 2.III (page 80) and 2.IV (page 95).

For use in on-bead screening assays, single peptides and peptoids were synthesized on TentaGel resin (for R5 peptoid, see Fig. 2.7, page 110, and for RC peptoid, see Fig. 2.9, page 115), as were on-bead peptoid libraries (described in Sections 2.I.B.2, page 41 and 2.I.B.3, page 46). To synthesize peptides and peptoids for attachment to maleimide-activated carrier proteins or to affinity purification column resin, synthesis on Rink Amide resin was employed (for R5, see Fig. 2.8, page 112 and for RC, see Fig. 2.10, page 116). This resin bears the Rink Amide linker, which allows simultaneous cleavage of the peptide or peptoid from the resin and removal of protecting groups from amino acid and/or peptoid monomer side chains using the TFA deprotection solution described in Section 2.I.B.4 (page 49).

2.I.C.2. Preparation of single peptides and peptoids on TentaGel resin

2.I.C.2.a. Synthesis of single peptides on TentaGel resin

Synthesis of single peptides (FLAG$^+$ and FLAG$^-$) on TentaGel resin, at the Molecular Foundry by Dr. Allison Case and Ms. Angela Collins was performed as described in Section

2.I.B.2 (page 41) for peptoid libraries, with the FMOC chemistry used to add the linker amino acids to peptoid library resin extended here to each peptide monomer, and with the omission of the split-pool technique (332).

At UTSW, single peptides ($FLAG^+$ and $FLAG^-$) were synthesized on TentaGel resin initially by Ms. Angela Collins and Dr. Allison Case as described previously (332). Later, these were synthesized by me using a Titan 357 automated synthesizer (AAPPTec, Louisville, KY), or by a combination of automated and manual synthesis. Synthesis using the Titan 357 was performed as for automated synthesis at the Molecular Foundry with the following modifications: the reactions took place at RT; the DMF used was as above or #DX1730-1, EMD Chemicals; all mixing was accomplished by the vortex motor of the reaction block at 650 rpm; DMF and 20% (v/v) 4-MP in DMF were delivered to the reaction vessels by robotic arms 1 and 2, respectively, from glass bottles outside the synthesizer but attached to the synthesizer *via* plastic tubing; 50% (v/v) DIC in DMF and 0.6 M BAA in DMF were placed in glass bottles or, in some instances for BAA, in a 50 mL conical tube, and aspirated by robotic arm 1 for delivery to the reaction vessels; and slightly different ratios of reagent volume to mmol of resin capacity were used [DMF for swelling and washing, 36 mL/mmol of resin capacity; 0.4 M FMOC-amino acids in 0.4 M HOBt in NMP, 18 mL/mmol of resin capacity; 50% (v/v) DIC in DMF, 2.55 mL/mmol of resin capacity; and 20% (v/v) 4-MP in DMF, 36 mL/mmol of resin capacity].

For manual synthesis of single peptides ($FLAG^+$ and $FLAG^-$) at UTSW, the following changes from automated synthesis using the Titan 357 were made: synthesis was conducted in 5 mL disposable fritted reaction vessels (#NC9160511, Fisher Scientific); mixing was accomplished by orbital shaking at RT in a ROSI 1000 Reciprocating/Orbital Shaking Incubator (Thermolyne, Dubuque, IA) at approximately 200 rpm for all steps other

than washing with DMF, during which the reaction vessels were shaken manually; reagents were drawn into and expelled from the reaction vessels through an attached needle (#305196, Beckton Dickinson) using the reaction vessel plunger; and the ratios of reagent volume to mmol of resin capacity used were the same as for the addition of linker amino acids to peptoid libraries using the automated synthesizers at the Molecular Foundry.

The following additional FMOC-protected amino acids were used in the synthesis of $FLAG^+$ and $FLAG^-$ peptides: FMOC-L-aspartic acid(OtBu)-OH (Asp, D; #20124, AnaSpec); FMOC-L-tyrosine(tBu)-OH (Tyr, Y; #00495, Chem-Impex), where tBu indicates a tBu group protecting the side chain alcohol; FMOC-L-alanine-OH (Ala, A; #8.52003.0100, Novabiochem, EMD Millipore, Billerica, MA); and FMOC-L-lysine(Boc)-OH (Lys, K; #8.52012.0025, Novabiochem, or #AFK105, AAPPtec, Louisville, KY), where Boc indicates a *tert*-butyloxycarbonyl (Boc) group protecting the side chain amine.

At the completion of synthesis, on-bead peptides synthesized using the automated synthesizer were transferred to 5 mL disposable reaction vessels (#NC9160511, Fisher Scientific) using a metal spatula, while on-bead peptides synthesized manually remained in their disposable reaction vessels. The reaction vessels were transferred to a chemical hood and the resin washed with DMF and dried with DCM as described in Section 2.I.B.5 (page 52) for Library 6 sub-aliquots. Following the last wash, the disposable reaction vessels were covered with parafilm or placed in 50 mL conical tubes with or without blanketing with argon gas (#AR UHP300, Airgas, Radnor, PA) and stored at 4°C.

2.I.C.2.b. Synthesis of single peptoids on TentaGel resin

Single peptoids were synthesized on TentaGel resin using an automated synthesizer, manually, or by a combination of the two as described for single peptides, with

several modifications. For manual synthesis, either 5 mL reaction vessels were used as for single peptides when 100 mg of resin were used per reaction vessel, or 10 mL disposable reaction vessels (#NC9003437, Fisher Scientific) were used when 250 mg of resin were used per reaction vessel. Linker amino acids (Met and two Ahex residues) were added to TentaGel resin prior to the synthesis of peptoid residues as described for amino acid residues in single peptides (Section 2.I.C.2.a, page 54). For peptoid residues added using the Titan 357, the peptoid-specific solutions were added using the following ratios of reagent volume to mmol of resin capacity: 0.6 M BAA in DMF, 16 mL/mmol of resin capacity; and 1 M amines in the solvents listed in Table 2.2 (pages 103-104), 18 mL/mmol of resin capacity. For peptoid residues added manually, the ratios of reagent volume to mmol of resin capacity used were the same as for peptoid library synthesis at the Molecular Foundry. Following synthesis, peptoids were washed with DMF and DCM, dried, and stored as described for single peptides in Section 2.I.C.1.a.

2.I.C.2.c. Quality control of single peptides and peptoids synthesized on TentaGel resin

To verify that the desired peptides or peptoids had been synthesized on TentaGel resin, a small amount of beads from each reaction vessel was subjected to test deprotection, cleavage, and sequencing by MS/MS as described in Section 2.I.B.4 (page 49) for peptoid libraries, with the modification that quality of synthesis was judged to be adequate when peaks were present in MS spectra at the correct m/z and when the desired sequence was determined from MS/MS spectra as described for peptoid libraries. Verification of synthesis for peptides synthesized at the Molecular Foundry was performed by Dr. Allison Case and Ms. Angela Collins as described previously (332).

2.I.C.2.d. Bulk deprotection of single peptides and peptoids synthesized on TentaGel resin

Prior to the use of on-bead peptides or peptoids in screening assays (described below), protecting groups were removed as described in Section 2.I.B.5 (Bulk deprotection of peptoid libraries, page 52), beginning with the addition of TFA solution. Bulk deprotection for peptides synthesized at the Molecular Foundry was performed by Dr. Allison Case and Ms. Angela Collins as described previously (332).

2.I.C.3. Preparation of single peptides and peptoids on Rink Amide resin

2.I.C.3.a. Synthesis of single peptides on Rink Amide resin

Synthesis of single peptides ($FLAG^+$ and $FLAG^-$) on polystyrene A Rink Amide resin (#HA 500560 23; particle size 500-560 µm; resin capacity 0.4-0.7 mmol/g or 80-110 nmol/bead; 11,500 beads/g; Rapp Polymere) was performed as described for single peptides synthesized on TentaGel resin at UTSW (Section 2.I.C.2.a, page 54), with several modifications. Following swelling in DMF, the FMOC group present on the Rink Amide resin was removed with 20% (v/v) 4-MP in DMF as described for deprotection of FMOC-protected amino acids in Section 2.I.B.2 (page 41) with the modification listed for synthesis at UTSW in Section 2.I.C.2.a. To facilitate downstream conjugation to carrier proteins or coupling to column resin via its sulfhydryl group, a cysteine (Cys) residue with its sulfhydryl protected with a triphenylmethyl (trityl or Trt) group was then added as the first monomer [FMOC-cysteine(Trt); #AFC105, AAPPTec], using FMOC chemistry as described in Section 2.I.C.2.a (page 54), followed by the synthesis of the desired peptide in the same manner.

2.I.C.3.b. Synthesis of single peptoids on Rink Amide resin

Synthesis of single peptoids (R5 and RC) was performed as described in Section 2.I.C.3.a (page 58) for single peptides synthesized on Rink Amide resin, with the modification that peptoid residues were added as described for single peptoids synthesized on TentaGel resin (Section 2.I.C.2.b, page 56). Synthesis as well as quality control (Section 2.I.C.3.c, page 59) and bulk deprotection (Section 2.I.C.3.c) was performed as described previously (332) by Dr. Allison Case at UTSW and by Dr. Allison Case and me during the early portion of the rabbit study (see Section 2.III.A, page 80). Later synthesis at UTSW was performed by me.

2.I.C.3.c. Quality control of single peptides and peptoids synthesized on Rink Amide resin

To verify that the desired peptides or peptoids had been synthesized on Rink Amide resin, small amounts of beads from each reaction vessel were placed in 1.5 mL or 0.5 mL microfuge tubes and subjected to test deprotection and simultaneous cleavage from the resin. A TFA solution was prepared as described for the deprotection of compounds synthesized on TentaGel resin (Section 2.I.C.2.c, page 57), applied to the resin, and incubated for 20 min with orbital shaking as described in Section 2.I.C.2.c. A stream of argon or nitrogen (#NI NF300, Airgas) gas was then used to evaporate the TFA solution, and the cleaved peptide or peptoid was resuspended in 20 μL of 50% (v/v) ACN in ultrapure water. If necessary, the resin was pelleted by pulse centrifugation (Picofuge, #400550, Stratagene, La Jolla, CA) before the supernatant was analyzed by MS/MS as described for compounds synthesized on TentaGel resin (Section 2.I.C.2.c). If additional peaks, representing side products, were present in the MS spectra, subsequent steps would still be

carried out as the purity of the desired peptide or peptoid would again be assessed following purification by reverse phase high performance liquid chromatography (RP-HPLC), described below in Section 2.I.C.3.e (page 61).

2.I.C.3.d. Bulk deprotection and cleavage of single peptides and peptoids from Rink Amide resin

Individual peptides and peptoids were cleaved from Rink Amide resin and their side chain protecting groups simultaneously removed as described in Section 2.I.C.3.c (page 59), with several modifications. As for bulk deprotection of compounds synthesized on TentaGel resin (Section 2.I.C.2.d, page 58), the reaction using the TFA solution was carried out in disposable reaction vessels and incubated for 1-2 h with orbital shaking. To collect the cleaved peptide or peptoid released into the TFA solution, the solution was expelled from the reaction vessels into 15 mL conical tubes (#352097, BD or #C1017-P, Denville, South Plainfield, NJ) and the resin was then washed with 1-2 mL TFA. In contrast to TentaGel resin, the TFA solution and wash containing the peptoid or peptide were retained (Fig. 2.1, page 101). As performed during quality control (Section 2.I.C.3.c, page 59), the TFA solution was evaporated using a stream of nitrogen or argon gas. In preparation for purification by RP-HPLC, the cleaved peptide or peptoid remaining on the walls of the tube was then stored at 4°C, was dissolved in the lowest percentage possible of ACN (v/v) in ultrapure water and stored at 4°C, or was dissolved in 50% (v/v) ACN in ultrapure water for freezing at -80°C prior to lyophilization (described in Section 2.I.C.3.f, page 63) to remove residual TFA solution. At this point or just prior to purification by RP-HPLC, successful deprotection of the

side groups and cleavage from the resin were confirmed by MS/MS as in Section 2.I.C.3.c (page 59).

2.I.C.3.e. Purification by RP-HPLC of single peptides and peptoids cleaved from Rink Amide resin

Desired peptides and peptoids were purified from contaminants arising from synthesis, deprotection, and cleavage by RP-HPLC. If not already dissolved in a solution of ACN in ultrapure water, the peptide or peptoid was dissolved in the lowest possible percentage of ACN in ultrapure water (total volume was approximately 2 mL per 100 mg resin used in synthesis). At the Molecular Foundry, preparative HPLC was carried out by Dr. Allison Case and me using Varian's (Palo Alto, CA) Prepstar pumps (#218), Dynamax C18 column (#CP28174), Prostar ultraviolet (UV) detector, Prostar fraction collector, and Galaxie software (version 1.9.3.2). The solution containing the crude compound was filtered using a 0.2-μm, 13-millimeter (mm) polytetrafluoroethylene (PTFE) syringe filter (Pall, Port Washington, NY), and purified over 60 min using a gradient of 3-95% buffer B in buffer A, where buffer B was 0.1% (v/v) TFA in ACN (#TX1277P-1, EMD Chemicals) and buffer A was ultrapure water to which 0.1% (v/v) TFA had been added. One 10 mL fraction was collected per minute, and fractions corresponding to peaks in the resulting spectrum were analyzed by MS and MS/MS as described in Section 2.I.C.2.c (page 57) to determine whether they contained the desired peptide or peptoid and were devoid of contaminants. Those fractions containing the purified desired peptide or peptoid were stored at 4°C until lyophilization (Section 2.I.C.3.f, page 63).

At UTSW, HPLC was carried out by Ms. Kelly Mapes and Ms. Kelly Dye in consultation with Dr. Allison Case [early rabbit work (332); see Section 2.III.A, page 80] or with me (depending on who performed the initial synthesis). Crude compounds were filtered using a syringe filter (#431215, Corning, Corning NY), then purified using a semi-preparative HPLC system consisting of a VersaGrad Prep 36 pump (#VGP105FT03HT, Scientific Systems, State College, PA), a Rheodyne injector and loop [#7725(i), IDEX Health & Science, Rohnert Park, CA], a Vydac C18 column (#218MS510, Grace, Columbia, MD), an Elite LaChrom L-2400 UV detector (#890-9401, Hitachi High Technologies America, Dallas, TX) with a preparative flow cell (#890-0502, Hitachi), and EZChrom Elite software (version 3.2, Agilent, Santa Clara, CA). A solution of 0.1% TFA (v/v) in ultrapure water or HPLC-grade water (#W5-4, Fisher, Fair Lawn, NJ) was used as buffer A and 0.1% (v/v) TFA in ACN (#A998-4, Fisher, Fair Lawn, NJ) was used as buffer B. If the compound was being purified for the first time, or if a general gradient was desired for purification, a volume of approximately 500 μL crude peptide or peptoid solution was purified using a gradient of 5-65% buffer B in buffer A over 50 min. Fractions were collected manually based on the appearance of a peak in the spectrum generated by the UV detector (milli-absorbance units vs. time). These fractions were analyzed by MS and MS/MS, then stored at 4°C or directly frozen at -80°C for lyophilization. Based on the retention time and percentage of buffer B at which the desired compound and contaminants eluted, a focused gradient was then designed to optimize purification. For $FLAG^+$ and $FLAG^-$ peptides, a gradient of 5%-30% buffer B in buffer A over 25 min was used, and for R5 and RC, a gradient of 5-30% buffer B in buffer A over 40 min was used.

2.I.C.3.f. Lyophilization of single peptides and peptoids

Crude single peptides and peptoids dissolved in 50% (v/v) ACN in ultrapure water to be lyophilized prior to purification by RP-HPLC, or HPLC fractions containing the desired purified single peptides and peptoids in a solution of ACN and ultrapure water with 0.1% (v/v) TFA, were frozen at -80°C at least overnight prior to lyophilization using a ModulyoD freeze dryer (Thermo Fisher, Waltham, MA) with 24-port drum manifold (#F05621000, Thermo Fisher). Where possible without exceeding approximately 10 mL per 15 mL conical tube or 30 mL per 50 mL conical tube, HPLC fractions containing the same desired compound were combined prior to freezing to reduce loss of the product. Lyophilization was continued for at least 24 h or until the solvent had been removed. The product was then either stored directly at -20°C with or without being blanketed by argon gas, or was first re-dissolved in an appropriate percentage of ACN (v/v) in ultrapure water, re-lyophilized in a pre-weighed container (15 mL conical tube or 1.5 mL microfuge tube), and the container and product re-weighed following lyophilization to determine the mass of the product prior to storage.

2.I.D. Preparation of protein-, peptide-, and peptoid-carrier protein conjugates

2.I.D.1. Overview

For animal immunization and for coating of plates used in ELISAs to determine the presence and concentration of antibodies against peptides and peptoids of interest, peptides and peptoids that had been synthesized with a C-terminal Cys residue on Rink Amide resin, purified by HPLC, and lyophilized were conjugated to maleimide-activated carrier proteins. The conjugation was carried out by Dr. Allison Case [early rabbit work (332); see Section

2.III.A, page 80] or by me. For immunization, keyhole limpet hemocyanin (KLH) was used as the carrier protein, whereas to detect antibodies against the peptide or peptoid itself (rather than the carrier protein), peptides and peptoids were conjugated to a different carrier protein, bovine serum albumin (BSA) and tested by ELISA. To detect antibodies against the structure linking the peptide or peptoid to the carrier and distinguish these antibodies from those against the peptide or peptoid itself, OVA was conjugated to BSA and used to coat an ELISA. Ovalbumin has been shown to contain 6 Cys residues, two of which form a disulfide bond, and four of which have free sulfhydryls (337) to enable maleimide conjugation. Subsequently, anti-linker antibodies were detected using the RC peptoid conjugated to BSA (RC-BSA).

2.1.D.2. Conjugation of proteins, purified peptides, or purified peptoids to maleimide-activated carrier proteins

If desired, quantification of free sulfhydryls in purified peptides or peptoids to be conjugated to maleimide-activated carrier proteins was first performed using Ellman's reagent (#22582, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions, at times with the assistance of Ms. Kelly Dye and Mr. Ali Saherwala. Briefly, a series of standards was first prepared by making a 1.5 millimolar (mM) solution of cysteine hydrochloride monohydrate (Cys•HCl•H_2O; #44889, Pierce) in Reaction Buffer [0.1 M sodium phosphate, pH 8.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA)] and diluting it to the following concentrations: 1.25 mM; 1 mM; 0.75 mM; 0.5 mM; 0.25 mM; and 0 mM Cys•HCl•H_2O. Four milligrams of Ellman's reagent were then dissolved in 1 mL Reaction Buffer to give a solution of Ellman's reagent, and unknowns (purified peptides or

peptoids containing a Cys residue) were then dissolved in Reaction Buffer to give 0.5 mM solutions. A peptide containing no Cys residues [threonine-leucine-glutamic acid-asparagine-serine-tryptophan-glycine-arginine-leucine-serine-threonine-alanine-isoleucine-glutamine-glutamic acid (TLENSWGRLSTAIQE)] was used as a negative control. To determine the free sulfhydryl content of both standards and unknowns using a UV spectrophotometer (DU 730, Beckman Coulter, Brea, CA) at 412 nanometers (nm), 250 μL of each standard or unknown were added to 50 μL Ellman's reagent solution in 2.5 mL Reaction Buffer in a glass test tube (#47729-570, VWR). The absorbance vs. concentration of Cys•HCl•H_2O for the standards was plotted and linear regression was calculated using GraphPad Prism (Version 6.0a for OS X, GraphPad Software Inc., La Jolla, CA). The concentration of free sulfhydryls in the unknowns was then calculated in two ways: 1.) using the linear regression equation from the standards; and 2.) using the molar extinction coefficient for Ellman's reagent (E = 14150 $M^{-1}cm^{-1}$) and the equation for Beer's Law, c = A/(*b*E), where c = concentration in moles/liter, A = absorbance, and *b* = the path length in centimeters, as described in the manufacturer's instructions. The percentage of free sulhydryls was then calculated by dividing the concentration of free sulfhydryls in the starting solution of the unknown by the concentration of the unknown in this solution (0.5 mM).

Proteins, peptides, and peptoids were conjugated to Imject maleimide-activated KLH [#77666 (kit) or #77606 (individual), Pierce] or BSA [#77667 (kit) or #77116 (individual), Pierce] according to the manufacturer's instructions, with several modifications. For OVA or when 2 mg of the purified peptide or peptoid to be conjugated was available, 2 mg of a maleimide-activated carrier protein, lyophilized by the manufacturer from phosphate buffered saline (PBS) containing EDTA and stabilizer, were reconstituted with 200 μL

ultrapure water delivered into the glass vial containing the carrier protein using a 1 mL syringe (#309602 or #309659, BD) and 20G1½ needle (#305176, BD). Two milligrams of lyophilized, purified OVA protein (#A5503, Sigma-Aldrich), peptide, or peptoid were then reconstituted with 200 μL Imject Maleimide Conjugation Buffer (included with kits or purchased separately as #77164, Pierce) or PBS [150 mM NaCl, 2 mM sodium phosphate monobasic (NaH_2PO_4; #BP329, Fisher or similar), 8 mM sodium phosphate dibasic (Na_2HPO_4; #BP332, Fisher or similar), pH 7.4]. The reconstituted protein, peptide, or peptoid was then added dropwise to the reconstituted carrier protein, and the conjugation reaction was allowed to proceed for 2 h. When less than 2 mg but greater than or equal to 1 mg of a single purified, lyophilized peptide or peptoid were available, 2 mg maleimide-activated carrier were reconstituted and then split such that 1 mg maleimide-activated carrier was conjugated to each of two 1 mg aliquots of peptide or peptoid. Meanwhile, Zeba spin desalting columns (included with kits or purchased separately as #89889, Pierce) were prepared by centrifuging for 2 min at 1000 x *g*, 4°C to remove the storage buffer. One milliliter of PBS was then added dropwise to the column and the centrifugation repeated. The addition of PBS followed by centrifugation was repeated three more times for a total of four washes. If the preparation of the columns finished prior to the end of the conjugation reaction, an additional 1 mL PBS was added to the column, the column was capped, and the PBS was centrifuged out of the column immediately before use. If no precipitate formed during the conjugation reaction, the entire conjugation solution was added dropwise to the desalting column, and, when this solution had entered the column bed, 40 μL PBS was added as a stacker. The column was centrifuged as above and the flow-through, containing the conjugate, was stored at 4°C directly or sterile-filtered using a Nalgene 0.2 μm syringe

filter (#180-1320, Thermo Fisher) prior to storage. If the conjugation reaction formed a precipitate, as often occurred during the conjugation of R5 peptoid to maleimide-activated BSA [regardless of whether Conjugation Buffer or PBS was used to reconstitute R5 peptoid or even if, as suggested by the manufacturer, the conjugation reaction was performed in up to 30% (v/v) DMSO in ultrapure water], only the supernatant was added to the desalting column and the flow-through was combined with the precipitate and saved as the conjugate. Notably, sterile filtration of R5-BSA flow-through and pellet that had been mixed by pipetting eliminated any visible precipitate and resulted in a product that, like the unfiltered column flow-through, could be bound by serum from a rabbit immunized with R5-KLH.

2.II. Peptide-optimized screening of peptoid libraries

2.II.A. Peptide-optimized screening of Peptoid Library 1 with bnMAbs against WNV and MNV-1

2.II.A.1. Overview

Two sequential on-bead screening assays were initially optimized using the FLAG peptide model system [described in Section 2.I.C.1, page 53 and (332)], a magnetic screen and a colorimetric screen (Fig. 2.11, page 118). Variations of these assays had been previously published by other groups, including magnetic screening of a OBOC D-peptide/peptoid hybrid library using MAb anti-FLAG (227) and a color screen of a OBOC peptoid library (229). In the magnetic screen, on-bead compounds (peptide or peptoid) were incubated with the screening antibody. Magnetic particles (Dynabeads) bearing Protein G, which binds to the Fc portion of antibodies, were then added. When this mixture was subjected to a strong magnetic field, complexes of on-bead peptide or peptoid bound by screening antibody in turn bound by Protein G Dynabeads (PGDs) were retained by the magnet and could be physically separated from the remaining on-bead peptides or peptoids.

Those on-bead peptides or peptoids retained by the magnet were then stripped of their screening reagents using a low pH solution and subjected to a second, colorimetric screening step. In this assay, on-bead peptides or peptoids were incubated with the screening antibody, followed by the addition of an HRP-conjugated species-specific secondary antibody. When an appropriate substrate (3,3', 5,5'-tetramethylbenzidine, TMB) was supplied, complexes of on-bead peptide or peptoid, screening antibody, and secondary antibody could be distinguished by a blue color change under a dissecting microscope, while the beads bearing peptides or peptoids not bound by antibody remained clear. On-bead peptides and peptoids to be sequenced were then stripped of screening reagents

using a low pH solution, after which the peptides or peptoids were cleaved from the beads using CNBr and their sequences were determined by MS/MS. Sequences of peptoids retained from peptoid libraries were then compared to determine whether informative patterns could be identified.

2.II.A.2 Preparation of MAbs used in peptoid library screening

The preparation of E16, a bnMAb raised against WNV E protein, was performed by the Diamond laboratory and has been described previously (133), as has the preparation of A6.2 (311), a neutralizing MAb raised against the MNV-1 capsid protein P domain. Table 1.4 (page 33) contains more details about these MAbs.

However, due to the concern that MAbs received from collaborators could be contaminated with bovine immunoglobulin (Ig) from fetal bovine serum (FBS) used in the hybridoma culture media, and to produce A6.2 in bulk, the hybridoma secreting A6.2 was obtained from the Wobus lab and A6.2 was prepared from it in-house. The process used has been described previously (338) with the following modifications. To generate a small batch of A6.2 (performed by Dr. John Gu and me) before preparing a larger batch, frozen hybridoma cells were thawed and cultured in 25 cm^2, then 75 cm^2 tissue culture flasks (#3056 and #431464, respectively, Corning) in RPMI-1640 medium formulated with L-glutamine and sodium bicarbonate ($NaHCO_3$) (#R8758, Sigma) and 10% (v/v) FBS (#SH30910, Thermo Scientific Hyclone, Logan, UT). Cells were then transferred to 2 liter (L) roller bottles (#353007, BD) and cultured with loosened caps for 2 days in medium as previously but containing 10-30% (v/v) bovine Ig-depleted FBS. Briefly, this bovine Ig-depleted FBS was prepared by Ms. Kelly Mapes by passing FBS over a polypropylene column (#732-1010, Bio-Rad, Hercules, CA) containing Protein G-Sepharose (#17-0618-05,

GE Healthcare BioSciences AB, Uppsala, Sweden) that had been washed with PBS, then medium. The flow-through was collected and the column was washed with medium, then PBS. Bound protein was then eluted by applying a solution of 0.1 M glycine-HCl, pH 2.6 (#G-7126, Sigma), and the column was re-equilibrated by washing with PBS, then medium. This process was then repeated by passing the flow-through over the column twice more, for a total of three passes. It was then filter-sterilized using a 0.22 μm filter (#SCGPU05RE, Millipore, Billerica, MA) and stored at 4°C as bovine Ig-depleted FBS. The need for three passes over the Protein G column to deplete bovine Ig from FBS was determined by Ms. Kelly Mapes using radioimmunoassays (RIA), in which rabbit anti-bovine IgG (#B5645, Sigma) at 20 micrograms (μg)/mL in PBS was used to coat the plates (#2401, Thermo Fisher) overnight at 4°C. The plates were washed by aspirating the coating solution, adding deionized (DI) water to each well, and pouring the DI water out for a total of six washes. The plates were then blotted on paper towels before being blocked with 1 mg/mL OVA (#A5503, Sigma) overnight at 4°C and washed as previously. To prepare the samples being analyzed, the flow-throughs from the first and third passes over the Protein G column were diluted from 1 to 1/20,000 in dilution buffer [PBS containing 20% weight/volume (w/v) OVA] and incubated on the plates for 2 h at RT. Fetal bovine serum alone that had not been passed over the Protein G column was included as a positive control, as were three samples into which 0.1 μg/mL bovine Ig (prepared in-house as described here) was added: FBS that was not passed over the Protein G column; the flow-through from FBS passage over the column once; and the flow-through after three passes. Bovine Ig at concentrations from 0-5 μg/mL in dilution buffer was included as a standard curve. The plates were then washed twice with PBS containing 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20; #P-1379,

Sigma) and three times with DI water before I^{125}-labeled rabbit anti-bovine IgG [radioiodinated as described previously (339)], at 100,000 counts per minute (cpm)/well, was added as a secondary antibody for 2 h at RT. Following washing as previously, the wells were cut from the plates and allowed to fall into 12 x 75 mm glass tubes (for example, #47729-570, VWR, West Chester, PA) using a hot wire plate-cutting device manufactured at UTSW. The cpm were measured for each tube using a gamma counter (Wallac 1470 Wizard Automatic Gamma Counter, Perkin Elmer, Turku, Finland). The average cpm for triplicate tubes were then calculated, and the background radioactivity when no sample was applied was subtracted. By performing linear regression of the linear portion of the bovine Ig standard curve, a concentration of 0.133 μg/mL bovine Ig remaining in the bovine Ig-depleted FBS was calculated. This might have been a subclass of IgG that did not bind to Protein G.

On the third day, the roller bottles were capped tightly and incubated in a rolling incubator (Roll In Incubator, Bellco, Vineland, NJ). On the fifth day, the A6.2 hybridoma supernatant was collected by centrifugation of the roller bottle contents for 10 min at 293 x *g*, RT, followed by removal of the supernatant and centrifugation of it for 10 min at 1831 x *g*, RT. A solution of 1% (w/v) sodium azide (NaN_3) in ultrapure water was then added to a final concentration of 0.005%. Prior to affinity purification of MAb A6.2 from the hybridoma supernatant, this supernatant was filtered as previously to remove particulates. Affinity purification of A6.2 from the hybridoma supernatant was then performed by passing this supernatant once over a Protein G-sepharose column as described for depletion of bovine Ig from FBS and by Coleman *et al*. (338). The Protein G column eluate was dialyzed into PBS, filter-sterilized using a 0.22 μm filter as previously, concentrated to 0.6 mg/mL using

centrifugal concentrator devices (Amicon Ultra-4, #UFC801024, or Amicon Ultra-15, #UFC901024, Merck Millipore, Carrigtwohill, Ireland), and filter-sterilized as previously before being separated into 1 mL aliquots and stored at -20°C. This A6.2 produced in-house was compared with A6.2 produced by the Wobus lab by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a PhastSystem. A 4-15% gel (#17-0678-01, Pharmacia, Uppsala, Sweden) was used along with PhastGel Blue staining (#17-0518-01, GE Healthcare, Little Chalfont, England), according to the manufacturer's instructions. Low molecular weight calibration standards (#17-0446-01, GE Healthcare) and high molecular weight calibration standards (#17-0615-01, GE Healthcare) were prepared according to the manufacturer's instructions and included for comparison. A large batch of A6.2 was then prepared in a similar manner by Dr. John Gu, concentrated to 1.6 mg/mL, and this was used for peptoid library screening as described below.

2.II.A.3. On-bead magnetic screening of Peptoid Library 1 with anti-WNV and anti-MNV-1 MAbs

One set of 10 sub-aliquots (A through J, according to the last peptoid monomer added) of Peptoid Library 1 that had been stored dry in 1.5 mL microfuge tubes following deprotection (see Section 2.I.B.5, page 52) was swollen in a solution of 0.01% (v/v) Tween 20 (#274348, Sigma) in PBS (PBST) by incubation on a Labquake shaker rotisserie (#400110, Barnstead Thermolyne, Dubuque, IA). The condition of the resin prior to screening was then assessed under an inverted light microscope (CK2, Olympus or Diaphot, Nikon) at 40X power. Each sub-aliquot, containing approximately 31,200 beads, yielded a swollen bead volume of approximately 250 μL. Two sub-aliquots at a time were

then transferred to 5 mL disposable reaction vessels (#NC9160511, Fisher Scientific) using a stream of PBST delivered by a wash bottle. The resin was washed twice with PBST by attaching 22G1 needles (#305155, BD) to the reaction vessels, drawing up approximately 10 times (~10X) the bead volume of PBST (~2.5 mL), shaking the reaction vessels by hand briefly, and ejecting the wash solution using the reaction vessel plunger, taking care not to crush the resin with the plunger. Antibody solution was then prepared at 10 μg/mL in PBST and 500 μL (~2X bead volume) was drawn into the syringe containing each library sub-aliquot. To preclear Peptoid Library 1 of peptoids binding to antibodies outside of the antigen binding site or to PGDs, a species- and isotype-matched control MAb was used to screen the library prior to use of the anti-viral screening MAbs. As shown in Tables 3.1-3.14 (pages 152-164), the following antibodies were used in successive rounds of magnetic screening: MOPC-141 (mouse IgG2b; #M8894, Sigma-Aldrich) was added to all ten sub-aliquots in Round 1a and to sub-aliquots E and F in Round 1b; E16 (also mouse IgG2b) was added to all ten sub-aliquots in Round 2; UPC-10 (mouse IgG2a; #M9144, Sigma-Aldrich) was added to all ten sub-aliquots in Round 3; A6.2 (also mouse IgG2a) produced by the Wobus lab was added to all ten sub-aliquots in Round 4; and A6.2 produced in-house was added to all ten sub-aliquots in Round 5. Note that in between Round 4 and Round 5 aliquots A and B were precleared with MOPC-21 (mouse IgG1, #M9269, Sigma-Aldrich) and UPC-10, and aliquot B was precleared twice more with MOPC-21.

