

Engineering modular platforms for rapid vaccine development



Karl Dietrich Brune

Department of Biochemistry

Medical Sciences Division

Corpus Christi College

University of Oxford

Trinity 2016

A thesis submitted to the University of Oxford in partial fulfilment of the
requirement for the degree of Doctor of Philosophy

Abstract

Vaccines have saved more lives than any other medical intervention. Recombinant vaccines provide unmatched safety profiles, but at the expense of reduced immunogenicity. Virus-like particles (VLPs) resemble viruses in size, shape and repetitive arrangement but are devoid of pathogenic genetic material and therefore safe. Poor immunogens can be rendered immunogenic by display on VLPs. Successfully decorating VLPs is still a major challenge. Genetic fusion or chemical modification is often time-consuming and can lead to misassembly or misfolding, which obstructs generation of the desired immune response. SpyCatcher is a genetically encodable protein, previously engineered to form a covalent isopeptide bond to its peptide-partner SpyTag. Presented in this thesis are SpyCatcher-VLPs, based on the fusion of SpyCatcher to the bacteriophage VLP AP205. SpyCatcher-VLPs can be conveniently conjugated with SpyTag fused antigens, simply by mixing. I demonstrate the modularity of this approach by covalently linking several complex, cysteine-rich malarial antigens to SpyCatcher-VLPs, such as the transmission-blocking antigen Pfs25 and the blood-stage antigen CIDR. A single administration of Pfs25-SpyTag conjugated to SpyCatcher-VLPs induced potent antibody generation against Pfs25, even in the absence of adjuvant. Anti-Pfs25 antibodies induced by this platform conveyed potent transmission-blocking activity in the mosquito vector. The thesis further demonstrates the feasibility of more complex Catcher-nanoparticle architectures. The previously engineered SnoopCatcher covalently reacts with SnoopTag peptide and is orthogonal to the SpyCatcher / SpyTag pair. IMX313 is an engineered chimera of the multimerization

domain of chicken complement inhibitor C4-binding protein. This work describes fusion of SnoopCatcher and SpyCatcher to IMX313, which yields independently addressable Catcher-moieties on a single IMX313 nanoparticle. Display of two antigens on one particle may enable single-particle, multi-disease vaccines as well as multi-stage vaccines to tackle immune evasion of parasites. The platforms presented should accelerate and enhance vaccine development and may create opportunities for imaging and metabolic engineering.

Authorship Declaration

I declare that this thesis is entirely my own work, and except where otherwise stated, describes my own research.

Part of works that have been performed by others:

Chapter 3

Immunogenicity of conjugated SpyCatcher-VLPs:

Initial Pfs25-SpyTag preparations were prepared by Andrew Ishizuka.

Immunisations and ELISAs were performed by Dr Darren Leneghan.

IFA imaging was performed by Iona Brian.

SMFA experiments were performed in Dr Carole Long's laboratories.

TEM images were taken by Dr Errin Johnson.

Publication arising from this thesis:

Brune, K.D., Leneghan, D.B., Brian, I.J., Ishizuka, A.S., Bachmann, M.F., Draper, S.J., Biswas, S., and Howarth, M. (2016). *Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization*. Scientific Reports 6, 19234.

Acknowledgements

I would like to thank Dr Mark Howarth for accepting me into his laboratory, providing expertise and guidance in what has been an intense journey and an immense opportunity. I am grateful to the Department of Biochemistry for their financial and outstanding support in my exceptional circumstances. I would also like to express my gratitude to the President and Scholars of the College of Corpus Christi in the University of Oxford for their financial support and providing a vibrant academic and social experience.

I greatly appreciate the help and support of the laboratory members of the Howarth group: Anselm Schneider, Dr Antonio Serrano, Dr Anthony Keeble, Can Buldun, Dr Christopher Schoene, Dr Gianluca Veggiani, Linda Tchatchouang, Marlene Pröschel, Max Jamilly, Dr Michael Brenner, Dr Michael Jacobsen, Dr Robert Wieduwild and Dr Samuel Reddington. I am also thankful to all the previous members of the Howarth group, who have helped build a foundation on which I could rest my work.

Next I would like to extend my gratitude to Professor Judith Armitage, Professor Lee Sweetlove, as well as Dr Travis Bayer for their contribution to my academic career. My acknowledgements also to the various laboratories who have helped my academic efforts in one way or another: the Armitage, Bachmann, Bayer, Berks, Biswas, Draper, Ferguson, Heap, Higgins, Sherratt and Whitby groups.

Furthermore I would like to thank Dr Grigorij Sutov, Dr Alexander Morris, Mattia Pastrolin, Dr Siobhan McMahon, Dr Dejana Jovićević and Chih-Chao 'Eric' Liang.

Finally I would like to thank my family for support throughout my studies, in particular my mother Mechthild Blömer-Brune and Wilfried Schlimm, Josef 'Jupp' Blömer and my sisters Dora Brune, Ruth Brune and Kathrin Schlimm.

Meiner Mutter

Mechthild Blömer-Brune

Im Gedenken an meinen Vater

Dr. Gerhard Brune

† 17 Mai 1998

Table of contents

Abstract	i
Authorship Declaration	iii
Acknowledgements	iv
Dedication	vi
Table of contents	vii
Abbreviations	xiii
List of Figures and Tables	16
Chapter 1 Introduction	20
1.1 Vaccines and Immunology	21
1.1.1 Vaccine History	21
1.1.2 VLPs & Nanoparticles	26
1.1.2.1 Chimeric VLP History	26
1.1.2.2 Size, geometry and repetitive arrangement	29
1.1.2.3 Nucleic acid content	32
1.1.2.4 Means of VLP production and industrial applicability	32
1.1.2.5 Heterologous antigen display and increasing interest in VLPs	33
1.2 Decoration of VLPs	34
1.2.1 The decoration challenge and motivation	34
1.2.2 Covalent approaches to VLP decoration	37
1.2.2.1 Arginine	37
1.2.2.2 Carboxylic acid amino acids – Aspartic acid and glutamic acid	37
1.2.2.3 Cysteine	39
	vii

1.2.2.4 Lysine and N-terminus	44
1.2.2.5 Tyrosine	46
1.2.2.6 Unnatural Amino Acid Incorporation	46
1.2.2.7 Reduction mutagenesis for unique (natural) amino acids side chains	50
1.2.2.8 Incorporation of Reactive Handles by post-translational modifications	50
1.2.2.9 Dock-and-Lock	52
1.2.2.10 Electrostatic Interaction Lock	53
1.2.2.11 HaloTag	54
1.2.2.12 Phosphopantetheinyl transferases (ACP/PCP)	54
1.2.2.13 SNAP-Tag	55
1.2.2.14 Sortases	56
1.2.2.15 Genetic fusion	57
1.2.3 Non-covalent coupling approaches	62
1.2.3.1 Antibody affinity through Protein A / Z	62
1.2.3.2 Avidins / biotin	63
1.2.3.3 Coiled coils	65
1.2.3.4 Affinity Binding	65
1.2.3.5 Lipid incorporation via GPI anchor and ER membrane localization sequences	66
1.2.3.6 Histidine tag – Ni-NTA / Co	67
1.2.3.7 RNA-directed loading of particles	67
1.2.4 Preceding work on Isopeptide-forming peptide Tags and Catchers	68
1.2.4.1 Intramolecular isopeptide bonds	68
1.2.4.2 Isopeptag(N) / pilin-(N/C)	70
1.2.4.3 SpyTag / SpyCatcher(Δ N1)	72
1.2.4.4 SnoopTag / SnoopCatcher	73
1.2.5 Thesis objectives	76

Chapter 2 Materials and Methods	77
2.1 Cloning	78
2.1.1 Polymerase chain reactions (PCR)	78
2.1.2 Isothermal DNA assembly (Gibson assembly)	79
2.1.3 Splicing by overlap extension (SOE-PCR)	80
2.1.4 Circular polymerase extension cloning (CPEC)	81
2.1.5 Desalting of DNA fragments by drop dialysis	81
2.1.6 Transformation of E. coli	81
2.1.7 Miniprep of plasmid and BAC DNA	81
2.1.8 Maxiprep of plasmid DNA	82
2.1.9 Genomic DNA isolation from bacteria	82
2.1.10 Sequencing	83
2.2 Protein expression and purification	84
2.2.1 Construction of bacterial expression strains	84
2.2.2 Media formulations and incubator parameters	86
2.2.3 Standard E. coli protein expression	87
2.2.4 Mammalian cell maintenance	87
2.2.5 Immobilized metal affinity chromatography (IMAC) of monomeric proteins	88
2.2.6 Amylose agarose chromatography	89
2.2.7 Gel filtration chromatography	89
2.2.8 Expression of SpyCatcher-VLPs	90
2.2.9 Purification of SpyCatcher-VLPs.	90
2.2.10 Improved expression and purification of SpyCatcher(Duo)-VLPs	92
2.2.11 Size-exclusion chromatography of SpyCatcher-VLPs for size determination	93
2.2.12 Transmission electron microscopy (TEM)	94
2.2.13 Calculation of maximum numbers of Catcher residues in cross section view	94

2.2.14 Expression of model proteins SpyTag (DA)-MBP	95
2.2.15 Expression of CIDR proteins	96
2.2.16 Alternative expression of CIDR proteins	96
2.2.17 Purification of CIDR proteins	97
2.2.18 Expression of Pfs25-SpyTag	98
2.2.18.1 Initial Pfs25-SpyTag expressions for SpyCatcher-VLP experiments	98
2.2.18.2 Subsequent expression for Pfs25-SpyTag:SpyCatcher-IMX experiments	99
2.2.19 Purification of Pfs25-SpyTag	100
2.2.20 Protein gel electrophoresis	100
2.2.20.1 SDS-PAGE	100
2.2.20.2 (Native) agarose gel electrophoresis (NAGE)	101
2.2.20.3 Coomassie blue-staining	102
2.2.21 SpyCatcher-VLP:SpyTag-protein reactivity	102
2.2.22 SpyCatcher-VLP:SpyTag-peptide reactivity	103
2.2.22.1 SpyTag-peptides	103
2.2.22.2 SpyCatcher-VLP:SpyTag-peptide reactivity	103
2.2.23 SpyCatcher-VLP reactivity after heating	104
2.2.24 Preparation of SpyCatcher-VLP immunogens	105
2.2.24.1 SpyCatcher-VLPs:SpyTag-CIDR and control immunogens	105
2.2.24.2 SpyCatcher-VLPs:Pfs25-SpyTag and control immunogens	106
2.2.25 Endotoxin quantification and removal	106
2.2.26 SpyCatcher-VLPs immunizations	107
2.2.27 Endpoint ELISA	107
2.2.28 Statistical analysis of immunizations	109
2.2.29 Immunofluorescence (Indirect fluorescent-antibody – IFA)	112
2.2.30 Standard membrane feeding assay (SMFA)	112

2.2.31 SpyCatcherDuo-VLP:SpyTag-protein reactivity	113
2.2.32 Expression of IMX nanoparticles	114
2.2.33 Size-exclusion chromatography of SpyCatcher-IMX-SnoopCatcher for size determination	115
2.2.34 SpyCatcher-IMX-SnoopCatcher:SpyTag-/SnoopTag-protein reactivity	115
Chapter 3 Plug-and-Display decoration of Virus-Like Particles	118
3.1 Generation of SpyCatcher-VLPs and their features	119
3.1.1 Chapter introduction	119
3.1.2 Expression studies of SpyCatcher-VLPs	122
3.1.3 Biochemical and biophysical characterization of SpyCatcher-VLPs	124
3.1.4 SpyCatcher-VLP:SpyTag reaction with model protein MBP	127
3.1.5 SpyCatcher-VLP:SpyTag reaction with complex SpyTag-antigens	128
3.1.6 SpyCatcher-VLP:SpyTag reaction with synthesized peptides	131
3.1.7 SpyCatcher-VLP reaction with protein internal SpyTag	133
3.1.8 Storage of SpyCatcher-VLPs and reactivity	136
3.2 Immunogenicity of conjugated SpyCatcher-VLPs	138
3.2.1 Immunogenicity of SpyTag-CIDR(IT4var07):SpyCatcher-VLPs conjugates	138
3.2.2 Immunogenicity of Pfs25-SpyTag:SpyCatcher-VLPs conjugates	143
3.3 Attempts at improving SpyCatcher-VLP yields	150
3.4 Dual-antigen load: SpyCatcherDuo-VLPs and their features	155
3.4.1 The case for increased antigen density	155
3.4.2 SpyCatcherDuo-VLPs and their features	156
3.5 Discussion	160
<i>Expression studies of SpyCatcher-VLPs</i>	160
Chapter 4 Plug-and-Display: Dually addressable Nanoparticles	182

4.1 Generation of dually addressable Nano-Particles	183
4.1.1 Chapter introduction	183
4.1.1.1 SnoopCatcher	183
4.1.1.2 Chimeric oligomerization domain from <i>Gallus gallus</i> complement inhibitor C4b-binding protein (C4bp) – IMX313	184
4.1.2 SpyCatcher-IMX-SnoopCatcher design	185
4.1.3 Purification of SpyCatcher-IMX-SnoopCatcher	186
4.1.4 SpyCatcher-IMX-SnoopCatcher reactivity with model proteins	191
4.1.5 Dual-conjugation of SpyCatcher-IMX-SnoopCatcher with complex antigens	192
4.1.6 Discussion	195
Chapter 5 Summary, General Discussion and Future Outlook	201
Bibliography	207
Appendix	242

Abbreviations

AU	Antibody units
AP	Alkaline phosphatase
AP	Acceptor peptide
Ab	Antibody
AmpR	Ampicillin resistance
APC	Antigen Presenting Cells
AX	Absorbance at X nm
BCA	Bicinchoninic acid
BirA	Biotin ligase
bp	base pair
BSA	Bovine Serum Albumin
CamR	Chloramphenicol resistance
CPMV	Cowpea Mosaic Virus
Da	Dalton
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
FDC	Follicular dendritic cell
FHV	Flock House Virus