Following addition of the antibody solution, the needles were removed from the reaction vessels and these ports were covered with parafilm or plastic caps (#731-1660, Bio-Rad, Hercules, CA, or similar). The mixture of resin and MAb solution was incubated for 1 h at RT on a rotisserie. Meanwhile, a 1:5 dilution of PGDs (#100.04D or #100.03D,

Invitrogen Dynal AS, or #10004D or #10003D, Life Technologies AS, Oslo, Norway) in PBST, 250 μL per library sub-aliquot, was prepared as follows. The stock solution of PGDs was vortexed to produce an even suspension of PGDs. Immediately, 50 μL of PGD stock solution (30 μg/mL PGDs) were then delivered by pipet into a 1.5 mL microfuge tube for each library sub-aliquot being screened. The microfuge tubes were placed on a magnet (#123-21D, Invitrogen Dynal AS, Oslo, Norway) until the solution clarified (less than 1 min) and the storage solution was removed by pipetting. Five hundred microliters of PBST (10X PGD stock solution volume) were then added to each microfuge tube and the solution was vortexed to resuspend the PGDs. The microfuge tubes were briefly centrifuged (QuikSpin, #MCS-2010, Phenix, Candler, NC), again placed on a magnet and, when the solution had clarified, the solution was removed by pipetting. This wash was repeated once more before each PGD pellet was resuspended in 250 μL PBST.

At the completion of the MAb incubation, needles were reattached to the disposable reaction vessels and the MAb solution was ejected using the reaction vessel plungers. The resin was then washed three times as previously with 10X reaction volume (~5 mL) PBST. Each library sub-aliquot was then transferred to a 15 mL conical tube (#62.554.002, Sarstedt, Nümbrecht, Germany) by removing the reaction vessel plunger, rinsing any peptoid library beads clinging to the plunger into the 15 mL conical tube by using a stream of PBST delivered by a wash bottle, pouring the bulk of the resin from the reaction vessel into the conical tube, and using a stream of PBST delivered by a wash bottle to transfer the remaining resin. The resin was then pelleted by centrifugation at 293 x *g* for 2 min at 4°C. The supernatant was removed by pipetting until ~1.5 mL total volume remained in the conical tube and this solution was used to transfer the resin by pipetting into a 1.5 mL

microcentrifuge tube for each sub-aliquot. The resin was allowed to settle and the supernatant was removed until 500 μL total volume remained (in theory, 250 μL bead volume and 250 μL PBST). Two hundred fifty microliters of the 1:5 dilution of PGDs in PBST prepared earlier were then added to each library sub-aliquot in 250 μL PBST by pipetting to give a 1:10 final dilution of PGDs. The solution was mixed gently by vortexing and incubated for 30 min on a rotisserie at RT.

The magnet was then used to separate peptoid library beads retained by the magnet ("positive" in magnetic screening) from those not retained ("negative" in magnetic screening). The tubes containing peptoid library beads were placed on the magnet and the magnet was immediately tilted approximately 45° from its upright position such that the tops of the tubes were tilted away from the screener. With one hand on each end of the magnet, the magnet was rotated slowly in a bicycle pedaling motion to allow the resin to pass along the magnet inside the tubes. The magnet was then gently placed upright on the bench top for at least 1 min to allow library beads not retained by the magnet to settle at the bottom of the tubes. A P1000 pipet was then used to stir the library beads not bound by the magnet ("negatives") while removing them from the microfuge tubes, until no less than 250 μL volume remained. The tubes containing remaining negatives as well as positives were then removed from the magnet, 500 μL of PBST were added, the solution was mixed by gentle vortexing, and the process was repeated at least twice before being modified to include the following: centrifugation following vortexing to collect any beads or solution clinging to the tube cap; lessening the degree of magnet tilt so that the solution did not contact the cap; and, once the tubes were placed on the magnet, twisting the tubes on the magnet to visualize peptoid library beads retained by the magnet and to loosen any library beads not

strongly retained. This process was continued until only the peptoid library beads retained by the magnet (“positives”) remained in the original tubes, and all peptoid beads not bound by the magnet (“negatives”) were removed. These negative peptoid library beads were either stored in 1.5 mL microcentrifuge tubes, or deposited directly in the fritted syringes according to library sub-aliquot for the next round of screening, which was begun by washing the resin three times with PBST as previously. Positive peptoid library beads were visualized in their tubes under an inverted light microscope at 40X power before being transferred by pipetting to 60 mm tissue culture dishes (Falcon, #3002, BD, Lincoln Park, NJ) to be counted under the microscope. Positive peptoid library beads were then transferred back into their microcentrifuge tubes and stored at 4°C, or, if continuing directly to color screening, into a new 1.5 mL microcentrifuge tube. Between Round 4 and Round 5, all sub-aliquots were stored in PBST to which 2% (w/v) NaN_3 (#S-8032, Sigma-Aldrich) in ultrapure water had been added to a final concentration of 0.02%. Following Round 5, 2% NaN_3 was again added to 0.02% prior to storage at 4°C.

2.II.A.4. Color screening of Peptoid Library 1 beads positive in magnetic screening with anti-WNV and anti-MNV-1 MAbs

Peptoid library beads retained by the magnet in magnetic screening (“positives”) were transferred to new 1.5 mL microcentrifuge tubes according to sub-aliquot and screening MAb. The number of peptoid library beads in each tube was counted using a light microscope at 40X power. The peptoid library beads, as single tubes or in pairs of tubes, were then subjected to stripping of magnetic screening reagents as follows. After removing as much PBST as possible by pipetting, 50 μL of 0.1 M glycine-HCl, pH 2.8 containing

0.01% (v/v) Tween 20 (glycine: #G-7126, Sigma-Aldrich; HCl: #A144, Fisher) were added to each tube and the solution was mixed by gentle finger flicking for 5 seconds. The glycine stripping solution was incubated for 1 min at RT before approximately 1 mL of PBST was added. The peptoid library beads were pelleted by centrifugation and as much supernatant as possible was removed by pipetting without disturbing the bead pellet. The presence of the peptoid library beads in the tube was verified under a dissecting microscope (Stereo Star Zoom, American Optical, Buffalo, NY) before the PBST wash was repeated twice more so as to restore the solution to physiologic pH and remove stripped screening reagents. After the final wash, approximately 1 mL PBST was added to each tube.

Peptoid library beads that were positive in magnetic screening with anti-viral MAbs were then color screened. For peptoid library beads positive in magnetic screening with preclearing antibodies, a few beads were randomly selected from each sub-aliquot for color screening (see Tables 3.2 and 3.3, page 153) and placed in new 1.5 mL microfuge tubes, maintaining separation by screening antibody and sub-aliquot. Those peptoid library beads selected by preclearing MAb UPC-10 were color screened by Ms. Kelly Dye through the step of observing the color change, while all others were color screened by me. For each tube of peptoid library beads to be color screened, a 1.5 mL microcentrifuge tube containing 50 μL of a 10 μg/mL solution of the screening antibody (the same antibody used for magnetic screening) in PBST was then prepared. The peptoid library beads from each tube were transferred by pipetting into a corresponding tube of screening antibody. In some color screening assays (Table 3.4, page 154), three beads bearing $FLAG^+$ peptide were transferred into a tube of MAb anti-FLAG (#F3165, Sigma-Aldrich) as a positive control, and three beads bearing $FLAG^-$ peptide were transferred into a tube of this antibody as a

negative control. Bead transfer was verified under a dissecting microscope, and the tubes were incubated for 1 h on a rotisserie.

Meanwhile, one tube of 50 μL HRP-conjugated goat anti-mouse IgG secondary antibody (#115-035-164, Jackson ImmunoResearch, West Grove, PA), diluted 1:5000 in PBST, was prepared for each tube of peptoid library beads being color screened. At the conclusion of incubation with the screening antibody, the peptoid library beads were then washed three times by adding 1 mL of PBST to each tube, pelleting the beads by centrifugation, removing the supernatant by pipetting, and verifying by microscopy that the beads remained in the tube. Following the last wash, the beads were transferred by pipetting in a minimum volume of liquid to the corresponding tube of secondary antibody and the tubes were again incubated for 1 h on a rotisserie. During the antibody incubations, TMB (#34028, Pierce) was allowed to equilibrate to RT. At the conclusion of the secondary antibody incubation, the beads were washed three times as previously. Following the final wash, 200 μL of PBST were added to each tube. On the stage of a dissecting microscope (Stereomaster, #1256212, Fisher), the beads from one tube of peptoid library beads at a time were transferred by pipetting to a 60 mm tissue culture dish. If included in that color screening assay (Table 3.4, page 154), $FLAG^+$ and $FLAG^-$ peptide beads were also transferred by pipetting to other spots on the tissue culture dish. For each set of beads transferred to the tissue culture dish, a 20 μL drop of TMB was added by pipetting to fresh spots on the dish. In a minimal volume of liquid and using a P2 pipet, each set of beads was then transferred into its own drop of TMB. If included in that assay (Table 3.4, page 154), a timer was started, and the beads were observed for color change for at least ten minutes.

Since it had been observed by other members of the peptoid team that TMB interferes with peptide or peptoid sequencing by MS/MS, on-bead peptides or peptoids were then stripped of screening reagents in the following manner. Each bead was transferred to its own 1.5 mL microfuge tube containing PBST, or beads of similar color change from the same sub-aliquot screened with the same MAb were pooled in a 1.5 mL microfuge tube. As much liquid as possible was removed by pipetting before 200 μL of the glycine stripping solution used above were added to each tube. The solution was mixed by vortexing for 30 seconds before as much liquid as possible was removed by pipetting without disturbing the bead pellet. The glycine wash was repeated twice more before any residual glycine was neutralized by adding 1 mL of PBST to each tube. Individual beads were then isolated into 0.5 mL microfuge tubes for cleavage of the peptide or peptoid from the bead using CNBr and sequencing by MS/MS as described in Sections 2.I.B.4 (page 49) and 2.I.C.2.c (page 57).

2.III. Generating a peptoid/antibody pair

2.III.A. Immunization of rabbits with a peptoid-carrier adsorbed to alum

2.III.A.1. Overview

To generate the first affinity-purified antibody against a peptoid with which to optimize assays for the identification of peptoid vaccine candidates, the R5 peptoid was designed as described in Section 2.I.C.1 (page 53). As shown in Fig. 2.8C (page 112), R5 peptoid was attached to the carrier protein KLH as described in Section 2.I.D (page 63), and, as described below, was adsorbed to the adjuvant alum for rabbit immunizations.

2.III.A.2. Preparation of R5-KLH immunizations for rabbits

Following conjugation of R5 peptoid to maleimide-activated KLH, the R5 peptoid-KLH conjugate was filter-sterilized as described in Section 2.I.D (page 63). For one rabbit immunization with R5-KLH, the desired mass of R5-KLH was 250-500 μg, while the final volume to be injected was capped at 250 μL. Approximately 30% extra volume was prepared to account for loss of the final solution in the syringe and needle. The desired mass of R5-KLH was added to a solution of 2 mg/mL alum (Alhydrogel 2%, Brenntag, Frederikssund, Denmark) such that in the final volume, the final concentration of alum would be 1 mg/mL. The mixture was then incubated for at least 20 min at 4°C on a rotisserie (Labquake Shaker, #415-110, Lab Industries, Berkeley, CA) to adsorb R5-KLH to alum. Following incubation, if the volume exceeded the maximum allowed (250 μL per immunization, and accounting for 30% as extra), the mixture was centrifuged at 14,000 x *g* for 2 min at 4°C to pellet the R5-KLH adsorbed to alum. The supernatant was then removed until the desired final volume was reached. The pellet was then resuspended, the solution

was drawn into a 1 mL syringe, and the syringe was capped with parafilm and stored at 4°C until use. Immunizations prior to Day 326 (Fig. 2.12, page 119) were prepared by Drs. Allison Case and Kate Yi; immunizations administered on Day 326 were prepared by Dr. Allison Case and me.

2.III.A.3. Rabbit test bleeds and immunizations for the production of anti-R5 peptoid antibodies

All animal work was carried out at the UTSW Animal Resources Center (ARC) with the approval of, and in accordance with, the policies of the Institutional Animal Care & Use Committee (IACUC). Two 16-week old female New Zealand White rabbits, designated **12D** and **13D**, were purchased from Myrtle's Rabbitry (Thompson Station, TN) and allowed to acclimate in the UTSW ARC for approximately 5 weeks before a pre-immunization test bleed was collected by ARC technicians (Day -120, Fig. 2.12, page 119). For test bleeds, approximately 5 mL of blood was drawn from the central ear artery. Sera were then prepared by Ms. Kelly Mapes and Ms. Phyllis Barron. Blood was transferred from the collection vacutainer into a sterile 15 mL conical tube and refrigerated overnight at 4°C to allow the blood to clot. The following day, the tubes were centrifuged at 659 x *g* for 15 min at 20°C. The supernatant (serum layer) was transferred to a new tube and centrifuged again as previously to remove any remaining debris. This supernatant was filter-sterilized using a 0.22 μm polyethersulfone (PES) syringe filter (25 mm diameter) and frozen at -20°C as aliquots in 1-5 mL cryovials until further use. The rabbits were again test bled on Day -8 prior to receiving their first immunization of R5-KLH adsorbed to alum (prepared as described in Section 2.III.A.2, page 80) on Day 0 (Fig. 2.12). Immunizations were

administered intramuscularly (IM) in the rabbits' hind legs. Booster immunizations were then administered and test bleeds were collected as shown in Fig. 2.12.

2.III.A.4. ELISAs to determine the serum antibody levels against the R5 peptoid, KLH, and linker

Periodically over the course of the rabbit immunizations, sera prepared from test bleeds were assessed by ELISA to monitor the concentrations of anti-R5 peptoid, anti-KLH, and anti-linker antibodies. The early portion of this work has been described previously (332) and was performed by Dr. Allison Case; following the immunizations on Day 326 (Fig. 2.12, page 119), it was performed with Dr. Allison Case. Ninety-six-well plates (#9018, Corning) were coated with 10 μg/mL solutions of the following proteins or conjugates in PBS at 50 μL per well: R5-BSA; BSA (#A8022, Sigma-Aldrich); KLH (#H7017, Sigma-Aldrich); OVA-BSA (to detect antibodies against the maleimide linker); OVA (#A5503, Sigma-Aldrich); and R5-KLH. Following coating for 1 h at RT or overnight at 4°C, plates were washed twice by removing the contents and adding 200 μL PBS to each well. After the final wash, the contents were again removed and the plates were dried by blotting on paper towels. The plates were then blocked with 100-200 μL Starting Block (#37538, Pierce) per well for at least 20 min at RT or overnight at 4°C, followed by washing as previously. Sera to be used as the primary antibody were then thawed and centrifuged at 14,000 x *g* for 10 min at 4°C to pellet immune complexes. The supernatant was transferred to a new tube, from which dilutions of sera in Dilution Buffer (1% Starting Block in PBST) were then prepared. A standard curve using affinity-purified PAb rabbit anti-KLH prepared in-house was also prepared in Dilution Buffer. These primary antibodies were added to triplicate wells of the

plates at 50 μL per well and incubated for 1 h at RT with gentle rocking (#7740-20020, Bellco, Vineland, NJ or RP1200, #R-2050-1, GeneMate). The plates were then washed three times with PBST and dried as described above. A secondary antibody solution, HRP-conjugated goat anti-rabbit IgG (#111-035-144, Jackson ImmunoResearch) at 1:2500 in Dilution Buffer, was then prepared and 50 μL per well were applied to the plates. The secondary antibody was incubated for 1 h at RT with gentle rocking. Meanwhile, TMB was equilibrated to RT. Following washing as described after the primary antibody incubation, TMB was applied to the plates at 50 μL per well and the color change was observed for approximately 2 min before the reaction was stopped with a solution of 2 M sulfuric acid in ultrapure water. The absorbance of each well when light at a wavelength of 450 nm was applied was immediately quantified using a microplate reader (THERMOmax, Molecular Devices, Sunnyvale, CA) with SOFTMax software (version #2.32, Molecular Devices). Background absorbance when primary antibody solution at 0 μg/mL was applied to the plate was subtracted from the absorbance for each well, the normalized absorbance from triplicate wells was averaged, and the results were displayed as average normalized absorbance versus log dilution or concentration using GraphPad Prism. Error bars representing standard deviation of normalized absorbance were calculated using Prism. To determine the concentrations of serum antibodies against the coating proteins or conjugates, linear regression of the linear portion of the standard curve was performed using Prism. The slope (m) and y-intercept (b) of this line were then used to calculate the unknown concentrations of antibodies in sera (x) from their average normalized absorbance (y) in the linear portion of the curve according to the equation $y = mx + b$.

2.III.A.5. Rabbit production bleeds and exsanguination

When the concentrations of anti-R5 peptoid and anti-KLH antibodies in rabbit sera from test bleeds on Day 337 (Fig. 2.12, page 119) were determined to exceed 1 mg/mL by ELISA as described in Section 2.III.A.4 (page 82), a large volume of rabbit blood was then collected by ARC technicians. Two production bleeds of 30 mL each were collected on Days 356 and 370 (Fig. 2.12), and sera were prepared as described in Section 2.III.A.3 (page 81). After each production bleed, the rabbits were allowed to recover. On Day 391 (Fig. 2.12), an additional 10 mL of blood were collected from each rabbit by ARC technicians and sera were prepared as described in Section 2.III.A.3. Finally, on Day 397 for rabbit 12D and on Day 398 for rabbit 13D (Fig. 2.12), the rabbits were sedated with ketamine and xylazine supplemented with isoflurane and oxygen, and the final volume of blood was collected through exsanguination by cardiac puncture by ARC technicians. Sera were then prepared as described in Section 2.III.A.3.

2.III.A.6. Pooling rabbit sera from first production bleed through exsanguination and ELISAs to determine anti-R5 peptoid, anti-KLH, and anti-linker antibody concentrations

Aliquots of sera frozen at -20°C from the first and second production bleeds and exsanguination for each rabbit (Fig. 2.12, page 119) were equilibrated to RT. Under sterile conditions, the sera from each rabbit were pooled by pipetting into 50 mL conical tubes (#352098, BD, Franklin Lakes, NJ), then centrifuging at 14,000 x *g* for 10 min at 4°C to pellet immune complexes. The supernatant for each rabbit serum sample was collected by pipetting into 50 mL conical tubes, then pooled and filter-sterilized using a 0.22 μm filter

(#SCGPU01RE or SCGPU02RE, Millipore) before being divided into aliquots of approximately 5 mL in 15 mL conical tubes (#352097, BD) and stored at -20°C. The final volumes of pooled sera from each rabbit were approximately 60 mL for rabbit 12D and 40 mL for rabbit 13D. The ELISA described in Section 2.III.A.4 (page 82) was then repeated in triplicate using the pooled immune sera from rabbit 12D and from rabbit 13D to determine the concentrations of antibodies against the R5 peptoid, KLH, and linker.

2.III.B. Preparation of polyclonal rabbit anti-R5 peptoid antibody from the pooled post-immunization serum of rabbit 12D

2.III.B.1. Overview

The pooled post-immunization serum from rabbit 12D was first passed over a KLH-sepharose column to remove anti-KLH antibodies. Subsequently, the flow-through from this column was passed over a column displaying R5 peptoid without KLH or the linker used to conjugate R5 to KLH for immunization. The use of such an R5 column was previously described without prior purification over a KLH column (332). The concentrations of anti-R5 peptoid, anti-KLH, and anti-linker antibodies from column eluates and final flow-throughs were monitored by ELISA, after which eluates from each column were pooled. Since the anti-R5 peptoid concentration in the pooled R5 column eluate was quite dilute, it was then concentrated by adding an equal volume of saturated ammonium sulfate to precipitate the globulins and the precipitates were collected by centrifugation.

2.III.B.2. Affinity purification of anti-KLH and anti-R5 antibodies from the rabbit 12D antiserum pool

To purify anti-KLH antibodies away from anti-R5 peptoid antibodies, a KLH-sepharose column was first constructed by Ms. Kelly Mapes. The KLH (#H7017, Sigma-Aldrich), lyophilized by the manufacturer in 31 mM sodium phosphate buffer, pH 7.4, containing 0.46 M NaCl, 2% polyvinylpyrrolidone (PVP), and 41 mM sucrose, was first dissolved in ultrapure water to give 60 mg KLH at a final concentration of 4 mg/mL. This KLH solution was dialyzed into 0.1 M $NaHCO_3$ (#S233-3, Fisher Scientific), pH 8.3 containing 0.5 M NaCl, at 4˚C overnight, after which absorbance at 280 nm indicated the presence of 48 mg of KLH present in the solution. To prepare a CNBr solution for sepharose activation, 2 g of CNBr were then dissolved in 20 mL distilled water (equivalent to the volume of packed sepharose to be activated). The 20 mL of sepharose to be activated (Sepharose CL-4B, #CL4B200, Sigma-Aldrich) were washed with distilled water at 10-20X the volume of the sepharose using a Buchner funnel apparatus (Pyrex Buchner funnel with a fritted disc of 40-60 μm porosity attached to a 1 L vacuum flask). The washed sepharose was then transferred to a small beaker, the CNBr solution was added, and the solution was mixed slowly by magnetic stirring. The pH of the mixture was then raised to 10 by the dropwise addition of 12.5 M NaOH (#SS254-4, Fisher Scientific) with continuous monitoring using a pH meter (UB-10, Denver Instrument, Arvada, CO), at which point 0.5 M NaOH was added dropwise until the pH remained stable between 10 and 11 for 15 min. The sepharose was then considered activated, and was returned to the Buchner funnel apparatus to be washed with 0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl at 10X the volume of activated sepharose. To conjugate KLH to this activated sepharose, the washed, activated sepharose was transferred to a 50 mL conical tube, the KLH solution prepared previously was added to

give 2.4 mg KLH/mL activated sepharose, and the mixture was incubated at 4°C overnight on a rotisserie. The mixture was then centrifuged at ~2300 x *g* for 10 min at 4°C and the supernatant was carefully removed. One milliliter of this supernatant was then centrifuged for ~2200 x *g* for 1 min and the absorbance at 280 nm was determined using a UV spectrophotometer (Ultrospec 3100 *pro*, #80-2112-31, Amersham Biosciences, Little Chalfont, England) to assess the amount of unbound KLH in the supernatant. To block any remaining sites on the activated sepharose, a 1 M solution of ethanolamine (#E9508, Sigma-Aldrich) in 0.02 M tris(hydroxymethyl)amino-methane (Tris; #T-1503, Sigma-Aldrich)-HCl, pH 8.0 was then added at an equal volume to the KLH-sepharose, and this mixture was incubated at 4°C for 1-2 h on a rotisserie. The mixture was centrifuged and the supernatant was removed as described previously. The UV spectrophotometer was then blanked with the ethanolamine solution and the absorbance at 280 nm was determined to detect unbound KLH. The KLH-sepharose was then washed twice by adding an equal volume of PBS, centrifuging as previously, removing the supernatant, and measuring the absorbance of the supernatant at 280 nm to detect unbound KLH. The UV spectrophotometer was then blanked with PBS and the KLH-sepharose was washed with PBS as previously until the absorbance at 280 nm dropped to zero. In total, 5.48 mg of KLH was found to be unbound after all steps, such that 42.5 mg of 48 mg KLH was conjugated to the sepharose. The KLH-sepharose was then poured into a polypropylene column (#732-1010, Bio-Rad, Hercules, CA) and washed with 5 column volumes of PBS. The absorbance at 280 nm of the final wash was measured to ensure that no protein was being released from the column resin. The column was then stored in 0.04% (w/v) NaN_3 (#S2002, Sigma-Aldrich) in PBS at 4°C until further use.

To construct an R5 peptoid affinity column to purify the anti-R5 peptoid antibodies from pooled immune rabbit serum, and to avoid using the same maleimide linker that was used to conjugate the R5 peptoid to KLH for immunization, an R5 peptoid-SulfoLink column was constructed with Dr. Allison Case and Mr. Ali Saherwala using the SulfoLink Immobilization Kit for Peptides (#44999, Pierce, Rockford, IL), according to the manufacturer's instructions with some modifications. Briefly, all components were allowed to warm to RT prior to use. One milligram of purified R5 peptoid including a C-terminal Cys residue (see Sections 2.I.C.3.b-2.I.C.3.f, pages 59-63, and Fig. 2.8B, page 112) was dissolved in 2 mL Coupling Buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5) and the absorbance at 280 nm of this starting solution was measured using a UV spectrophotometer (DU 730, Beckman Coulter). To reduce any disulfide bonds that might have formed between Cys sulfhydryls, 0.2 mL tris(2-carboxyethyl)phosphine (TCEP) was then added to the solution of R5 peptoid in Coupling Buffer to give a final concentration of 50 mM TCEP. This mixture was incubated for 30 min at RT. Meanwhile, the column resin was centrifuged at 1,000 x *g* for 1 min at 4°C to remove the storage buffer, then washed three times by adding 2 mL Coupling Buffer and centrifuging as previously. The reduced R5 peptoid solution was then added to the column resin and mixed for 15 min at RT with gentle rocking (#7740-20020, Bellco, Vineland, NJ), followed by standing for 30 min at RT. The column was then centrifuged as previously to collect unbound R5 peptoid. This unbound R5 peptoid was compared by absorbance at 280 nm to the starting solution of R5 peptoid to ensure that coupling of R5 to the column resin had occurred. The column was then washed four times with Wash Solution (1 M NaCl, 0.05% NaN_3) by adding 2 mL Wash Solution and centrifuging as previously, followed by two washes with 2 mL Coupling Buffer each. The absorbance at 280 nm of the column flow-through from each wash was monitored and found to have

dropped to zero by the third wash with Wash Solution, demonstrating that unbound R5 peptoid had been removed. Unreacted SulfoLink resin was then capped by adding 2 mL of 50 mM Cys (as L-cysteine hydrochloride) in Coupling Buffer to the column, then rocking the column for 15 min and allowing the column to stand upright for 30 min as previously. The Cys solution was then allowed to drain from the column and the column was equilibrated by adding 2 mL PBS that had been degassed by magnetic stirring in a flask connected to vacuum using a stopper and tubing and to which had been added 0.05% (w/v) NaN_3. The column was then centrifuged as previously and the flow-through was discarded. This equilibration step was repeated three additional times before 2 mL degassed PBS with 0.05% (w/v) NaN_3 was added and the column was stored at 4°C until further use. The following day, this R5-SulfoLink column was tested to determine whether it could purify anti-R5 peptoid antibodies from rabbit 12D exsanguination serum. All reagents were allowed to equilibrate to RT before the column was centrifuged as previously to remove the storage solution, and 6 mL PBS were then allowed to pass over the column by gravity flow. Two milliliters of PBS were then used to dilute 1.4 mL of rabbit 12D exsanguination serum. This mixture was added to the column and mixed by rocking gently as previously for 60 min at RT. The column was then centrifuged as previously and the flow-through designated flow-through zero (FT#0). The column was then washed nine times by adding 2 mL PBS, centrifuging, and collecting the flow-through to determine the absorbance at 280 nm; by FT#7, the absorbance had dropped to zero. To elute protein bound to the column, 2 mL of Elution Buffer (0.1 M glycine-HCl, pH 2.5) were applied to the column and the column was centrifuged in a 15 mL conical tube containing 100 μL Neutralization Buffer (1 M Tris-HCl, pH 8.0) to collect the eluate. This elution step was repeated two more times before the

column was re-equiilibrated by adding 4 mL PBS, which were allowed to pass through the column by gravity flow. The column was then stored at 4°C in 4 mL PBS containing 0.05% (w/v) NaN_3 until further use. Meanwhile, column eluates were transferred to dialysis tubing (Spectra/Por4, #132700, Spectrum Laboratories, Rancho Dominguez, CA), which had been rinsed with and soaked in DI water to remove glycerol, for dialysis into PBS at 4°C with at least two buffer changes. Column FT#0 through FT#6 and Eluate #3 were analyzed by ELISA as described in Section 2.III.A.4 (page 82) to confirm that the R5 peptoid-SulfoLink column could purify anti-R5 antibodies from rabbit 12D immune serum.

A small volume (5 mL) of 12D pooled immune serum was then used to test the purification protocol prior to purification of anti-R5 antibodies using a larger volume (25 mL) of this serum. The KLH column was equilibrated to RT, its outflow was connected to a UV detector (UVicord SII, Pharmacia LKB, Uppsala, Sweden) attached to a chart recorder (#2210-061, LKB, Bromma, Sweden), and the column was washed with at least 20 column volumes of PBS by gravity flow. Serum was diluted 1:1 with PBS and allowed to pass over the column by gravity flow. When absorbance was detected by the UV detector as demonstrated by the chart recorder, the column outflow was collected as the flow-through until fractions collected showed that the absorbance at 280 nm had fallen below 0.05 units using a UV spectrophotometer (Ultrospec 3100 *pro*, #80-2112-31, Amersham Biosciences). The column was then washed with at least 20 column volumes of PBS as previously until the absorbance of fractions collected fell to zero. Elution Buffer (0.1 M glycine-HCl, pH 2.8) was then applied to the column by gravity flow to elute bound protein. Fractions were collected using a fraction collector (2110, #731-8122, Bio-Rad) in tubes containing 10% Neutralization Buffer (1 M Tris-HCl, pH 8) until the absorbance dropped below 0.05, when all

fractions above 0.05 were pooled and the absorbance of the pool was determined. The pool was immediately placed in dialysis tubing (Spectra/Por4, #132700, Spectrum Laboratories) that had been rinsed with and soaked in DI water to remove glycerol and was dialyzed into PBS at 4°C with at least two buffer changes. At the completion of dialysis, the eluates were filter-sterilized using a 10 mL syringe (#309604, BD), needle (#305155, BD) and 0.22 μm syringe filter (Millex-GP, #SLGPR25CS, Millipore, Bedford, MA) and the absorbance was determined as previously. Eluates were then stored at 4°C. Meanwhile, Elution Buffer was allowed to continue flowing over the column until the absorbance of the column outflow dropped to zero, at which point at least 20 column volumes of PBS were applied to re-equilibrate and wash the column. The pH of the column outflow was monitored using pH paper (1.0-12.0 Hydrion, #140, Micro Essential Laboratory, Brooklyn, NY). For the large volume of rabbit 12D pooled immune serum, the serum was passed over the KLH column, the flow-through was set aside, the bound protein was eluted, and the column was re-equilibrated. The flow-through was then passed over the column again as Pass #2, and the process was repeated for a total of four passes. The flow-through from the fourth pass over the KLH column was then passed over the R5 column. The flow-through from this first pass over the R5 column was then passed over the R5 column again, and this process was repeated for a total of six passes over the R5 column. The sixth flow-through was then filter-sterilized and stored at 4°C. The concentrations of anti-R5, anti-KLH, and anti-linker antibodies in the eluates from each column pass, as well as the flow-through from the final pass over each column, were determined by ELISA as described in Section 2.III.A.4 (page 82). The eluates from each column were then combined, split into two 50 mL tubes each, and stored at 4°C.

2.III.B.3. Concentration of R5 column pooled eluate

Each half of the R5 column pooled eluate was concentrated. The absorbance at 280 nm was determined as described in Section 2.III.B.2 (page 86), as well as using an additional UV spectrophotometer (DU 730, Beckman Coulter) capable of determining absorbance using only 100 μL of liquid. A supersaturated solution of ammonium sulfate (#A4915, Sigma-Aldrich) was prepared by adding 200 g ammonium sulfate to 250 mL ultrapure water with vigorous magnetic stirring. When magnetic stirring was stopped, ammonium sulfate was visible as a layer of translucent white crystals at the bottom of the glass bottle, indicating the saturation point had been surpassed. To each half of the R5 column pooled eluate in a 50 mL conical tube, an equal volume of supersaturated ammonium sulfate was added dropwise by pipetting. The solution was mixed for 30 min using an Adams Nutator mixer (#1105, Beckton Dickinson, Parsippany, NJ), after which the 50 mL conical tube containing the mixture was stored upright at RT for almost 24 h. The mixture was then centrifuged at 900 x *g* for 30 min at 4°C. Meanwhile, a MINI Dialysis Device (#88402, Pierce) was prepared according to the manufacturer's instructions by adding 1 mL ultrapure water and observing the device for leaks for 2 min. The water was removed from the device and the device was kept wet with PBS until needed. At the conclusion of centrifugation, the supernatant from the ammonium sulfate precipitation was pipetted into a 50 mL conical tube until approximately 5 mL remained, when the pellet was re-centrifuged as previously. Following centrifugation, as much of the remaining supernatant as possible was removed without disturbing the pellet and combined in the 50 mL conical tube with the initial supernatant. The pellet was then resuspended in a minimal volume of ultrapure water and transferred to the MINI Dialysis Device along with a small volume of

ultrapure water used to wash out any remaining pellet from the centrifuged 50 mL conical tube; the volumes of the resuspended pellet and wash were determined using the pipet used for transfer. Dialysis into PBS was carried out according to the manufacturer's instructions, incubating for 2 h at RT with gentle orbital shaking (ROSI 1000 Reciprocating/Orbital Shaking Incubator, Thermolyne) before the buffer was changed and the device was transferred to 4°C for overnight incubation with orbital shaking (Spindrive Orbital Shaker Platform, #F37041-0000, Bel-Art Products, Wayne, NJ on top of a Cimarec magnetic stir plate, Barnstead Thermolyne). The following day, the buffer was changed once more before the volume and absorbance were determined as described previously. The sample was stored at 4°C. Meanwhile, a centrifugal concentrating device (Amicon Ultra-15, #UFC901024, Merck Millipore, for the first half of the R5 column eluate; Amicon Ultra-4, #UFC801024 for the second half of the R5 column eluate) was prepared by adding ultrapure water and centrifuging at 4000 x *g* for 10-20 min at 4°C. The ultrapure water was removed and the ammonium sulfate precipitation supernatant was applied. The device was centrifuged at 4000 x *g* for 20 min at 4°C. The flow-through was removed, additional ammonium sulfate precipitation supernatant was applied, and the process was repeated until all of the supernatant had been concentrated by the device. The volume and absorbance of concentrated supernatant was determined as previously and the concentrated supernatant was then dialyzed as described for the ammonium sulfate pellet. The volume and absorbance of the dialyzed, concentrated supernatant was then determined as described previously. The dialyzed, concentrated ammonium sulfate precipitation pellet and supernatant were then combined and concentrated further using a smaller centrifugal concentrating device (#42406, Millipore). The final volume was measured and the

concentration was determined by measuring absorbance as described previously. Each half of the concentrated R5 column eluate was then filter-sterilized using a 1 mL syringe (#309659, BD), 22G1 needle (#305155, BD) and 0.22 μm syringe filter (Millex-GV, #SLGV004SL, Millipore, Carrigtwohill, Ireland). The anti-R5 peptoid, anti-KLH, and anti-linker antibody concentrations of the first half of the R5 column pooled eluate were examined by ELISA as described in Section 2.III.A.4 (page 82), and the two halves of the concentrated R5 column eluate were then combined, split into two aliquots, and stored at 4°C.

2.III.B.4. Analysis of pooled, concentrated R5 column eluate by ELISA

The anti-R5 peptoid, anti-KLH, and anti-linker antibody concentrations in the concentrated R5 column eluate were determined by ELISA as described in Section 2.III.A.4 (page 82), but with two modifications: RC-BSA was included to serve as an irrelevant control peptoid and to determine the anti-linker antibody concentration instead of OVA-BSA; and serum prepared from a pre-immunization test bleed (Day -8, Fig. 2.12, page 119) and pooled post-immunization serum (described in Section 2.III.A.6, page 84) were included for comparison.

2.III.B.5. Analysis of pooled, concentrated R5 column eluate by SDS-PAGE

The purity and molecular weight of the concentrated R5 column eluate were examined by SDS-PAGE under non-reducing and reducing conditions using the PhastSystem as described in Section 2.II.A.2 (page 69) by Ms. Kelly Mapes in consultation with me and with Dr. Laurentiu Pop.

2.IV. Optimization of on-bead magnetic screening using the R5 peptoid model system

2.IV.A. Small-scale assay

Small-scale magnetic screening assays were performed with Mr. Stephen Ruback and Mr. Edward Neill. Barrier pipet tips were used when pipetting peptoid TentaGel beads (see Table 3.21, page 173). Dry R5 and RC peptoid TentaGel beads from which the protecting groups had been removed as described in Section 2.I.C.2.d (page 58) and shown in Figs. 2.7B (page 110) and 2.9A (page 115), respectively, were transferred to 15 mL conical tubes and swollen in PBST overnight on a rotisserie at RT, after which 2% (w/v) NaN_3 was added to a final concentration of 0.02% and the resin was stored at 4°C until further use. To prepare 1.5 mL microfuge tubes containing 27 RC peptoid TentaGel beads, the beads were removed from the stock and transferred into a 60 mm tissue culture dish by pipetting. Additional PBST was added to the dish by pipetting or from a wash bottle if necessary. To transfer the appropriate number of beads to each microfuge tube by pipetting, the beads were visualized using an inverted light microscope. This process was repeated to add three R5 peptoid TentaGel beads to each tube for a total of 30 peptoid TentaGel beads in each tube. The resin was then washed twice to remove the storage solution by adding 1 mL PBST, pelleting the resin by pulse centrifugation, and removing the supernatant by pipetting. For each tube of resin, a 1.5 mL microfuge tube of 20 μL screening antibody [either rabbit anti-R5 peptoid (RAR5), generated as described in Section 2.III (page 80), or an irrelevant species-matched control antibody, rabbit anti-mouse Ig (RAMIg), generated in-house] was prepared at the desired dilution in PBST. While visualizing the beads using a dissecting microscope (Stereo Star Zoom, American Optical), the beads were transferred into the appropriate tube of antibody solution by aspirating the beads with a P20 pipet,

placing the pipet tip in the antibody solution, and allowing the beads to fall out of the pipet tip into the antibody solution (shaking the pipet tip allowed the beads to fall out faster). The tubes were then incubated for 1 h on a rotisserie at RT. Meanwhile, a 1.5 mL microfuge tube containing PGDs was prepared for each tube of resin by vortexing the PGD stock solution, then immediately removing the desired volume of PGDs to give 20 μL for each resin tube at the desired PGD dilution and placing these PGDs in a 1.5 mL microfuge tube (or tubes, depending on the volume needed for 10X washes of the PGDs as described in Section 2.II.A.3, page 72). The tube(s) of PGDs were placed on the magnet, the PGD storage solution was removed by pipetting, and the PGDs were washed as described in Section 2.II.A.3. The PGDs were then resuspended in the volume of PBST that would give the desired starting dilution of PGDs, and further dilutions of PGDs were made in PBST if needed. Twenty microliter aliquots of PGD solutions at the desired dilutions were distributed to new 1.5 mL microfuge tubes. Where indicated, the full 500 μL of diluted PGDs (the volume used for screening peptoid library aliquots of ~20,000 beads as described in Section 2.II.A.3) was prepared per 1.5 mL microfuge tube instead of 20 μL.

At the conclusion of the incubation with the screening antibody, the resin was washed three times with PBST, transferred into the tubes of diluted PGDs as described for the addition of beads to the primary antibody solution, and incubated for 30 min on a rotisserie at RT. If 20 μL of PGD solution were used, PBST was then added to 500 μL before placing the resin-containing 1.5 mL microfuge tubes on the magnet. The numbers of positive (retained by the magnet) and negative (not retained by the magnet) TentaGel beads in each 1.5 mL tube, as well as the PGD coverage of the TentaGel resin, were then observed using an inverted light microscope at 40X power and recorded. TentaGel beads

were then separated individually into 0.5 mL microfuge tubes for CNBr cleavage and analysis by MS (and sequencing by MS/MS if desired) as described in Section 2.I.C.2.c (page 57).

2.IV.B. Large-scale assay

As described in Section 2.II.A.3 (page 72), peptoid library sub-aliquots of ~250 μL TentaGel bead volume (~20,000 beads) had been screened in 1.5 mL microfuge tubes in peptide-optimized magnetic screening. Accordingly, ~250 μL bead volumes of RC TentaGel beads or Peptoid Library 1 sub-aliquots were screened in 1.5 mL microfuge tubes in these large-scale assays. Tubes containing this volume of RC TentaGel beads were prepared by pipetting beads swollen in PBST as described in Section 2.IV.A (page 95) into new 1.5 mL microfuge tubes, while Peptoid Library 1 sub-aliquots had been stored in 1.5 mL microfuge tubes following the completion of the experiments described in Section 2.II (page 68). The tubes containing resin were fitted with nylon mesh with 80 μm pores (#CMN-0074-10YD, Small Parts, Inc., Miami Lakes, FL) that had been cut into squares large enough to cover the tube opening, but with some extra to allow a microfuge tube cap, into which a hole had been punched and widened using a needle, to be fitted to the tube. To drain the solution under vacuum from the resin in these tubes while retaining the beads, the tubes were inverted such that the hole in the cap aligned with a serological pipet passing through the stopper of a vacuum flask. The vacuum was allowed to drain the tubes, and when drainage was complete, the tubes were centrifuged briefly to pellet the resin. One milliliter of PBST was then added to each tube by pipetting through the hole in the added cap and the solution was mixed by manual shaking. The tubes were then inverted over the vacuum flask again and

the process was repeated twice more for a total of three washes. Following the last wash, the caps with the holes were removed and set aside according to their tube of origin. The mesh was then removed and immediately inspected for clinging TentaGel beads under a dissecting microscope (Stereo Star Zoom, American Optical). Any beads found were returned to the appropriate tube by pipetting and the mesh was set aside in 60 mm tissue culture dishes labeled with the appropriate tube number. If the pores of the mesh were beginning to widen as seen under the dissecting microscope, that piece of mesh was replaced. The supernatant in each tube was then adjusted by pipetting until the meniscus reached 500 μL (250 μL PBST and 250 μL bead volume). In later rounds of screening, if PBST needed to be added to bring the volume in the tube to 500 μL, the PBST was added before the cap and mesh were removed to help wash down any clinging beads. For each tube of resin to be screened, a 1.5 mL microfuge tube containing 250 μL screening antibody (either RAR5 or RAMIg) diluted in PBST to 2X the desired final concentration was prepared. When this antibody was applied to the appropriate tube of resin, dilution in 250 μL PBST gave 500 μL antibody solution at the desired final concentration. The tubes were then capped with their attached cap (without the hole) and incubated for 1 h on a rotisserie at RT. Meanwhile, tubes of 250 μL PGDs in PBST at 2X the desired final concentration were prepared as described in Section 2.II.A.3 (page 72). At the conclusion of the incubation, the resin was washed five times with PBST as described above, and the appropriate PGD solution was applied. Tubes were then incubated for 30 min on a rotisserie at RT before the positives (TentaGel beads retained by the magnet) and negatives (TentaGel beads not retained by the magnet) were separated using a magnet as described in Section 2.II.A.3. Once all the negatives had been removed, they were stored at 4°C in 1.5 mL microfuge

tubes in PBST to which 2% NaN_3 had been added to a final concentration of 0.02%, as described in Section 2.II.A.3. Meanwhile, the number of positives in each 1.5 mL tube, as well as the density of PGD coverage of each TentaGel bead, were then observed using an inverted light microscope at 40X power and recorded. These TentaGel beads were then ranked in groups according to the density of PGD coverage and, while visualizing them with the inverted light microscope, were transferred by pipetting into new 1.5 mL microfuge tubes containing PBST. This diluted the PGDs prior to separating each TentaGel bead into its own 0.5 mL microfuge tube in preparation for CNBr cleavage and analysis by MS/MS as described in Section 2.I.C.2.c (page 57), performed with Mr. Edward Neill, Ms. Kelly Dye, and Mr. Stephen Ruback.

2.V. Peptoid-optimized magnetic screening of Peptoid Library 6 with a neutralizing MAb against MNV-1

This procedure was performed by Mr. Stephen Ruback after consultation with me based on the results of the experiments described in Section 2.IV (page 95). As described in Section 2.I.B.3 (page 46), Peptoid Library 6 was not pooled after the last monomer addition during synthesis and instead remained separated in aliquots by the last monomer. Sub-aliquots were made for aliquots having 2-aminoethanol and glycine (Table 2.2, pages 103-104 and Fig. 2.6, page 109) as the amines that formed their N-terminal monomers by pipetting resin into 5 mL disposable reaction vessels, then washing with DMF and drying with DCM prior to and after bulk deprotection as described in Section 2.I.B.5 (page 52). After quality control was performed as described in Section 2.I.B.5, the resin was swollen in PBST as described in Section 2.IV.A (page 95) and was first precleared using PGDs alone (screening antibody concentration was 0 μg/mL), as in Table 3.18 (page 170). Based on the results of the experiments in Table 3.18 using the R5 peptoid model system, 1 μg/mL of the neutralizing MAb against MNV-1, bovine-Ig free A6.2 (prepared in Section 2.II.A.2, page 69 and used in Round 5 of the peptide-optimized magnetic screening as described in Section 2.II.A.3, page 72), was used in magnetic screening of these Peptoid Library 6 sub-aliquots as described in Section 2.IV.B (page 97). Beads retained in magnetic screening were split into two groups, then glycine stripped and color screened in a similar manner to that described in Section 2.II.A.4 (page 76), including recording the time to color change and including on-bead $FLAG^+$ peptide controls, through sequencing by MS/MS.

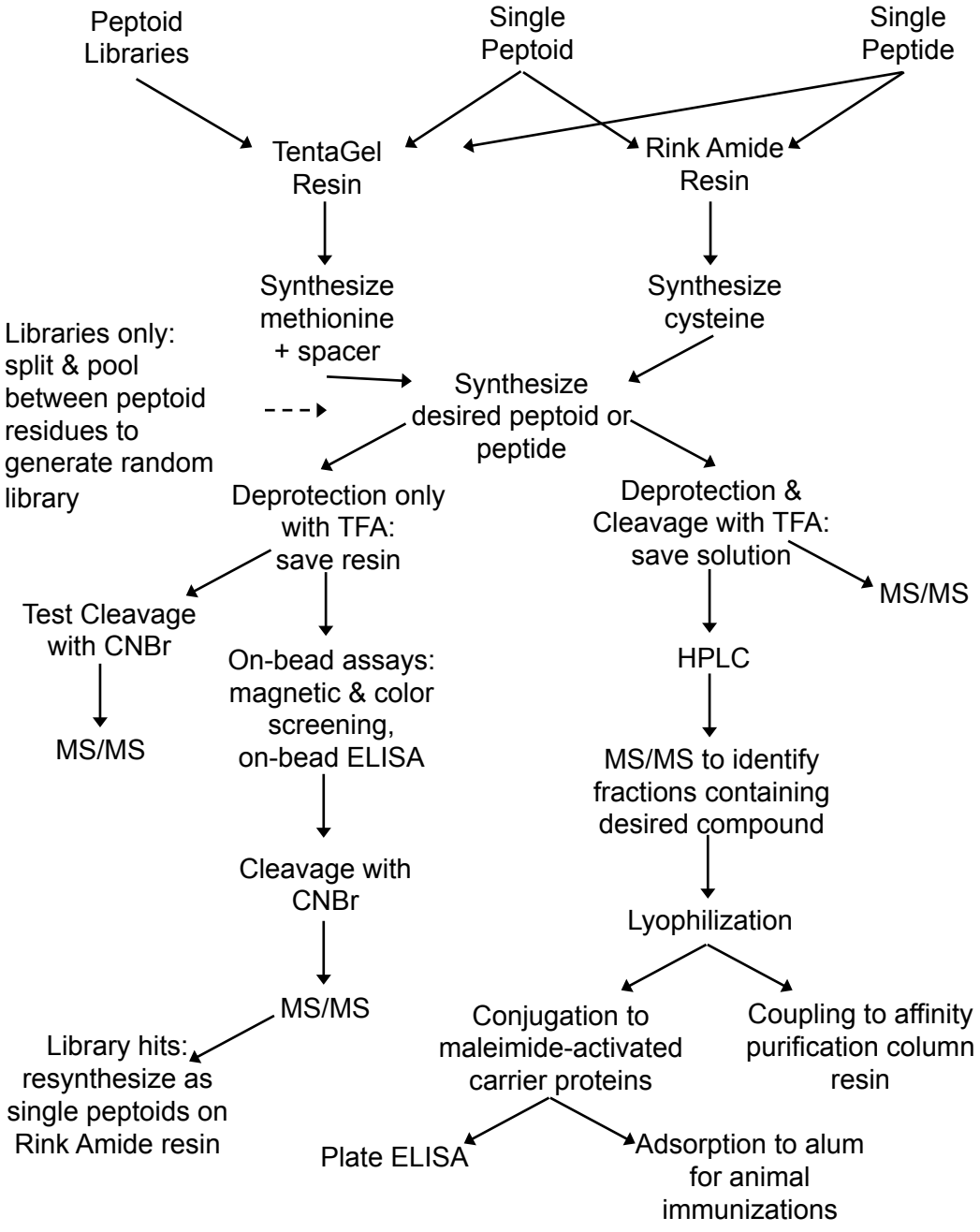


Figure 2.1. General protocol for preparation and use of peptoid libraries, single peptoids, and single peptides. Steps are detailed in Section 2.I (page 38).

Table 2.1. Vitetta lab peptoid libraries synthesized to date.

Library	1	2	3	4	5	6
Linker	Met-Ahex-Ahex	Met-2-aminoethanol*	Met-2-aminoethanol*	Met-methylamine	Met-Ahex-Ahex	Met-methylamine
Length (number of monomers)	5	5	6	6	10	8
Number of monomers used	10	17	17	16	Two alternating groups of 8‡	18
Theoretical compounds	10^5 = 100,000	17^5 = ~1.4 x 10^6	17^6 = ~2.4 x 10^7	16^6 = ~1.6 x 10^7	8^{10} = ~1.1 x 10^9	18^8 = ~1.1 x 10^{10}
Number of beads	1.56 x 10^{6}†	1.82 x 10^6	1.3 x 10^6	1.3 x 10^6	2.1 x 10^6	1.1 x 10^7
Grams TentaGel Macrobead HL beads (140-170 μm)	3	3.5	2.5	2.5	4	22
Theoretical redundancy	~15.6 (split 5 ways)	~1.3	~0.054	~0.08	~0.002	~0.001
Following completion of synthesis, pooling was omitted to allow the library to remain as aliquots according to last peptoid monomer?	Yes	No	No	No	Yes	Yes

*As peptoid monomer nomenclature is still being debated by the field, peptoid monomers are listed by the name of the amine used to provide the peptoid monomer side group.

†Each aliquot (A through J, according to the last monomer added in each reaction vessel) was split into 5 sub-aliquots so that several screening antibodies could be used to screen fresh peptoid library resin. Each of the 10 aliquots (A through J) therefore contained 1.56 x 10^6 beads divided by ten, or approximately 156,000 beads, with approximately 31,200 beads in each sub-aliquot. Since the entire Library 1 had a theoretical redundancy of approximately 15 copies of each possible peptoid, each sub-aliquot contained approximately three theoretical copies of each possible peptoid, or 31,200/3 = 10,400 theoretical different compounds.

‡Of the 8 monomers in each set, one was shared between both sets for a total of 15 different monomers included.

Table 2.2. Amines used in the synthesis of Vitetta lab peptoids and peptoid libraries, adapted from Case 2012.

Number	Amine (protected or protected salt if applicable)	Product number (for protected or protected salt if applicable)	Supplier	CAS Number		Molecular Weight (MW)		Library				
				Deprotected and/or desalted if applicable	Protected or protected salt if applicable	Amine (protected and/or desalted if applicable)	Monomer, deprotected if applicable	1	2 & 3	4	5	6
1	2-aminoethanol (B)* (O-*tert*-butyl-2-aminoethanol)†	53001	CSPS Pharmaceuticals, San Diego, CA	141-43-5	CAS number not available	61.08 (117.19)	101.08	X	X (also linker)	X	X	X
2	**isobutylamine**	A11017, L14469	Alfa Aesar, Ward Hill, MA	78-81-9		73.14	113.14	X	X	X		
3	**glycine (A, B)** (glycine *tert*-butyl ester hydrochloride)‡	03072	Chem-Impex, Wood Dale, IL	56-40-6	27532-96-3	75.07 (131)	115.07	X	X	X	X	X
4	**1,4-butanediamine (B)** (N-Boc-1,4-butanediamine)	15404	Sigma-Aldrich, St. Louis, MO	110-60-1	68076-36-8	88.15 (188)	128.15	X	X	X	X	X
5	benzylamine	185701; 13180	Sigma-Aldrich; Fluka, Buchs, Switzerland	100-46-9		107.16	147.16	X	X	X		X
6	1-aminoindan	A1309	TCI, Tokyo, Japan	34698-41-4		133.19	173.19	X				X
7	**tyramine**§	T90344	Sigma-Aldrich	51-67-2		137.18	177.18	X	X			X
8	1-(3-aminopropyl)-2-pyrrolidinone (A)	136565	Sigma-Aldrich	7663-77-6		142.2	182.2	X			X	
9	2,2-diphenethylamine	D206709	Sigma-Aldrich	3963-62-0		197.28	237.28	X	X	X		X
10	4-(2-aminoethyl)benzenesulfonamide (B)	275247	Sigma-Aldrich	35303-76-5		200.26	240.26	X			X	
11	*allylamine (A)*	220730500; 220732500	Acros Organics, Geel, Belgium	107-11-9		57.09	97.09		X	X	X	
12	ethylenediamine (A) (N-Boc-ethylenediamine)	15369	Sigma-Aldrich	107-15-3	57260-73-8	60.1 (160.21)	100.1				X	
13	butylamine (A)	L03575 or 42778	Alfa Aesar	109-73-9		73.14	113.14				X	
14	cyclohexanemethylamine‖ (B)	101842	Sigma-Aldrich	3218-02-8		113.2	153.2				X	
15	2-(1-cyclohexenyl)ethylamine‖ (B)	255866; 243150250, 243250250	Sigma-Aldrich; Acros	3399-73-3		125.22	165.22			X	X	X

Table 2.2. Continued.

Number	Amine (protected or protected salt if applicable)	Product number (for protected or protected salt if applicable)	Supplier	CAS Number		Molecular Weight (MW)		Library				
				Deprotected and/or desalted if applicable	Protected or protected salt if applicable	Amine (protected and/or desalted if applicable)	Monomer, deprotected if applicable	1	2 & 3	4	5	6
16	2-thiopheneethylamine(A)	423270	Sigma-Aldrich	30433-91-1		127.21	167.21				X	
17	3-butoxypropylamine (A)	123544	Sigma-Aldrich	16499-88-0		131.22	171.22				X	
18	2-(4-chlorophenyl)-ethylamine (A)	C65408	Sigma-Aldrich	156-41-2		155.63	195.63				X	
19	3,4-dimethoxybenzylamine = veratrylamine (B)	V1309	Sigma-Aldrich	5763-61-1		167.21	207.21		X	X	X	X
20	4-phenylbenzylamine (B)	552313	Sigma-Aldrich	712-76-5		183.26	223.26				X	X
21	cyclobutylamine	225185	Sigma-Aldrich	2516-34-9		71.12	111.12		X	X		X
22	3-methoxypropylamine	M25007; 257520050	Sigma-Aldrich; Acros	5332-73-0		89.14	129.14		X	X		X
23	tetrahydrofurfurylamine (racemic)	131911	Sigma-Aldrich	4795-29-3		101.15	141.15		X	X		
24	4-(aminomethyl)pyridine	A65603	Sigma-Aldrich	3731-53-1		108.14	148.14		X			X
25	histamine	H7125	Sigma-Aldrich	51-45-6		111.15	151.15		X			
26	4-(aminomethyl)piperidine [1-Boc-4-(aminomethyl)piperidine]	641472	Sigma-Aldrich	7144-05-0	144222-22-0	114.3 (214.30)	154.3		X	X		
27	**piperonylamine**	131360250	Acros	2620-50-0		151.1	191.1		X	X		
28	tryptamine	19,374-7	Sigma-Aldrich	61-54-1		160.22	200.22		X			X
29	furfurylamine	119802500; 48120	Acros; Fluka	617-89-0		97.06	137.06			X		
30	(R)-(+)-α-methylbenzylamine	11,554-1	Sigma-Aldrich	3886-69-9		121.18	161.18			X		
31	propargylamine	P50900	Sigma-Aldrich	2450-71-7		55.08	95.08					X
32	*exo*-2-aminonorbornane	179604	Sigma-Aldrich	7242-92-4		111.18	151.18					X
33	2-(aminomethyl)-5-methylpyrazine	A1154	TCI	132664-85-8		123.16	163.16					X
34	4-(aminomethyl)benzonitrile [4-(aminomethyl)benzonitrile hydrochloride]‡	631396	Sigma-Aldrich	10406-25-4	15996-76-6	(132.62)	172					X
35	*2-methoxyethylamine*	241067; 162582500	Sigma-Aldrich; Acros	109-85-3		75.11	115.11	(included in RC only)				
36	methylamine as 2 M solution in tetrahydropuran (THF)	395056	Sigma-Aldrich	74-89-5		31.06	71.06			(linker only)		(linker only)

*(A) and (B) denote set A and set B amines used to synthesize Peptoid Library 5; to construct the library, these sets of amines were used as follows: B-A-B-A-B-A-B-A-B-A-Ahex-Ahex-Met-TentaGel resin (see Table 2.1, page 102 and Section 3.II.A, page 123 for additional details)

†Product information is for *tert*-butyl protected, but THP-protected 2-aminoethanol (see Section 2.I.A.1, page 38) was used in Peptoid Library 1

‡Purchased as a salt (hydrochloride); free base must be prepared prior to use in peptoid synthesis (see Section 2.I.A.2, page 38)

§Diluted in DMSO for peptoid synthesis (not DMF)

||Diluted in NMP for peptoid synthesis (not DMF)

Bold = included in R5 peptoid

italics = included in RC peptoid; 2-methoxyethylamine was not included in peptoid libraries because its monomer MW, 115 (shown in red), is the same as glycine, and each monomer in a library must have a unique MW

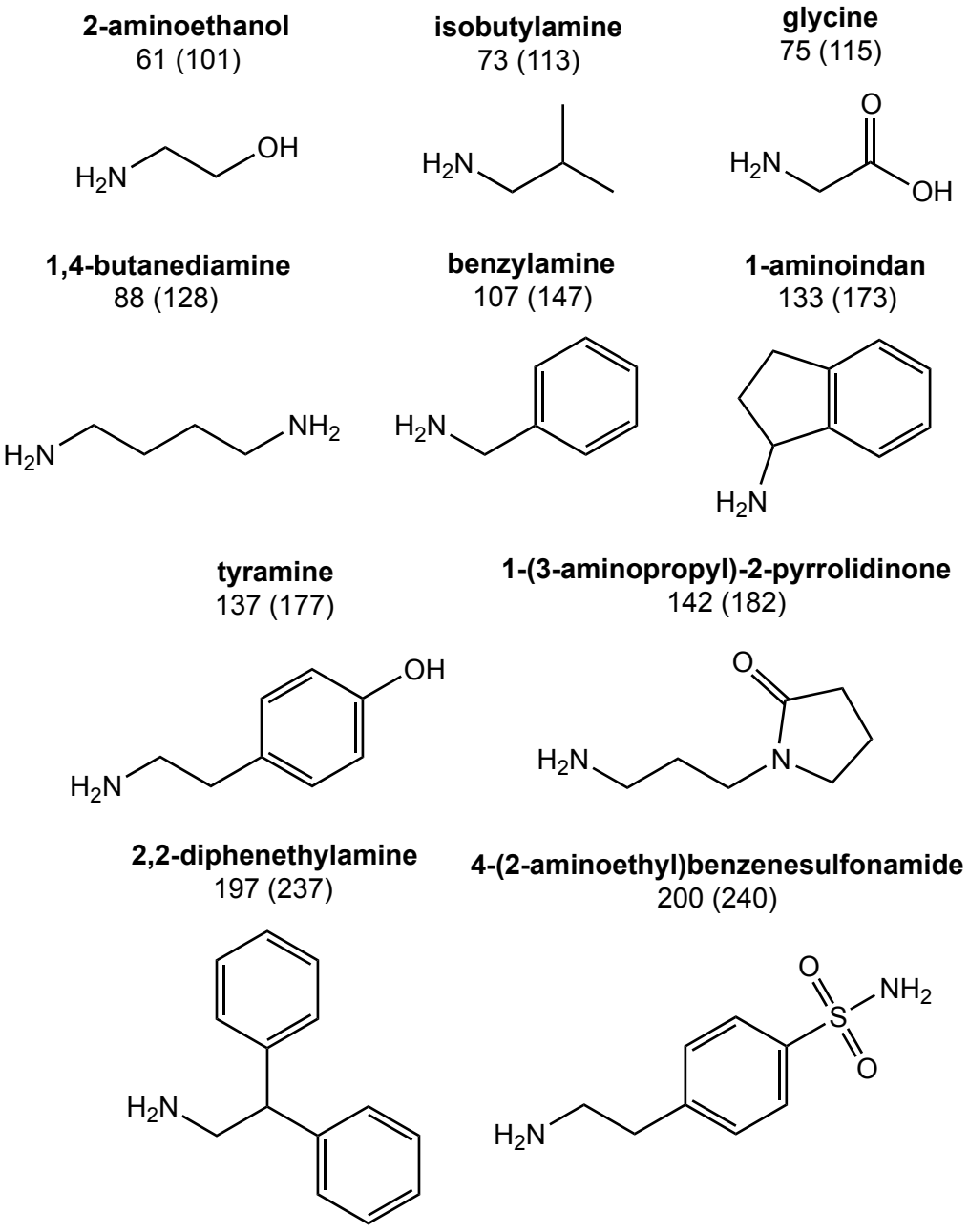


Figure 2.2. Structures of amines included in Peptoid Library 1.
Amines were chosen to cover chemical space (for example, aliphatic, aromatic, hydrophobic, hydrophilic, and positive or negative charge), and to maintain at least one dalton difference in mass such that the unique mass of each amine could be used to distinguish between peptoid monomers when sequencing library peptoids by MS/MS. The mass of the amine is shown below its name with the mass of the peptoid monomer synthesized using that amine shown in parentheses.

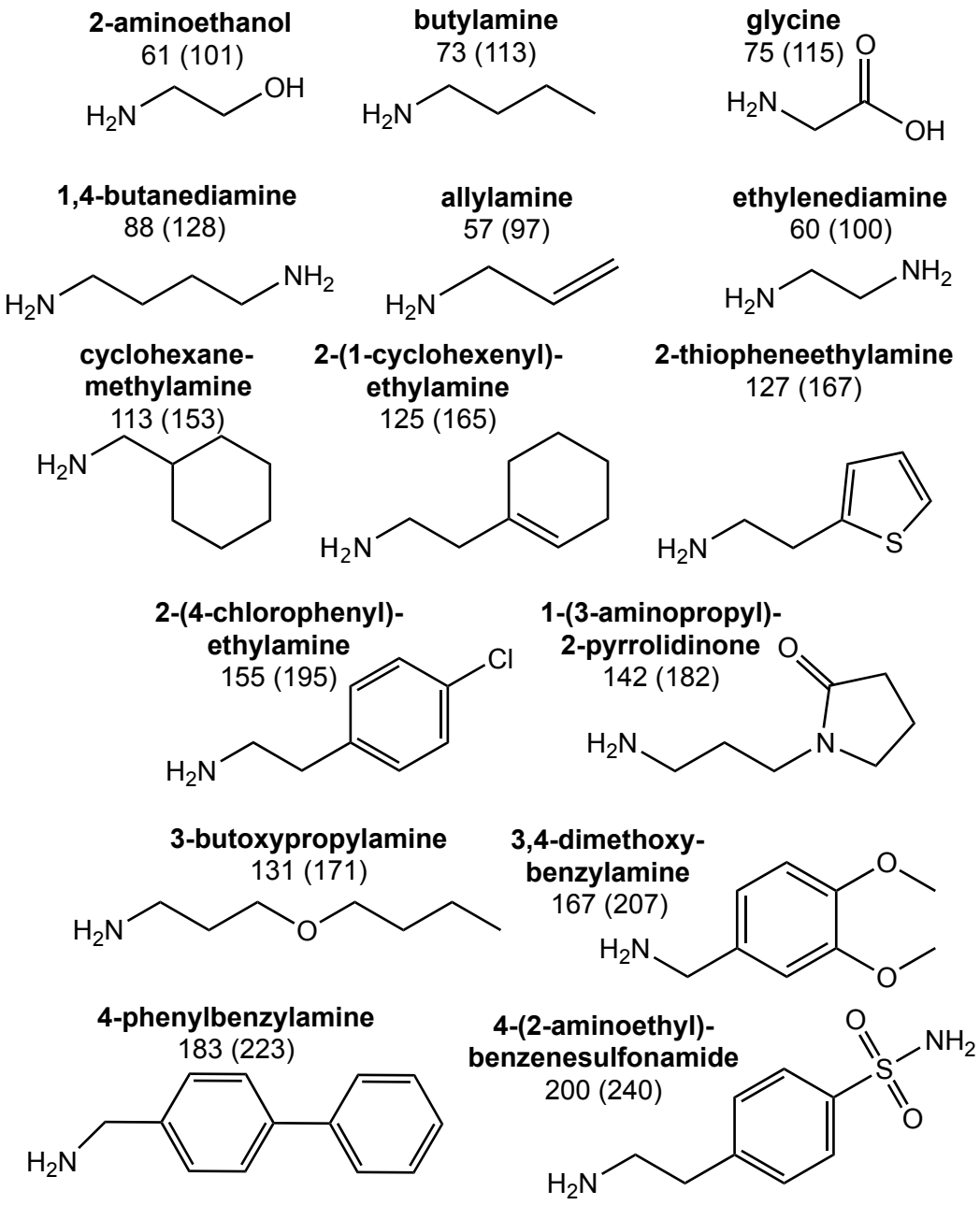


Figure 2.3. Structures of amines included in Peptoid Library 5.
Amines were chosen to cover chemical space (for example, aliphatic, aromatic, hydrophobic, hydrophilic, and positive or negative charge), and to maintain at least one dalton difference in mass such that the unique mass of each amine could be used to distinguish between peptoid monomers when sequencing library peptoids by MS/MS. The mass of the amine is shown below its name with the mass of the peptoid monomer synthesized using that amine shown in parentheses.