HBsAg	Hepatitis B surface antigen
HBcAg	Hepatitis B core antigen
h	Hour
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl- β -D-thiogalactopyranoside
KanR	Kanamycin resistance
kb	kilobases
K_d	Equilibrium dissociation constant
LB	Luria Bertani broth
mAb	Monoclonal antibody
MBP	Maltose Binding Protein
MHC	Major histocompatibility complex
MS	Mass spectrometry
NCL	Native Chemical Ligation
NHS	N-hydroxysuccinimide
Ni-NTA	Nickel-nitrilotriacetic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pDC	peripheral dendritic cell

PMSF	Phenylmethanesulfonyl Fluoride
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SN	Supernatant
STD	Standard deviation
TBS	Tris buffered saline
TCR	T-cell receptor
TEV	Tobacco Etch Virus
TMAO	Trimethylamine N-oxide
TNF- α	Tumor necrosis factor alpha
uAA	Unnatural amino acid
pNPP	p-nitrophenylphosphate
yr	Year

List of Figures and Tables

Figures

Figure 1.1 Crystal structures (cartoon) of representatives of the <i>Leviviridae</i> family.	28
Figure 1.2 Published items in each year.	33
Figure 1.3 EDC mediated coupling to carboxylic acids.	39
Figure 1.4 Disulfide bond.	41
Figure 1.5 Mechanism of the native chemical ligation reaction.	42
Figure 1.6 Maleimide mediated conjugation to a sulfhydryl via thioether bond formation.	43
Figure .7 Dock-and-Lock (DNL) mechanism.	52
Figure 1.8 Electrostatic interaction (lock).	53
Figure 1.9 Mechanism for Sortase A-mediated ligation (SML).	56
Figure 1.10 Antibody mediated affinity conjugation.	62
Figure 1.11 Streptavidin complex.	63
Figure 1.12 Coiled coil.	65
Figure 1.13 Crystal structure of Spy0128 colour-annotated with two different splits.	70
Figure 1.14 Isopeptide triads of Spy0128.	71
Figure 1.15 Structure of SpyCatcher Δ N1 and SpyTag.	72
Figure 1.16 Structure of SnoopCatcher and SnoopTag.	74
Figure 3.1 Overview of Plug-and-Display VLP assembly.	121

Figure 3.2 Schematic of pGEM-SpyCatcher-AP205cp3 (pKB_215) used to generate first generation SpyCatcher-VLPs.	123
Figure 3.3 SDS-PAGE of SpyCatcher-VLPs.	124
Figure 3.4 Biophysical characterization of SpyCatcher-VLPs.	126
Figure 3.5 SpyCatcher-VLPs:SpyTag reaction with model protein SpyTag-MBP.	128
Figure 3.6 SpyCatcher-VLPs reacted with range of malarial protein antigens. ...	130
Figure 3.7 SpyCatcher-VLPs:SpyTag-peptide reactivity.	131
Figure 3.8 Structural basis of TLR5:flagellin recognition and flagellin SpyTag loop-insertion.....	133
Figure 3.9 SpyCatcher-VLPs can react with an internally fused SpyTag.....	135
Figure 3.10 SpyCatcher-VLPs reacted after storage at elevated temperature. ...	136
Figure 3.11 SpyCatcher-VLPs:SpyTag-CIDR(IT4var07) immunogens.	138
Figure 3.12 Negatively-stained TEM images of SpyCatcher-VLPs.....	140
Figure 3.13 Plug-and-Display immunization with CIDR immunogens.	141
Figure 3.14 SpyCatcher-VLPs:Pfs25-SpyTag immunogens.	143
Figure 3.15 Plug-and-Display immunization with Pfs25 immunogens (I).....	144
Figure 3.16 Plug-and-Display immunization with Pfs25 immunogens (II).....	145
Figure 3.17 Plug-and-Display immunization with Pfs25 immunogens (III).....	147
Figure 3.18 Schematic representation of SpyCatcher-CP3 vector inserts.	151
Figure 3.19 IMAC elution profiles of SpyCatcher-VLP expression systems.	152
Figure 3.20 Comparison between pKB_215 and pKB_337 derived SpyCatcher-VLPs.	153
Figure 3.21 SpyCatcherDuo-VLPs for increased antigen display.	156

Figure 3.22 SpyCatcherDuo-VLPs.	157
Figure 4.1 Overview of SpyCatcher-IMX-SnoopCatcher dual Plug-and-Display nanoparticle assembly.	185
Figure 4.2 SpyCatcher-IMX-SnoopCatcher purification.	186
Figure 4.3 SpyCatcher-IMX-SnoopCatcher elution from size-exclusion chromatography (I).	189
Figure 4.4 Size-exclusion chromatography of SpyCatcher-IMX-SnoopCatcher (II).	190
Figure 4.5 SpyCatcher-IMX-SnoopCatcher reaction with Spy/Snoop-model proteins.	191
Figure 4.6 SpyCatcher-IMX-SnoopCatcher reacted with the antigens Pfs25-SpyTag and SnoopTag-CIDR.	194

Tables

Table 2.1 Parental strains employed for purposes of protein expression.....	84
Table 2.2 Strains constructed for purposes of protein expression.....	85
Table 2.3 Descriptive statistics for Mann–Whitney U test for Figure 3.13.	110
Table 2.4 Descriptive statistics for Mann–Whitney U test for Figure 3.14.	111
Table 3.1 <i>E. coli</i> strains and observed clumping or lysis during initial expression screen.....	122

Chapter 1 Introduction

1.1 Vaccines and Immunology

1.1.1 Vaccine History

Vaccines are among the greatest advances in medical history, having saved more lives than any other intervention (De Gregorio and Rappuoli, 2014). The first documented examples of vaccinations date back some 1,000 years ago. Variolation, the purposeful infection of healthy individuals with live virulent smallpox virus, was practiced in India and China, perhaps spurred by observations that prior smallpox exposure protected against subsequent infections (Plotkin, 2005; Leung, 2011). Although very successful in reducing death from smallpox, variolation also induced severe and even sometimes fatal reactions, slowing its spread and use. Edward Jenner's use of cowpox (*Variolae vaccinae*) at the end of the 18th century to prophylactically protect humans against smallpox infections was probably motivated by observation of these sometimes fatal side-effects (Smith, 2011).

Jenner's vaccinia likewise stimulated efforts in what would become the science of immunology, resulting in the development of the first inactivated (anthrax) vaccine by Toussaint in 1877 and the chance discovery of attenuated chicken cholera in Pasteur's laboratory in 1879 (Bazin, 2011).

The 20th century saw an explosion in the field of vaccine development. In the 1920s Calmette and Guérin used passage in artificial media to attenuate *Mycobacterium bovis*, the causative bacterial agent of tuberculosis in cattle, while Madsen initiated work on inactivated whole-cell vaccines, in particular pertussis (bacterial, whooping cough) vaccine in the 1930s. Theiler used passage in mice and chicken embryos to attenuate yellow fever virus in 1937 (Plotkin, 2014). Madsen's work was further refined by Kendrick and Eldering, who cemented the concept of large-scale,

controlled clinical trials in the 1940s (Shapiro-Shapin, 2010). In terms of basic academic research, Fagraeus reported in 1948 that plasma B cells were specifically involved in antibody generation and Burnet and Talmage reported their clonal selection hypothesis in 1957.

Landmark developments in the 1950s to 1980s were achieved when virologists successfully attenuated many of the viruses responsible for crippling childhood diseases, including those of polio (Salk and Sabin), measles (Enders and Peeble), rubella (Hilleman), mumps (Hilleman) and varicella (Takahashi) (De Gregorio and Rappuoli, 2014). Advances in understanding and analysis of proteins spurred efforts to refine chemical inactivation and isolation of proteins. Sato and Sato established the concept of acellular (protein vaccines) by developing a safer alternative to the whole-cell pertussis vaccine (Sato and Sato, 1999). Though effective, this vaccine caused rare but adverse reactions which led to serious social backlash against vaccination efforts in Japan and elsewhere (Sato and Sato, 1999). Porter, Hilschmann and Edelman elucidated the primary and secondary structure of antibodies (Edelman and Gall, 1969; Hilschmann, 1969; Porter, 1959), which set the stage for crystal structure elucidation of the antibody (Poljak et al., 1973; Amit et al., 1986) and the advent of monoclonal antibodies in 1975 by Köhler and Milstein (Köhler and Milstein, 1975; LeBien and Tedder, 2008).

Throughout the 20th century, interest in Metchnikoff's cellular immunity had declined. In the 1960s and 70s however, detailed characterization of cells according to their protein expression became more feasible and resulted in the elucidation of the origin and clear definition of the two major lymphocyte populations: the antibody-producing B cells (Cooper et al., 1965) as well as the

helper (Miller and Mitchell, 1968) and cytotoxic T cells (Zinkernagel and Doherty, 1974). These and following discoveries sparked revival of great interest in Metchnikoff's work and would ultimately unite Ehrlich's and Metchnikoff's school of thoughts (Kaufmann, 2008). Mitchell and Miller demonstrated the role of thymus derived helper (T_H) cells on antibody responses (Miller and Mitchell, 1968; Crotty, 2015). Snell introduced the notion of histocompatibility genes and started the systematic breeding of congenic mice, which would later be used by Zinkernagel and Doherty to elucidate the biological function of MHC proteins and restriction as a surveillance method to distinguish self from non-self (Zinkernagel and Doherty, 1974, 1979).

Armed with this knowledge, starting in the late 1970s, several waves of ground-breaking technologies enabled the development of novel vaccine classes. New technologies and increasing insight into cellular and humoral immunity steered the field from an empirical to a more rational approach (De Gregorio and Rappuoli, 2014).

First glycoconjugate vaccines revolutionized the polysaccharide vaccine field by conferring T-cell mediated immunity attributes of the carrier protein(s) to attached polysaccharides. Robbins seminal *Haemophilus influenzae b* vaccine (Schneerson and Robbins, 1975) inspired meningococcal (Lepow et al., 1977), pneumococcal (Shapiro et al., 1991) glycoconjugate vaccines. Curiously, conjugate polysaccharide vaccine candidates had been reported by Avery and Goebel in 1929 but fell into obscurity until Robbins renewed advances (Plotkin, 2014).

Secondly genetic engineering (Cohen et al., 1973) emerged and changed everything. Influenza haemagglutinin (HA), even today predominantly produced in

embryonated chicken eggs, could now be expressed in insect cells and is now licensed, which is of particular interest to individuals with allergy to egg proteins (Mullard, 2014). Pioneered by Rappuoli, reverse vaccinology led to the development of recombinant meningococcal vaccines by selecting proteins likely to provide protection via genomic dissection of the pathogen (Sette and Rappuoli, 2010). Many candidate antigens have since been recombinantly expressed in bacterial, yeast, animal, insect and plant cells with an unmatched safety profile but at the expense of immunogenicity (Bachmann and Jennings, 2010).

Genetic engineering also enabled precise deletions of toxicity factors from pathogens, resulting in genetically attenuated, safe and highly immunogenic oral typhoid (Germanier and Fiirer, 1975) and cholera vaccines (Plotkin, 2014). Amongst others *Bacillus Calmette–Guérin* (attenuated *Mycobacterium bovis*), pox- (Antoine et al., 1998; Panicali et al., 1983) and adenoviruses have also been equipped with protective antigens from other pathogens inserted into their genomes, in order to (also) convey immunogenicity to these heterologous antigens (Ewer et al., 2016). Modified carriers are administered and either undergo abortive or complete replication, whilst expressing the desired genes as part of their outer appearance or for MHC class I display (Hansen and Bouvier, 2009). Examples include the licensed Japanese encephalitis (JE) virus vaccine based on an attenuated yellow fever strain. This strain induces protective JE-immunity due to heterologous expression of the JE genes prM and gene E, that now encode the coat proteins for the chimeric virus (Guy et al., 2010).

Expression of antigens *in vivo* in the vaccinee has been of great interest, as intracellular expression is known to promote loading of antigen epitope on MHC

class I, and thus display to CD8⁺ T cells. DNA vaccines and viral vectored vaccines have been investigated to deliver instructions for antigen expression to cells (Kutzler and Weiner, 2008). Vectored vaccines, in particular heterologous prime boost regimens such as replication-deficient adenovirus vectored followed by modified Ankara virus (MVA), have proven superior to DNA vaccines in inducing potent T-cell, as well as humoral responses (Ewer et al., 2016).

Hilleman also attempted to produce a vaccine against hepatitis B virus (HBV) and succeeded in purifying and further inactivating naturally occurring VLPs from plasma of chronically infected individuals (Buynak et al., 1976). The associated safety risks and the continuous need for infected individuals imposed major limitations on this approach (Buynak et al., 1976; Valenzuela et al., 1982). Genetic engineering allowed Rutter and Valenzuela to clone the surface antigen encoding gene of HBV (HBsAg) and express it in *Saccharomyces cerevisiae*. The resulting recombinant VLP particulate vaccine solved all problems associated with Hilleman's approach (Valenzuela et al., 1982). The demonstration of recombinant HBsAg VLP production paved the way for future VLP vaccines, enabling production of the human papilloma virus (HPV) vaccine (Cook et al., 1999). Recently, more complex VLP scaffolds were enabled by novel expression systems such as *Baculoviridae* (baculovirus) (Latham and Galarza, 2001) and alternative yeast systems, e.g. *Pichia pastoris* (Falcón et al., 1999) and *Hansenula* (Janowicz et al., 1991). Further VLP developments and relevant immunology are described in the following sections.