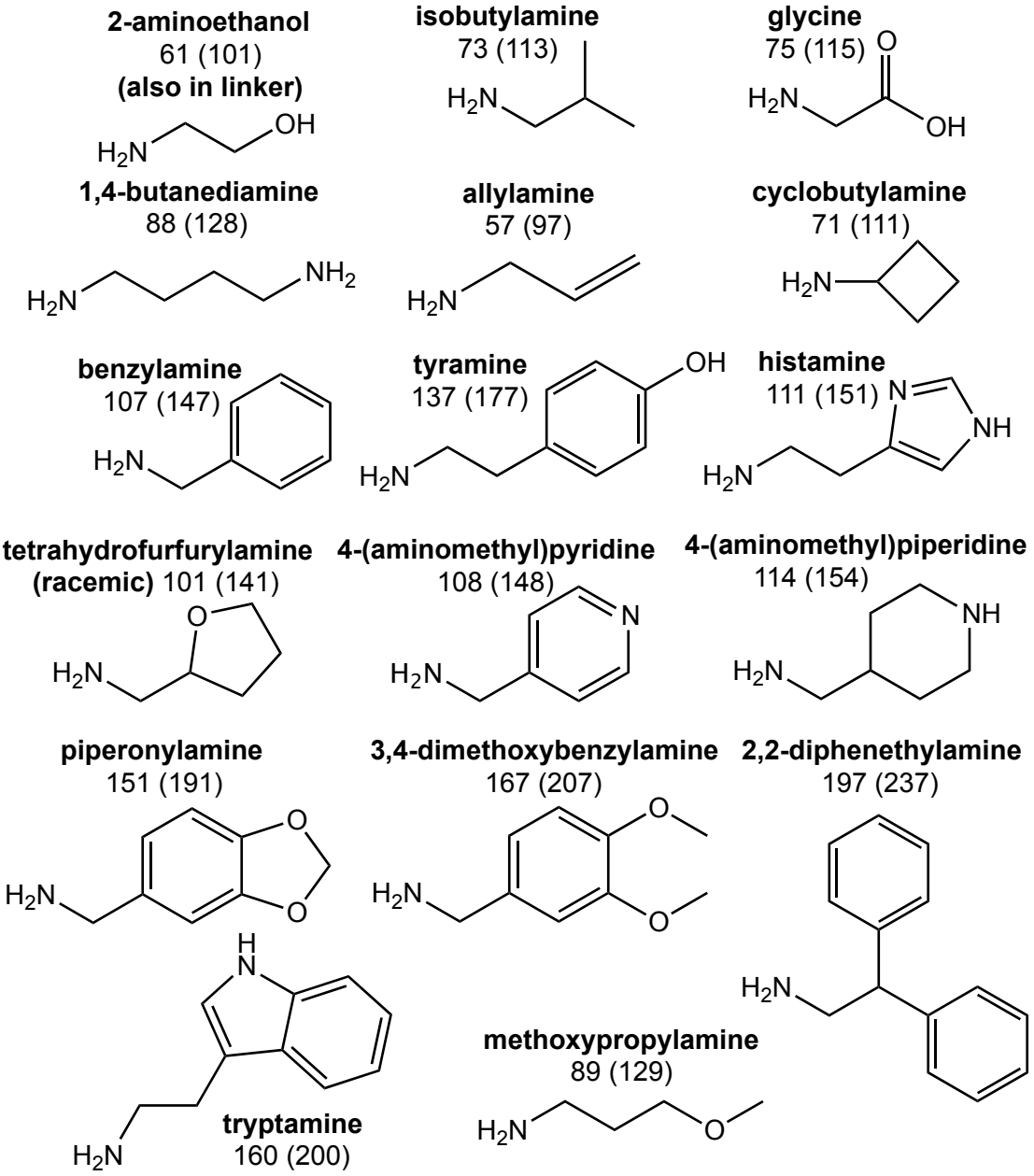


Figure 2.4. Structures of amines included in Peptoid Libraries 2 and 3.
Amines were chosen to cover chemical space (for example, aliphatic, aromatic, hydrophobic, hydrophilic, and positive or negative charge), and to maintain at least one dalton difference in mass such that the unique mass of each amine could be used to distinguish between peptoid monomers when sequencing library peptoids by MS/MS. The mass of the amine is shown below its name with the mass of the peptoid monomer synthesized using that amine shown in parentheses.

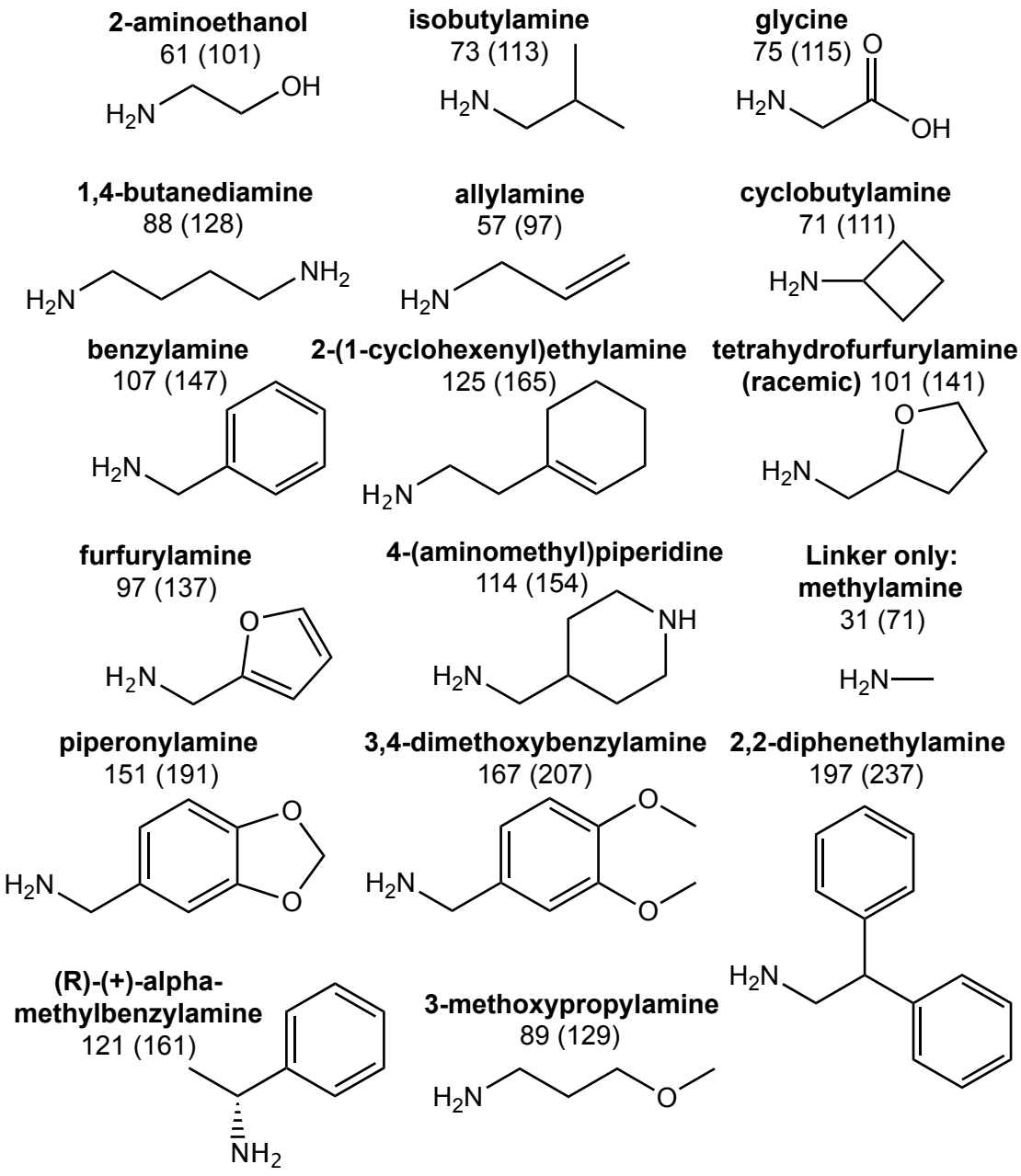


Figure 2.5. Structures of amines included in Peptoid Library 4.
Amines were chosen to cover chemical space (for example, aliphatic, aromatic, hydrophobic, hydrophilic, and positive or negative charge), and to maintain at least one dalton difference in mass such that the unique mass of each amine could be used to distinguish between peptoid monomers when sequencing library peptoids by MS/MS. The mass of the amine is shown below its name with the mass of the peptoid monomer synthesized using that amine shown in parentheses.

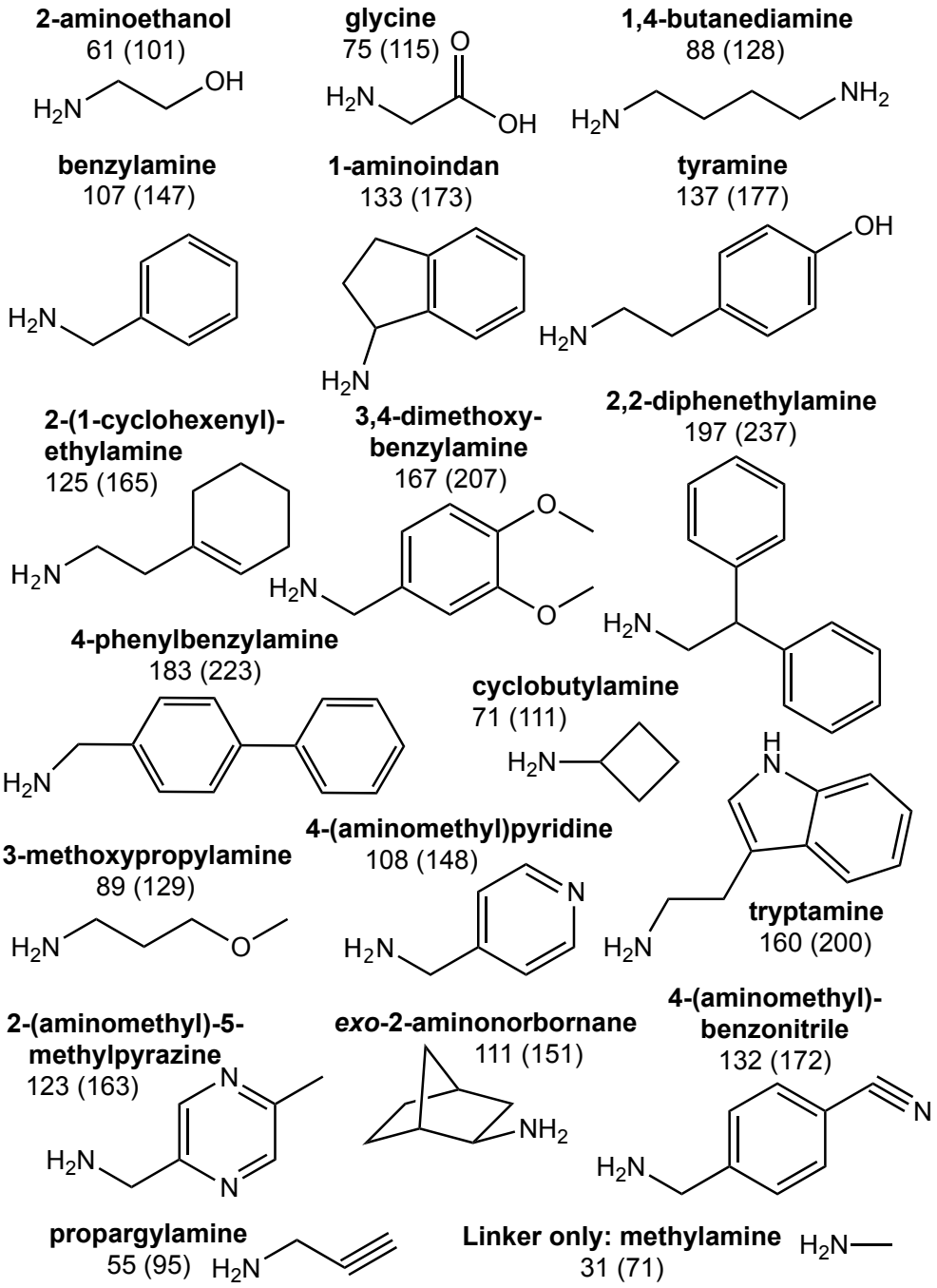
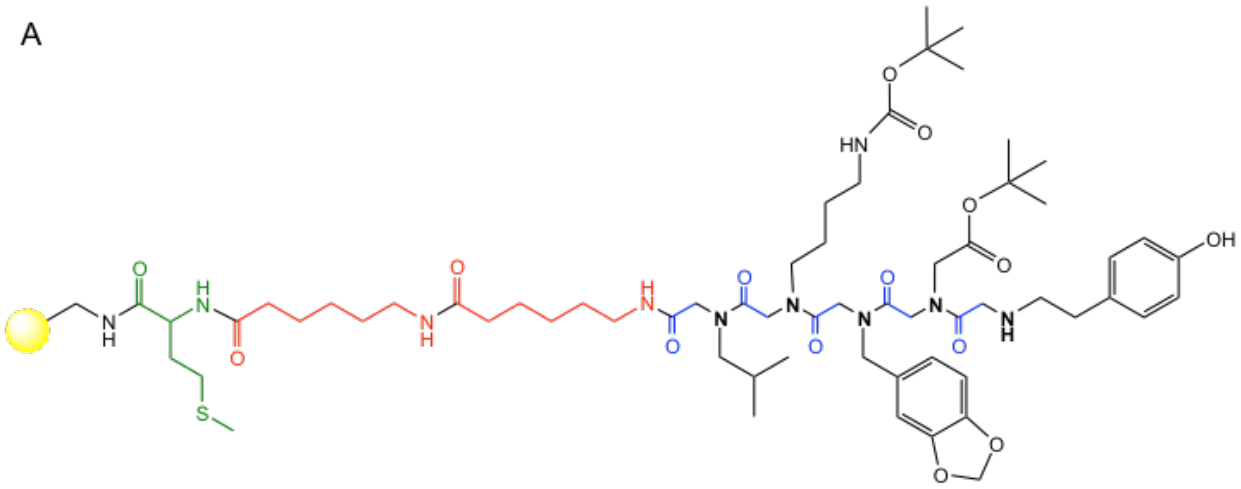
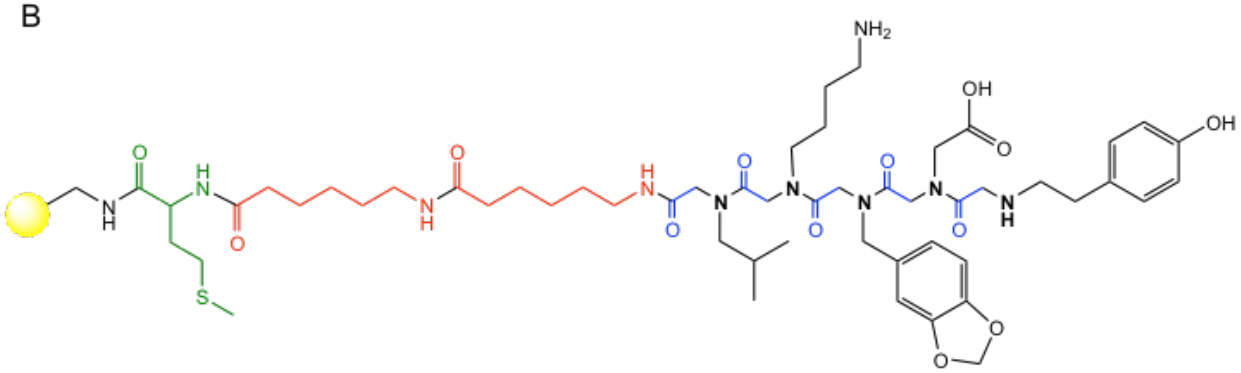


Figure 2.6. Structures of amines included in Peptoid Library 6.
Amines were chosen to cover chemical space (for example, aliphatic, aromatic, hydrophobic, hydrophilic, and positive or negative charge), and to maintain at least one dalton difference in mass such that the unique mass of each amine could be used to distinguish between peptoid monomers when sequencing library peptoids by MS/MS. The mass of the amine is shown below its name with the mass of the peptoid monomer synthesized using that amine shown in parentheses.

A



B



C

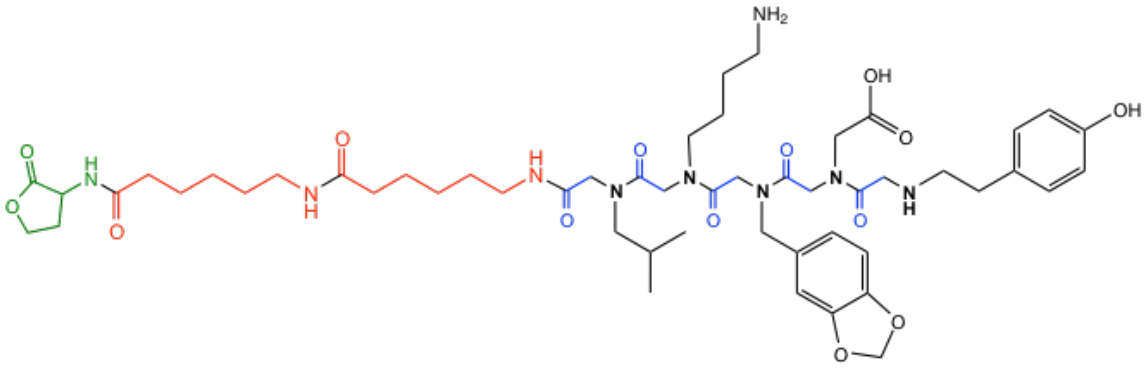
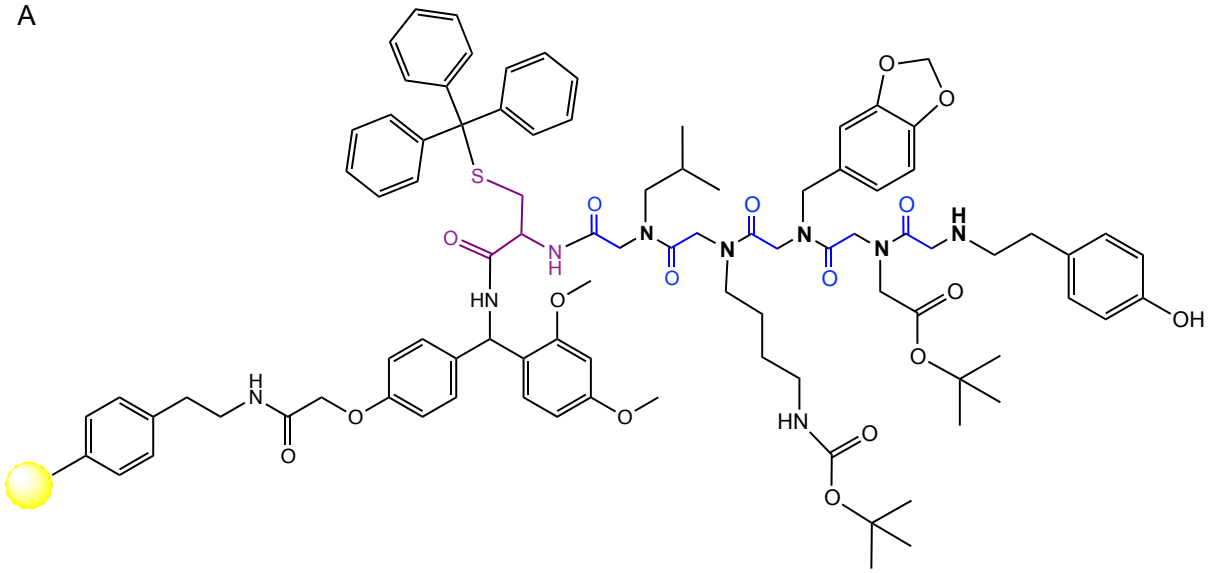
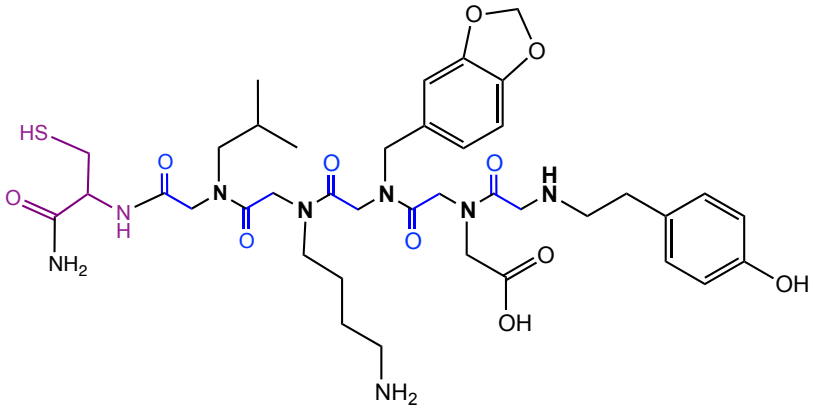


Figure 2.7. Rabbit 5-mer (R5) peptoid for on-bead assays.
A. R5 peptoid immediately following synthesis on TentaGel resin (Section 2.I.C.2.b, page 56). R5 peptoid was synthesized from C terminus to N terminus on amino-functionalized TentaGel resin. A Met residue (shown in green) was first added to allow eventual cleavage of the compound from the resin. Two residues of Ahex (shown in red) were then added to act as a spacer between the resin and the peptoid and, with the Met residue, provide a known mass for ease of sequencing by MS/MS. R5 peptoid itself was then synthesized using the two-step submonomer method (Fig. 1.3, page 35). Amines with side chains requiring protecting groups to avoid side reactions are shown with their protecting groups in place. The amines used to synthesize R5 peptoid are listed in Table 2.2 (pages 103-104). Synthesis of the correct compound was verified by deprotection using a TFA solution, cleavage of the peptoid from representative beads at the Met residue using a CNBr solution, and sequencing by MS/MS as described in Fig. 2.1 (page 101) and Section 2.I.C.2.c (page 57); see panel C for structure. **B.** R5 peptoid synthesized on TentaGel resin following the removal of protecting groups. Deprotection was carried out using a strong solution of the acid TFA and scavengers as described in Sections 2.I.C.2.c (page 57) and 2.I.C.2.d (page 58). **This was the form of R5 peptoid used in on-bead assays.** Deprotection was verified by cleavage of the peptoid from representative beads using a CNBr solution and sequencing by MS/MS as described in Fig. 2.1 and Sections 2.I.C.2.c and 2.I.C.2.d (see panel C for structure). **C.** R5 peptoid cleaved from TentaGel resin. For verification of synthesis and/or deprotection (Sections 2.I.C.2.c and 2.I.C.2.d), or following screening assays, a solution containing CNBr was used to cleave R5 peptoid from the TentaGel resin. The products of this reaction were then mixed with CHCA matrix for verification of the correct mass by MALDI-TOF MS and sequencing by MALDI-TOF/TOF MS/MS (Sections 2.I.C.2.c and 2.I.C.2.d).

A



B



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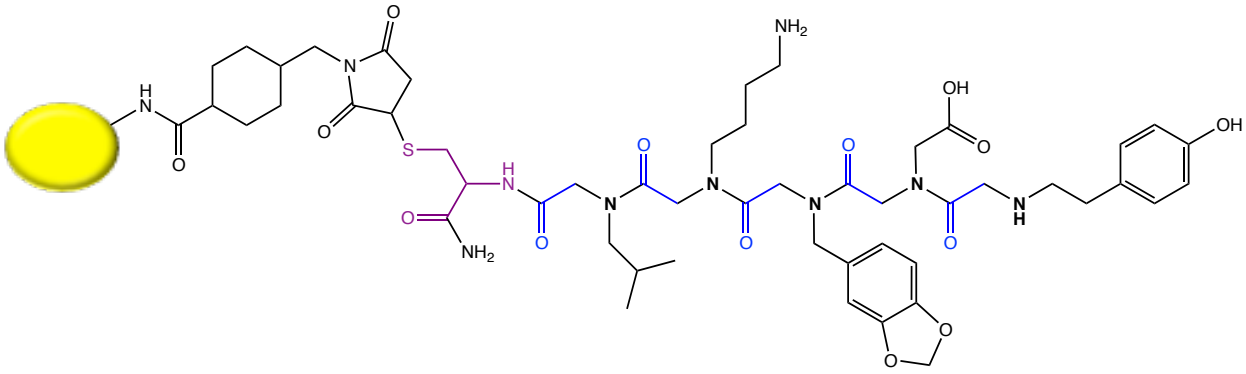


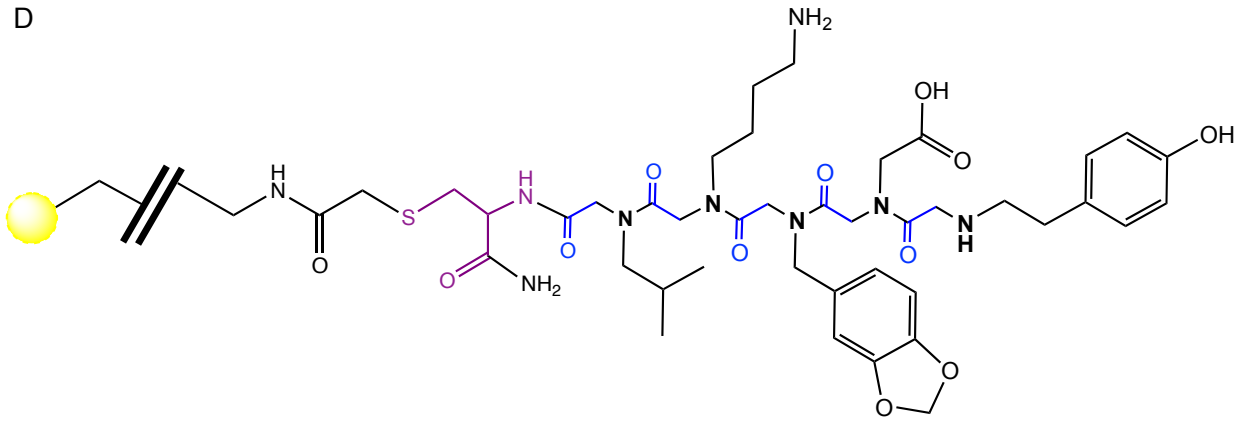
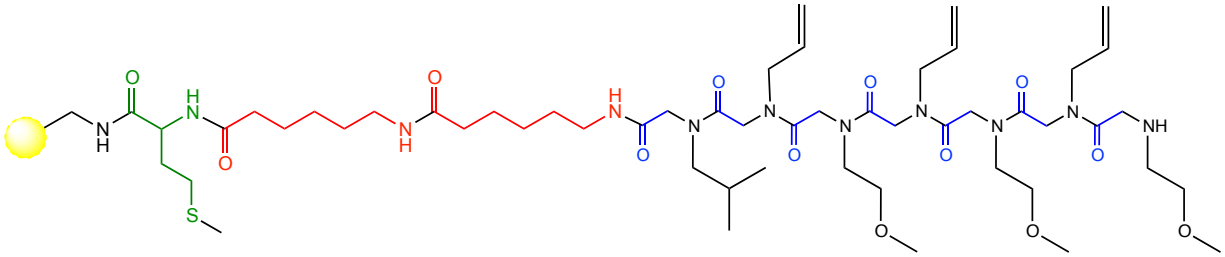


Figure 2.8. R5 peptoid for conjugation to carrier proteins or coupling to SulfoLink resin for affinity purification.
A. R5 peptoid immediately following synthesis on Rink amide resin (Section 2.I.C.3.b, page 59). R5 peptoid was synthesized from C terminus to N terminus on Rink Amide resin. A Cys residue (shown in purple) was first added to allow eventual conjugation of the compound to maleimide-activated carrier proteins (panel C and Section 2.I.D.2, page 64) or coupling to SulfoLink resin (panel D and Section 2.III.B, page 85). This Cys residue also provided a known mass for ease of sequencing by MS/MS. R5 peptoid itself was then synthesized using the two step-submonomer method as described in Fig. 2.7 (page 110). Synthesis of the correct compound was verified by simultaneous deprotection and cleavage of the peptoid from representative beads followed by sequencing by MS/MS as described in Fig. 2.1 (page 101) and Sections 2.I.C.3.c (page 59) and 2.I.C.3.d (page 60) (see panel B for structure). **B.** R5 peptoid following the removal of protecting groups and simultaneous cleavage from Rink Amide resin. Simultaneous deprotection and cleavage was carried out using a strong solution of the acid TFA and scavengers. To verify deprotection and cleavage, the products of this reaction were mixed with CHCA matrix for verification of the correct mass by MALDI-TOF MS and sequencing by MALDI-TOF/TOF MS/MS as described in Fig. 2.1 and Sections 2.I.C.3.c. and 2.I.C.3.d. To purify R5 peptoid from contaminants, RP-HPLC was then employed (Section 2.I.C.3.e, page 61), and the eluted fractions identified by MS as containing purified R5 peptoid were lyophilized as described in Fig. 2.1 and Section 2.I.C.3.f (page 63). **C.** R5 peptoid conjugated to a maleimide-activated carrier protein, represented by the yellow oval. R5 peptoid was incubated with a maleimide-activated carrier protein (KLH or BSA) before being passed through a desalting column to remove contaminants (Section 2.I.D.2). **This was the form of R5 peptoid used to coat ELISA plates** (R5-KLH and R5-BSA; Sections 2.III.A.4, page 82 and 2.III.B.4, page 94) **and in rabbit immunizations** (R5-KLH only; Section 2.III.A.2, page 80). **D.** R5 peptoid coupled to SulfoLink resin [fig. adapted from the manufacturer's protocol (340)]. SulfoLink resin consists of agarose beads functionalized with a 12-atom spacer (omitted from the drawing but indicated by the double black lines) followed by an iodoacetyl group. By nucleophilic displacement of the iodoacetyl iodine with the sulfur of the C-terminal Cys residue (shown in purple) of R5 peptoid, a thioether bond was formed that linked R5 peptoid to the SulfoLink resin. **This is the form of R5 peptoid used for affinity purification of rabbit anti-R5 peptoid antibodies** (Section 2.III.B.2, page 86) from the serum of a rabbit immunized with

R5-KLH adsorbed to alum. The SulfoLink linker was specifically chosen for this affinity purification because it is different from the maleimide linker shown in panel C used for conjugation of R5 peptoid to KLH for rabbit immunizations. Therefore, anti-R5 peptoid antibodies could be purified away from antibodies against the maleimide linker.

A



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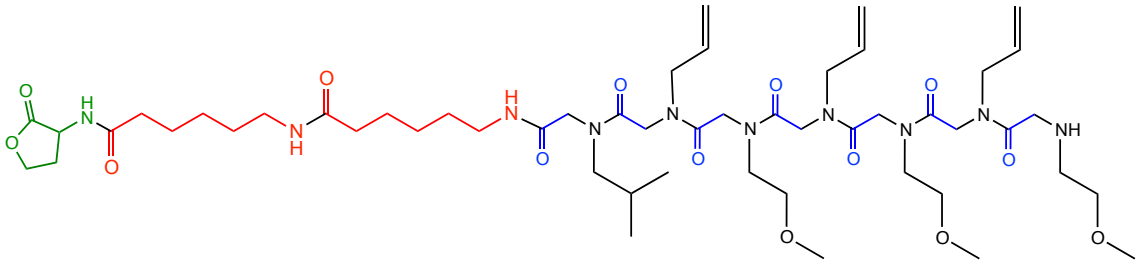
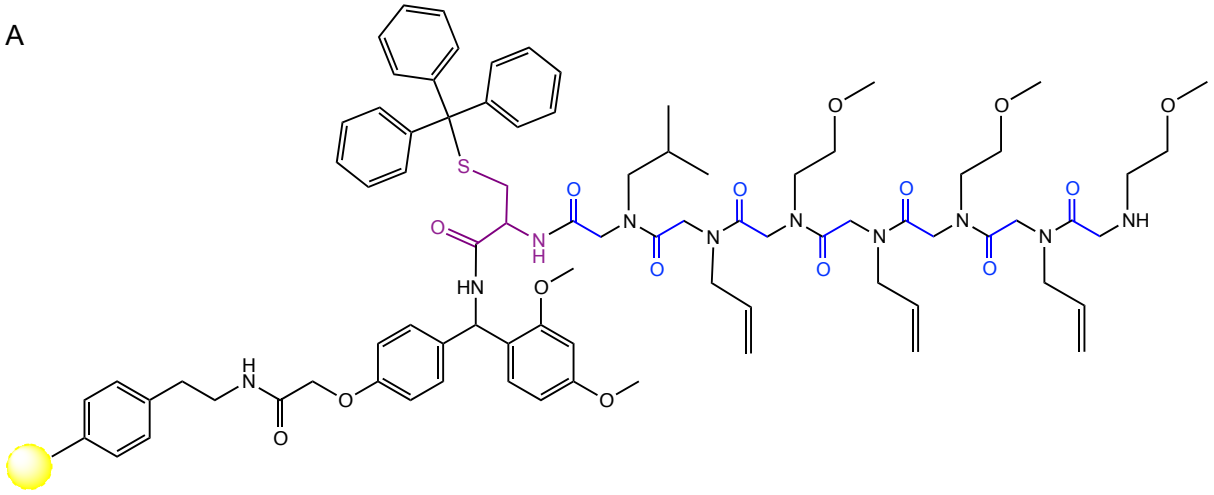


Figure 2.9. Rabbit control (RC) peptoid for on-bead assays.
A. RC peptoid immediately following synthesis on TentaGel resin (Section 2.I.C.2.b, page 56). RC peptoid was synthesized and the correct synthesis was verified as described for R5 peptoid synthesized on TentaGel resin in Fig. 2.7 (page 110). Note that none of the amines used in the synthesis of RC peptoid required the use of protecting groups (see Table 2.2, pages 103-104). However, prior to use in on-bead assays, deprotection was carried out as described for R5 peptoid (Fig. 2.7) in order to allow this compound to serve as a control for R5 peptoid. **This was the form of RC peptoid used in on-bead assays. B.** RC peptoid cleaved from TentaGel resin. For verification of synthesis (Section 2.I.C.2.c, page 57) and/or mock deprotection (Section 2.I.C.2.d, page 58), or following screening assays, RC peptoid was subjected to CNBr cleavage and analysis by MS and MS/MS as described for R5 peptoid (Fig. 2.7).

A



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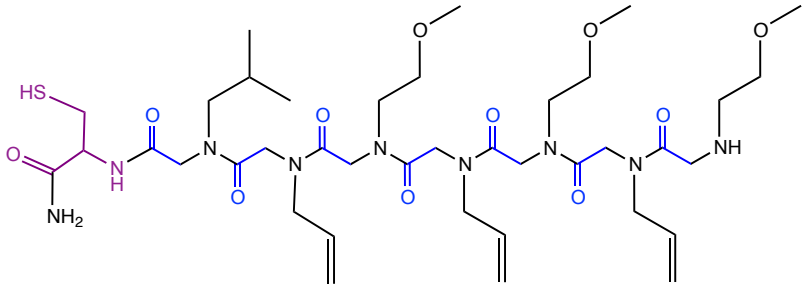

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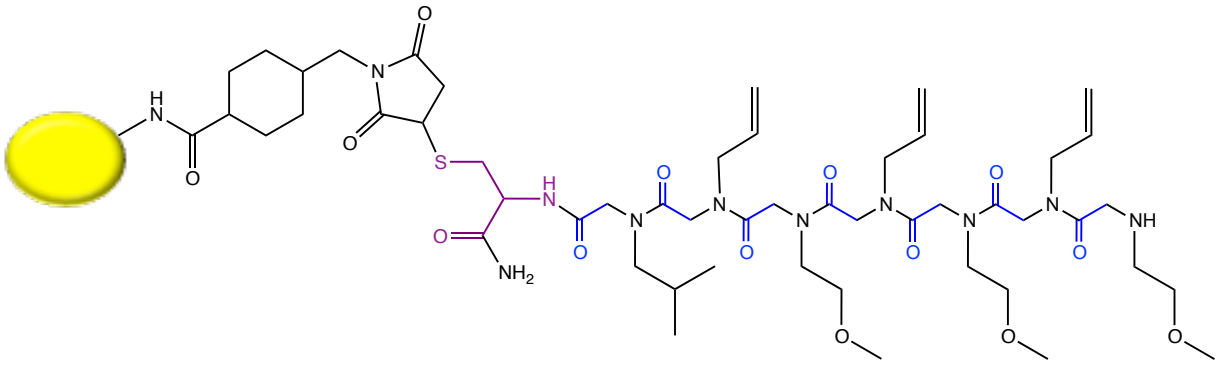

Figure 2.10. RC peptoid for conjugation to carrier proteins.
A. RC peptoid immediately following synthesis on Rink Amide resin (Section 2.I.C.3.b, page 59). RC peptoid was synthesized and the correct synthesis was verified as described for R5 peptoid synthesized on Rink Amide resin (Fig. 2.8, page 112). Note that none of the amines used in the synthesis of RC peptoid required the use of protecting groups. **B.** RC peptoid following cleavage from Rink amide resin. Cleavage, purification, and lyophilization were carried out as described for R5 peptoid (Fig. 2.8). **C.** RC peptoid conjugated to a maleimide-activated carrier protein, represented by the yellow oval, as described for R5 peptoid (Fig. 2.8). **This was the form of RC peptoid used to coat ELISA plates** (RC-BSA; Sections 2.III.A.4, page 82 and 2.III.B.4, page 94).

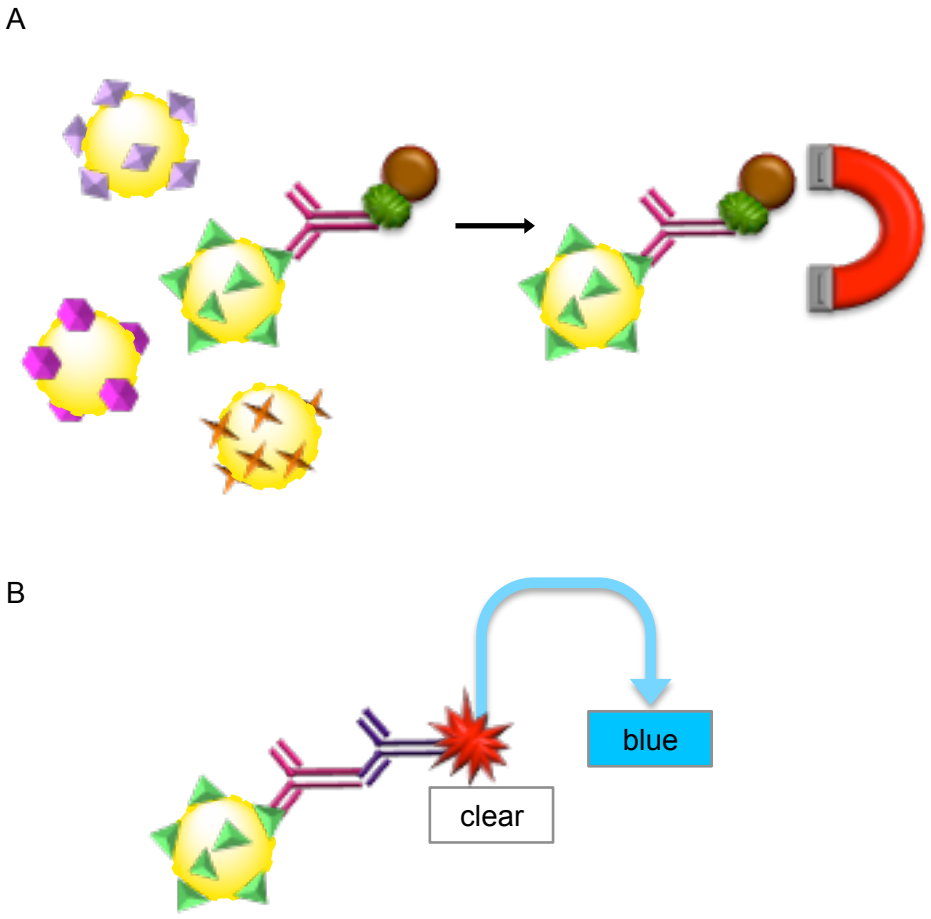

Figure 2.11. Sequential magnetic- and color-based assays used to screen on-bead peptoid libraries.
A. In the magnetic screening assay, on-bead peptoids or peptides (represented by the yellow circles bearing colored shapes) were incubated with the screening MAb (shown in pink; in some optimization experiments, a PAb was used). On-bead peptoids or peptides bound by the screening MAb were then selected using PGDs. Protein G (shown in dark green) has a high affinity for the Fc or tail portion of antibodies. The iron oxide core of the PGDs (represented by the small brown circle) is magnetic, and upon application of a magnet, the antibody-bound, PGD-bound peptoid or peptide beads were isolated from on-bead peptoids or peptides not bound by the screening antibody. **B.** In the color screening assay, on-bead peptoids or peptides selected in the magnetic screening assay were re-exposed to the screening MAb, then incubated with a species-specific secondary antibody (shown in purple) conjugated to the enzyme HRP (represented by the red burst). When an appropriate color-changing substrate (3,3',5,5'-tetramethylbenzidine; TMB) for HRP was added, complexes of on-bead peptoid or peptide, screening antibody, and secondary antibody showed a color change from clear to blue that was visible often by the naked eye and more clearly using a dissecting microscope. By using the magnetic and color screening assays in succession, the number of false positives may be reduced.

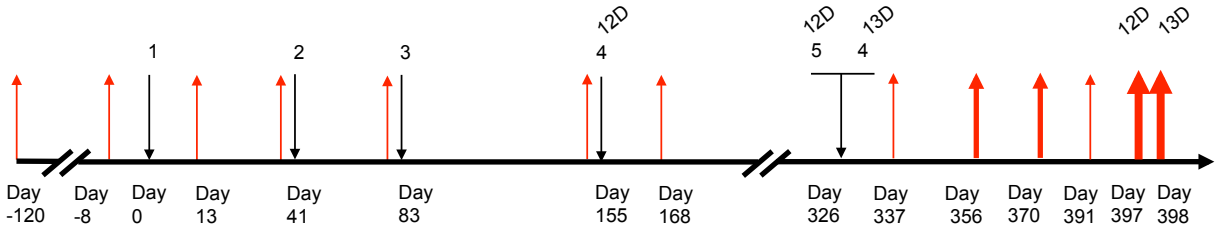

Figure 2.12. Immunization of rabbits with R5 peptoid conjugated to the carrier protein KLH and adsorbed to alum to produce affinity-purified rabbit PAb anti-R5 peptoid. Timeline of rabbit bleeds and immunizations with R5-KLH adsorbed to alum. Two rabbits, designated 12D and 13D, were bled (red arrows) on Day -120 and Day -8 prior to receiving any immunizations. Sera from these bleeds, prepared by allowing the blood to clot overnight at 4°C followed by centrifugation and removal of the supernatant as the serum component, were designated pre-immunization sera. The rabbits were then immunized on the days shown (black arrows) with 250-500 μg R5-KLH adsorbed to alum. Rabbit 12D received a total of five immunizations (as indicated above the black arrows), while rabbit 13D received four. Throughout the process of immunization, test bleeds (thinnest red arrows) of approximately 5 mL were collected (Days 13, 41, 83, 155, 168, and 337). Sera prepared from these test bleeds were used to monitor the anti-R5 peptoid and anti-KLH antibody concentrations by ELISA. When the ELISAs indicated that anti-R5 peptoid and anti-KLH antibody concentrations exceeded 1 mg/mL (sera from Day 337), two production bleeds of approximately 30 mL (Days 356 and 370, thicker red arrows) were collected, along with one additional test bleed on Day 391, to accumulate as much blood as possible before the rabbits were exsanguinated (thickest red arrows) on Day 397 (rabbit 12D) and 398 (rabbit 13D). Sera from the two production bleeds and exsanguination were prepared and tested by ELISA as previously, then pooled for each rabbit and designated post-immunization sera. Approximately 60 mL of post-immunization serum were obtained for rabbit 12D and 40 mL for rabbit 13D.

CHAPTER THREE

Results

3.I. Project objectives

The long-term goal of the peptoid vaccine development project in the Vitetta laboratory is to identify peptoids that mimic the shape of B cell epitopes that elicit broadly protective antibodies against toxins or pathogens, and to use these mimetic peptoid B cell epitopes to make prophylactic vaccines. The strategic plan we adopted to achieve these goals included several steps, several of which were addressed in this dissertation research.

1. Synthesize large OBOC peptoid libraries.
2. Screen peptoid libraries with *known* broadly protective MAbs against toxins or pathogens, isolate candidate peptoids, and determine their sequences.
3. Resynthesize the candidate peptoids in bulk.
4. Conjugate the candidate peptoids to carrier proteins.
5. Adsorb these peptoid-carriers to alum and use them to immunize mice in the boosting phase of a prime-boost model. In the priming phase, mice are immunized with the native antigen that the screening MAb recognizes. In preparation for these priming immunizations, the native antigen is conjugated to a carrier protein if necessary, but always adsorbed to alum. Following priming immunizations, the animals are rested until their circulating antibody levels returned to baseline. They are then boosted with the candidate peptoid-carrier conjugates adsorbed to alum. This prime-boost model should provide a sensitive test for peptoid mimicry of the native antigen, since memory B and T cells developed during the priming phase

should be more easily stimulated by the peptoids in the boosting phase if the peptoids mimic any epitopes on the priming antigen. Throughout the prime-boost study, mice are bled and their sera prepared for testing in an ELISA to detect antibodies against the candidate peptoids and the native antigen.

6. If the candidate peptoids do boost the immune response against the native antigen used in priming in step 5, naïve mice are then immunized with these candidate peptoid-carriers adsorbed to alum. Blood is collected, and sera is prepared and analyzed by ELISA to determine whether the candidate peptoids, conjugated to carrier, elicit antibodies that cross-react with the native antigen.
7. Those sera that recognize the native antigen in step 6 would then be tested for the ability to neutralize the toxin or pathogen in question.
8. Those peptoid-carriers that elicited neutralizing sera as demonstrated in step 7 would be adsorbed to alum and used to immunize an appropriate vertebrate model. These animals would then be challenged with the toxin or pathogen in question to determine whether the peptoids can provide protection, and hence make good vaccine candidates.

Since it is anticipated that steps 7 and 8 in the strategic plan will be carried out in conjunction with collaborators, except when involving ricin toxin, with which the Vitetta lab has extensive experience (341-348), and because these steps require vaccine candidates at a stage of testing that has not yet been reached, these steps will not be discussed further. However, experiments have been done to test steps 1 through 5 of the strategic plan, and were performed in four phases. Much of the work in the first phase was conducted by a team led by Drs. Allison Case and Kate Yi, Ms. Angela Collins, and myself, and involved the

synthesis of peptoid libraries and the use of a model *peptide* B cell epitope, unrelated to pathogens, to optimize many different assays. Some of this work has been described previously (332). In the second phase, I applied the assays developed using the model peptide B cell epitope to screen a OBOC library for peptoid vaccine candidates against WNV and MNV-1. Vaccine candidates for MNV-1 were identified that await further testing. Furthermore, improvements in these screening assays emerged from this phase of my work and from work conducted in parallel by Dr. Kate Yi and by Dr. Joan Smallshaw and Mr. Stephen Ruback, who had performed similar screens for vaccine candidates against HIV and ricin toxin, respectively. The third phase of the project extended previous investigations of the immunogenicity of peptoids (300), and involved the immunization of rabbits to generate anti-peptoid antibodies. This study was begun by Dr. Allison Case, as reported previously (332), and was continued by me. This portion of my work resulted in the generation of the first affinity-purified anti-peptoid antibody and the demonstration of its specificity, confirming that peptoids are haptens, *i.e.*, they elicit specific antibodies when attached to carrier proteins. This confirmation of peptoid immunogenicity may be considered step 0 of the strategic plan. Finally, in the fourth phase of the project, I began using the affinity-purified anti-peptoid antibody, and its associated model *peptoid* B cell epitope, in further optimization of the assay used in the first round of screening to identify peptoid vaccine candidates. With Mr. Stephen Ruback, these observations have been applied to screening portions of a OBOC library for additional vaccine candidates against MNV-1. Meanwhile, Dr. Allison Case had begun performing experiments to validate step 5 of the strategic plan using the peptide model B cell epitope and potential peptoid mimetics of it that had been previously identified (332).

In essence, my work and that of others has developed the technology necessary for a new way of making vaccines, enabling the lab to begin developing vaccines against toxins and pathogens, such as those listed in Table 1.3 (page 32). As described in Section 1.II (page 3), the selection of these pathogens and toxins for peptoid vaccine development is based on the fact that neutralizing MAbs were available and there is evidence that antibodies are critical for protection. In the remainder of this chapter, I will provide a brief summary of the peptide-optimized screening platform that was developed in phase 1, which has been described previously (332) but informs the work that followed, and present in detail the results of my major contributions in screening for WNV and MNV-1 vaccine candidates, generating the first affinity-purified anti-peptoid antibody, which was raised against a well-defined model peptoid, and using this antibody in screening optimization. Lastly, I will present the preliminary results from experiments by Dr. Allison Case testing step 5 of the strategic plan and the results of screening performed by Mr. Stephen Ruback to identify vaccine candidates against MNV-1, as these have implications for the future directions of my work and the peptoid vaccine development project as a whole.

3.II. Summary of the peptide-optimized screening platform

3.II.A. Synthesis of large OBOC peptoid libraries

To begin step 1 of the strategic plan, six OBOC peptoid libraries, shown in Table 2.1 (page 102), were synthesized using the split-pool technique (222-224) as described in Section 2.I.B (page 40) and Figure 1.4 (page 37). The libraries are numbered from least complex to most complex. Library 1 was the first library synthesized, and was designed to allow enough redundancy to be split five ways and still contain, theoretically, three copies of every peptoid in the library. Accordingly, only ten peptoid monomers, a relatively small

number, were included, and the library length was set at five peptoid monomers. This led to a theoretical size of 100,000 compounds (10 monomers ^ 5 monomers long = 10^5 = 100,000). When this OBOC library was synthesized on 1.56 x 10^6 beads, the desired approximate theoretical 15-fold redundancy was produced (1.56 x 10^6 beads/100,000 theoretical compounds ≈ 15 beads on which each theoretical compound was synthesized). In contrast, Library 6 was designed to achieve a much larger theoretical size. With 18 monomers used at each position of this 8-mer library, the theoretical size of the library was approximately 18^8 or 1.1 x 10^{10} compounds. Synthesis of the library on approximately 1.1 x 10^7 beads led to the desired under-sampling, or theoretical redundancy of approximately 0.001 (1.1 x 10^7 beads/1.1 x 10^{10} theoretical compounds ≈ 0.001 beads on which each theoretical compound was synthesized). In other words, in Library 1, the library was synthesized on more beads than theoretical compounds, leading to theoretical oversampling or redundancy, while in Library 6, the library was synthesized on fewer beads than the number of theoretical compounds, leading to under-sampling. In addition to over- versus under-sampling, another parameter that varied in the design of these libraries was the number of monomers used at each position in the library. In all libraries except Library 5, all monomers were used at all positions. In contrast, in Library 5, two sets of eight monomers (with some overlap between the monomers used in each set) were used alternately at each position in the 10-mer peptoid (set A was used at positions 1, 3, 5, 7, and 9, and set B at positions 2, 4, 6, 8, and 10). In all libraries synthesized, the amines used to provide peptoid monomer side chains (Table 2.2, pages 103-104, and Figs. 2.2-2.6, pages 105-109) were selected based on previous success in peptoid synthesis (220), properties that cover chemical space (for example, aliphatic, aromatic, hydrophobic, hydrophilic, and positive or negative charge), and at least one dalton difference in mass (to distinguish each peptoid

monomer by its unique mass using MS/MS to sequence the library constituents). As can be seen from the structures of the amines used to synthesize each peptoid library in Figures 2.2-2.6, these peptoid libraries provided a large number of shapes or potential B cell epitopes from which to identify vaccine candidates.

3.II.B. Optimization of assays to screen peptoid libraries using known *broadly protective MAbs against toxins or pathogens*

To begin optimizing assays by which peptoid B cell epitopes for broadly protective MAbs against toxins or pathogens could be identified, as described in step 2 of the strategic plan, a simple experimental system was needed. This would consist of an on-bead B cell epitope against which a MAb was available, as well as an on-bead negative control to which this antibody did not bind. In the absence of an affinity-purified anti-peptoid antibody, a commercially available mouse MAb [clone M2; (335)] that bound to FLAG peptide [N-DYKDDDDK-C; (334)], but did not bind to a version of this peptide in which one residue was replaced with alanine [N-DAKDDDDK-C; (336)] was chosen for use in initial experiments during the first trip from our laboratory (by Dr. Allison Case and Ms. Angela Collins) to the Molecular Foundry. To indicate this difference in binding by MAb anti-FLAG, FLAG peptide was designated as **FLAG^{+} peptide** and the alanine-replacement peptide was designated as **FLAG^{-} peptide**. This FLAG peptide model system was also chosen because the length of FLAG peptide (eight monomers) was similar to the length of peptoids in our libraries (five to ten monomers; Table 2.1, page 102). Using these two peptides and MAb anti-FLAG, two sequential assays (Fig. 2.11, page 118), with which to screen libraries of peptoids for those that bind to the screening MAb's antigen binding sites were optimized: 1.) magnetic screening, in which magnetic PGDs were used to isolate complexes of on-bead compounds

that were bound by the screening MAb; and 2.) color screening, in which on-bead peptoids isolated in magnetic screening were incubated with the screening MAb and complexes were detected using an enzyme-conjugated, species-specific secondary antibody supplied with an appropriate “color-changing” substrate.

3.II.B.1. Optimization of the magnetic screening assay using the FLAG peptide model system

To optimize the magnetic screening assay, Dr. Allison Case and I determined the binding capacity of radioiodinated MAb anti-FLAG for on-bead $FLAG^+$ versus on-bead $FLAG^-$ peptides; a species- and isotype-matched control antibody was included as another negative control. The binding capacities of PGDs for radioiodinated MAb anti-FLAG (mouse IgG1) and a species- and isotype-matched control MAb against human CD25, RFT5 (also a mouse IgG1 MAb) were then determined by Dr. Kate Yi and Ms. Angela Collins; radioiodinated BSA, which lacks the Fc portion of an antibody to which PGDs bind, was also included as a negative control. Experiments were then performed by my colleagues to determine the order in which the three components used in the magnetic screening assay (on-bead compounds, MAb, and PGDs) should be added for optimal retention of these complexes. These experiments indicated that the addition of MAb anti-FLAG to on-bead $FLAG^+$ peptide, followed by the addition of PGDs, resulted in superior retention of these complexes by the magnet as compared to mixing PGDs and MAb anti-FLAG, followed by the addition of on-bead compounds. Meanwhile, regardless of the order that on-bead compounds, MAb, and PGDs were added, on-bead compounds were not retained when the species- and isotype-matched control IgG, RFT5, was added to $FLAG^+$ peptide beads, or when MAb anti-FLAG was added to $FLAG^-$ peptide beads. Finally, to demonstrate the

sensitivity and specificity of the magnetic screening assay at the scale on which peptoid libraries would be screened (namely, the maximum number of on-bead compounds that could fit in the 1.5 mL microfuge tubes for which the magnets were designed, while allowing space for adequate washing), we performed experiments in which 3 or 5 $FLAG^+$ peptide beads were spiked into 20,000 or more $FLAG^-$ peptide beads, and my colleagues performed experiments in which 1, 3, or 5 $FLAG^+$ peptide beads were spiked into 20,000 or more peptoid library beads. Based on the optimization experiments described above, MAb anti-FLAG was added in these spiking assays at 10 μg/mL. After washing, PGDs were added at a 1:10 dilution of the stock solution (3 mg/mL final concentration). From $FLAG^-$ peptide beads, 80-88% of the $FLAG^+$ peptide beads were retained in six experiments (three experiments in which 3 $FLAG^+$ peptide beads were spiked into 20,000 $FLAG^-$ peptide beads and three experiments in which 5 $FLAG^+$ peptide beads were spiked into 20,000 $FLAG^-$ peptide beads), as confirmed by cleavage of the peptides from the beads and analysis by MS. Meanwhile, from these six experiments, 7 total $FLAG^-$ peptide beads were retained, as confirmed by cleavage and MS. Similarly, from peptoid library beads, 67-100% of the $FLAG^+$ peptide beads were retained in nine experiments (three experiments each spiking 1, 3, or 5 $FLAG^+$ peptide beads into 20,000 peptoid library beads). Meanwhile, candidate peptoid mimetics of $FLAG^+$ peptide were also retained. Taken together, these experiments demonstrated that the magnetic screening assay could identify on-bead B cell epitopes with sensitivity, specificity, and reproducibility.

3.II.B.2. Optimization of the color screening assay using the FLAG system

Since any assay has the potential of giving false positive results, a second, color-based screening assay or on-bead ELISA was then developed by my colleagues. First, the

dilution of an HRP-conjugated species-specific secondary antibody added to complexes of MAb anti-FLAG and 20 $FLAG^+$ peptide beads was titrated, as compared to adding dilutions of this secondary antibody to 20 $FLAG^+$ peptide beads that had been incubated with RFT5 as a negative control. The substrate TMB was then added and the reaction was incubated until a blue color change was observed. At that point, sulfuric acid was added to stop the reaction and the absorbance at 450 nm was recorded using a plate reader. In similar experiments, the concentration of MAb anti-FLAG added to on-bead $FLAG^+$ peptide was then optimized. While these color screening optimization experiments in many ways mirrored the assay that was intended for use in screening, it is important to note that in a true screening assay, the addition of sulfuric acid and quantitation of absorbance using a plate reader would not be used. In a true screening assay, the sequences of the peptoids being screened would have to be determined by MS/MS, and it was observed by members of the peptoid team that sulfuric acid could interfere with this analysis. Likewise, in a true screening assay, only one bead bearing a particular peptoid might be available with which to perform the color screening assay, precluding the use of the plate reader. Therefore, the read-out of a true color screening experiment would be the observation of blue color change following the addition of TMB using a light microscope.

3.II.C. Summary

Taken together, the libraries synthesized and the experiments described to optimize magnetic and color screening assays constituted a peptide-optimized screening platform for peptoid vaccine development. They have been described in detail previously (332). They represent the starting point for the studies described in detail below.

3.III. Peptide-optimized screening to identify peptoid vaccine candidates

3.III.A. Peptide-optimized magnetic screening of a peptoid library with neutralizing MAbs against WNV and MNV-1 resulted in the retention of on-bead peptoids

To begin applying the peptide-optimized screening assays to the discovery of peptoid vaccine candidates, as described in step 2 of the strategic plan, a MAb against WNV E protein (E16) and a MAb against the P domain of the MNV-1 capsid protein (A6.2) (Table 1.4, page 33) were used sequentially to screen Peptoid Library 1 (Fig. 2.2, page 105 and Tables 2.1, page 102 and 2.2, pages 103-104) as shown in Table 3.1 (page 152). Prior to screening Peptoid Library 1 with E16, the library was "precleared" with a species- and isotype-matched control MAb for E16, MOPC-141, to remove peptoid library beads that could have bound to sites on E16 outside of the antigen binding sites (Round 1, Table 3.1). This preclearing step could have removed on-bead peptoids that bound to Protein G on PGDs or bound nonspecifically to any screening reagent. Following the preclearing step and screening with E16 (Round 2), the library was precleared again, this time with a species- and isotype-matched control MAb for A6.2, UPC-10 (Round 3, Table 3.1), before screening with A6.2 (Round 4). The numbers of on-bead peptoids retained in these four rounds of magnetic screening are shown separated by library aliquot in Table 3.2 (page 153), as Peptoid Library 1 remained separated according to the last peptoid monomer added to each of the ten aliquots, A through J (see Section 2.I.B.2, page 41 and Fig. 1.4, page 37). These data demonstrated that the peptide-optimized magnetic screening assay can retain candidate peptoids.

3.III.B. Peptide-optimized color screening of on-bead peptoids retained in magnetic screening substantially reduced the number of candidates for peptoid vaccines against WNV or MNV-1

Following the selection of on-peptoid beads using the four rounds of magnetic screening, color screening was then performed for the retained candidate peptoids (Table 3.3, page 153). For candidate peptoids retained in magnetic screening with anti-pathogen MAbs (Rounds 2 and 4), all retained on-bead peptoids were subjected to color screening. For peptoids retained in magnetic screening with preclearing MAbs (Rounds 1 and 3), a random sample of on-bead peptoids was subjected to color screening as indicated by the difference between the number of on-bead peptoids retained in magnetic screening (Table 3.2, page 153) and subjected to color screening (Table 3.3, page 153). The method used to record the color screening results (Table 3.4, page 154) was improved over the course of the four rounds of screening, with the addition of timing the color change, using a color palette, and screening and timing the color change of on-bead $FLAG^+$ and $FLAG^-$ peptide with MAb anti-FLAG in the same assay as positive and negative controls, respectively. The results of this color screening, separated by library aliquot, are shown in Tables 3.5-3.14 (pages 155-164), demonstrating that of the many on-bead peptoids retained during magnetic screening, few exhibited the rapid and robust color change of the bead itself that was observed in color screening of on-bead $FLAG^+$ peptide with MAb anti-FLAG. Of those beads that did show color change of the beads themselves (shown together in Table 3.15, page 165), none was identified by screening with the MAb E16, while several were identified using the MAb A6.2. In the preclearing rounds, two weakly color-changed beads were identified by screening with MOPC-141, and none was identified by screening with UPC-10. For all MAbs used in screening, some beads identified did not show any color change of the

bead itself or surrounding solution, while many produced a relatively weak color change of the surrounding solution, designated a "**halo**". This suggested that few robust peptoid vaccine candidates had been selected using the peptide-optimized magnetic and color screening assays.

3.III.C. Sequence determination using MS/MS of on-bead peptoids retained in magnetic screening and subjected to color screening revealed the need for further screening optimization

To begin investigating the relationship between the sequences of the on-bead peptoids and the magnetic and color screening results, and perhaps to identify consensus motifs that could help with the design of future libraries to screen for more robust vaccine candidates, on-bead peptoids that had been color screened were stripped of screening reagents, then cleaved from the beads and sequenced using MS/MS, as described in Section 2.II.A.4 (page 76). The sequences of these peptoids, designated by the unique mass of each monomer (see Fig. 2.2, page 105 and Table 2.2, pages 103-104) listed after the cleaved linker (mass of 328), are shown in Tables 3.5-3.14 (pages 155-164). The sequences were arranged to show consensus and color-coded by monomer mass for easier visualization of patterns. In some cases, an alternative sequence is listed when the MS/MS spectra suggested two possible sequences.

Several observations emerged from these analyses:

i. Of those beads that themselves showed some color change following selection using A6.2 (shown together in Table 3.15, page 165), some part of the sequence could be determined from the MS/MS spectra, although the interference of screening reagents in analysis by MS/MS cannot be ruled out for those sequences that could not be

determined or that were incomplete. Furthermore, the sequences may have revealed some consensus, particularly the monomer designated by a mass of 115 and color-coded bright blue (synthesized using glycine and resembling the amino acid aspartic acid; Table 2.2, pages 103-104 and Fig. 2.2, page 105) appearing at the C or N terminus of the peptoid sequence;

ii. Sequences could be obtained from MS/MS spectra for most peptoids, but the sequence at the N terminus of on-bead peptoids from Aliquot E was particularly difficult to determine. This suggested that the terminal monomer for Aliquot E, designated by a mass of 113 and color-coded dark blue-green (synthesized using isobutylamine, and resembling the amino acid leucine; Table 2.2, pages 103-104 and Fig. 2.2, page 105) may cause problems at this position at the level of synthesis, deprotection, screening, cleavage from the resin, or MS/MS;

iii. Some duplicate sequences were selected by the same screening MAb, for example, in Table 3.5 (page 155) for Aliquot A, sequences 7 and 8 and sequences 20 and 21, in Table 3.12 (page 162) for Aliquot H, sequences 4 and 5 and sequences 32 and 33, and in Table 3.13 (page 163) for Aliquot I, sequences 10, 11, and 12, highlighting the advantage of this redundant peptoid library (see Section 3.II.A, page 123) in providing built-in reproducibility;

iv. Those peptoids that showed a halo in color screening, regardless of the screening MAb used to select them, frequently included the positively-charged monomer designated by 128 and color-coded purple (synthesized using 1,4-butanediamine and resembling the amino acid lysine; Table 2.2, pages 103-104 and Fig. 2.2, page 105). This may indicate the involvement of this residue in non-specific binding to

screening reagents, or that patterns involving this residue bound to a common epitope in screening reagents; and

v. Peptoids of the same sequence were retained by more than one MAb, for example, in Table 3.5 (page 155) for Aliquot A, sequence 1 retained by MOPC-141 and sequence 19 retained by A6.2, as well as in Table 3.10 (page 160) for Aliquot F, sequence 4 retained by MOPC-141 and sequence 17 retained by E16. These peptoids were not those that exhibited color change of the bead itself, and tended to be those showing halos in color screening, with the frequent presence of the monomer designated by a mass of 128 in their sequence.