1.1.2 VLPs & Nanoparticles

1.1.2.1 Chimeric VLP History

The first chimeric VLPs were based on HBsAg (Valenzuela et al., 1985), Hepatitis B core antigen (HBcAg) (Borisova et al., 1989; Clarke et al., 1987), the plant ribonucleic acid (RNA) virus TMV (Haynes et al., 1986) and bacteriophage fr (Kozlovskaja et al., 1988). The work on HBsAg demonstrated the safe and superior immunogenicity of VLP vaccines compared to recombinant monomeric protein (Valenzuela et al., 1985). After these landmark papers, a search for higher yielding VLP scaffolds took place. Work performed in the Kingsman laboratory in the Biochemistry Department in Oxford University yielded the first mosaic chimeric HIV:VLP vaccine from the yeast retrotransposon Ty (Adams et al., 1987), spurring efforts to develop other multivalent VLP vaccines. Insertion of the poliovirus neutralization epitope into HBsAg and co-expression with HBsAg yielded an immunogenic mixture of both HBV and HBsPolioAg VLPs (Delpeyroux et al., 1988). Co-expression of HBsAg with Circumsporozoite(CS)-HBsAg yielded what would become not only the world's first licensed chimeric VLP and malaria vaccine, but also the first licensed vaccine against a parasitic disease of any kind: RTS,S/AS01 (Rutgers et al., 1988; RTS,S Clinical Trials Partnership, 2015).

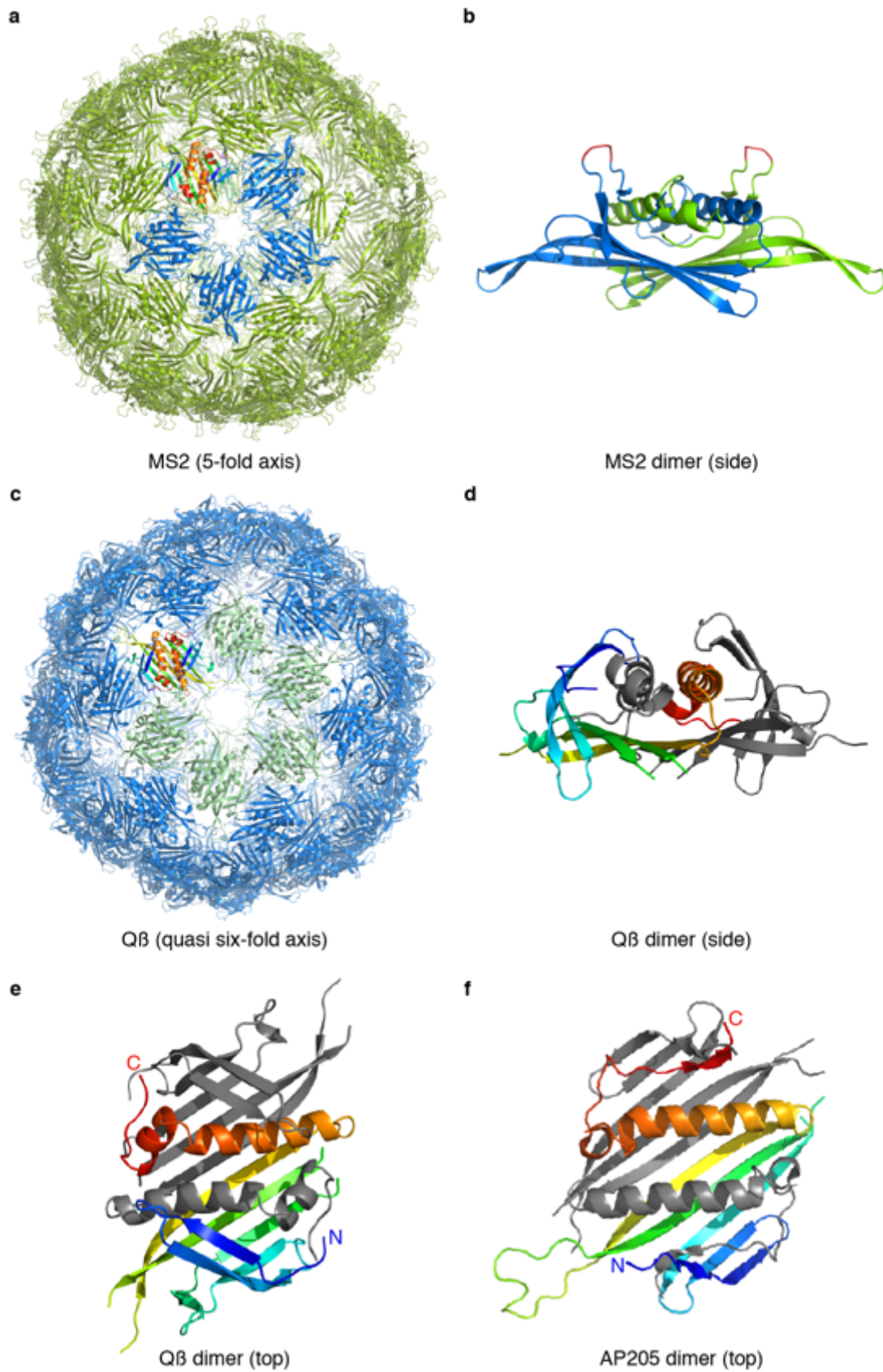


Figure 1.1 Crystal structures of the *Leviviridae* family. (a) Crystal structure for MS2 (PDB 2MS2); 5 anti-parallel dimers form a 5-fold axis; (b) MS2 dimer (PDB 2MS2), red regions indicate typical loop insertion position; (c) Q β (PDB 1QBE), 6 anti-parallel dimer from a quasi 6-fold axis; (d,e) Q β dimer (PDB 1QBE). (f) AP205 dimer (PDB 5FS4) Single coat protein monomers are represented in cartoon, a single dimer set is shown in rainbow colouring. Disulfides / Cysteine represented as stick model and in yellow.

Bacteriophages as VLP scaffolds gained traction, as phages had served as models for virus replication, infection and assembly in the previous decades (Khudyakov and Pumpens, 2016). Methods to produce large quantities of pure and soluble VLPs were already known (Mateu, 2013). Bacteriophages represent the most basic forms of viruses and only employ a minimal set of proteins to allow replication, significantly reducing complexity compared to enveloped VLPs (Mateu, 2013). Some of the first bacteriophage scaffolds explored were phage fr (Kozlovskaya et al., 1988 [in Russian]; Sominskaya et al., 1992), its structural relative MS2 (Figure 1.1a) (Mastico et al., 1993; Liljas et al., 1994) and Q β (Figure 1.1c, d, e) (Kozlovskaya et al., 1993, 1996), all members of the *Leviviridae* family. Not surprisingly, other members of the family were also explored, e.g. PP7 (Caldeira and Peabody, 2007) and lastly AP205 (Figure 1.1f) (Tissot et al., 2010), which served as the scaffold for SpyCatcher-VLPs described in this thesis. In contrast to all other solved phages of the *Leviviridae* family, the AP205 terminal beta hairpins are composed by two dimers, whereas this beta hairpin is part of a single monomer in Q β , see Figure 1.1e)) (Shishovs et al., 2016). Consequently the termini are exposed to the outside (Figure 1.1f) and are amenable to fusion of long epitopes to the surface of AP205 VLPs (Tissot et al., 2010; Shishovs et al., 2016).

The underlying reasons for the strong immunogenicity induced by VLPs are described in the following paragraphs.

1.1.2.2 Size, geometry and repetitive arrangement

VLPs are self-assembling multi-protein particles that mimic the structural organization and conformation of viruses, but lack pathogenic genetic material (Smith et al., 2013a). After injection, VLPs can directly enter lymphatic vessels and drain to local lymph nodes (LN) due to their size, in addition to active transport by peripheral dendritic cells (pDC) (Manolova et al., 2008; Reddy et al., 2006). Structural and geometrical features also allow intranasal VLP administration via the lung to splenic B cell follicles, an active process mediated by low-affinity B-cells (Bessa et al., 2012; Richert et al., 2012). The optimal size for efficient induction of drainage is ~40 nm (Fifis et al., 2004), while bigger particles above 200 nm mainly associate with administration site DCs (Manolova et al., 2008). Attempts to elucidate shape effects on circulation have been performed using Tobacco Mosaic Virus (TMV). TMV naturally assumes a cylindrical shape of ~ 300 nm length and ~ 18 nm diameter (Bruckman et al., 2014). Thermal shaping allows for icosahedral-like assembly of the same TMV protein composition with diameter ranging from 50 to 800 nm (Bruckman et al., 2014). It was found that TMV rods circulate longer than icosahedral particles, but display a higher rate of spleen clearance (Bruckman et al., 2014). Several factors in the TMV bio-distribution study could not be controlled for or kept constant, such as zeta potential difference between the different shapes, or integrity and conformation of the protein monomers, which may well influence the observations (Bruckman et al., 2014). It is not clear how spleen clearance may effect antigenicity; literature reports the successful use of both spherical and rod-like influenza M2-displaying VLPs. Hence shape may not be a significant antigenicity contributor and the main motivation for icosahedral VLPs

may be reduced complexity and increased rigidity (Mastico et al., 1993; Fehr et al., 1998; Tissot et al., 2010; Carignan et al., 2015).

In the periphery of lymphoid organs, VLPs are taken up by antigen presenting cells, in particular DCs and macrophages, but VLPs can also bind directly to B-cells. Binding occurs by two distinct mechanisms, which both depend on the geometry and repetitive display of protein: The first binding mechanism allows direct B-cell receptor (BcR)-crosslinking due to multivalent interactions between B-cells and VLPs. BcR-crosslinking can be sufficient for B-cell activation and class switching (Bachmann and Zinkernagel, 1997; Fehr et al., 1997; Jegerlehner et al., 2007; Link et al., 2012). Optimal spacing of particulate compound antigens has been suggested to be 5-10 nm; VLPs fulfil this criteria with their large, repetitive surface area (Bachmann et al., 1993; Fehr et al., 1997). Several groups have attempted to determine the minimal and optimal number of antigen repeats for crosslinking; the reported values range widely from at least 2, 3 to 4-6 (Klaus and Humphrey, 1976), to 12-16 (Dintzis et al., 1976) or above 20 repeats (Dintzis et al., 1983). Thus, uncertainty still exists in regards to how many repeats are actually required. Mechanistically, this question may now be revisited from an academic point, as new technologies have enabled control of mixed linear and branched chain protein polymers (Veggiani et al., 2016). In practice, both VLPs (which far exceed 20 repeats per particle) but also nanoparticles (with “only” 7 repeats) are similarly effective in inducing potent antibody responses (Jones et al., 2013; Li et al., 2016). The second binding mechanism of VLPs to B-cells is mediated by the innate immune system. Natural antibodies and complement are multivalent components of the innate humoral immune system and can interact with the repetitive structures

of VLPs (Bottazzi et al., 2010). Even though affinity may be low, avidity effects enable natural IgM (decamer) to bind VLPs, fix complement component C1q and subsequently deposit VLPs on follicular dendritic cells (FDCs) for interaction with B-cells (Link et al., 2012). Interestingly, BcR-crosslinking on FDCs proceeds at a lower degree of cross-linking, enabling increased B-cell activation (Jegerlehner et al., 2002a). The combination of direct BcR-crosslinking by VLPs and FDC display mediated BcR-crosslinking allows for strong antibody responses via T cell-independent type I activation (Fehr et al., 1998; Jegerlehner et al., 2002a; Bachmann and Zabel, 2016).

More conventional generation of antibody responses against VLPs is further achieved by FDCs and T helper cells in B cell follicles (Keller et al., 2010). FDCs, retain intact VLPs and can select VLP specific B cells into the germinal center (GC)(Bachmann and Zabel, 2016). GCs are located within secondary lymph nodes and facilitate B-cell proliferation, differentiation (class-switching) and maturation (somatic hyper-mutation) (Crotty, 2015). Again the geometrical features of VLPs benefit the response further, as monomeric antigen is not normally deposited on FDCs due to lack of complement activation (Bachmann and Zabel, 2016). Antigen presenting cells (APCs) are also thought to have evolved to detect, process and present virus-derived epitopes to T cells via MHC class I and II (Kündig et al., 2014). MHC class I and II regulate adaptive immune responses via display of VLP epitopes to CD4⁺ T-helper, epitope-specific CD8⁺ cytotoxic T cells (CTL response), which in turn stimulate Ag-specific B cells, as well as non-Ag-specific macrophages (Kurosaki et al., 2015).

1.1.2.3 Nucleic acid content

The other key feature of VLPs is that they may encapsulate nucleic acid, which enhances antibody responses via Toll-like receptor (TLR) signalling (Schmitz et al., 2012). VLP-derived nucleic acids (unless produced in mammalian systems) are detected by TLRs in endosomes of processing APCs, in particular DCs but also B-cells. Cognate receptors for nucleic acids are TLR 3 [(double stranded (ds)RNA)], TLR 7/8 (ssRNA) and TLR 9 [unmethylated CpG, ds deoxyribonucleic acid (DNA) motifs]. All of these ligands can be chemically synthesized and tailored for vaccine applications (Zabel et al., 2013; Hua and Hou, 2013; Liu and Irvine, 2015).

1.1.2.4 Means of VLP production and industrial applicability

VLPs have been conveniently produced at high quantities and commercial scale through heterologous recombinant gene expression in a range of expression systems, including *Escherichia coli*, yeast, plant, baculovirus and mammalian cells (Roldão et al., 2010). Yields can be in the range of hundreds of mg per litre by bacterial culture of cells, while plant viruses such as TMV can be produced at up to 3000 mg/kg plant material (Khudyakov and Pumpens, 2016). Noteworthy licensed and commercially viable prophylactic VLP-based vaccines are GlaxoSmithKline's (GSK) Cervarix as well as Merck's Gardasil against human papillomavirus (HPV) (Mateu, 2013).

1.1.2.5 Heterologous antigen display and increasing interest in VLPs

Importantly VLPs also induce strong B-cell responses against chemically or genetically linked epitopes that are not native to the VLP itself. Several of these hybrid VLPs are vaccine candidates currently in development (Roldão et al., 2010; Kushnir et al., 2012; Pitoiset et al., 2015; Lee et al., 2016). The prime example is the previously mentioned adjuvanted subunit malaria vaccine RTS,S/AS01 (RTS,S Clinical Trials Partnership, 2015; Rutgers et al., 1988). AS01 stands for Adjuvant System number 1 and is a liposome-based vaccine adjuvant containing 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and the glycoside saponin QS-21 (Didierlaurent et al., 2014). VLPs have become a very active area of research as can be seen in Figure 1.2.

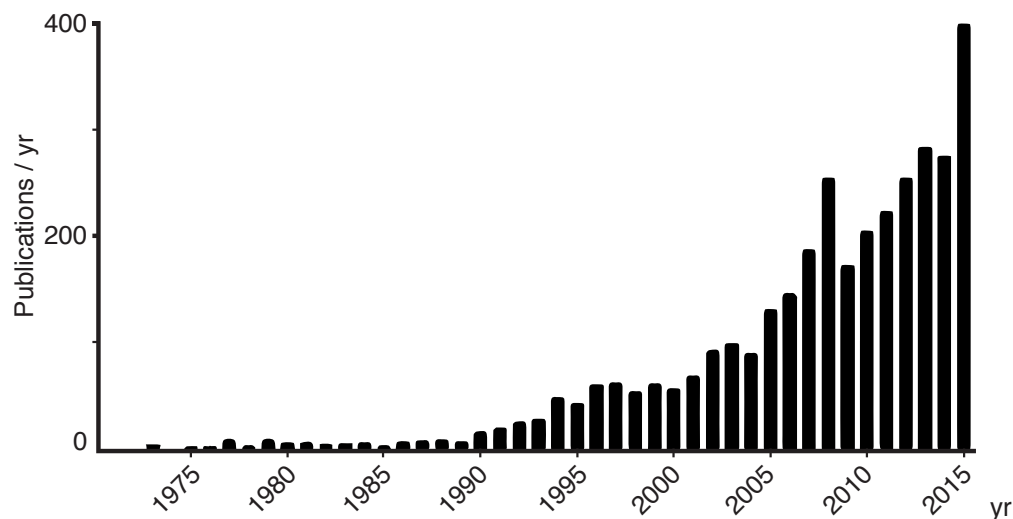


Figure 1.2 Published items in each year. Data obtained from Web of Science in July 2016. Total items ~9200, Keywords were: VLP, Virus-like particle, VNP and virus-nano particle.

1.2 Decoration of VLPs

1.2.1 *The decoration challenge and motivation*

Antigen display has been successfully achieved by genetic fusion of heterologous epitopes or antigen on the inside or outside face of the self-assembling coat protein (Mateu, 2011). Genetic fusion of short peptides has been successful in several cases (Mastico et al., 1993; Tissot et al., 2010), whereas insertion of larger sequences generally prevents VLP-assembly (Stockley and Mastico, 2000). Even if VLP-assembly can be achieved by refolding or buffer-screening, chimeric VLPs are often unstable. Also, the structural conformation of the inserted epitope may not be retained, if it ever assumed correct folding in the first place (Manzenrieder et al., 2011; Jain et al., 2014). Thus, genetic fusion is subject to tedious trial-and-error optimization and is largely confined to small epitopes, necessitating the elucidation of such determinants in the (possibly exotic) antigen. Even when strong epitopes can be identified and their transplantation to VLP carriers is successful, these epitopes may not provide sufficient immunity. An example is Malarivax, an *E. coli*-expressed VLP vaccine based on HBcAg genetically fused to one B cell and two CD4⁺ T cell circumsporozoite (CS) protein epitopes. Malarivax was not successful in phase II clinical trials (Schwartz et al., 2012). In this context, the malarial parasite has repeatedly evolved drug-resistance. The capacity for adaptive evolution of *Plasmodium* now appears historically underestimated (Anderson et al., 2016).

Even in the blue-sky scenario of a safe and efficient epitope-restricted vaccine; the naturally occurring parasite population may have already generated vaccine-escape variants. If an epitope-restricted vaccine does not confer 100% protection,

selection will favour vaccine escapees. Mutants that are not affected will propagate and the vaccine will be less effective over time (Barry and Arnott, 2014).

It is more likely for a pathogen to escape antibodies recognizing a single epitope compared to escaping antibodies that recognizes multiple, diverse sets of epitopes or even structural motifs (Barry and Arnott, 2014). Mutated pathogen proteins likely need to retain a similar structural conformation in order to function, as recently demonstrated for the PfEMP1 family (Lau et al., 2015). Therefore display of larger antigens is generally preferable but is difficult to achieve by genetic fusion (section 1.2.2.14) (Thrane et al., 2016).

When direct fusion to the VLP subunit is not possible due to complexity or size of the antigen, the antigen must be installed in a secondary step. Chemical conjugation to pre-assembled VLPs typically relies on exploitation of reactive amino acid side chains in the antigen and coat protein (Strable and Finn, 2009a; Wen and Steinmetz, 2016). However, VLPs and antigens can contain multiple reactive residues, hindering the homogenous directional coupling of the antigen to the VLP required for optimal immunogenicity (Strable and Finn, 2009a; Sletten and Bertozzi, 2009). Furthermore, chemical reactions often yield only partial conjugation. Even if full conjugation is not required, batch-to-batch variation imposes costly quality control measures in production (Stephanopoulos and Francis, 2011). Matching and minimizing reaction time and amount of reagents is important for multiple reasons:

- (i) long reaction times are impractical,
- (ii) large excess of reagents may promote side-reactions or be expensive for difficult antigens,

- (iv) reproducibility requirements are extraordinarily high for vaccine production,
- (v) remaining reactivity must be quenched to avoid reaction with self-antigen *in vivo* and thereby generation of auto-antibodies.

An ideal covalent VLP decoration method would therefore selectively modify a defined residue or target site of interest (Sletten and Bertozzi, 2009). Ideally this reaction could also occur in the presence of non-target proteins, competing side-chains, and in conditions optimal for protein integrity: e.g. aqueous buffers, low to ambient temperatures, and at or near neutral pH (Chalker et al., 2009). The reaction should be irreversible and tolerate a range of excipients, i.e. salts, surfactants and other stabilizer required for protein integrity. The underlying practical challenge for all VLP conjugation approaches is to achieve quantitative coupling, that is complete conversion (Ng et al., 2012).

In the following sections, I will discuss the major approaches used for display of antigen on VLPs and their uses, strengths and limitations.

1.2.2 Covalent approaches to VLP decoration

Covalent approaches predominantly rely on chemical conjugation through the naturally-occurring abundant amino groups (Stephanopoulos and Francis, 2011). High reaction yields are difficult to achieve as most natural amino acid side-chain reactions show slow kinetics. Yields can be difficult to characterize due to multiple possible reaction sites, typically yielding Gaussian-distributed degrees of conjugation (Ng et al., 2012).

1.2.2.1 Arginine

Arginine (Arg) residues are relatively rare and can be modified with 1,2-dicarbonyl reagents (Hermanson, 1996). Other nucleophiles, such as Lysine (Lys) residues are also labelled but these residue modifications are reversible. Arg-specific modification depends on the irreversibility of the guanidinium side chain modification. Yields are low, around 30% typically and only up to 60% under optimized conditions. Therefore this type of modification remains largely unused in the VLP field (Horowitz et al., 2011; Ng et al., 2012).

1.2.2.2 Carboxylic acid amino acids – Aspartic acid and glutamic acid

Aspartic acid (Asp) and glutamic acid (Glu) offer carboxylic acids groups that can be reacted with primary amines in the presence of carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC, see Figure 1.3, (Hermanson, 1996)]. This method results in stable formation of an amide bond (Lee et al., 2016). The major drawback of carboxyl labelling is the ubiquitous presence of both carboxyls and primary amines in VLP proteins, making it impossible to avoid cross-linking

between proteins and particles, often resulting in aggregation (Hermanson, 1996). Nonetheless, VLPs have been decorated using this approach, but conjugation is limited to single amine- or carboxyl-containing small molecules, such as synthesized peptides or nicotine amine derivatives that can be added in great excess to avoid VLP self-reaction (McCluskie et al., 2015).

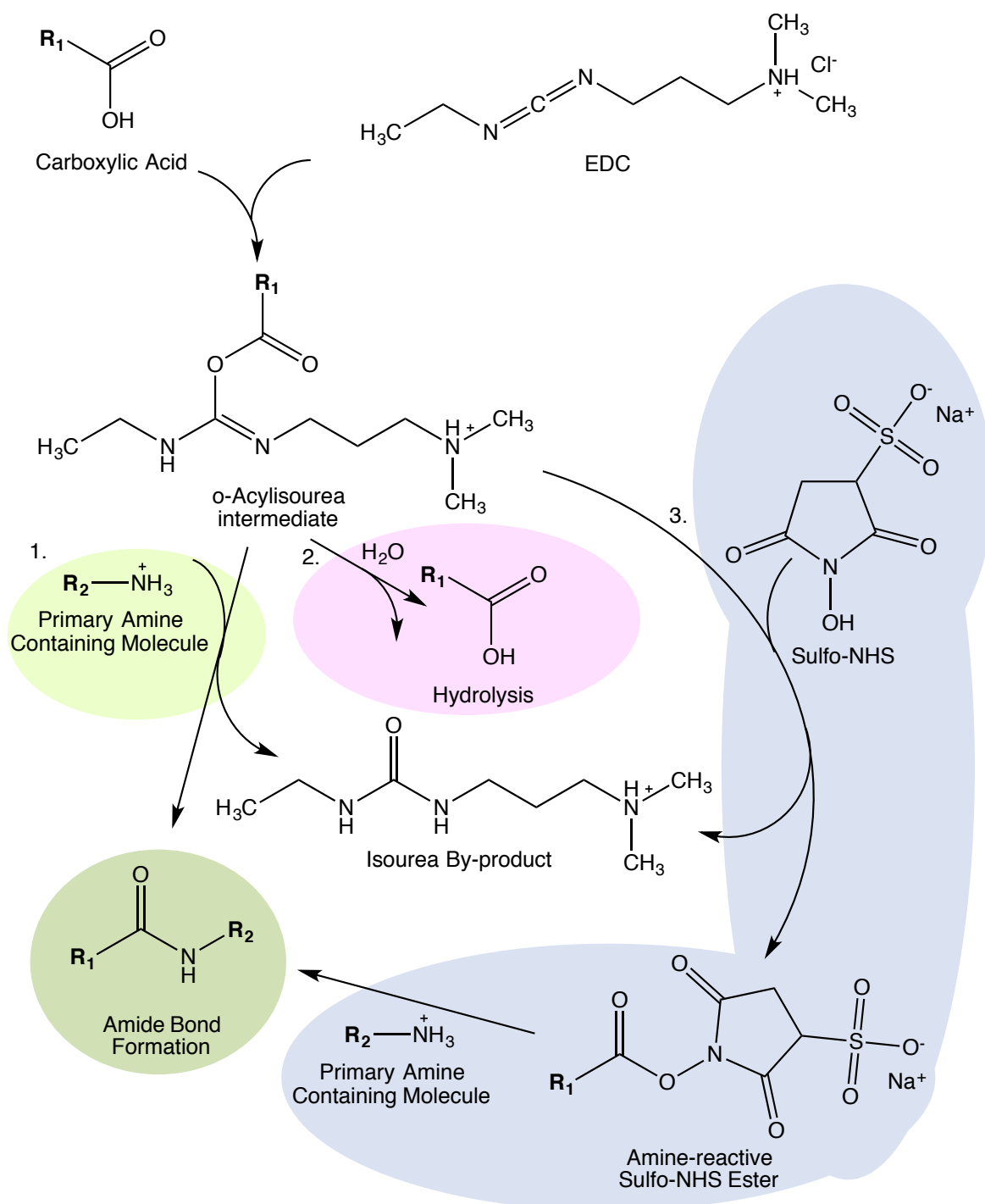


Figure 1.3 EDC mediated coupling to carboxylic acids. Reaction 1: An amide bond is formed after a nucleophilic attack of a primary amine containing molecules to the active-ester intermediate. Reaction 2: The intermediate is hydrolysed to the starting carboxylic acid and the EDC is lost to the isourea by-product. Reaction 3: Sulfo-NHS reacts with the isourea intermediate to yield the amine-reactive Sulfo-NHS ester intermediate, which further reacts with a primary amine containing molecule to yield the amide bond. Adapted from (Hermanson, 1996).

1.2.2.3 Cysteine

One of the least abundant but most commonly modified amino acids is Cysteine (Cys). The most common approach is decoration of an either natively unpaired or genetically installed, unique internal cysteine (Stephanopoulos and Francis, 2011). Cysteines can be alkylated using maleimide reagents (section 1.2.2.3.3), iodoacetamides, vinyl sulfones and acrylamides, in addition to being equipped with other functionality through disulfide formation with activated thiol reagents (section 1.2.2.3.1). The major drawback with cysteine conjugation is the typical requirement for reducing agent as disulfides are non-reactive. VLPs that do not rely on disulfide bonds for (intermediate) stability have been mutated to display unique cysteine handles on the outside of their capsids, notably Cowpea Mosaic Virus (CPMV) (Wang et al., 2002a). As expected, mutants aggregated due to disulfide bond formation in absence of reducing agent, but were stable after alkylation with maleimide and haloacetamide (Wang et al., 2002a). Conversely, a lysine residue was installed in the major immunodominant region (MIR of Hepatitis B core antigen (HBcAg) and the particle was rendered devoid of any cysteines (Jegerlehner et al., 2002b). Antigens with a single terminal cysteine could then be preferentially reacted on to the exposed MIR of the lysine modified HBcAg particle using a maleimide / NHS ester bifunctional chemical cross-linker [see section 1.2.2.3.3, 1.2.2.4 and Figure 1.3 (Jegerlehner et al., 2002b)].

N-terminal Cys is very uncommon among natural proteins but may be readily installed by genetic modification. N-terminal Cys residues in combination with the proximal amine can undergo reactions that are orthogonal to those of internal Cys, such as native chemical ligation (see section 1.2.2.3.2).