Since the same or similar peptoid sequences were retained in magnetic screening by both the screening and preclearing MAbs, but these on-bead peptoids did not exhibit a robust color change of the beads themselves in color screening, several possible explanations are suggested:

i. These peptoids were binding weakly outside of the antigen binding sites of these antibodies, to the PGDs, or nonspecifically; or

ii. All MAb solutions used contained a common contaminant that resulted in on-bead peptoid retention but weak color screening color change.

One possible contaminant that seemed to fit the criteria for the second explanation was bovine Ig, which may have been present in the FBS used by our collaborators to culture the hybridomas producing E16 (133) and A6.2 (311), and was perhaps used in the production of the control MAbs as well, although these were listed as being prepared from plasmacytomas grown in the peritonea of mice (349, 350) (in which case irrelevant mouse

IgG would be present in the final MAb). If bovine Ig were present in the solutions of all MAbs used in these screening experiments, it could in theory result in the retention of on-bead peptoids in magnetic screening because its Fc region could be bound by PGDs. In color screening, use of a species-specific secondary antibody, which may not be cross-reactive with bovine Ig, may have resulted in weak color change. Preliminary experiments, including an ELISA in which the preclearing and screening MAbs were used to coat plates, followed by addition of either HRP-goat anti-mouse IgG or HRP-goat anti-bovine Ig, as well as re-color screening of on-bead peptoids (prior to cleavage and sequencing) with the screening MAb and HRP-goat anti-bovine Ig, suggested little or no contamination with bovine Ig (data not shown). However, to avoid the possibility that contamination was not detectable but still present, a large stock of A6.2 was prepared using FBS that had been depleted of bovine Ig as described in Section 2.II.A.2 (page 69). This batch of A6.2 was then used to screen Peptoid Library 1. The results from magnetic screening are shown in Tables 3.1 and 3.2 (Round 5; pages 152 and 153). Taking into account the results from screening Rounds 1–4, only the aliquot in which an on-bead compound densely covered with PGDs (as observed when MAb anti-FLAG was applied to on-bead $FLAG^+$ peptide), Aliquot B, was subjected to color screening as shown in Tables 3.2 and 3.3 (page 153). The on-bead compound that was densely covered with PGDs in magnetic screening demonstrated a blue color change of the bead itself (Table 3.6, page 156). This compound was then shown by MS/MS to be on-bead $FLAG^+$ peptide, present in this library aliquot because of cross-contamination. Meanwhile, the remaining on-bead peptoids retained in magnetic screening from this aliquot showed a halo in color screening, indicating that the retention of such peptoids in magnetic screening had not been eliminated by using bovine Ig-free A6.2. In fact, one of these peptoids (sequence 70 in Table 3.6, page 156) was found to share its sequence with a

peptoid identified using A6.2 in Round 4 (sequence 44 in the same table). These data demonstrated that despite the use of a screening MAb that was not contaminated with bovine Ig, no strong candidates for peptoid vaccines, *i.e*. peptoids retained in magnetic screening that exhibited rapid and robust color change in color screening, were identified.

3.III.D. Summary

Applying the peptide-optimized magnetic screening assay to the identification of peptoid vaccine candidates mimicking the epitopes of an anti-WNV MAb, E16, and an anti-MNV-1 MAb, A6.2, resulted in the retention of on-bead peptoids from Peptoid Library 1. However, the on-bead peptoids retained by E16 did not demonstrate rapid and robust color change in the color screening assay. In contrast, a few on-bead peptoids retained by A6.2 showed color changes of beads similar to those observed when MAb anti-FLAG was applied to on-bead $FLAG^+$ peptide, albeit most showed a weaker and/or slower color change. Of these peptoid vaccine candidates identified using A6.2, most could be sequenced from MS/MS spectra. **These peptoids could be considered for further validation as vaccine candidates beginning with step 3 of the strategic plan outlined in Section 3.I (page 120).**

While applying peptide-optimized magnetic and color screens in succession did narrow down the number of potential peptoid vaccine candidates as desired, these screens provided several important observations:

i. Sequencing peptoids from Peptoid Library 1 Aliquot E, in which isobutylamine was used to synthesize the N-terminal peptoid monomer, demonstrated that particular monomers such as that synthesized from isobutylamine could cause difficulty in interpreting peptoid library sequences;

ii. Preclearing Peptoid Library 1 by magnetic screening with species- and isotype-matched control MAbs prior to magnetic screening with anti-pathogen MAbs resulted in the retention of on-bead peptoids of very similar or even, in some cases, the same sequence as on-bead peptoids retained by the anti-pathogen MAbs. In color screening, these beads did not show color changes of the beads themselves, but rather showed a halo, and in sequencing, many were found to contain the positively-charged lysine-like peptoid monomer;

iii. To explore the possibility that all MAb solutions used in screening may have been contaminated with bovine Ig, which might result in this on-bead peptoid retention by PGDs in magnetic screening but produce a weak color change or halo when color screened with a species-specific secondary antibody, A6.2 was prepared without bovine Ig contamination. Nevertheless, on-bead peptoids were still retained in magnetic screening with this bovine Ig-free A6.2 that produced a halo in color screening. Alternative explanations for these results, such as that the halo-producing peptoids retained in magnetic screening were binding weakly outside of the antigen binding sites of the preclearing and screening MAbs, or that the peptoids were binding to the PGDs or nonspecifically, remained to be explored;

iv. Regardless of the cause, the retention of the same or similar on-bead peptoids in magnetic screening with the preclearing and anti-pathogen MAbs suggested that these peptoids were not binding specifically to the antigen binding sites of an anti-pathogen MAb. Accordingly, these on-bead peptoids were removed from consideration as vaccine candidates;

v. Since the FLAG model system includes $FLAG^{+}$ peptide beads, which turned blue within one minute when color screened using MAb anti-FLAG, and $FLAG^{-}$ peptide

beads, which remained clear when color screened using MAb anti-FLAG, the appearance in color screening of an on-bead compound that bound to the screening MAb weakly but specifically was not apparent in optimization assays. When the formation of a halo was observed for some on-bead peptoids during this screening experiment, it was possible that this halo indicated peptoid vaccine candidates with weak but specific binding to the screening MAb. The sequences of these peptoids could then be used to design sublibraries for screening to identify peptoids with improved binding. However, a halo formed for those peptoid sequences selected using both the preclearing and anti-pathogen MAbs, indicating that peptoids that bind specifically but weakly have not yet been identified; and

vi. The labor involved in color screening and sequencing the on-bead peptoids in this experiment was significant. Future sequencing efforts should concentrate on those on-bead peptoids that turn blue in color screening, and not those that produce halos; and for those on-bead peptoids that turned blue, the significance of the time-to-color-change or intensity of color change in relation to peptoid mimicry of the native antigen for the screening MAb is not yet understood. However, recording the time to color change in comparison with FLAG model system controls in the same assay, and ranking the color change using a color palette (Table 3.4, page 154), should be included in the protocol for color screening.

Taken together, these results suggested that further optimization of the peptoid vaccine development platform was needed to allow efficient identification of robust peptoid vaccine candidates.

3.IV. Generation of an affinity-purified anti-peptoid antibody with which to optimize assays for screening peptoid libraries

The FLAG peptide model system used to develop the peptide-optimized vaccine platform had several strengths. A MAb, rather than a PAb, was used, which reflected the proposed strategy of using broadly protective MAbs raised against toxins or pathogens to identify peptoid vaccine candidates. A MAb against a peptide, as opposed to a peptoid, was used, reflecting our intention of using MAbs raised against toxins or pathogens, which would not be composed of peptoids, to identify peptoids that mimic the epitopes of these MAbs. Finally, both a peptide that bound to the anti-FLAG MAb ($FLAG^+$ peptide) and a negative control peptide that did not bind ($FLAG^-$ peptide) could be synthesized easily on the same resin as the peptoid libraries. To continue optimizing the peptoid vaccine development platform, a new model system was developed that retained certain strengths of the FLAG peptide model system while conferring other advantages.

3.IV.A. Immunization of a rabbit with a peptoid conjugated to a carrier protein and adsorbed to alum elicited antibodies against the peptoid, the carrier, and the linker

To ensure that the screening assays for identifying peptoid vaccine candidates could result in the isolation of a specific, known peptoid, a peptoid was designed, conjugated to a carrier protein, and adsorbed to alum to immunize rabbits and generate the first affinity-purified anti-peptoid antibody. While this plan would produce a PAb, rather than a MAb, against the peptoid of interest, it would likely result in the generation of a useful antibody without having to develop a MAb. Furthermore, rabbits were chosen for immunization to produce this PAb because their larger blood volumes would allow production of greater volumes of antisera, and therefore antibody against the peptoid. Although MAbs could be

made from these rabbits, the immortalized B lymphocyte cell lines needed to make the hybridomas for MAb production are proprietary (351-353). Nevertheless, following exsanguination at the end of the immunization series with peptoid-carrier adsorbed to alum, the draining lymph nodes and spleens of the immunized rabbits were harvested and frozen as single cell suspensions in case development of a MAb against the peptoid became possible.

To immunize the rabbits, a peptoid sequence five monomers in length (Figs. 2.7 and 2.8, pages 110-114) was designed by the peptoid team, myself included, using similar principles to those guiding the selection of amines for peptoid libraries. Amines were chosen that had been successfully used in peptoid synthesis (220) and that varied in their chemical properties. The peptoid monomers were arranged such that tyramine, used in peptoid synthesis without a protecting group on its hydroxyl group, would be added at the last position during synthesis to avoid undesirable side reactions. This peptoid was designated **rabbit 5-mer** or **R5** to indicate that the peptoid would be used to immunize rabbits. A second peptoid was designed as a negative control and designated **rabbit control** or **RC** (Figs. 2.9 and 2.10, pages 115-117). The amines selected for the synthesis of RC were chosen in part because they had been used with success in peptoid synthesis (220) and were relatively inexpensive. Moreover, the first peptoid monomer in RC was designed to be the same as that used in R5 to aid in the detection of antibodies against the chemical structure that would link R5 to the carrier protein for immunization, while the remaining amines included in RC were chosen because they were chemically distinct from those used to make R5. The length of RC, at seven monomers, gave RC approximately the same molecular weight as that of R5. Together, R5 peptoid, the rabbit PAb against it, and RC peptoid comprised the R5 peptoid model system, which would provide insights into peptoid

immunogenicity and allow further optimization of the peptoid vaccine development assays. The design of these peptoids and the early part of the rabbit immunizations, as well as some affinity purification and optimization experiments, have been described previously (332). However, the end of the rabbit immunizations (from Day 326 onward, Fig. 2.12, page 119) and several subsequent steps are newly reported here.

To raise a rabbit PAb against the R5 peptoid, R5 was first synthesized with a C-terminal cysteine on Rink Amide resin, simultaneously deprotected and cleaved from the resin, purified by HPLC, and lyophilized as described in Sections 2.I.C.3.b-2.I.C.3.f (pages 59-63), conjugated to the maleimide-activated carrier protein KLH as described in Section 2.I.D (page 63), and adsorbed to alum as described in Section 2.III.A.2 (page 80). One rabbit, designated 12D, was then immunized five times with R5-KLH adsorbed to alum, while another rabbit, designated 13D, received four immunizations as shown in Fig. 2.12 (page 119) and described in Section 2.III.A.3 (page 81). Throughout this immunization series, sera prepared from periodic test bleeds as shown in Fig. 2.12 were analyzed by ELISA to monitor the development of antibodies against R5, KLH, and the maleimide linker as described in Section 2.III.A.4 (page 82). At the completion of this immunization series, two bleeds of larger volume (production bleeds) were collected for each rabbit before the rabbits were exsanguinated as shown in Fig. 2.12 and described in Section 2.III.A.5 (page 84). Sera prepared from each of these bleeds were then pooled according to the rabbit of origin and designated **post-immunization sera**. Since the serum from rabbit 12D had demonstrated higher levels of anti-R5 peptoid antibody in previous ELISAs (data not shown), post-immunization serum from rabbit 12D was compared by ELISA with **pre-immunization serum** from this rabbit (Day -8, Fig. 2.12) as described in Section 2.III.B.4 (page 94). The results of this ELISA are shown in Fig. 3.1A (page 166). Pre-immunization

serum did not bind to R5 when conjugated to an irrelevant carrier, BSA, nor did it bind to RC-BSA, BSA alone, KLH alone, the original immunogen R5-KLH, or an irrelevant protein, OVA. Likewise, post-immunization serum did not bind to the irrelevant protein, OVA. In contrast, post-immunization serum did show robust binding to the coating antigens R5-BSA, KLH, and R5-KLH, with some binding to RC-BSA, suggesting binding to the maleimide linker. These results indicated that repeated immunization of a rabbit with R5-KLH adsorbed to alum elicited antibodies against R5 peptoid, the carrier protein KLH, and the maleimide linker.

3.IV.B. Anti-R5 peptoid antibodies could be purified from the serum of an immunized rabbit

To affinity purify antibodies against the R5 peptoid from the post-immunization serum of rabbit 12D, this serum was first passed repeatedly over a KLH column to remove antibodies against KLH as described in Section 2.III.B.2 (page 86). The final flow-through from this column was then passed repeatedly over a column displaying R5 peptoid without the maleimide linker used to make the R5-KLH used for immunization. The goal of these experiments was to retain antibodies against the R5 peptoid itself. The pooled eluate from the R5 column was then concentrated as described in Section 2.III.B.3 (page 92) and analyzed by ELISA as described in Section 2.III.B.4 (page 94), within the same experiments shown in Fig. 3.1A (page 166) and described in Section 3.IV.A (page 138), such that this concentrated eluate was compared with pre- and post-immunization serum from the same rabbit. The results of the ELISA analyzing this **post-purification** concentrated R5 column eluate are shown in Fig. 3.1B (page 166). The concentrated R5 column eluate bound to R5-BSA and R5-KLH, suggesting the presence of anti-R5 antibodies. In contrast to post-immunization serum, the concentrated R5 column eluate no longer bound to RC-BSA, BSA,

or KLH, suggesting that antibodies against R5 had been successfully purified and that anti-linker and anti-KLH PAbs had been removed. The concentrations of the antibodies detected against R5-BSA, KLH, and RC-BSA in the pre-immunization and post-immunization sera and in the post-purification concentrated R5 column eluate are shown in Fig. 3.1C (page 166). In Fig. 3.1D (page 166), concentrated R5 column eluate analyzed by SDS-PAGE under non-reducing conditions (right-hand panel) showed a band at approximately 150 kilodaltons (kD), consistent with intact IgG, while under reducing conditions (left-hand panel), bands of approximately 50 kD and 25 kD corresponding to gamma chains and light chains, respectively, were observed. In the absence of other bands, these results demonstrated that antibodies against R5 peptoid were successfully purified from post-immunization rabbit serum. This affinity-purified **PAb rabbit anti-R5 peptoid (RAR5)** was then ready for use in optimization assays for peptoid vaccine development.

3.IV.C. Magnetic screening using RAR5 resulted in the specific, sensitive, and reproducible retention of on-bead R5 peptoid

To begin optimizing the magnetic screening assay using the R5 peptoid model system, small-scale experiments were first conducted in which tubes containing three R5 peptoid beads spiked into 27 RC peptoid beads were prepared, and to which a series of concentrations of RAR5 were then added. Following washing, the on-bead peptoids were incubated with PGDs at a 1:10 dilution of stock, washed, and subjected to separation using a magnet as described in Section 2.IV.A (page 95). In these experiments, R5 peptoid beads were retained when RAR5 was added at a concentration of 0.1 μg/mL or higher, as shown in Table 3.16 (page 168). In contrast, only 2 RC peptoid beads in total were retained by the magnet in three similar experiments. Similarly, addition of an irrelevant rabbit PAb, RAMIg,

did not result in the retention of any on-bead peptoids (data not shown). Taken together, these results indicated that RAR5 bound specifically to R5 peptoid in this on-bead assay, confirming the results of the ELISA described in Section 3.IV.B (page 141) and shown in Fig. 3.1B (page 166).

These small-scale experiments also provided a starting point for larger, screening-scale experiments using tubes containing three R5 peptoid beads spiked into approximately 20,000 RC peptoid beads or 20,000 beads from Peptoid Library 1. Increasing concentrations of RAR5 were added in each round of screening until the three R5 peptoid beads were retained. As shown in Table 3.17 (page 169), screening without RAR5 in Round 1, which may be considered preclearing with PGDs, did not result in the retention of R5 peptoid beads from either RC or peptoid library beads. However, from the tube of peptoid library beads, some peptoid library beads were retained, which may be attributed to the tendency of broken TentaGel beads to be bound by PGDs, or may indicate binding of on-bead peptoids to PGDs themselves. In contrast, only one RC bead was retained from the tube of RC beads. At 0.01 μg/mL RAR5 in Round 2, again no R5 beads were retained from RC or peptoid library beads and a peptoid library bead was retained from the peptoid library tube. This peptoid library bead may have been retained due to binding to PGDs or by RAR5 itself, binding either to the antigen binding sites or elsewhere on the antibodies. However, no RC beads were retained from the RC tube, supporting the binding specificity of RAR5 for R5 peptoid. At 0.1 μg/mL RAR5 in Round 3, the three R5 beads were retained from the tube containing many RC beads, while no RC beads were retained. This indicated that RAR5 not only recognized R5 peptoid with specificity, but also with sensitivity. In contrast to the 0.1 μg/mL RAR5 needed to retain three R5 beads from many RC beads, 1 μg/mL RAR5 was

required to retain the three R5 beads from the tube of beads from Peptoid Library 1 (Round 4), and the retention of R5 was accompanied by the retention of peptoid library beads. These three R5 peptoid beads could be distinguished from the retained peptoid library beads by increased PGD coverage when viewed under a light microscope at 40X power, with the exception of one retained peptoid bead that rivaled the R5 peptoid beads in PGD coverage. The increased PGD coverage of R5 peptoid library beads suggested that RAR5 bound with higher affinity to R5 peptoid than to the vast majority of the retained peptoid library beads, or that the PAb RAR5 bound multiple epitopes on or composed of R5 peptoid. Meanwhile, applying RAR5 at 1 μg/mL to the tube of RC beads from which the three R5 beads had been removed in Round 3 at 0.1 μg/mL RAR5 did not result in the retention of RC beads. This indicated that even at 1 μg/mL, RAR5 did not result in retention of irrelevant peptoid beads. Finally, over the course of all four rounds, no R5 beads were retained when an irrelevant rabbit PAb, RAMIg, was applied to three R5 beads spiked into approximately 20,000 RC beads, although one RC peptoid bead was retained in total from the four rounds of screening (data not shown).

Taken together, these results suggested that a higher concentration of RAR5 would be required to retain R5 beads from a pool of related peptoid sequences in Peptoid Library 1, which contains four of the five peptoid monomers used in the synthesis of R5 (Table 2.2, pages 103-104 and Fig. 2.2, page 105) and is a library of limited diversity (Table 2.1, page 102), versus the concentration of RAR5 needed to retain R5 beads from many irrelevant RC peptoid beads. Moreover, it was encouraging that R5 peptoid beads, presumably the best possible ligand for RAR5 in these experiments, could be visibly distinguished from almost all other peptoid beads isolated in the same screen. This suggested that even if many peptoid

beads (too many to be practical to cleave, sequence, and resynthesize) were isolated in a magnetic screen, the best possible vaccine candidates isolated by magnetic screening could still be identified as those peptoid beads covered with the highest density of PGDs. Given this observation and that 1 μg/mL RAR5 did not result in retention of irrelevant RC peptoid beads, 1 μg/mL was adopted as the concentration of RAR5 for use in further experiments.

To continue optimizing the magnetic screening assay, and to begin exploring why so many on-bead peptoids were retained from Peptoid Library 1 along with R5 peptoid beads in the previous experiments, small-scale experiments of three R5 beads spiked into 27 RC beads were again carried out as described in Section 2.IV.A (page 95), this time using 1 μg/mL RAR5 along with a series of PGD dilutions at the 500 μL volume. As shown in Table 3.18 (page 170), a solution of PGDs as dilute as 1:10,000 could result in the retention of R5 peptoid beads when few other beads were present to interfere with retention by the magnet (in the two other similar experiments performed, a solution of PGDs as dilute as 1:25,000 could result in the retention of R5 peptoid beads). A PGD solution as dilute as 1:5,000 could retain 100% of the spiked R5 peptoid beads across three similar experiments. Across all three experiments, only two RC beads were retained, indicating that RAR5 bound specifically to R5 peptoid, as had been observed in previous experiments. In addition, an irrelevant rabbit PAb, RAMIg, did not result in the retention of peptoid beads, further indicating the specificity of binding of RAR5 for R5 peptoid (data not shown).

Taken together, these results suggested that when the concentration of antibody against an on-bead ligand is high enough, the PGD binding threshold for retention of that on-bead ligand by the magnet is quite low compared with the dilution of PGDs that had been used based on the peptide-optimized screening experiments. At the time the peptide-

optimized screening experiments were performed, we intentionally chose concentrations of antibody and PGDs for our further experiments that were higher than the concentrations that appeared saturating, to ensure that any on-bead peptoids of interest in magnetic screening would be isolated. However, in accounting for the results presented here, an increased affinity of PGDs for rabbit antibodies as compared with MAbs from other species, such as the mouse MAb anti-FLAG used in the initial optimization of the magnetic screening assay, cannot be ruled out as the explanation for this observation. However, such low concentrations of PGDs isolating complexes of on-bead peptoid and screening antibody indicated that the magnetic screening assay is a sensitive initial screen to identify peptoid vaccine candidates. The pool of peptoid vaccine candidates can then be narrowed using a second screening assay such as the color screening assay used in Section 3.III (page 129). Moreover, while dilute solutions of PGDs resulted in the retention of complexes of RAR5 and R5 peptoid, using a high concentration of PGDs, such as the 1:10 dilution used in previous experiments, should ensure that any peptoid beads bound by the screening antibody are retained by the magnet. This is critical when peptoid libraries will be screened *sequentially* with different broadly protective anti-toxin or anti-pathogen MAbs as performed in Section 3.III (page 129).

3.IV.D. Summary

In magnetic screening assays, affinity-purified RAR5 demonstrated specificity, sensitivity, and reproducibility in identifying R5 peptoid beads and not irrelevant RC peptoid beads in small-scale assays useful for initial optimization, and in larger, screening-scale assays. Furthermore, applying RAR5 to a mixture of a small number of R5 peptoid beads and many peptoid library beads resulted in the retention of these R5 peptoid beads, as well

as some peptoid library beads, when PGDs were added and a magnet was applied. Importantly, among the beads retained, the R5 peptoid beads could be distinguished from almost all peptoid library beads by the density of PGD coverage visible under a light microscope, suggesting that RAR5 bound with higher affinity to the R5 peptoid beads than to the peptoid library beads or that RAR5 bound to multiple epitopes within R5 or composed of multiple copies of R5. With this R5 system in hand, including affinity-purified RAR5, R5 peptoid, and RC peptoid, this R5 peptoid model system can now be used as appropriate positive and negative controls for any additional assays performed in the course of developing peptoid vaccines, including the validation of peptoid vaccine candidates identified using broadly protective MAbs against toxins or pathogens.

3.V. Future directions

3.V.A. Preliminary experiments suggest that the screening conditions used to isolate the MNV-1 peptoid vaccine candidates can identify mimetic peptoids

To test the hypothesis that peptoids selected from large OBOC libraries by a MAb can act as B cell epitopes, mimicking the shape of the native antigen for that screening MAb, some preliminary data was recently collected by my colleagues, led by Dr. Allison Case, that I report here because it provides an example of how the MNV-1 vaccine candidates could be tested in the prime-boost mouse model (step 3 of the strategic plan) and suggests that the screening conditions used herein to identify the MNV-1 peptoid vaccine candidates can isolate mimetic peptoids. In this experiment, portions of all six peptoid libraries listed in Table 2.1 (page 102) were subjected to magnetic screening with MAb anti-FLAG, and on-bead peptoids retained in magnetic screening were then subjected to color screening. On-bead peptoids that demonstrated a rapid and robust color change in

color screening were cleaved from the beads and sequenced by MS/MS. This process has been described in detail previously (332). Two of these peptoid sequences were resynthesized in bulk as described in step 3 of the strategic plan to develop peptoid-based vaccines, and each was then conjugated to the carrier protein KLH as described in step 4 of the strategic plan. These peptoid-KLH conjugates were each adsorbed to alum before being mixed together to immunize mice that had been primed with $FLAG^+$ peptide conjugated to KLH and adsorbed to alum, then rested until levels of circulating anti-$FLAG^+$ peptide antibody returned to baseline, as described in step 5 of the strategic plan and as shown in Fig. 3.2A (prime-boost timeline; page 171). Sera prepared from blood collected prior to administration of any immunizations (**pre-immunization**), from blood collected following the priming immunizations (**FLAG-primed**), from blood collected following the rest period (**rested**), and from blood collected following the boosting immunizations with candidate peptoids conjugated to KLH and adsorbed to alum (**peptoid-boosted**) were then analyzed in an on-bead ELISA. This on-bead assay enabled the presentation of $FLAG^+$ peptide without the presence of the maleimide linker used to conjugate peptides and peptoids to carrier proteins both for immunization and for coating plates in conventional, plate-bound ELISAs. Therefore, the confounding effects of antibodies raised against the linker (and the carrier protein) were avoided (see Section 3.IV, page 138, in which immunization of a rabbit with R5-KLH elicited antibodies against the peptoid, carrier, and linker). The results of this experiment are shown in Fig. 3.2B (page 171). Pre-immunization serum showed no binding to on-bead $FLAG^+$ peptide, indicating that the mice did not have pre-existing antibodies against $FLAG^+$ peptide prior to immunization. In contrast, the FLAG-primed serum showed binding to on-bead $FLAG^+$ peptide, demonstrating that priming with $FLAG^+$ peptide-KLH elicited antibodies that were specific for FLAG peptide. This binding was not observed when

rested serum was applied to on-bead FLAG$^+$ peptide, indicating that the level of circulating antibody against anti-FLAG$^+$ peptide had returned to baseline. Finally, binding to on-bead FLAG$^+$ peptide was restored when peptoid-boosted serum was tested. These preliminary results demonstrate that boosting FLAG$^+$ peptide-KLH-primed mice with peptoids selected by MAb anti-FLAG in the magnetic and color screening assays elicited antibodies that bound to FLAG$^+$ peptide, the native antigen for the screening MAb. Since the blood from which the peptoid-boosted serum was produced was collected 8 days after the final boost, whether these anti-FLAG antibodies were elicited in a primary antibody response against the boosting peptoids, were produced by memory B cells elicited by priming with FLAG$^+$ peptide and restimulated by boosting with the candidate peptoids, or represented the products of both primary and memory responses has yet to be determined. Of course, since this experiment was performed for two mice and presented some technical challenges, it must be reiterated that these data are preliminary and must be confirmed in additional experiments. The next step will be to perform primary immunizations with the candidate peptoid mimetics of FLAG$^+$ peptide attached to carrier proteins and adsorbed to alum according to step 6 of the strategic plan.

3.V.B. Peptoid-optimized magnetic screening of Peptoid Library 6 sub-aliquots with a neutralizing MAb against MNV-1 resulted in the retention of on-bead peptoids that rapidly turned blue in color screening

To continue applying what I learned from proof-of-principle experiments using the FLAG peptide and R5 peptoid model systems to the selection of on-bead peptoids with broadly protective MAbs against toxins or pathogens, Mr. Stephen Ruback screened two sub-aliquots of the peptoid library shown in Table 2.1 (page 102) with largest theoretical

size, Peptoid Library 6, with bovine Ig-free A6.2, using the magnetic screening protocol I had modified for the experiments shown in Table 3.17 (page 169), as described in Section 2.V (page 100). This anti-MNV-1 antibody was chosen to be used in the first of our new rounds of screening because a large batch had been produced (see Section 2.II.A.2, page 69), because the role of antibodies in protection from infection is well established (see Section 1.II, page 3), and because, as a pathogen of mice, the animal model for MNV infection is also established (see Section 1.II). Together, these factors should facilitate the validation of steps 7 and 8 in the strategic plan to develop peptoid-based vaccines. The results of this screening to date are shown in Tables 3.19 and 3.20 (page 172). As observed in previous screens (for example, see Section 3.III, page 129), the number of candidate on-bead peptoids retained in magnetic screening was reduced when these on-bead peptoids were subjected to color screening. The on-bead peptoids that demonstrated a rapid and robust color change in color screening were then cleaved from the beads and analyzed by MS/MS. The sequences that could be interpreted from MS/MS spectra are listed in Table 3.20. These peptoids, along with additional candidate peptoids selected in Section 3.III, should be resynthesized, conjugated to carrier proteins, and adsorbed to alum for testing in the prime-boost model as described in steps 3, 4, and 5 of the strategic plan and in Section 3.V.A (page 147).

3.V.C. Summary

Preliminary experiments conducted by peptoid team members using the prime-boost mouse model suggested that the screening conditions used here in Section 3.III (page 129) to identify MNV-1 vaccine candidates are capable of selecting peptoids that can mimic the structure of a native, peptide antigen, *i.e.*, these peptoids induced cross-reactive antibodies.

This is encouraging for the MNV-1 peptoid vaccine candidates that have already been identified and for future screens to identify peptoid vaccine candidates using the neutralizing antibodies shown in Table 1.4 (page 33).

To that end, summarized in Table 3.21 (pages 173-176) are some suggestions for the “best practice” in screening peptoid libraries to identify vaccine candidates using broadly protective MAbs, compiled from the results of the experiments presented in this dissertation and some relevant literature. Some of these points will be discussed further in the next chapter.

Table 3.1. Peptide-optimized magnetic screening of Peptoid Library 1 with neutralizing MAbs against WNV and MNV-1.

Round		MAb			Number of beads retained in magnetic screening
		Name	Mouse Isotype	Description	
1A	Preclear	MOPC-141	IgG2b	Antigen unknown - isotype control MAb	139
1B*					90
2	Screen	E16	IgG2b	Anti-WNV E protein	87
3	Preclear	UPC-10	IgG2a	Beta-2,6-fructosan - isotype control MAb	90
4	Screen	A6.2 produced by Wobus lab	IgG2a	Anti-MNV-1 P domain	183
5	Screen	A6.2 produced in-house from Wobus lab hybridoma	IgG2a	Anti-MNV-1 P domain	122

*Aliquots E and F only.

Table 3.2. Peptide-optimized magnetic screening of Peptoid Library 1 with neutralizing MAbs against WNV and MNV-1, separated by aliquot.

Round		MAb	Mouse isotype	Beads retained by magnetic screening separated by aliquot										Total
				A	B	C	D	E	F	G	H	I	J	
1A	preclear	MOPC-141	IgG2b	16	7	29	2	36	13	2	3	16	15	139
1B				-	-	-	-	41	49	-	-	-	-	90
2	screen	E16	IgG2b	7	2	0	9	11	11	5	17	16	9	87
3	preclear	UPC -10	IgG2a	15	14	6	8	36	5	1	5	0	0	90
4	screen	A6.2	IgG2a	14	53	3	21	8	9	17	25	8	25	183
5	screen	A6.2 (bovine Ig-free)	IgG2a	8	8	13	7	11	40	16	4	3	12	122
Total	-	-	-	60	84	51	47	143	127	41	54	43	61	711

Table 3.3. Color screening of beads retained in magnetic screening of Peptoid Library 1 with neutralizing MAbs against WNV and MNV-1, separated by aliquot.

Round		MAb	Mouse isotype	Beads retained by magnetic screening and color screened separated by aliquot										Total
				A	B	C	D	E	F	G	H	I	J	
1A*	preclear	MOPC-141	IgG2b	5	6†	5	2	5	5	2	3	5	5	43
1B*				-	-	-	-	5	5	-	-	-	-	10
2	screen	E16	IgG2b	7	2	0	9	11	11	5	17	16	9	87
3*	preclear	UPC-10	IgG2a	5	6	6	5	5	5	1	5	0	0	38
4	screen	A6.2	IgG2a	14	54†	5†	16†	8	9	17	25	8	24†	180
5‡	screen	A6.2 (bovine Ig-free)	IgG2a	0	7†	0	0	0	0	0	0	0	0	7
Total	-	-	-	31	75	16	32	34	35	25	50	29	38	365

*Randomly selected a few beads for color screening

†Compared with the number of beads retained in magnetic screening, extra bead(s) were found in the tube at the start of color screening or beads were lost in color screening

‡Selected only the beads retained in magnetic screening from sub-aliquots in which at least one bead was bound by a high density of PGDs

Table 3.4. Peptide-optimized color screening of Peptoid Library 1 with neutralizing MAbs against WNV and MNV-1.