Expression of capsid proteins with an exposed single N-terminal Cys can be difficult because unpaired Cys may disrupt folding of capsomers by formation of non-native disulfides with internal Cys residues. Internal Cys can be removed but engineering may require tedious assessment of replacements or evolutionary approaches (Kather et al., 2005). To overcome these limitations, N-terminal Cys can be generated *in vitro* after VLP formation, e.g. by expression as an intein fusion: self-splicing generates particles bearing N-terminal Cys residues. Alternatively proteases, such as Tobacco Etch Virus (TEV) protease, can be employed to generate N-terminal Cys at modified cleavage sites. Proteolytic cleavage can be achieved at high efficiency (95% completion in 24 h), but typically requires a reducing environment to ensure sufficient cleavage as well as subsequent removal of protease (Tolbert and Wong, 2002).

Common reactions with Cys are described in the following paragraphs (Chalker et al., 2009).

1.2.2.3.1 Disulfide bonds

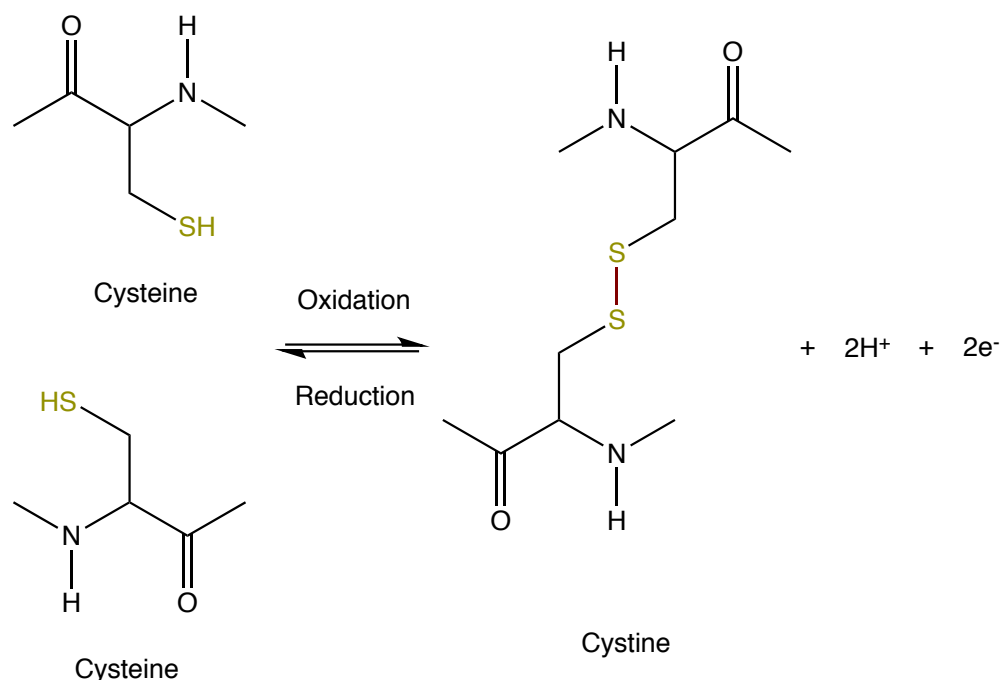


Figure 1.4 Disulfide bond. Oxidation of two reduced cysteine residues yields a cystine, a disulfide bond. This bond is reversible by a reduction reaction. Adapted from (Berg et al., 2015).

One of nature's covalent intra- and inter-molecular bonds is the disulfide bond. They are found across all kingdoms and are one of the major forces responsible for correct folding and maintenance of proteins in the correct conformation (Berg et al., 2015; Sevier and Kaiser, 2002). Disulfides can be very important for VLP assembly and stability of the underlying assembly but have also been engineered to convey thermal stability to otherwise less stable particles (Bundy and Swartz, 2011; Caldeira and Peabody, 2011; Lu et al., 2015). Formation of disulfides between surface-exposed cysteines of VLP and antigen proceed slowly in the absence of catalysts but can be accelerated using the disulfide locking mechanism described in section 1.2.2.9 and 1.2.2.10.

1.2.2.3.2 Native Chemical Ligation

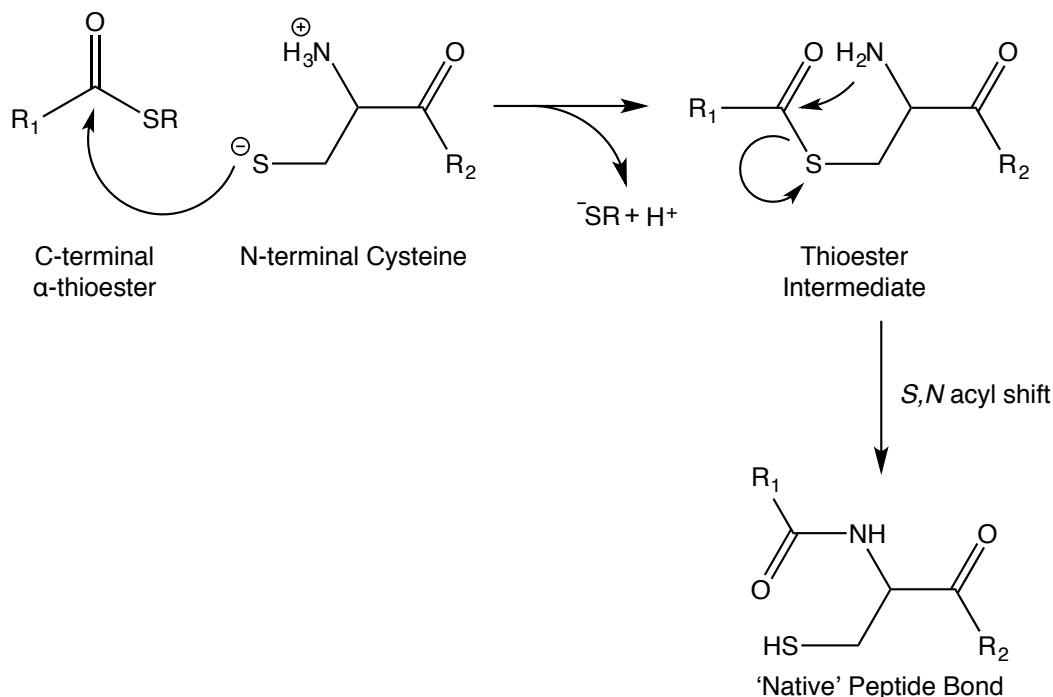


Figure 1.5 Mechanism of the native chemical ligation reaction. The thiol of an N-terminal Cys residue attacks the carbonyl group of a C-terminal thioester peptide, yielding a thioester intermediate. The intermediate spontaneously resolves by an intramolecular $S \rightarrow N$ acyl shift, resulting in the formation of a 'native' peptide bond. Adapted from (Malins and Payne, 2014)

Native chemical ligation (NCL) enables the selective conjugation of two unprotected polypeptides (Malins and Payne, 2014). The reaction is exceptionally specific and results in the formation of an amide bond between the C-terminal α -thioester with an N-terminal Cys. Importantly internal Cys residues are unable to participate in the reaction (Lu et al., 1996). The NCL reaction derives its chemoselectivity from more than one functional group, namely the proximal amine group and the adjacent thiol (Spicer and Davis, 2014). NCL has been employed for protein cyclization (Berrade and Camarero, 2009), labelling (Busch et al., 2008) and prominently to synthesize proteins from solid phase synthesis-derived peptides (Hackeng et al., 1999; Weinstock et al., 2014).

Flock House Virus (FHV) VLPs are based on a non-enveloped insect RNA virus with $T = 3$ icosahedral capsids, made up of 180 identical coat proteins. NCL has been used to install a target protein on FHV (Venter et al., 2011). NCL occurs under mild conditions and only leaves a minimal scar, a Cys residue at the ligation site. This unpaired Cys may be problematic for certain antigens and VLPs as outlined in section 1.2.2.3 and maybe converted to a non-problematic residue, such as Ala (Spicer and Davis, 2014).

1.2.2.3.3 Maleimide-mediated conjugation to sulfhydryls

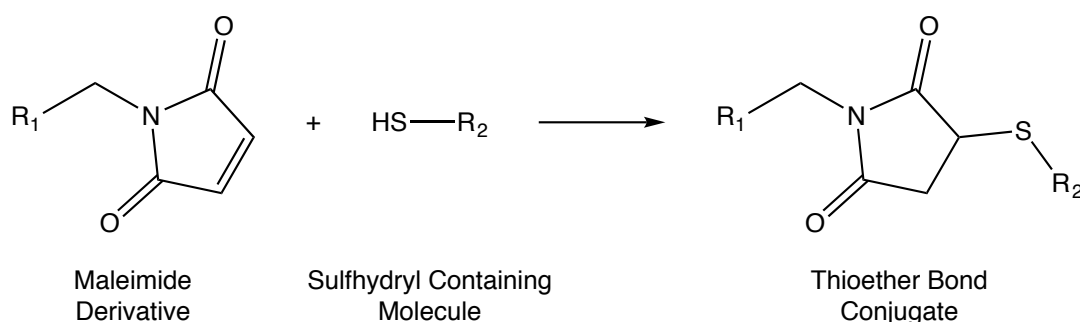


Figure 1.6 Maleimide mediated conjugation to a sulfhydryl via thioether bond formation. R_1 may exemplifies a labelling reagent or one terminus of a cross-linker presenting the maleimide reactive group. R_2 exemplifies a protein or other compound that contains the target functional group (i.e., sulfhydryl, $-SH$). Adapted from (Hermanson, 1996).

The use of maleimides is one of the most widely used methods in cysteine alkylation (Chalker et al., 2009). As an electrophile, maleimide reacts with the nucleophilic cysteine. The reactive species is the thiolate anion (S^-), with a theoretical pK_a of 8.2. Most maleimides are employed in heterobifunctional chemical cross-linkers, such as Succinimidyl 6-((beta-maleimidopropionamido)hexanoate) (SMPH), which has been used to install a variety of recombinantly produced proteins, such as Fel d1 [the major cat allergen,

(Schmitz et al., 2009)], domain III of the West Nile virus E (Spohn et al., 2010) and the self-antigens eotaxin and IL-5 (Zou et al., 2010).

Drawbacks of maleimide cross-linking are its potential to spontaneously hydrolyse (Kalia and Raines, 2007), disruptive cleavage by thiol exchange (Baldwin and Kiick, 2011), or stabilizing ring opening over time, all resulting in undesirable heterogeneity (Fontaine et al., 2015). *In vivo* thiol exchange has been demonstrated and may confer VLP properties to exchanged self-antigens, potentially causing autoimmunity (Shen et al., 2012). Analysis of conjugates can be difficult as bifunctional linkers have been shown to generate unexpected high molecular weight species, presumably due to inter capsid monomer cross-linking, when sensitive assays are employed (Cantin et al., 2011). Nonetheless, maleimide remains one of the common-standards in bioconjugation, presumably due to commercial availability (Chalker et al., 2009).

1.2.2.4 Lysine and N-terminus

Modification of primary amines is another popular bioconjugation strategy (Smith et al., 2013c). Lysine residues and N-termini provide reactive moieties and have been conjugated with N-hydroxysuccinimidyl-esters (NHS-esters) or the more water soluble sulfoNHS-ester derivatives [see Figure 1.2 (3)]. VLPs have been successfully coupled with a wide range of ligands, such as transferrins (Galaway and Stockley, 2013), polymerization initiators (Pokorski et al., 2011), polymers (Manzenrieder et al., 2011) or influenza epitopes (Cantin et al., 2011; Lechner et al., 2003). NHS-esters are readily commercially available. Lys residues are ubiquitous in proteins and so conjugates are typically inhomogeneous. Accessibility and

sequence-context can constrain possible conjugation sites dramatically however, as demonstrated for CPMV (Wang et al., 2002b). Canonical replacement with similarly-charged residues such as Arg and Histidine (His) can be used to further constrain potential target sites, but may come at cost of reduced or defective VLP assembly (Harrison et al., 2012). Targeting of the N-terminus is more site-selective but competes with intra-protein Lys, which are ideally buried. As an added complexity, N-termini are not always surface-exposed, which may be desirable, depending on the approach.

1.2.2.5 Tyrosine

Tyrosine provides a unique coupling moiety in the form of the electron-rich phenol groups, which in the presence of strong oxidizers and appropriate catalysts crosslink with other tyrosines within the VLP or heterologous proteins (Horowitz et al., 2012). An azo-derivative of maleimide has been shown to chemo-selectively target Tyr residues and has been utilized to display stabilized trimeric Env spikes on nanoparticles (Ban et al., 2010).

1.2.2.6 Unnatural Amino Acid Incorporation

Engineering of unnatural amino acids (UAA) into the phage capsids can introduce uniquely addressable residues, as the side chains of these UAAs provide novel reactivity, enabling stable conjugates under relatively mild conditions (Patel and Swartz, 2011). Over 50 UAAs have been incorporated into recombinant proteins via genetic means (Hallam et al., 2015).

Unique reaction handle amino acid derivatives may be substituted in chemically defined media to ensure exclusive global incorporation of the bioorthogonal UAA in place of the left out natural amino acid (section 1.2.2.6.1 and 1.2.2.6.2).

Amber-suppression may be used to create single, orthogonal residues (section 1.2.2.6.3). First a protein variant is generated with an Amber (TAG) non-sense mutation at the desired position for incorporation of the UAA (Wals and Ovaa, 2014). Suppressor tRNA, directed against the non-sense codon, is loaded with the desired unnatural amino acid using *in vitro* aminoacylation (Noren et al., 1989). Addition of the aminoacylated tRNA to a cell free protein synthesis (CFPS) system results in incorporation of the unnatural amino acid in the position of the UAG

codon (Noren et al., 1989). Full length protein can be purified via C-terminal affinity tags; VLP coat proteins may be labelled by strategically positioning the stop-codon so that lack of incorporation of the amino acid results in an early truncated protein unable to associate into capsids and only suppressed proteins are assembly competent.