Magnetic screening round		MAb	Mouse isotype	Color recording	Timer used	Color palette used	FLAG peptide model system controls used within assay
1A 1B	preclear	MOPC-141	IgG2b	faint, halo, no change	yes	yes	no
2	screen	E16	IgG2b	positive, positive later, no change	no	no	no
3	preclear	UPC-10	IgG2a	beads yellow, halo, no change	yes	no	no
4	screen	A6.2	IgG2a	beads blue, halo, no change	yes	yes	yes
5	screen	A6.2 (bovine Ig-free)	IgG2a	beads blue, halo, no change	yes	yes	yes

Table 3.5. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot A using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1†	1A	MOPC 141	**halo**	**1145**	**147**	**128**	**128**	**177**	**237**	**328**
2			halo	1115	147	128	147	128	237	328
3			no change	934	147	128	101	115	115	328
4			halo	1150	147	128	237	182	128	328
5			no change	988		147	237	147	128	328
6	2	E16	Positive	1145	147	128	177	128	237	328
7			**Positive**	**1141**	**147**	**128**	**128**	**173**	**237**	**328**
8			**Positive**	**1141**	**147**	**128**	**128**	**173**	**237**	**328**
9			no change	1053		147	101	237	240	328
10			no change	1032	147	240	?‡	147	113	328
11			no change	1357?					?	328
12			no change	1162?					?	328
13	3	UPC 10	halo	1141	147	128	128	237	173	328
14			halo	904		147	128	173	128	328
15			halo	1137	147	240	128	147	147	328
16			halo	1017		147	237	128	177	328
17			halo	?	?	?	?	237	128	328
18	4	1A6.2.1	halo	1107	147	177	101	177	177	328
19			**halo**	**1145**	**147**	**128**	**128**	**177**	**237**	**328**
20			halo	1208	147	240	128	128	237	328
21			**halo**	**1150**	**147**	**182**	**128**	**128**	**237**	**328**
22			**halo**	**1150**	**147**	**182**	**128**	**128**	**237**	**328**
23			halo	1137	147	182	128	115	237	328
24			halo	1208	147	128	128	240	237	328
25			halo	1096	147	128	128	237	128	328
26			halo	1096	147	128	237	128	128	328
27			halo	1342	147	240	147	240	240	328
28			halo	1041		147	147	237	182	328
29			halo	?						328
30			halo	?						328
31			halo	(lost)						

*Sequences are indicated by the unique mass of each monomer in the library (see Table 2.2, pages 103-104), with 328 designating the mass of the cleaved linker. Each monomer was also assigned a color for easier visualization of patterns in the sequences, listed by the amine used to make each monomer as follows: 2-aminoethanol, pink; isobutylamine, turquoise; glycine, bright blue; 1,4-butanediamine, light purple; benzylamine, orange; 1-aminoindan, bright green; tyramine, maroon; 1-(3-aminopropyl)-2-pyrrolidinone, pale blue; 2,2-diphenethylamine, yellow; and 4-(2-aminoethyl)benzenesulfonamide, pale green. See Fig. 2.2 (page 105) for the structure (and molecular weight) of each amine.

†Bold text across a row indicates that the exact sequence appears elsewhere in the same table.

‡? indicates total mass (determined by mass spectrometry) or monomers that could not be determined or could not be determined definitively from MS and/or MS/MS spectra.

§Red text indicates the order of monomers within one sequence could not be determined definitively from MS/MS spectra.

Table 3.6. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot B using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	no change	1000	101	101	115	182	173	328
2			halo	1160	101	237	128	128	237	328
3			halo	1070	101	128	128	147	237	328
4			halo	1159	101	128	128	237	237	328
5			halo	983[§]	101	173	177	?	?	328
6			halo	(lost)						
7	2	E16	no change	896		101	173	147	147	328
8			no change	893?		101	147	?[‡]	113	328
9	3	UPC 10	no change	1044	101	128	177	182	128	328
10			halo	1149	101	237	182	128	173	328
11			halo	?	?	?	?	128	237	328
12			bead itself yellow	?						328
13			bead itself yellow	?						328
14			bead itself yellow	?						328
15	4	1A6.2.1	halo	964		101	182	240	113	328
16			no change	1053		101	240	237	147	328
17			**bead itself blue by 2 min**	958?		101	177	237	115	328
18			halo	916		101	177	182	128	328
23 (truncation)			(halo)	965		101	177	182	177	328
19			halo	974		101	128	240	177	328
20			halo	1020		101	237	177	177	328
21			halo	1064	101	101	147	147	240	328
22			halo	881?	101	101	?	147	147	328
23			halo	1066	101	101	177	182	177	328
24			halo	950	101	128	101	115	177	328
25			halo	1121	101	128	177	147	240	328
26			?	1063	101	128	182	177	147	328
27			halo	1033	101	128	147	182	147	328
28			halo	1083	101	128	173	240	113	328
29			halo	1061	101	147	101	237	147	328
30			halo	1018	101	147	101	240	101	328
31			halo	1027	101	173	101	177	147	328
32			halo	1095	101	173	128	128	237	328
33			halo	997	101	177	101	177	113	328
34			halo	1134	101	177	115	240	173	328
35			halo	1009	101	177	147	128	128	328
36			halo	1012	101	177	177	101	128	328
37			halo	1197	101	177	177	177	237	328
38			halo	1147	101	177	240	173	128	328
39			halo	1145	101	182	182	115	237	328
40			halo	1091	101	182	115	128	237	328
41			halo	982	101	182	128	128	115	328
42			no change	1001	101	182	147	128	115	328
43			**bead itself slightly green by 4 min**	1140	101	182	237	177	115	328
44[†]			**halo**	**1104**	**101**	**182**	**128**	**237**	**128**	**328**
45			halo	1262	101	237	237	177	182	328
46			halo	1211	101	237	128	240	177	328
47			no change	?	101	237	?	?	182	328
48			halo	1032	101	240	101	147	115	328
49			halo	1058	101	240	101	173	115	328
50			**bead itself blue by 1 min**	?	101	240	101	?	115	328
51			no change	1071	101	240	128	101	173	328
52			halo	1108	101	240	237	101	101	328
53			halo	1230	101	240	237	177	147	328
54			halo	808?	101	113?	?	?	?	328
55			halo	?	?	?	?	115	128	328
56			no change	?	?	?	?	101	177	328
57			**bead itself blue/green by 7 min 40 sec**	?	?	?	?	240	115	328
58			halo	1010?	?	?	?	113	101	328
59			**bead itself blue by 1 min**	?	?	?	240?	173?	115?	328
60			halo	?						328
61			halo	?						328
62			halo	?						328
63			no change	?						328
64			halo	?						328
65			halo	?						328
66			**bead itself blue by 10 sec**	?						328
67			no change	?						328
68			halo	?						328
69	5	1A6.2.1	**bead itself blue by 1 min**	1322			FLAG+			328
70			halo	**1104**	**101**	**182**	**128**	**237**	**128**	**328**
71			halo	1005	101	128	128	147	173	328
72			halo	922		101	128	128	237	328
73			halo	1029	101	237	101	147	115	328
74			halo	?						328
75			halo	?						328

For footnotes, see Table 3.5 (page 155).

Table 3.7. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot C using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*				C	Linker
1	1A	MOPC 141	halo	1140	177	128	177	147	182	328
2			halo	1176	177	128	177	128	237	328
3			halo	1239	177	128	240	128	237	328
4			halo	1145	177	128	128	147	237	328
5			halo	1172	177	128	128	173	237	328
-	2	E16	-	-	-	-	-	-	-	-
6	3	UPC10	halo	1158	177	237	128	115	173	328
7			no change	1161	177	182	182	115	177	328
8			no change	1334	177	240	237	115	237	328
6 (truncation)			(halo)	1042		177	237	128	173	328
9			halo	1077	177	?	?	177	128	328
10			halo	1130	177	173	147	177	128	328
11			no change	?‡						328
12	4	1A6.2.1	halo	1184	177	147	115	240	177	328
13			halo	1223	177	173	240	128	177	328
14			**no change by 2 min; bead itself very slight blue by 5 min; bead itself yellow by 9 min**	1230	177	237§	240	147	101	328
15			**no change by 2 min; bead itself very slight blue by 5 min; bead itself yellow by 9 min**	1099	177	128	128	101	237	328
16			no change				FLAG-			328

For footnotes, see Table 3.5 (page 155).

Table 3.8. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot D using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	halo	1096	147	128	128	128	237	328
2			halo	1186	237	128	128	128	237	328
3	2	E16	positive‖	1307	237	240	115	240	147	328
4			positive‖	1159	237	101	128	128	237	328
5			positive‖	1186	237	128	128	237	128	328
6			positive‖	1050	237	128	128	101	128	328
7			positive‖	1240	237	128	128	182	237	328
8			no change	1195	237	128	147	115	240	328
9			no change	1076	237	115	182	101	113	328
10			no change	1057		237	182	128	182	326
11			no change	1186?	237	182	182	147	?‡	326
12	3	UPC 10	bead itself yellow	1023	237	115	115	115	113	328
13			bead itself yellow	1247	237	240	147	182	113‡	328
14			halo	1175	237	128	177	128	177	328
15 (alternate sequence)			(halo)	1169?	237	173	128	128	173	328
15			halo	994		237	128	128	173	328
16			halo	?						328
17	4	1A6.2.1	halo	1162	237	177	115	177	128	328
18			halo	1141	237	147	128	173	128	328
19			halo	1189	237	128	128	240	128	328
20			halo	1077	237	128	128	128	128	328
21			halo	1186	237	128	128	237	128	328
22			halo	1126	237	128	177	128	128	328
23			halo	1141	237	128	147	173	128	328
24			halo	1195	237	182	147	173	128	328
25			halo	1258	237	182	182	182	147	328
26			no change?	1240	237	147	240	115	173	328
27			halo	1350	237	240	128	177	240	328
28			no change?	1410	237	240	128	237	240	328
29			halo	1288	237	240	240	128	115	328
30			no change	1186	237	101	237	182	101	328
31			halo	1159	237	101	128	128	237	328
32			bead itself slightly blue/green by 4 min 30 sec	928		237	101	147	115	328

‖One bead from this set showed no change but which one is not known. For remaining footnotes, see Table 3.5 (page 155).

Table 3.9. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot E using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N		Sequence*		C	Linker
1	1A	MOPC 141	**faint**	923?‡	113	128	?	?	115	328
2			no change	888?			?	101	147	328
3			halo	1198?	?	?	128	237	237	328
4			halo	1197?	?	?	128	237	237	328
5			halo	1134?	?	?	173	128	237	328
6	1B	MOPC 141	halo	1138?	?	?	128	128	237	328
7			halo	1089?	?	?	128	128	237	328
8			halo	1198?	?	?	237	128	237	328
9			halo	1134?	?	?	173	128	237	328
10			**faint**	1133?	?	?	128	173	237	328
11	2	E16	no change	1088?	?	?	237	128	128	328
12			positive	1088?	?	?	128	237	128	328
13			positive	994?	?	?	?	177	173	328
14			no change	1240?	?	?	237	177	177	328
15			no change	919?	?	?	?	147	177	328
16			no change	1063?	?	?	128§	182	177	328
17			no change	1254?	?	?	240	237	182	328
18			positive	1142?	113	?	182	128	237	328
19			positive	969?	?	113	173	115	240	328
20			no change	1306?	?	?	240?	?	?	328
21			no change	1137?						328
22	3	UPC 10	halo	1110?	?	?	237	101	128	328
23			halo	1076?	?	?	128	182	173	328
24			halo	1194?	?	?	240	182	177	328
25			halo	1096?	?	?	177	128	177	328
26			halo	979?	?	?	?	147	237	328
27	4	1A6.2.1	halo	?	?	?	128	182	177	328
28			halo	?	?	?	?	237	177	328
29			halo	?						328
30			halo	?						328
31			halo	?						328
32			halo	?						328
33			halo	?						328
34			halo	(lost)						

For footnotes, see Table 3.5 (page 155).

Table 3.10. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot F using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	halo	1232	173	128	128	237	237	328
2[†]			**halo**	**1167?**[‡]	**173**	**128**	**128**	**173**	**237**	**328**
3			**halo**	**1167?**	**173**	**128**	**128**	**173?**	**237?**	**328**
4			**halo**	**1167**	**173**	**128**	**173**	**128**	**237**	**328**
5			no change	1231	173	128	237	128	237	328
6	1B	MOPC 141	halo	1096	173	128	101	128	237	328
7			halo	1125	173	128	128	128	240	328
8			halo	1063	173	128	128	177	128	328
9			**halo**	**1171?**	**173**	**177**	**128**	**237**	**128**	**328**
10			halo	1063	173	177	128	128	128	328
11	2	E16	positive	1021		173	237	101	182	328
12			positive	1107	173	173	128	128	177	328
13			**positive**	**1095**	**173**	**101**	**128**	**128**	**237**	**328**
14			**no change**	**1095**	**173**[§]	**101**	**128**	**128**	**237**	**328**
15			no change	1141	173	147	128	128	237	328
16			positive	1107	173	128	173	128	177	328
17			**positive**	**1167**	**173**	**128**	**173**	**128**	**237**	**328**
18			positive	1167	173	128	237	128	173	328
19			positive	1167	173	128	237	173	128	328
20			no change	1334	173	237	182	177	237	328
21			**positive**	**1171**	**173**	**177**	**128**	**237**	**128**	**328**
22	3	UPC 10	no change	947	173	115	101	115	115	328
23			no change	1035		173	182	115	237	328
24			halo	1157		173	240	177	240	328
25			halo	1253	173	147	237	128	240	328
26			no change	?						328
27	4	1A6.2.1	no change	995	173	177	101	115	101	328
28			halo	1228	173	177	182	240	128	328
29			halo	1005	173	101	128	147	128	328
30			halo	1067	173	128	128	182	128	328
31			halo	1013	173	128	128	128	128	328
32			halo	1112	173	173	182	128	128	328
33			halo	1027?	?	?	?	128	128	328
34			halo	1228	173	240	182	128	177	328
35			**bead itself slightly blue**	1058	173	101	101	173	182	328

For footnotes, see Table 3.5 (page 155).

Table 3.11. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot G using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	halo	1241	182	128	237	237	128	326
2			halo	(lost)						
3	2	E16	no change	1050	182	115	177	101	147	326
4			positive	1204	182	128	237	182	147	326
5			positive	1240	182	128	128	237	237	326
6			positive	1180	182	128	128	177	237	326
7			no change	1289	182	128	237	177	237	326
8	3	UPC 10	crack turned blue	991?	?	?‡	128?	128?	128?	326
9	4	1A6.2.1	halo	1143	182	240	115	101	177	328
10			halo	1077	182	177	128	147	115	328
11			halo	1022	182	128	128	128	128	328
12			halo	1107	182	101	128	240	128	328
13			halo	1131	182	128	128	237	128	328
14			halo	1180	182	128	128	237	177	328
15			halo	1082	182	182	147	128	115	328
16			**bead itself blue**	1092	182	177	177	113	115	328
17			halo	1089	182	173	177	101	128	328
18			halo	1221	182	237	177	115	182§	328
19			halo?	1284	182	237	182	182	173	328
20			halo	1114	182	128	182	147	147	328
21			halo	1284	182	115	182	237	240	328
22			halo	927			182	177	240	328
23			halo	1128?	182	237	?	?	177	328
24			halo	?						328
25			halo	?						328

For footnotes, see Table 3.5 (page 155).

Table 3.12. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot H using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	halo	1098	240	128	173	101	128[§]	328
2			halo	1079	240	128	128	128	128	328
3			halo	1163	240	101	128	128	237	328
4[†]	2	E16	**positive**	**1189**	**240**	**128**	**128**	**237**	**128**	**328**
5			**positive**	**1189**	**240**	**128**	**128**	**237**	**128**	**328**
6			positive	1211	240	147	128	240	128	328
7			positive	1178	240	128	128	177	177	328
8			positive	1301	240	240	128	128	237	328
9			positive	1238	240	177	128	128	237	328
10			positive	1208	240	147	128	128	237	328
11			positive	1189	240	128	128	128	237	328
12			positive	1238	240	128	177	128	237	328
13			no change	1117?[‡]	240	?	177	128	147	328
14			no change	1110	240	147	147	101	147	328
15			positive	1197	240	147	177	177	128	328
16			positive	1301	240	128	240	237	128	328
17			positive	1279	240	177	182	237	115	328
18			no change	1201	240	177	240	101	115	328
19			no change	1349	240	182	240	177	182	328
20			positive	(lost)						
21	3	UPC 10	slight yellow	1217	240	177	177	182	113	328
22			slight yellow	1242?	?	?	237	237	115	328
23			slight yellow	1127	240	147	182	115	115	328
23 (alternate sequence)			slight yellow	1127		240	147	240	173	328
24			slight yellow	1155		240	173	237	177	328
25			slight yellow	?						328
27	4	1A6.2.1	halo	?	?	?	?	?	101	328
28			halo	1225	240	237	177	128	115	328
29			halo	1342	240	240	237	182	115	328
30			halo	1195	240	128	237	115	147	328
31			halo	1272	240	173	237	147	147	328
32			**halo**	**1253**	**240**	**173**	**128**	**237**	**147**	**328**
33			**halo**	**1253**	**240**	**173**	**128**	**237**	**147**	**328**
34			halo	1296	240	240	240	101	147	328
35			halo	1508?	?	?	?	182	177	328
36			halo	1143	240	101	115	182	177	328
37			halo	1272?	?	?	113	128	182	328
38			halo	1297	240	237	182	128	182	328
39			halo	1233	240	128	115	240	182	328
40			halo	1374	240	147	237	240	182	328
41			halo	1216	240	182	101	128	237	328
42			halo	1198	240	128	147	115	240	328
43			halo	1113?	240	101	240	?	?	328
44			halo	1136?	240	237	?	?	?	328
45			halo	1136?		240	182	147	?	328
46			halo	?						328
47			halo	?						328
48			halo	?						328
49			**bead itself blue**	?						328
50			**bead itself slightly blue**	?						328

For footnotes, see Table 3.5 (page 155).

Table 3.13. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot I using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	halo	1186?‡	128	128	237	237	128	328
2†			**halo**	**1186?**	**128**	**128**	**128**	**237**	**237**	**328**
3			no change	1096	128	128	128	147	237	328
4			halo	1097	128	128	147	128	237	328
5			halo	?						328
6	2	E16	positive	1126	128	128	128	177	237	328
7			no change	1159	128	101	128	237	237	328
8			**positive**	**1186**	**128**	**128**	**128**	**237**	**237**	**328**
9			positive	1126	128	128	177	128	237	328
10			**positive**	**1186**	**128**	**128**	**237**	**128**	**237**	**328**
11			**positive**	**1186**	**128**	**128**	**237**	**128**	**237**	**328**
12			**positive**	**1186**	**128**	**128**	**237**	**128**	**237**	**328**
13			no change	1122	128	128	173	128	237	328
14			positive later	932		128	147	147	182	328
15			positive later	944		128	240	147	101	328
16			no change	1051	128	173	128	147	147§	328
17			**no change**	**1122**	**128**	**173**	**128**	**237**	**128**	**328**
18			**positive**	**1122**	**128**	**173**	**128**	**237**	**128**	**328**
19			positive	1186	128	237	128	128	237	328
20			positive later	(lost)						
21			positive later	(lost)						
-	3	UPC 10	-	-	-	-	-	-	-	-
22	4	1A6.2.1	halo	1050	128	128	237	128	101	328
23			halo	1118	128	147	237	177	101	328
24			halo	1237	128	240	182	182	177	328
25			halo	1243	128	182	240	128	237	328
26			halo	1167	128	173	173	128	237	328
27			halo	1077	128	128	128	128	237	328
28			halo	1129	128	128	128	177	240	328
29			no change	?	128	173	115	?	?	328

For footnotes, see Table 3.5 (page 155).

Table 3.14. Color screening and sequencing of on-bead peptoids retained from magnetic screening of Peptoid Library 1 Aliquot J using the indicated MAbs.

Number by aliquot	Round	MAb	Color screening	m/z	N	Sequence*			C	Linker
1	1A	MOPC 141	no change	1032	115	177	182	115	115	328
2[†]			**halo**	**1004**	**115**	**177**	**128**	**128**	**128**	**328**
3			no change	929?	115	173	?[‡]	?	128	328
4			no change	1113	115	128	128	237	177	328
5			halo	1114	115	128	177	128	237	328
6 (alternate sequence)	2	E16	positive	1122	115	128	128	240[§]	182	328
7			**positive**	**1064**	**115**	**128**	**128**	**237**	**128**	**328**
6			**positive**	**1064**	**115**	**128**	**128**	**237**	**128**	**328**
8			positive	1053	115	177	128	177	128	328
9			positive	1083	115	147	128	128	237	328
10			positive	1083	115	128	147	128	237	328
11			positive later	1072	115	101	240	115	173	328
12			positive	?						328
13			positive	?						328
14			positive	?						328
-	3	UPC 10	-	-	-	-	-	-	-	-
15	4	1A6.2.1	**halo**	**1004**	**115**	**177**	**128**	**128**	**128**	**328**
16			halo	974	115	128	147	128	128	328
17			halo	1063	115	182	182	128	128	328
18			halo	1054	115	173	182	128	128	328
19			halo	881		115	182	128	128	328
20			halo	?	?	?	240	128	128	328
21			halo	1113	115	177	237	128	128	328
22			halo	1026	115	101	177	177	128	328
23			halo	1112	115	128	240	173	128	328
24			halo	1023	115	177	101	173	128	328
25			halo	973	115	101	128	173	128	328
26			halo	1058	115	177	128	182	128	328
27			halo	974	115	128	128	147	128	328
28			halo	1067	115	128	128	240	128	328
29			halo	1173	115	237	128	237	128	328
30			halo	1040	115	101	128	128	240	328
31			halo	1334	115	240	237	237	177	328
32			**bead itself blue by 1 min**	1198	115	240	101	237	177	328
33			halo	1002?	?	?	?	237	237	328
34			**bead itself blue by 30 sec**	?	?	?	?	182	237	328
35			**bead itself blue by 30 sec**	1077?	115	182	115	101	237	328
36			halo	?	?	?	?	?	147	328
37			halo	?						328
38			**bead itself by 30 sec, then very blue**	?						328

For footnotes, see Table 3.5 (page 155).

Table 3.15. Sequencing of peptoids retained from magnetic screening and positive in color screening of Peptoid Library 1 using the indicated MAbs.

Aliquot	Number by Aliquot	Round	MAb	Color Screening	m/z	N	Sequence*				C
E	1	1	MOPC 141	faint	923?‡	113	128	?	?	115	328
	10			faint	1133?	?	?	128	173	237	328
B	17	4	1A6.2.1	**bead itself blue by 2 min**	958?		101	177	237	115	328
	43			**bead itself slightly green by 4 min**	1140	101	182	237	177	115	328
	50			bead itself blue by 1 min	?	101	240	101	?	115	328
	57			bead itself blue/green by 7 min 40 sec	?	?	?	?	240	115	328
	59			bead itself blue by 1 min	?	?	?	240?	173?	115?	328
	66			bead itself blue by 10 sec	?						328
C	14	4	1A6.2.1	no change by 2 min; bead itself very slight blue by 5 min; bead itself yellow by 9 min	1230	177§	237	240	147	101	328
	15			no change by 2 min; bead itself very slight blue by 5 min; bead itself yellow by 9 min	1099	177	128	128	101	237	328
D	32	4	1A6.2.1	bead itself slightly blue/green by 4 min 30 sec	928		237	101	147	115	328
F	35	4	1A6.2.1	bead itself slightly blue	1058	173	101	101	173	182	328
G	16	4	1A6.2.1	**bead itself blue**	1092	182	177	177	113	115	328
H	49	4	1A6.2.1	bead itself blue	?						328
	50			bead itself slightly blue	?						328
J	32	4	1A6.2.1	**bead itself blue by 1 min**	1198	115	240	101	237	177	328
	34			bead itself blue by 30 sec	?	?	?	?	182	237	328
	35			**bead itself blue by 30 sec**	1077?	115	182	115	101	237	328
	38			bead itself by 30 sec, then very blue	?						328

For footnotes, see Table 3.5 (page 155).

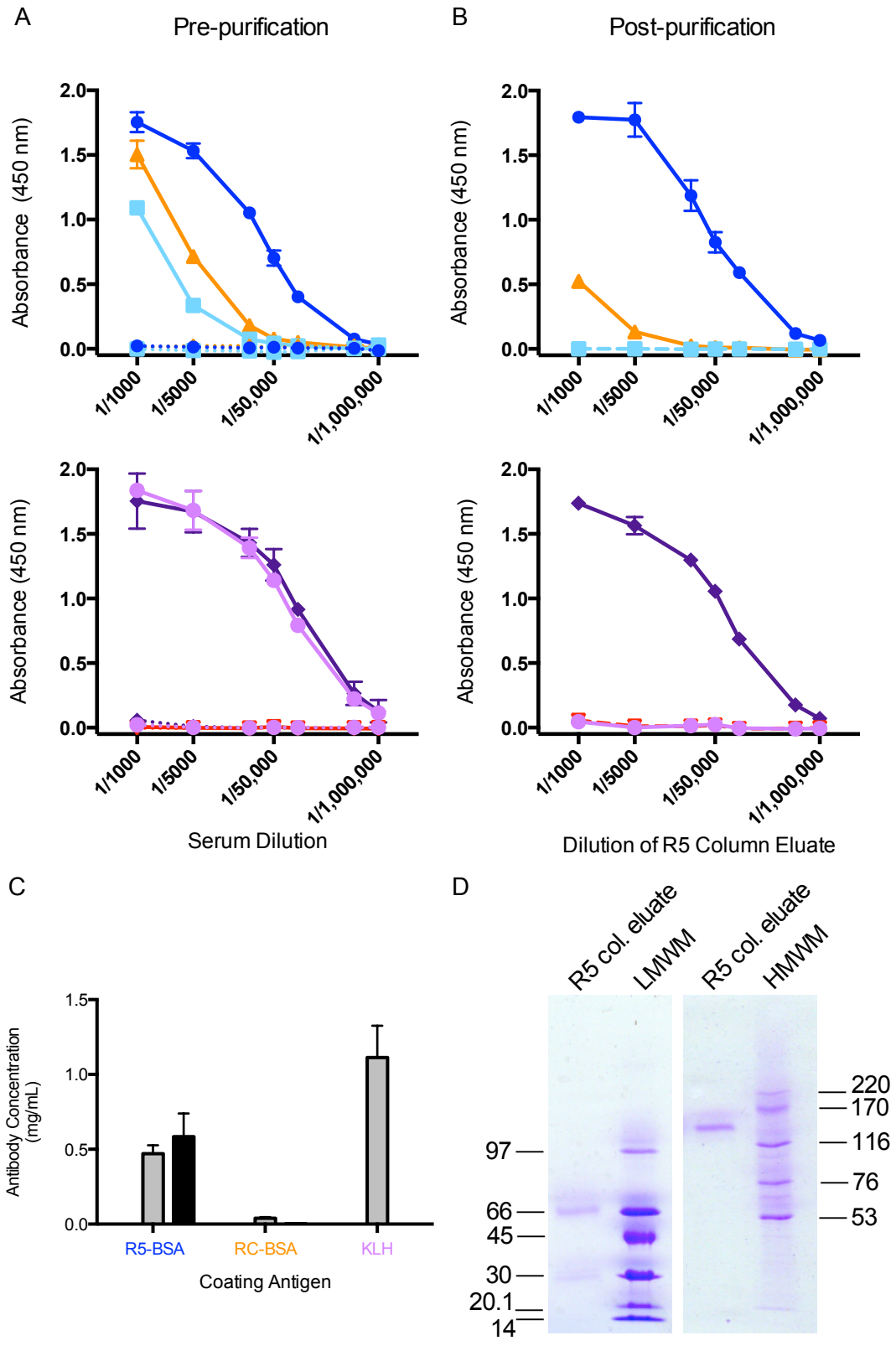
A
Pre-purification
B
Post-purification
Absorbance (450 nm)
2.0
1.5
1.0
0.5
0.0
1/1000
1/5000
1/50,000
1/1,000,000
Serum Dilution
Dilution of R5 Column Eluate
C
Antibody Concentration (mg/mL)
1.5
1.0
0.5
0.0
R5-BSA
RC-BSA
KLH
Coating Antigen
D
R5 col. eluate
LMWM
R5 col. eluate
HMWM
97
66
45
30
20.1
14
220
170
116
76
53

Figure 3.1. Purification of anti-R5 peptoid antibodies.
A. Rabbit 12D pre-immunization serum (*dotted lines*; Day -8 in Fig. 2.12, page 119) and post-immunization serum (*solid lines*; pooled from Days 356, 370, and 397) were analyzed by ELISA for the presence of serum antibodies. Plates were coated with 10 μg/mL of the following antigens: R5 peptoid conjugated to a carrier protein irrelevant to the original immunization (R5-BSA; ●); an irrelevant peptoid conjugated to BSA (RC-BSA; ▲); BSA alone (■); the immunogen, R5-KLH (◆); KLH alone (●); and an irrelevant protein, OVA (▼). Following blocking, dilutions of sera were applied and bound antibody was detected using an HRP-conjugated goat anti-rabbit IgG secondary antibody and the substrate TMB. The reaction was stopped with 2 M sulfuric acid and the absorbance at 450 nm was recorded for each well. The normalized absorbance averaged from triplicate wells is shown from one representative experiment of three performed. Error bars represent mean normalized absorbance ± standard deviation. **B.** To affinity purify antibodies against R5 peptoid, as described in Section 2.III.B.2 (page 86), rabbit 12D post-immunization serum was passed over a KLH-sepharose column to remove anti-KLH antibodies. The final flow-through from this column was then passed over an R5-SulfoLink column, which allowed the presentation of R5 peptoid on the column resin without the maleimide linker used to conjugate R5 peptoid to KLH for immunization. Anti-R5 peptoid antibodies were eluted from the column, dialyzed into PBS, then concentrated using ammonium sulfate precipitation and centrifugal concentrator devices. The concentrated R5 column eluate was analyzed by ELISA simultaneously with the sera in panel A. Data shown are from one representative experiment of three performed. Error bars represent mean normalized absorbance ± standard deviation. **C.** Quantification of anti-R5 peptoid, anti-linker, and anti-KLH antibody concentrations in pre-immunization serum (*white bars*) and post-immunization serum (*gray bars*) from panel A, and R5 column eluate (*black bars*) from panel B, using a standard curve included in those ELISAs as described in Section 2.III.B.4 (page 94). Since the R5-BSA conjugate used to determine the concentration of anti-R5 peptoid still contained the maleimide linker used to conjugate R5 to KLH for immunization, the antibody concentration against an irrelevant peptoid conjugated to BSA (RC-BSA) was calculated to indicate the concentration of anti-linker antibodies. Data shown represent the average of three experiments; error bars represent mean plus standard deviation. **D.** Concentrated R5 column eluate was analyzed by SDS-PAGE using a PhastSystem to assess the purity of anti-R5 antibodies. Under reducing conditions [left-hand panel, showing the low molecular weight marker (LMWM) in the second lane], heavy and light chains were visible in the R5 column eluate, whereas non-reducing conditions [right-hand panel, showing the high molecular weight marker (HMWM) in the second lane] suggested that the R5 column eluate contained intact IgG.

Table 3.16. RAR5 binds specifically and reproducibly to on-bead R5 peptoid versus irrelevant (RC) peptoid beads in small-scale magnetic screening assays.

	Number of TentaGel beads after magnetic screening*		
	Retained		
Antibody Concentration (ug/mL)	Total (PGD coverage†)	Confirmed as R5 by MS	Not retained
0	0	0	30
0.0001	0	0	30
0.001	0	0	30
0.01	0	0	30
0.1	1 (sparse)	0	29
1	2 (sparse)	2	28
10	3 (covered)	3	27

*Three R5 peptoid beads were added to tubes of 27 RC peptoid beads to give a total of 30 beads in each tube. Data shown are from one representative experiment of three performed. In parallel, 3 R5 peptoid beads were added to tubes of 27 RC peptoid beads and screened with an irrelevant rabbit PAb, RAMIg, at the concentrations shown; no on-bead peptoids were retained by the magnet (data not shown).

†PGDs were added a 1:10 dilution (3 mg/mL) to peptoid beads that had been previously incubated with RAR5.

Table 3.17. Selection of R5 peptoid beads by RAR5 from a mixture of many on-bead peptoids requires a higher concentration of RAR5 than selection of R5 peptoid beads from many irrelevant (RC) peptoid beads.

		Number of TentaGel beads						
		Added			Retained by magnetic screening			
						Confirmed by MS*		
Round	Antibody Concentration (ug/mL)	R5	RC	Peptoid Library 1	Total	R5	RC	Peptoid Library 1
1	0	3	20,000	-	0	-	-	-
2	0.01				0	-	-	-
3	0.1				3	3	-	-
4	1				0	-	-	-
1	0	3	-	20,000	11	0	-	11
2	0.01				1	0	-	1
3	0.1				2	0	-	2
4	1				51	3	-	48

*If R5 or RC was suspected based on MS, MS/MS was performed. Data shown are from one representative experiment of three performed.