The limitations of CFPS may be overcome by using evolutionary engineered orthogonal aminoacyl-tRNA-synthetases, which are capable of UAA-tRNA charging *in vivo* [section 1.2.2.6.3 (Hallam et al., 2015; Sletten and Bertozzi, 2009)]. Challenges concerning UAAs have been extensively reviewed (Algar et al., 2011; Hoesl and Budisa, 2011; Sapsford et al., 2013; Sletten and Bertozzi, 2009; Wang et al., 2006). Examples are increased complexity of protein expression and infidelity of unnatural amino acid codons. Hence purity of UAA-containing proteins requires careful examination (Aerni et al., 2015). In particular for VLPs, capsid protein synthesis and assembly is a spatially and temporally orchestrated process. The UAA must not only be efficiently incorporated but must also not alter steric properties of the capsid protein or kinetics of synthesis and assembly. Furthermore commonly used chemistries are prone to side-reactions, e.g. azide / alkyne / tetrazine groups (van den Bosch et al., 2013; Sasmal et al., 2012; Versteegen et al., 2013).

Nonetheless, in my opinion UAAs are an extremely interesting and quickly evolving field with huge potential, strongly driven by the antibody-drug conjugate industry (Patel and Swartz, 2011; Strable and Finn, 2009a; Tong et al., 2009).

I briefly summarize the key examples of UAA incorporation into VLPs in the following paragraphs.

1.2.2.6.1 Selenocysteine

The natural 21st amino acid selenocysteine (Sec) is more nucleophilic than cysteine and reacts at lower pH (pKa of 5.2 for Sec vs. 8.1 for Cys). Thus a Sec-bearing VLP can be derivatized selectively in the presence of other potential side-chain nucleophiles. Sec has been incorporated into M13 phage using a cysteine auxotroph strain and reacted with iodoacetyl-PEO₂-biotin (Love et al., 2006). Though easy to produce, SecH is rapidly oxidized and requires glovebox handling under inert atmosphere, rendering this approach technically challenging (Love et al., 2006).

1.2.2.6.2 Azidohomoalanine (Met → Aha)

Methionine (Met) replacement with isosteric azidohomoalanine (Aha) is one of the least invasive UAA incorporations (Ng et al., 2012). Supplementation of Aha to (costly) Met-free media replaces every Met with Aha. Undesired capsid internal Met codons can be typically replaced at will, as Met-Leu or Met-Ile mutations are well tolerated, the exception being the N-terminal start codon (AUG), which is typically translated as *N*-formyl-Met. Substitution thus may yield the terminal *N*-formyl-Aha unless N-terminal processing cleaves this group. In *E. coli* this process can be controlled by choosing neighbouring residues accordingly to either encourage or inhibit deformylation and subsequent processing by methionyl aminopeptidase (MetAP) and / or utilizing knockout-strains (Wang et al., 2008a). This approach requires expression in either methionine auxotroph or CFPS (Strable et al., 2008). VLP decoration examples of this approach have been pioneered by the Swartz group for up to 3 simultaneous ligands via CFPS (Patel and Swartz, 2011).

1.2.2.6.3 *p*-acetophenylalanine and *p*-azidophenylalanine

Another method for incorporation of UAA is nonsense suppression. *p*-Azidophenylalanine (AzPhe) and *p*-acetophenylalanine (AcPhe), the most commonly used UAAs, have been incorporated into many different phages and VLPs (Bundy and Swartz, 2010a; Tsao et al., 2005).

AcPhe site-specifically incorporated into a protein can be orthogonally reacted on its ketone moiety as an electrophile with an aminoxy nucleophile, generating a moderately stable oxime linkage (Wang et al., 2003). In buffered aqueous solution and the presence of an aniline catalyst, this reaction progresses to high yield (Wang et al., 2003). The major drawback of this method is that a pH below 5 is required for optimal reaction, disqualifying acid-sensitive proteins and VLPs (Hallam et al., 2015). The reaction may also be performed at pH 6 but will proceed slower (Hallam et al., 2015).

AzPhe has been used to incorporate the azide moiety required to perform a copper-catalysed cycloaddition between the azide and a terminal alkyne (Hallam et al., 2015). Q β and MS2 were expressed in CPFS with AzPhe and AcPhe and assembled into particles, albeit at reduced yield. While bioorthogonal and compatible in aqueous buffers at neutral pH, the presence of copper in these reactions can cause oxidative damage to proteins (Ramirez et al., 2005). To avoid this problem, strained cyclic alkynes allow the reaction to proceed in the absence of copper catalysis (Jewett and Bertozzi, 2010; Sletten and Bertozzi, 2009).

Yet another example is incorporation of *p*-amino-L-phenylalanine into MS2 for oxidative coupling, yielding 60-75% conjugation with peptides (Carrico et al., 2008).

In vivo suppression for AA replacement is still not widely used, presumably due to the expression bias against proteins containing UAAs (Liu and Schultz, 2010; Ng et al., 2012). The advent of Genetically Recoded Organisms (GROs) is helping to address this issue, as evolutionary forces can be applied to further improve UAA incorporation as well to install multiple handles in the same protein for dually addressable particles and proteins (Lajoie et al., 2013).

1.2.2.7 Reduction mutagenesis for unique (natural) amino acids side chains

Incorporation of unnatural amino acids with orthogonally-reactive side chains is an attractive option, but side-chains of natural amino acids can also serve as unique reactive groups, with the benefit of decreased production cost. Using commonly occurring amino acids as unique reactive moieties requires deletion of non-target residues in the coat protein or target protein. Reduction mutagenesis may also be applied in global replacements strategies with UAAs to achieve reduced number or single reaction sites (Bundy and Swartz, 2010b).

1.2.2.8 Incorporation of Reactive Handles by post-translational modifications

Unique reactive handles can also be derived from post-translational modifications (PTM). Carbohydrates on VLPs can be oxidized to yield aldehydes using sodium periodate (NaIO_4). The aldehydes are reactive toward hydrazide conjugates and undergo a facile coupling reaction, resulting in the formation of covalent hydrazone linkage. This has been demonstrated with a thermophilic phage, but neither oxidation nor subsequent conjugation was complete (Steinmetz et al., 2008).

The biotinylation machinery can be manipulated to incorporate ketone-containing analogues, reactive towards hydrazide- or hydroxylamine-functionalized molecules. This approach requires the incorporation of the 15-amino-acid acceptor peptide (AP) – also known as Avi-Tag, (Chen et al., 2005; Ng et al., 2012) (see section 1.2.3.2 for biotinylation). A common problem with Avi-Tag is that it may be proteolysed during or after purification, which may be countered by addition of protease inhibitors (Thorner et al., 2000).

Incorporation of sequences that can be recognized by specific enzymes but typically require genetic encoding are discussed in the following sections.

1.2.2.9 Dock-and-Lock

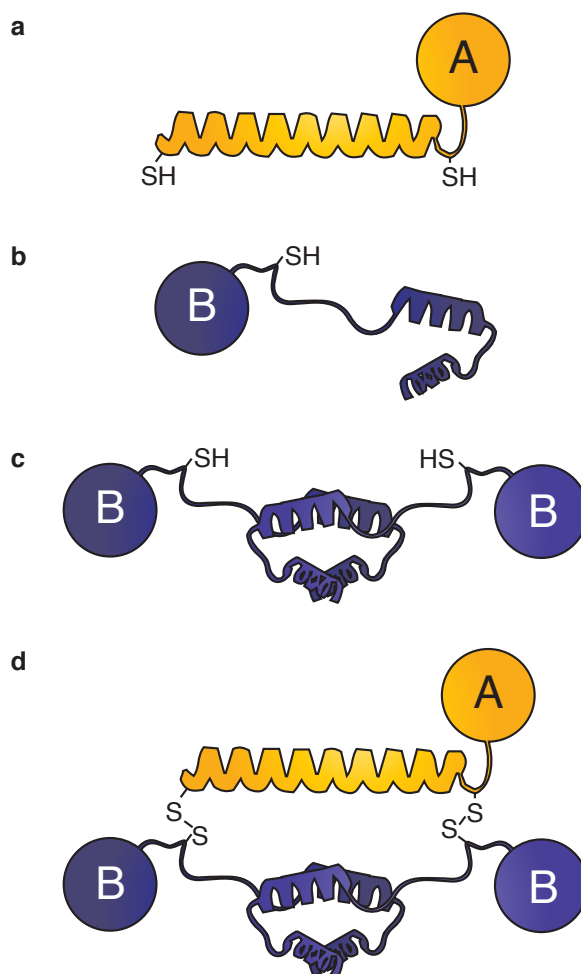


Figure .7 Dock-and-Lock (DNL) mechanism. (a) Anchor domain (AD) component of precursor A. (b) DDD component of precursor B. (c) dimerization and docking domain (DDD) mediated dimer of precursor B. (d) Stably tethered DNL conjugate comprising two units of precursor B and one unit A. DDD (blue helix) and AD (yellow helix). 'Locking' disulfide bonds and their originating sulfhydryls are highlighted. Adapted from (Rossi et al., 2012).

As outlined in section 1.2.2.3.1, disulfides are one of nature's way to covalently cross-link proteins intra- and intermolecularly. Though disulfides are possible to engineer, particularly when structural information is known (Craig and Dombkowski, 2013) and are flexible in terms of location, they are not an ideal crosslink since they are not only formed but also reversed under reducing conditions. Reducing reagents may only be required temporally, but extracellular

proteins of most human pathogenic organisms contain multiple Cys residues (Anelli et al., 2003; Baneyx and Mujacic, 2004). Thus after reduction, there can be promiscuous reactions between sulfhydryl groups.

To address this problem, disulfides have been used in combination with non-covalent affinity approaches to increase the likelihood of correct disulfide bond formation. The Dock-and-Lock (DNL) system is based on the heterotrimerization of the Protein Kinase A (PKA) and the A-Kinase Anchoring Protein (AKAP) (Chang et al., 2007). The two proteins dimerize via α -helical binding domains. They mutated spatially matching residues in the α -helices to Cys, such that after the initial binding, disulfide bridges form specifically at the interface (Chang et al., 2007). To my knowledge, no peer-reviewed academic literature exists that describes the use of DNL for VLPs, but patent literature lists examples with alternative DNL platforms (Padgett, 2013).

1.2.2.10 Electrostatic Interaction Lock

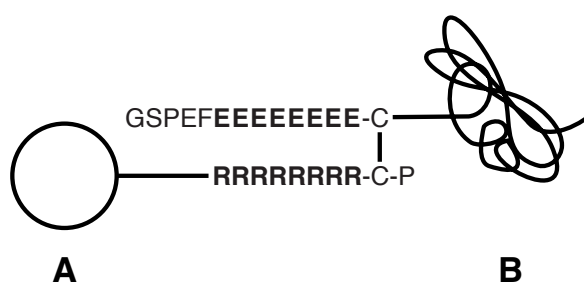


Figure 1.8 Electrostatic interaction (lock). Molecule A is fused to the polyionic peptide Arg₈(Cys)Pro. Affinity is achieved by appending an oppositely charged peptide sequence GlySerProGluPheGlu₈(Cys) to molecule B. Covalently coupling can be achieved by a disulfide bond in non-reducing conditions. Adapted from (Lilie et al., 2013).

Analogous to the DNL approach, electrostatic interactions have been exploited to align cysteine residues to form preferred disulfides, giving Electrostatic Interaction

Locks (EILs) (Lilie et al., 2013). EILs have been used to load VLPs with drug payloads (Ren et al., 2007) or to display 20 amino acid peptides on chimeric bovine papillomavirus (BPV) VLPs (Pejawar-Gaddy et al., 2010). In addition to the problems of DNLs, EILs can interfere with capsid assembly as electrostatic interactions are important for VLP assembly. EIL display on BPV resulted in reduced particle quality (Pejawar-Gaddy et al., 2010). Nucleic acid affinity is thought to be at least partially mediated by electrostatic interactions, as is the zeta potential on the solvent exposed surface of the VLP. Introduction of charged residue may interfere with nucleic acid binding and change the zeta potential, leading to poor particle quality (Basnak et al., 2010; Lu et al., 2015; Pejawar-Gaddy et al., 2010; Samandoulgou et al., 2015; Stockley et al., 2013).

1.2.2.11 HaloTag

HaloTag is a modified haloalkane dehalogenase (DhaA) which specifically and covalently binds to functionalized haloalkane ligands. DhaA normally cleaves halides from alkyl halide chains (Los et al., 2008). HaloTag has mutations to abolish hydrolysis of the covalent intermediate (Los et al., 2008). V4 and V5 Variable Loops of HIV-1 Envelope Glycoprotein were fused with HaloTag (Nakane et al., 2015) and HaloTag was also employed to display luciferase on a 60-mer non-VLP nanoparticle, E2 from *Geobacillus stearothermophilus* (Sun et al., 2015).

A drawback of the HaloTag system is its size (33 kDa), rendering even monomeric fusions often disruptive (Los et al., 2008).

1.2.2.12 SNAP-Tag

SNAP-Tag originates from the DNA repair enzyme O⁶-alkylguanine-DNA alkyltransferase (AGT) and can be covalently labeled with O⁶-benzylguanine derivatives (Gautier et al., 2008). Modified nucleotide analogues react irreversibly with SNAP-tag (mutated AGT). The system is particularly attractive for *in situ* labelling of proteins in live cells; first by expression of SNAP-tag fusions and subsequent exposure to nucleotide-dye analogues for protein tracking (Eckhardt et al., 2011; Gautier et al., 2008). SNAP-tag, being 20 kDa in size, was largely designed for fluorescence labelling of proteins *in vivo*, rather than a general system for protein ligation (Gautier et al., 2008). Therefore compatible nucleotide analogues equipped with bioorthogonal handles are desirable but not commercially available at this time. Addressing VLPs with the system is however possible, as SNAP-tagged HIV-1 virions assemble, can be labelled *in vivo* and remain infectious, albeit with reduced replication capacity (Eckhardt et al., 2011).

1.2.2.13 Sortases

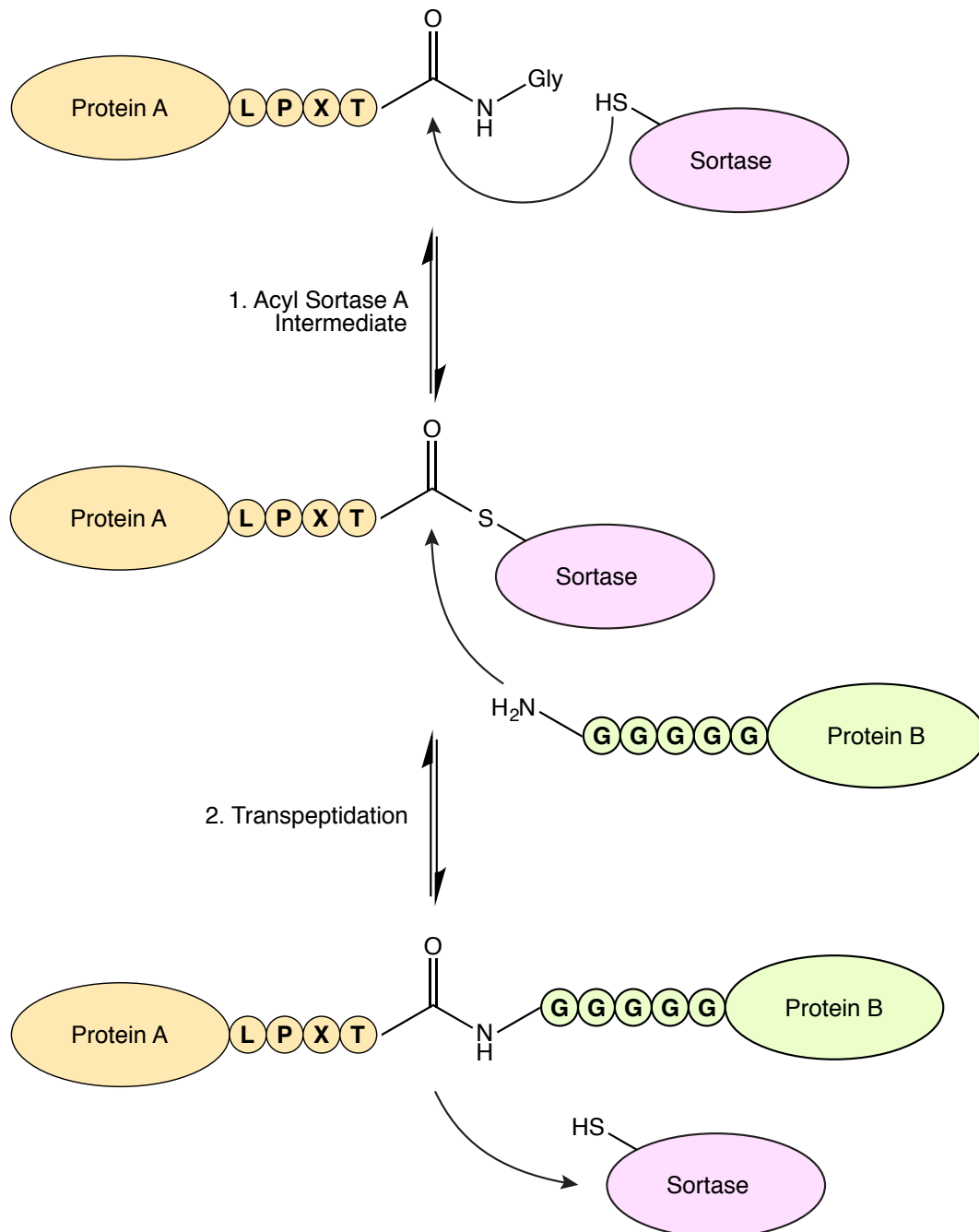


Figure 1.9 Mechanism for Sortase A-mediated ligation (SML). The catalytic Cysteine residue of Sortase A nucleophilically attacks the Threonine-Glycine amide bond of the LPXTG recognition sequence via its sulfhydryl group, yielding the acyl-enzyme intermediate (Step 1). The thioester intermediate is resolved by an attack mediated by an amino group of the Gly-containing protein B, resulting in the ligation of two proteins through a peptide bond. Adapted from (Tsukiji and Nagamune, 2009)

Sortase A-mediated ligation (SML) has been used to decorate a genetically-installed LPETGG motif on the surface of Hepatitis B Virus variant protein [SplitCore (Walker et al., 2011) see section 1.2.2.14.2] with 18 AA peptides (Tang et al., 2016)]. Reactivity was relatively high (90 %), but required 6-12 fold molar excess of peptides. 40 % material loss occurred from pre-to-post sortase conjugation, due to technical problems [not further specified (Tang et al., 2016)]. Sortase decoration of multimers had also been demonstrated for the E2 nanoparticle with proteins <150 kDa, yielding only sparsely decorated 60-mers (Chen et al., 2015).

1.2.2.14 Genetic fusion

Genetic fusion of epitopes to VLPs has been successfully used in numerous examples and remains one of the most common approaches in VLP decoration (Khudiyakov and Pumpens, 2016). In general, (poly)peptides are fused either into exposed loop regions or are appended N- or C-terminally (Mateu, 2013).

1.2.2.14.1 Hepatitis B surface antigen

One of the earliest and successful examples is the RTS,S vaccine pioneered by Rutgers (Rutgers et al., 1988). RTS,S induces high antibody titres that block the parasite from infecting the liver (Foquet et al., 2014). The vaccine is a genetic fusion of circumsporozoite protein (CSP, *Plasmodium falciparum*) NANP repeat epitope to HBsAg (Rutgers et al., 1988). Co-expression with HBsAg in yeast yields VLPs closely resembling the licensed HBV vaccine Engerix-B (GSK) based on Rutter and

Valuenzula's work but importantly also conveys the particulate immunological properties to CSP (RTS,S Clinical Trials Partnership, 2015).

1.2.2.14.2 Hepatitis B core antigen / SplitCore / Tandem Fusion of Hepatitis B Core Antigen

HbcAg, derived from HBV, forms icosahedral VLPs when expressed in either prokaryotic or eukaryotic expression systems. The VLPs assemble from dimers of the 183–185 amino acid HBcAg protein and occur with either $T = 3$ (180-mer) or $T = 4$ (240-mer) symmetry (Pumpens and Grens, 2001). HBcAg protein has a remarkably high α -helical content and the dimer contacts form four-helix bundles which appear as spikes on the surface of VLPs (Crowther et al., 1994; Wynne et al., 1999). These spikes have served as the major insertion region and are the major immunodominant region (MIR) in the wildtype (Conway et al., 1998; Pumpens and Grens, 1999). HBcAg can accept small epitopes but insertion of large or hydrophobic sequences interferes with its ability to assemble, with a consequent loss of antigenic and immunogenic properties (Jegerlehner et al., 2002b; Pumpens and Grens, 2001). An example of a promising HBc VLP was Malarivax that failed in phase III (Schwartz et al., 2012).

Several approaches have been attempted to overcome the size limit of insertions, such as co-expression with wildtype HBcAg (Vogel et al., 2005), a split fusion (SplitCore) allowing simultaneous display of two large proteins (Walker et al., 2011), and tandem repeat fusion of HBcAg (Peyret et al., 2015). Woodchuck House Virus

(WHV), also a Hepadnavirus, is slightly more tolerant to insertions (Billaud et al., 2005).

1.2.2.14.3 Bacteriophage Leviviridae

Several VLPs have been derived from the bacteriophages of the *Leviviridae* family. The phages infect *Enterobacteriaceae*, which are commonly used laboratory strains such as *E. coli*. Expression of wildtype phage coat protein in their native or closely-related hosts can thus yield high quantities of soluble, assembled VLPs (>100 mg / L culture) (Khudyakov and Pumpens, 2016).

Family representatives that have been successfully genetically decorated with small peptides are AP205 (Tissot et al., 2010), fr (Kozlovskaja et al., 1988), MS2 (Mastico et al., 1993), PRR7 (Caldeira and Peabody, 2007), and Q β (Kozlovskaja et al., 1993).

MS2, an icosahedral, positive-sense single-stranded RNA virus infects *E. coli* (Fiers et al., 1976). Atomic resolution structures of the capsid help insertion strategies (Golmohammadi et al., 1993; Valegård et al., 1990). MS2 VLPs accept insertions up to 24 residues (Mastico et al., 1993) but the octapeptide FLAG sequence did not lead to soluble VLPs (Peabody, 1997). Generally, insertions of more than 12 residues typically results in formation of insoluble, semi-crystalline protein which may be rescued by refolding in urea, stepdown dialysis and a final size exclusion chromatography (Heal et al., 1999; Stockley and Mastico, 2000). Due to the dimeric arrangement of the coat proteins and fortuitous positioning of the two termini, a “single-chain dimer” fusion allows for assembly of more thermostable and insert-tolerant particles (Peabody, 1997). Peptides displayed on MS2 and on the MS2-

single-chain dimer VLP are typically very immunogenic and the MS2 single-chain dimer VLP has been used in conjunction with affinity-based selection to evolve short epitope mimetics (mimotopes) of the challenging malarial antigen RH5 (Ord et al., 2014). Genetic fusion of larger inserts (>30aa) into this VLP scaffold remain elusive however.

Examination of the structurally-solved *Leviviridae* shows that the N-termini consist of β -hairpins that face back towards the capsids (Golmohammadi et al., 1993, 1996; Tars et al., 2000). Therefore fusion at the N-terminus may disrupt the β -hairpin and cause steric interference with the adjacent monomers, causing failure to assemble (an exception is AP205, discussed further below).

Interestingly several phages display read-through extensions at their C-termini, which are essential for producing infectious particles, but their precise functions are unknown (Rumnieks and Tars, 2011). For example, Q β phage has three to five copies of a 196 aa extension termed A1 at the C-terminus of the coat protein (Rumnieks and Tars, 2011; Weiner and Weber, 1971). Pumpens created VLPs that incorporated chimeric C-terminal extensions. by fusing either to full-length or truncations of A1 in a suppressor strain (Koletzki et al., 1997). Incorporation of A1 extensions varied inversely as a function of extra length, from ~10 per particle to a maximum of ~80 per particle for extensions of 11–24 amino acids (Koletzki et al., 1997). Finn improved on this by separating the expression of the unmodified coat protein required for assembly and the modified A1 coat protein onto two different plasmids. This enabled on average ~ 20 incorporations of a 50 aa protein (Brown et al., 2009). Though an improvement, the invariably inhomogeneous nature of

particles and lack of full conjugation is not ideal for pharmaceutical grade vaccines, nor for defined immunogen design in mechanistic studies.

Fusion to AP205 is special, allowing display of fragments of at least 55 amino acid to the termini (Tissot et al., 2010),

Conclusion on genetic fusion

Predicting the outcome of the genetic fusion of antigen to VLPs is difficult, if not impossible. Hence genetic fusion for vaccine development remains a costly time-consuming trial-and-error approach (Mateu, 2011). Further drawbacks can include limited accessibility of the fused epitope, constraints on epitope size, enforcement of non-native structures on the epitope, improper glycosylation, and most frequently the failure to assemble into homogeneous VLPs (Smith et al., 2013c; Strable and Finn, 2009b).

1.2.3 Non-covalent coupling approaches

1.2.3.1 Antibody affinity through Protein A / Z

The functional fragment of the *Staphylococcus aureus* protein A (133 aa) was displayed on TMV as a C-terminal fusion to the coat protein via a 15 aa linker for improved antibody binding purification capacity (Werner et al., 2006). Similarly, the VP1-Z system is based on the polyoma virus coat protein VP1 and protein Z (Protein A mutant) fusion (Gleiter, 2001). The system has been pioneered for changing tropism of the polyoma virus, whilst displaying antibodies that target the hybrid to cells via their cognate extracellular targets (Gleiter, 2001). Though neither systems has been engineered for immunization purposes, it may be feasible to display an antigen of choice via a monoclonal antibody on the surface as a mediator. This should also allow orientating the antigen in relation to the VLP as a function of available structural and mAb availability, exposing epitopes which yield desired antibodies.

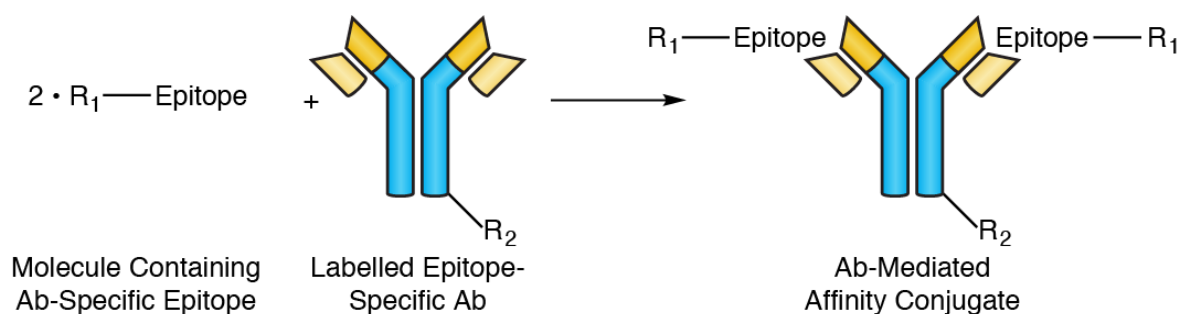


Figure 1.10 Antibody mediated affinity conjugation. An antibody is fused via a chemical means to ligand 2. Affinity of the antibody for its epitope allows non-covalent association of covalently epitope-associated ligand 1.