Table 3.18. Dilute solutions of PGDs specifically and routinely select complexes of on-bead R5 peptoid and RAR5 from irrelevent (RC) peptoid beads in small-scale assays.

Dilution of PGD stock†	Number of TentaGel beads after magnetic screening*		
	Retained		Not retained
	Total (PGD coverage)	Confirmed as R5 by MS	
0	0	-	30
1:100000	0	-	30
1:50000	0	-	30
1:25000	0	-	28
1:10000	3 (hardly any)	3	27
1:5000	3 (sparse)	2	27
1:2500	3 (sparse)	3	27
1:1000	3 (2 covered, 1 sparse)	3	27
1:10	3 (covered)	2 (1 bead lost)	25

*Three R5 peptoid beads were added to tubes of 27 RC peptoid beads to give a total of 30 beads in each tube. Data shown are from one representative experiment of three performed.

†PGDs were added at a range of dilutions from 0 to 1:10 (0-3 mg/mL) to peptoid beads that had been previously incubated with 1 μg/mL RAR5. In parallel, 3 R5 peptoid beads were added to tubes of 27 RC peptoid beads, then screened with an irrelevant rabbit PAb, RAMIg, at 1 μg/mL and the range of PGD dilutions shown; no on-bead peptoids were retained by the magnet (data not shown).

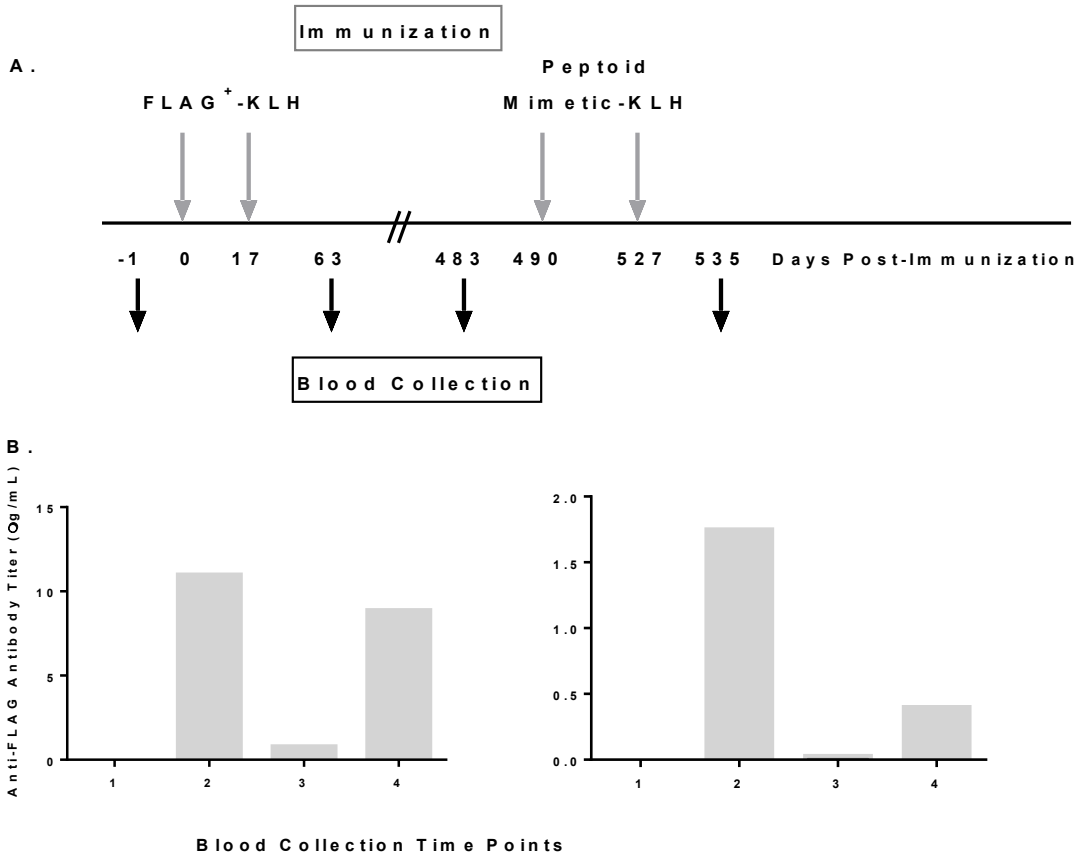

Figure 3.2. Preliminary data in a model where mice were primed with FLAG$^+$-KLH and boosted with peptoids selected using MAb anti-FLAG suggests peptoid mimicry of FLAG$^+$ peptide.

A. Schedule of immunizations and blood collection. On Day -1, mice were bled and sera prepared (pre-immunization). In the priming phase, mice received two immunizations with FLAG$^+$-KLH adsorbed to alum on Days 0 and 17, and blood was collected on Day 63 to prepare FLAG-primed sera. Mice were then rested until their antibody levels against FLAG$^+$ peptide returned to background levels as determined by traditional plate ELISA using sera prepared from blood collected on Day 483 (rested sera). Finally, mice were boosted on Days 490 and 527 with two peptoids selected by screening with MAb anti-FLAG, separately conjugated to KLH and adsorbed to alum, and mixed for immunization (peptoid-boosted sera). **B.** The concentrations of anti-FLAG$^+$ antibodies in pre-immunization sera (1), FLAG-primed sera (2), rested sera (3), and peptoid-boosted sera (4) were measured for two mice (#C5M3, left-hand panel, and #C6M4, right-hand panel) by on-bead ELISA. Data courtesy of Dr. Allison Case.

Table 3.19. Peptoid-optimized magnetic screening of sub-aliquots of Peptoid Library 6 using a neutralizing MAb against MNV-1.

Sub-aliquot	Amine forming N-terminal monomer	Number of beads retained in magnetic screening	Number of beads blue within 1 min in color screening
1	2-aminoethanol	33	5
3	glycine	11	2

Table 3.20. Sequencing of peptoids retained from magnetic screening and positive in color screening of Peptoid Library 6 sub-aliquots using a neutralizing MAb against MNV-1.

Sub-aliquot	Amine forming N-terminal monomer	Bead blue within 1 min in color screening	m/z	N	Sequence*							C	Linker
1	2-aminoethanol	A	1262	101	128	177	101	163	128	163	128	173	
		B	1328	101	163	128	223	148	147	128	?‡	173	
		C		FLAG+									
		D	1338	101	128	148	148	237	128	128	147	173	
		E	1259	101	101	128	129	147	115	165	200	173	
3	glycine	A	1299	115	177	128	128	151	148	128	151	173	
		B	1248	115	128	128	151	173	101	151	128§	173	

*Sequences are indicated by the unique mass of each monomer in the library (see Table 2.2, pages 103-104), with 173 designating the mass of the cleaved linker. Monomers appearing more than once were also assigned a color for easier visualization of patterns as follows. Monomers included in Tables 3.4-3.15 (pages 154-165) are colored the same here. Listed according to their amine name, they are: 2-aminoethanol, pink; glycine, bright blue; 1,4-butanediamine, light purple; benzylamine, orange; 1-aminoindan, bright green; tyramine, maroon; and 2,2-diphenethylamine, yellow. Monomers not included in Library 1 but that appeared more than once in the sequences shown here were assigned colors: 2-aminomethyl-5-methylpyrazine, dark pink; 4-aminomethylpyridine, pale turquoise; and histamine, pale green. See Fig. 2.6 (page 109) for the structure (and molecular weight) of each amine.

‡? indicates total mass (determined by mass spectrometry) or monomers that could not be determined or could not be determined definitively from MS and/or MS/MS spectra.

§Red text indicates the order of monomers within one sequence could not be determined definitively from MS/MS spectra.

Table 3.21. Suggestions for magnetic and color screening assays from this dissertation research and some relevant references.

Step	Suggestion	Rationale	Applicable dissertation section(s) and references
General tips	Use barrier tips	The TentaGel resin currently used for library synthesis and on-bead assays has a diameter of 140-170 μm. The beads are therefore small enough to be aspirated by a micropipette and lost or inadvertently transferred to another tube.	Tables 3.6 (page 156), 3.7 (page 157), and 3.20 (page 172)
	Store peptoid libraries or other TentaGel beads that have been swollen in aqueous solution (for example, PBST) with sodium azide	This is important to prevent microbial growth after the resin has been swollen in aqueous solution.	Section 2.II (starts on page 68)
Magnetic	Preclear with PGDs	This will help to remove members of the library that bind to the PGDs and not the screening MAb.	Table 3.17 (page 169)
	Consider preclearing with an irrelevant species- and isotype-matched control MAb	Here, a signature of nonspecific binding was identified by comparing the sequences of the peptoids retained in preclearing with those retained in screening with anti-viral MAbs even without preclearing until no more beads were retained by the preclearing MAb. This can be referred to as "limited preclearing."	Round 1A and 1B (Tables 3.1, page 152 and 3.2, page 153), Tables 3.5-3.15 (pages 155-165), Section 4.II (starts on page 181)

Step	Suggestion	Rationale	Applicable dissertation section(s) and references
Magnetic, continued	Consider beginning with a low concentration of the screening MAb and increasing it in successive rounds	This may help avoid retaining a large number of false positives or low-affinity binders and may save valuable reagents	Table 3.17 (page 169)
	Rank retained peptoid beads according to density of PGD coverage	This will help prioritize the best candidates, especially if many peptoid beads are retained in magnetic screening	Table 3.17
	Remember that the magnetic screen is designed to be inclusive rather than exclusive	A peptoid bead can be retained by the magnet even when a very dilute solution of PGDs is applied, particularly if few other beads are present to cause mechanical forces to interfere with bead retention. Using a high concentration of PGDs and selecting some false positives is preferable to failure to isolate peptoid beads bound by the screening MAb. If these remain in a library that is subsequently screened with a different MAb, this could confound the results of that screen.	Section 3.II (starts on page 123), Table 3.19 (page 172)

Step	Suggestion	Rationale	Applicable dissertation section(s) and references
Magnetic, continued	Before applying a new MAb to a used library, add PGDs alone. This may be called a "postclear" (as opposed to a preclear).	This could help remove from the library any beads bound by MAb that have not been retained by the magnet.	Section 3.II (starts on page 123), Section 4.IV (starts on page 188), (227)
	Consider postclearing if a large number of beads are retained	The upper limit of the number of beads that can be retained under each set of screening conditions is not always known. For example, roughly 50-100 peptoid library beads were retained along with R5 peptoid beads in each of three experiments screening Peptoid Library 1 sub-aliquots with RAR5 at 1 μg/mL and a 1:10 dilution of PGDs.	Table 3.17 (page 169)
Color	Include FLAG peptide model system controls in every color screen (3 $FLAG^+$ beads and 3 $FLAG^-$ beads work well)	This is critical to the interpretation of the assay.	Section 3.II (starts on page 123), Tables 3.4-3.15 (pages 154-165)
	Use a timer to time bead color change	Absolute time as well as time relative to FLAG model system controls should be recorded.	Section 3.II, Tables 3.4-3.15

Step	Suggestion	Rationale	Applicable dissertation section(s) and references
Color, continued	Use a color palette to rank color change of beads	This ensures a consistent interpretation of what "blue" means and allows this to be communicated to others. Furthermore, the blue color may change over time as the reaction progresses and this should be noted.	Section 3.II (starts on page 123), Tables 3.4-3.15 (pages 154-165)
	Include as controls some library peptoids that were not selected in magnetic screening	These could serve as a useful control.	Section 4.II (starts on page 181), (354)
	Color change of the bead itself (as exhibited by $FLAG^+$ peptide screened with MAb anti-FLAG) indicates positivity in this assay, while halos may indicate nonspecific binding	Many of the peptoids that produced halos were found to include the lysine-like peptoid monomer generated using 1,4-butanediamine. A recent study has shown that such charge may lead to nonspecific binding when the compounds on a bead are closely spaced (355).	Section 3.II, Tables 3.4-3.15

CHAPTER FOUR

Discussion

4.I. Project goals and major findings

The long-term goal of this research is to develop novel, prophylactic vaccines using peptoids that mimic the shape of B cell epitopes. It is hypothesized that by screening large libraries of peptoids with broadly protective MAbs against pathogens or toxins, peptoids will be selected that will elicit broadly protective antibodies. By coupling these peptoids to carrier proteins and mixing them with adjuvants, antibody responses should be class-switched and of high affinity.

The goal of this dissertation was to confirm and extend previous work to examine the immunogenicity of peptoids, as well as to optimize the screening of on-bead libraries to select peptoids that mimic native B cell epitopes. The major findings to emerge from this study are listed chronologically, and then discussed thematically, as follows:

1. The magnetic screening assay that had been previously optimized using on-bead $FLAG^+$ peptide, on-bead $FLAG^-$ peptide, and MAb anti-FLAG was applied to screen OBOC peptoid libraries with neutralizing MAbs against WNV and MNV-1. Using these experimental conditions, peptoids were retained. However, peptoids were also retained by the species- and isotype-matched irrelevant control MAbs that were used to preclear the library of those peptoids that bound to other portions of the antibody, such as the Fc.
2. Most of the on-bead peptoids isolated in magnetic screening using preclearing MAbs and neutralizing MAbs against WNV and MNV-1 did not show color changes in the second screening assay, which had also been tested using the FLAG peptide model

system. However, a few on-bead peptoids selected by the anti-MNV-1 neutralizing MAb did show color changes. The time elapsed until this color change occurred ranged from 10 seconds to 7 minutes 40 seconds, as compared to 30 seconds to 1 minute for on-bead $FLAG^+$ peptide identified with MAb anti-FLAG. **Those on-bead peptoids that changed color rapidly as well as peptoids identified in screening portions of Peptoid Library 6 using the neutralizing MAb against MNV-1 should be pursued as potential vaccine candidates according to the strategic plan outlined in Section 3.I (page 120).**

3. When sequencing by MS/MS was performed for those peptoids that were retained in magnetic screening and found to produce “halos” in color screening, some of the same sequences, or sequences in which the monomers were the same but arranged differently, were isolated using the irrelevant preclearing MAbs and the anti-viral MAbs. Additionally, the sequences of the on-bead peptoids that produced a halo in color screening tended to include the positively charged peptoid residue that is the peptoid analog of the amino acid lysine. These peptoids were considered to be nonspecific and were removed from consideration as potential peptoid vaccine candidates.
4. As a result of the concern with specificity in these studies, as well as in other published studies using proteins to screen OBOC libraries in magnetic screening assays, a peptoid/anti-peptoid antibody pair was generated for use in further optimization of the screening assays. To this end, R5 peptoid was designed, synthesized, and deprotected/cleaved from the resin. It was then purified, lyophilized, conjugated to the carrier protein KLH, adsorbed to alum, and used to immunize

rabbits. Rabbits were chosen since reasonable volumes of antisera could be obtained. The early part of this work has been reported previously (332).

5. Serum antibodies against the R5 peptoid, the carrier, and the linker were detected by ELISA following immunization. Subsequent affinity purification was first performed using a KLH-sepharose affinity column to remove anti-KLH antibodies. An R5-SulfoLink affinity column was then used to enrich antibodies against the R5 peptoid without the presence of the linker used for immunization. This resulted in the first affinity-purified antibody (RAR5) raised against a peptoid. The removal of anti-carrier and anti-linker antibodies demonstrated that the peptoid itself (when coupled to carrier) could elicit antibodies, and hence, was a typical hapten.
6. The specificity of RAR5 for R5 peptoid was demonstrated by ELISA. The PAb RAR5 bound to R5-BSA but not to the irrelevant control RC-BSA. Specificity was further confirmed by on-bead magnetic screening assays in which RAR5 bound to on-bead R5 peptoid but not to on-bead RC peptoid.
7. The sensitivity and reproducibility of RAR5 binding to R5 peptoid was demonstrated in on-bead magnetic screening assays, in which 3 R5 peptoid beads could be reproducibly isolated when spiked into ~20,000 control RC peptoid beads. In parallel, 2–3 R5 peptoid beads were reproducibly isolated from ~20,000 peptoid library beads. Additionally, peptoid library beads were isolated along with these R5 peptoid beads. However, the R5 peptoid beads could be distinguished from the vast majority of these peptoid library beads because they had the **highest density** of bound PGDs as determined by light microscopy.
8. In small-scale magnetic screening assays, complexes of RAR5 and on-bead R5 peptoid were specifically and routinely retained by solutions of PGDs as dilute as

1:5000. The magnetic screening assay also allowed the isolation of some complexes of on-bead R5 peptoid bound specifically by RAR5 when a solution of PGDs as dilute as 1:25,000 was applied. However, based on preliminary large-scale experiments, previous optimization using the FLAG peptide model system, and the decision that selecting some false positives in magnetic screening was preferable to the failure to capture low affinity antibody-bound on-bead peptoids and remove them from the library prior to additional screening, a 1:10 dilution of PGDs was adopted for future screens.

9. As an example of how the prime-boost mouse model will be used to test peptoid candidates for MNV-1 vaccines, and in support of the screening methods used to identify these candidates, preliminary experiments using on-bead ELISAs were conducted. In these studies, two peptoids identified previously (332) by magnetic and color screening with MAb anti-FLAG were sequenced, synthesized, conjugated to KLH, adsorbed to alum and used to boost mice. These mice had been previously immunized with $FLAG^+$ peptide-KLH and then rested until levels of serum antibodies against $FLAG^+$ peptide returned to baseline. Immunizing the mice with the combination of these two peptoids resulted in a “cross-boost” of the antibody response against $FLAG^+$ peptide, as detected by on-bead ELISA (designed to avoid a read-out of anti-linker and anti-carrier antibodies). Since these two peptoids shared no similarity in sequence with $FLAG^+$ peptide, this suggested that magnetic and color screening could have identified a peptoid or peptoids that mimicked the conformation of $FLAG^+$ peptide. **While preliminary, these data are encouraging for the MNV-1 candidate peptoids that were isolated using similar screening conditions.**

4.II. Screening a OBOC library to identify peptoid vaccine candidates for WNV and MNV-1 identified lead compounds for MNV-1 and provided further insights into screening assay optimization

To identify peptoid vaccine candidates using the on-bead magnetic and color screening assays that were optimized using the FLAG peptide model system (332), Peptoid Library 1 was first subjected to magnetic screening with neutralizing MAbs against WNV and against MNV-1. On-bead peptoids were isolated by each MAb. This was not unexpected in light of the previous optimization efforts using the FLAG peptide model system, as well as previous work demonstrating that a MAb raised against a *peptide* could bind to members of a peptoid library (294, 298). While there are many other studies using proteins to screen OBOC libraries of peptoids or related compounds, some of which are described in the Introduction to this dissertation, two studies stand out for having used magnetic screening as the first step in screening a OBOC library with proteins to identify ligands. These were published before (227) and after (354) the magnetic screens described in this dissertation were carried out. In the first study, a OBOC library of D-peptide/peptoid hybrids was screened using MAb anti-FLAG and Dynabeads coated with sheep anti-mouse IgG (227). In the second study, a OBOC library of cyclic peptides was screened with biotinylated calcineurin and streptavidin-coated Dynabeads (354). Notably, both groups of investigators might have benefitted from the use of on-bead controls like the FLAG peptide model system described here and previously (332) to optimize their magnetic screening assays. While this is critical to demonstrate that a protein can identify ligands specific for that protein within the on-bead magnetic screening assay, these investigators did not use magnetic screening as their sole assay to establish specificity, but rather delayed the emphasis on specificity until downstream screening steps. Although this approach may still result in the identification of

specific ligands if appropriate specificity is later established (for example, through competition assays, which both groups performed), the efficiency of the process might be improved or candidates that might be missed in the first round of screening could be discovered if a magnetic screening assay optimized with on-bead controls is used.

Accordingly, as performed in this dissertation research, both groups of investigators evaluated all on-bead compounds selected in magnetic screening in subsequent screening assays. Astle et al. tested lead compounds by microarray screening (227). In such studies, the irrelevant Myc peptide and MAb anti-Myc antibody served as controls. The outcome of these experiments will be discussed further below. In contrast, the lead compounds identified by Liu et al. were subjected to color screening (354), as were all WNV and MNV-1 peptoid vaccine candidates identified here. In the experiments with MAbs against WNV and MNV-1, it became clear that unlike the FLAG peptide model system, in which on-bead $FLAG^+$ peptide rapidly turned blue but $FLAG^-$ peptide remained clear when screened with MAb anti-FLAG, library screening required timing the color change of the on-bead peptoids in direct comparison with FLAG peptide model system controls. Those on-bead peptoids that exhibited the most rapid color changes were determined to be the best vaccine candidates. Again, the inclusion of such controls was not reported by Liu et al. (354). They did, however, report that “an intense turquoise color developed on positive beads in 15 min,” and ranked their lead compounds by intensity of color change, although they did not describe in detail the method they used to do so. The Pei group has evidently used various colorimetric on-bead screening assays for many years (356-358), and in a previous study, they described ranking the intensity of the bead color as “‘intense’, ‘medium’, and ‘light’” (357). In another study, they reported the use of a negative control protein that lacked the fused, functional portion of their screening protein, and also mentioned controlling the

conditions of their assay (staining time, washing, and protein concentration), such that ~10–100 beads are considered positive from each batch of approximately 286,000 beads screened (356). Unfortunately, these intriguing methods, particularly the inclusion of the control screening protein, were not reported for the color screening assay by Liu et al. Based on the studies presented in this dissertation, I would recommended timed, direct comparison to both positive and negative controls for color screening assays, although admittedly, how the batch-to-batch variation may affect the FLAG peptide model system used here has not been adequately addressed. Such studies should be performed so that these controls can increase the confidence with which screens of OBOC libraries for peptoid vaccine candidates are performed. Finally, the use of a color palette, which was employed for some of the color screening described in this dissertation but not reported in the Results section due to lack of uniform application, could be combined with measurements of time to color change in direct comparison with FLAG model system controls in future color screens. The use of the more defined color screening readouts suggested by this dissertation research and by the Pei group could be helpful in color screening applications for many investigators.

A few additional points regarding the identification of the best candidates from on-bead screening emerging from this dissertation research and the study by Liu et al. deserve mention here. In the final evaluation of the MNV-1 vaccine candidates performed to date, the sequences of the MNV-1 peptoid vaccine candidates, which changed color rapidly, were compared with the sequences of peptoids that did not change color or produced “halos.” This analysis reinforced the specificity of the results obtained with color screening. The MNV-1 vaccine candidate sequences did not show the predominance of the lysine-like peptoid monomer found in many of the sequences that did not change color but were

selected in magnetic screening by the preclearing and anti-viral MAbs. This may be of interest to investigators who have used the lysine-like peptoid monomer as component of their linkers coupling peptoids to resin for on-bead screening (299). Moreover, although Liu et al. did not perform a sequence comparison between color-changing compounds and those that did not change color, they did perform several subsequent screening steps following color screening. In their next step, fluorescently labeled calcineurin bound to on-bead compounds selected in previous rounds of screening but not to on-bead compounds that were not selected in magnetic screening. This could be a useful control for future color screening assays to identify peptoid vaccine candidates and should be included. Whether any of the other extensive downstream assays performed by Liu et al. should be incorporated into the strategic plan to identify peptoid vaccine candidates, as outlined in Section 3.I (page 120), remains unclear. The assays performed by Liu et al. did help these investigators to reduce their number of lead compounds at almost every stage, and may prove useful, particularly if prime-boost mice in which to test the MNV-1 vaccine candidates are not yet available. Especially attractive is their fluorescence anisotropy assay, which might allow the measurement of binding affinity using single beads. With regard to making peptoid vaccines, it is important to use only the very best peptoid candidates since the work involved in downstream vaccination is time-consuming.

Finally, an additional comment on the role of preclearing in on-bead screening is warranted. Preclearing with screening reagents had been utilized by investigators screening OBOC peptoid libraries in other types of assays [for example, (299)], but was not performed in magnetic screening by Astle et al. (227) or Liu et al. (354). Furthermore, exhaustive preclearing was not performed here so as to save possible wear and tear on the peptoid library; even though more peptoids were retained from two library aliquots in a second

preclear with the same MAb, the anti-WNV bnMAb was applied in the next round. However, this “limited preclearing” sampled the on-bead peptoids in the library and revealed a signature of nonspecific binding, the prevalence of the lysine-like peptoid monomer, that enabled a more confident endorsement of the color screening results identifying MNV-1 vaccine candidates. Therefore, limited preclearing of a OBOC library can serve a useful role.

In summary, these experiments have identified peptoid vaccine candidates for MNV-1 that require further testing in the prime-boost mouse model to demonstrate mimicry of the corresponding native antigen for the screening MAb. If these candidates continue to be positive when tested in the downstream functional assays outlined in the strategic plan, this would represent a significant step forward. Comparison of this work with the relatively few related studies using magnetic screening as the first step to identify proteins binding to members of OBOC libraries highlighted some strengths of the approach presented here (optimization, recording time-to-blue color in direct comparison with controls, limited preclearing), as well as opportunities to incorporate new techniques into the strategic plan to identify peptoid vaccine candidates.

As an optimistic coda to this story, my colleagues have generated some preliminary data demonstrating that screening protocols similar to those used here may have selected peptoid(s) that mimic the shape, but not the sequence, of FLAG peptide. The importance of this concept for peptoid vaccine development is even more apparent in view of the study by Astle et al. (227). All 27 lead compounds selected from the OBOC library of D-peptide/peptoid hybrids by magnetic and microarray-based screening with MAb anti-FLAG were found to contain a D-lysine (Dlys) or D-glutamine (Dgln) residue followed by a D-tyrosine (Dtyr) residue. Unfortunately, Dlys and Dgln had the same molecular weight and could not be distinguished in sequencing by MS/MS. However, if these residues were Dlys,

the N-Dlys-Dtyr-C pattern would form the retro-inverso D-peptide for a portion of $FLAG^+$ peptide known to be critical for MAb anti-FLAG binding, as $FLAG^+$ peptide has the sequence of N-D**YK**DDDDK-C, while the $FLAG^-$ peptide used in the present study has the sequence of N-D**AK**DDDDK-C (336). Considering that L-peptides and their corresponding retro-inverso D-peptides have similar arrangements of their side chains in space, and further considering that in these 27 candidate compounds, the N-Dlys or Dgln-Dtyr-C sequence was often but not always preceded or followed by a D-aspartic acid (Dasp) residue, the similarity between these candidate compounds and $FLAG^+$ peptide is apparent. This finding validates the screening methodology developed by Astle and colleagues. However, it may stand in contrast to the hypothesized strength of using peptoid libraries to identify B cell epitope mimetics, which is that the great diversity of shapes afforded by peptoids does not require this side-chain correspondence for mimicry, and will hopefully allow mimetics of complex conformational epitopes to be identified. Of course, if a mimetic peptoid were identified that shared side chain structure with the native antigen, this would not preclude its utility in vaccines.

4.III. The generation of the first-affinity purified antibody against a peptoid confirmed that peptoids are haptens

The lack of immunogenicity ascribed to free peptoids (300) has been viewed as a desirable attribute for the use of peptoids as therapeutics, but the ability to induce an anti-peptoid immune response is essential to the goal of using peptoids in vaccines. To test the immunogenicity of peptoids and generate the first affinity-purified anti-peptoid antibody for use in optimizing assays to discover peptoid vaccine candidates, rabbits were immunized with the peptoid R5 after conjugation of R5 to the carrier KLH and adsorption to alum. This

R5 peptoid was designed to include side chains with diverse characteristics, including charge, aromaticity, and hydrophobicity. Affinity purification of the resulting pooled immune rabbit serum was then performed to separate anti-R5 peptoid antibodies from other serum proteins and, in particular, from antibodies that were made against the carrier and the linker used to bind the peptoid to the carrier for immunization. The affinity-purified antibody, RAR5, bound to the R5 peptoid, but not to an irrelevant peptoid, in both a traditional plate ELISA and in on-bead magnetic screening assays.

In the first study designed to test whether peptoid-carriers could elicit antibodies against the peptoid itself, the assay used in one set of experiments was unable to distinguish between antibodies made against the linker, the peptoid, or both. In other experiments, an irrelevant control peptoid was not included. However, both sets of experiments showed that free peptoids did not elicit an antibody response (300). The experiments described here complete this first study to show definitively that antibodies are generated against both the linker and the peptoid. Furthermore, the specificity of the anti-peptoid antibodies was confirmed by testing them on an irrelevant peptoid. Taken together, these results demonstrated that peptoids are immunogenic only when attached to carriers, *i.e*. they are typical haptens. While this is essential for peptoid vaccines, investigators developing peptoids as therapeutics should revisit any assumption that free peptoids cannot *become* immunogenic, since the possibility of attachment to self proteins and resulting hypersensitivity in some individuals must be considered. In humans, this often happens with small drug molecules such as penicillin*, i.e.,* some individuals display types I and IV hypersensitivity and cannot take penicillin or any of its many analogs.

Additionally, the generation of antibodies against R5 peptoid, a 5-mer, supports the hypothesis that a peptoid containing only 5 monomers is sufficient to elicit an antibody

response. Indeed, this is the designed length of our shortest library peptoids, although truncation may lead to the synthesis of shorter sequences. Moreover, peptoids of this length have been shown to be capable of displaying secondary structure, with a particular sequence adopting a polyproline type I helix (359). Such secondary structure may contribute to the “shapeliness” of peptoid B cell epitopes, although the peptoid in the cited study was composed of monomers with chiral side chains, in contrast to most of the peptoid monomers here.

4.IV. The R5 model system in the magnetic screening assay demonstrated specific, sensitive, and reproducible retention of on-bead R5 peptoid: implications for future screening

To ensure that future screens with broadly protective MAbs against toxins or pathogens would efficiently identify the best peptoid vaccine candidates, the affinity-purified anti-peptoid antibody, RAR5, was then used in further optimization of the magnetic screening assay. These experiments demonstrated the recovery of a known on-bead peptoid spiked into a large number of irrelevant peptoid or peptoid library beads. This has not been demonstrated in reports by other groups using magnetic screening with a protein to identify ligands from OBOC libraries (227, 354). Furthermore, these investigators did not rank their retained on-bead compounds according to PGD density, but this should be considered based on the experiments presented here to improve the efficiency of screening. This is especially important given the finding here that very dilute solutions of PGDs retained complexes of RAR5 and on-bead R5 peptoids in small-scale assays, which may contribute to the isolation of false positives in magnetic screening, and supports the need for validation in subsequent screening assays. It is also possible that false positives could be reduced by

employing the strategy used here of starting with a low concentration of the screening antibody and increasing the concentration in each round of magnetic screening until a reasonable number of on-bead peptoids are retained. However, the selection of false positives at this stage is preferable to not retaining even low affinity on-bead peptoids bound by the screening MAb, which may confound subsequent screens of the same OBOC library. To this end, another group added PGDs to those on-bead compounds that had not been retained by the magnet after they had been separated from on-bead compounds retained by the magnet (227). This double PGD addition or “post-clear” should be considered for inclusion in the peptoid vaccine development platform magnetic screening assay, particularly if large numbers of on-bead peptoids are retained by the magnet or before screening a used library with a new MAb.

In future studies, the utility of this R5 model system could be extended in several important ways:

i. First, further optimization of other assays in the vaccine development platform, such as the color screening assay (332), should be extended with the present R5 model system. For example, in the current color screening protocol, the screening antibody is re-added to on-bead compounds isolated in magnetic screening, and then positive beads are detected with a labeled secondary antibody. The necessity of this reapplication should be compared to adding secondary antibody alone;

ii. The immunogenicity of peptoids could be further explored by comparing the antibody response against R5 to that of truncated versions of R5 to determine the minimal length necessary to induce an immune response;

iii. The frozen single cell suspensions of the spleens and lymph nodes from the rabbits immunized with R5 could also be used to develop a rabbit MAb against a peptoid; and

iv. Finally, use of the R5 model system, which includes the first affinity-purified anti-peptoid antibody, may provide additional tools to the field of peptoid chemistry.

In summary, this dissertation has presented studies that contribute to the long-term goal of developing novel, prophylactic vaccines in three important ways. First, peptoid vaccine candidates were identified by screening OBOC peptoid libraries with a neutralizing MAb against MNV-1. These candidates await further functional testing. In the best outcome, these candidates may protect mice from MNV-1 and provide protection from the related viruses that infect humans. However, even if this is not the case, the improvements to on-bead screening suggested by these studies may allow peptoid vaccines for this and other pathogens or toxins to be developed. Furthermore, since on-bead screening assays are used in many applications, these technical insights may also be of use to investigators in other fields, as may the first affinity-purified anti-peptoid antibody that was developed here. Finally, these studies confirmed that peptoids elicit a specific antibody response when conjugated to carriers, and thus are haptens. This concept is fundamental to the development of peptoid-based vaccines and speaks to the power of the immune system to recognize diverse molecules not found in nature. Should this work lead to the development of protective vaccines, I will consider my many hours of viewing and transferring peptoid beads from one tube to another to have been well spent.

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