1.2.3.2 Avidins / biotin

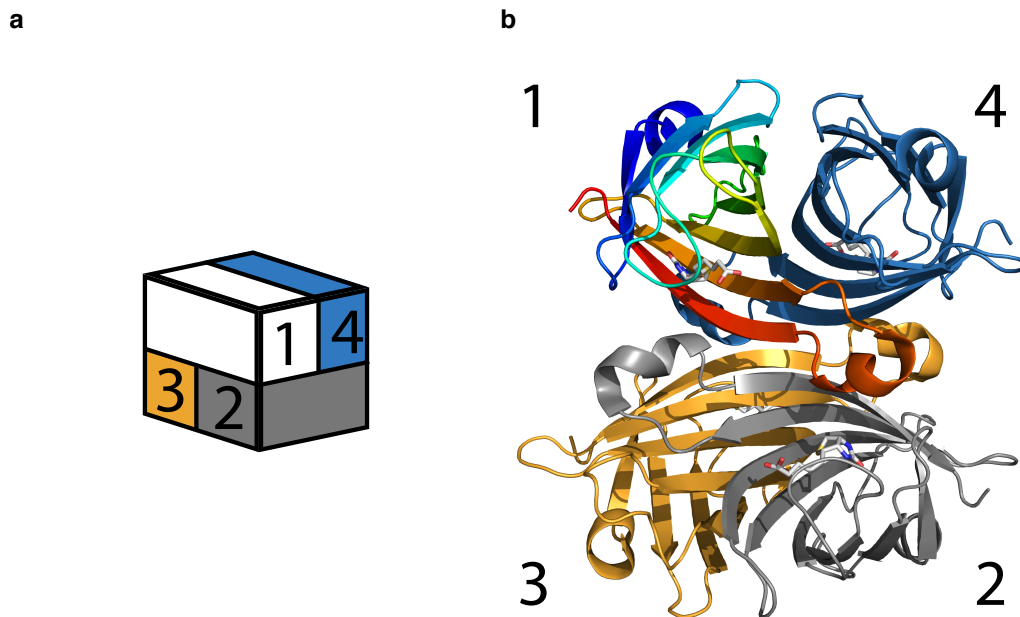


Figure 1.11 Streptavidin complex. (a) Schematic of 3-dimensional arrangement of the four streptavidin monomers. (b) Quaternary structure of tetrameric streptavidin depicted with bound biotins along dyad symmetry axis. Adapted from (Fairhead et al., 2014a; Weber et al., 1989).

t

The strong affinity interaction between streptavidin and biotin has been leveraged to generate chimeric VLPs. Sulfo-NHS-biotin has been used to derivatize both VLP (recombinant vaccinia expressing simian immunodeficiency virus envelope protein, rVV-SIV Env) and antigen (Cholera Toxin Subunit B, CTB) prior to affinity-association through tetrameric Streptavidin [SA, see Figure 1.11 (Kang et al., 2003; Weber et al., 1989)}. CTB binds to cell membrane GM1 gangliosides, thus enhancing its uptake by mucosa-associated lymphoid tissue, when administered mucosally. VLP-conjugated CTB enhanced humoral and cellular immunity against the conjugate, likely due to helper epitopes in CTB (Kang et al., 2003).

Independently, BPV VLPs were first derivatized using NHS-biotin and then complexed with tetrameric streptavidin-fused tumour necrosis factor alpha (TNF-

α) peptide (Chackerian et al., 2001). The conjugates were successfully in breaking self-tolerance, resulting in neutralizing anti-TNF-α antibodies (Bachmann and Jennings, 2010).

Salanti improved on this approach by using the smaller monomeric streptavidin / rhizavidin hybrid [mSA (Lim et al., 2013)] with HPV VLPs displaying the AviTag in the MIR region (Thrane et al., 2015). This approach came at the expense of reduced biotin affinity of the streptavidin variant: mSA K_d 2.8 nM vs. tetramer SA K_d 10 fM (Green, 1990). The loss in affinity may be overcome using the monovalent tetrameric SA A1D3 (Howarth et al., 2006).

The clear advantage of the avidin / biotin system is its modular nature (*Fairhead et al., 2014a; Livnah et al., 1993*). The inherent problems of non-covalent interactions, which can re-arrange over time, is of particular importance to multivalent biotinylated particles. Temporarily loss of affinity for their avidin ligand may initiate cross-binding with neighbouring particles after re-saturation with undesired avidin. This eventually yields larger and larger compound VLP multimers prone to aggregation, as well as heterogeneous mixtures with different pharmacologically active species. Therefore this approach entails extra challenges and quality control requirements, imposing additional regulatory hurdles (Mateu, 2013). To my knowledge, so far no group has reported the inverse arrangement; e.g. SA-displaying VLPs which may well alleviate aggregation problems. With recent advances this should now be possible (Fairhead et al., 2014a, 2014b).

Problems commonly encountered are proteolysis of the AviTag (Thorner et al., 2000) and incomplete labelling with BirA *in vitro* or *in vivo* (Fairhead and Howarth, 2015).

1.2.3.3 Coiled coils

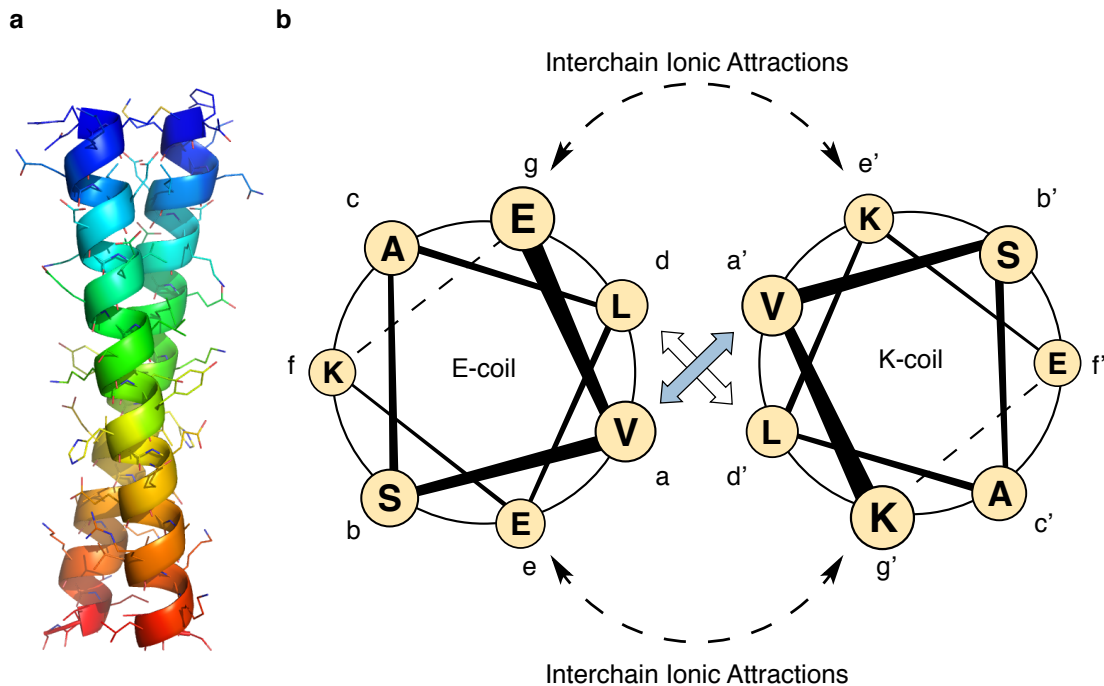


Figure 1.12 Coiled coil. (a) Representative crystal structure for a coiled coil (PDB 2ZTA). (b) Top-down representation of a heterodimeric E/K coiled-coil. The coils propagate into the page from the N- to the C-terminus and heptad repeats are depicted. Adapted from (Chao et al., 1998; Woolfson, 2005).

Use of coiled coils has not been reported for vaccine VLPs but has been demonstrated for phage M13-surface attachment on specific locations of patterned gold surfaces (White et al., 2012).

1.2.3.4 Affinity Binding

Non-covalent display of epitope can also be engineered using affinity of peptides to native or engineered capsids. For example wildtype Hepatitis B core antigen (HBcAg) and Hepatitis B surface antigen (HBsAg) interaction is blocked by oligopeptides with the motif ALLGRMKG (Dyson and Murray, 1995). A high affinity peptide (GSLGRMKGA) (Böttcher et al., 1998), which binds to the major

immunodominant region (MIR) of HBcAg was used as a binding tag to allow *in vitro* scaffolding of HBcAg with such tagged proteins (Blokhina et al., 2013). Though successful at inducing a potent antibody response against the tagged protein (influenza M2e), the interaction was not strong enough to withstand sucrose gradient ultracentrifugation (Blokhina et al., 2013).

1.2.3.5 Lipid incorporation via GPI anchor and ER membrane localization sequences

VLPs expressed in eukaryotic systems can be encapsulated in lipid bilayers and are thus amenable to lipid-conjugated protein incorporation. Glycosylphosphatidylinositol (GPI)-anchored proteins can be expressed in one cell-type and then transferred into separately purified VLPs, a process also known as “painting” or “re-insertion” (Metzner et al., 2008a, 2008b, 2013). However GPI-linked protein purification and transfer is non-trivial. Alternatively, the VLP production host can be engineered to co-express target-proteins with genetically encoded GPI-anchor (Kueng et al., 2007; Patel et al., 2015; Skountzou et al., 2007). The GPI-anchored target protein can then *in vivo* co-assemble into the VLP during budding (Kueng et al., 2007; Patel et al., 2015; Skountzou et al., 2007).

Other membrane-targeting sequences are available (e.g. via prenylation, palmylation etc.) and have been applied to enveloped VLPs (Nishimura et al., 2012). Though attractive due to its simplicity, lipid targeting approaches are constrained in choice of expression system for both VLP and target protein (Cardinale and Michon, 2015).

1.2.3.6 Histidine tag – Ni-NTA / Co

Attaching Ni-NTA residues to VLPs for linkage of His-tagged antigens has been demonstrated (Koho et al., 2015). Nickel(II)-chelated nitrilotriacetic acid (NTA) conjugated ligands to introduced His-tags have also been described.(Chatterji et al., 2005; Cheung et al., 2006). Ni²⁺ toxicity and allergenic potential rules out this approach from a regulatory perspective.

1.2.3.7 RNA-directed loading of particles

RNA-mediated loading of VLPs, exploiting coat protein-RNA interaction, has been demonstrated for RNA encapsulating VLPs. First an RNA aptamer is raised against a small peptide tag (rev-tag). Second, the RNA for the RNA aptamer is reverse-transcribed and fused to the DNA sequence encoding the coat protein of the VLP. No in-frame fusion to the coat protein is required, as the affinity to the rev-tag is mediated by the RNA aptamer-coat_protein RNA transcript. The RNA, which encodes the coat protein, has affinity for the proteinaceous CP it encodes. Therefore, the coat protein encoding RNA co-assembles into the VLP. Due to the genetic linkage of coat protein DNA sequence and RNA aptamer sequence, the transcribed, packaged RNA also contains the RNA aptamer. Co-expression of te RNA RNA(CP)-RNA aptamer with a protein that has the rev-tag fused to it, leads to co-assembly into the VLP through the RNA-aptamer to rev-tag affinity (Fiedler et al., 2010).

1.2.4 Preceding work on Isopeptide-forming peptide Tags and Catchers

Splitting of covalent bond-forming proteins is a technology pioneered by Howarth group (Zakeri and Howarth, 2010). The underlying proteins for the Catcher/Tag systems are derived from the family of Gram-positive bacterial cell surface adhesin and pilin proteins. These proteins are unusual in that they form spontaneous intramolecular isopeptide bonds. As the thesis and development of SpyCatcher-VLP technology relies on this phenomenon, I will describe the history, development and underlying features of Catcher technology in the following paragraphs.

1.2.4.1 Intramolecular isopeptide bonds

Structural analysis of the major pilin of *Streptococcus pyogenes*, Spy0128, revealed two self-catalysed intramolecular isopeptide bonds, both cross-linking a Lys to an Asn through their side chains (Kang et al., 2007). Intramolecular isopeptide bonds can confer astonishing resilience to extreme chemical (~ pH 2), thermal (> 100 °C) and mechanical (~ 700 pN) insults, as well as to proteolytic degradation (Echelman et al., 2016; Hagan et al., 2010; Kang and Baker, 2011; Schoene et al., 2014). These imparted features may explain the unexpectedly widespread occurrence of intramolecular isopeptide bonds, as they may help in sustaining bacterial adhesion against strong mechanical forces and help to ward off proteolytic and chemical attacks of the host (Alegre-Cebollada et al., 2010; Echelman et al., 2016; Schwarz-Linek and Banfield, 2014). Indeed bioinformatics and further crystallography identified further isopeptide bonds in various cell surface proteins of Gram-positive bacteria, all characterized by collagen-binding

adhesion CnaA or CnaB domains (Krishnan et al., 2007; Nelson et al., 2007a; Budzik et al., 2008; Kang et al., 2009).

CnaA domains are composed of ~10 β -strands whereas CnaB is composed of ~7 β -strands (Symersky et al., 1997; Deivanayagam et al., 2000; Vengadesan and Narayana, 2011). Isopeptide bonds in this fold are most commonly formed by Lys reacting with Asn, but can also be formed between Lys and Asp (Kang and Baker, 2011). A proximal carboxyl group provided by either Glu or Asp is required to promote formation of the reactive triad (Figure 1.14) (Vengadesan and Narayana, 2011).

Split proteins can be obtained by genetically dissecting a protein into two or more inactive fragments that may reassemble and regain function. Split proteins have been widely employed to study protein-protein interactions; prominent examples are split Green Fluorescent Protein (GFP) or split Dihydrofolate Reductase (DHFR) (Shekhawat and Ghosh, 2011). The Howarth group previously pioneered splitting isopeptide bond containing proteins, yielding highly efficient and bioorthogonal covalent conjugation system, which I describe in the following paragraphs.

1.2.4.2 Isopeptag(N) / pilin-(N/C)

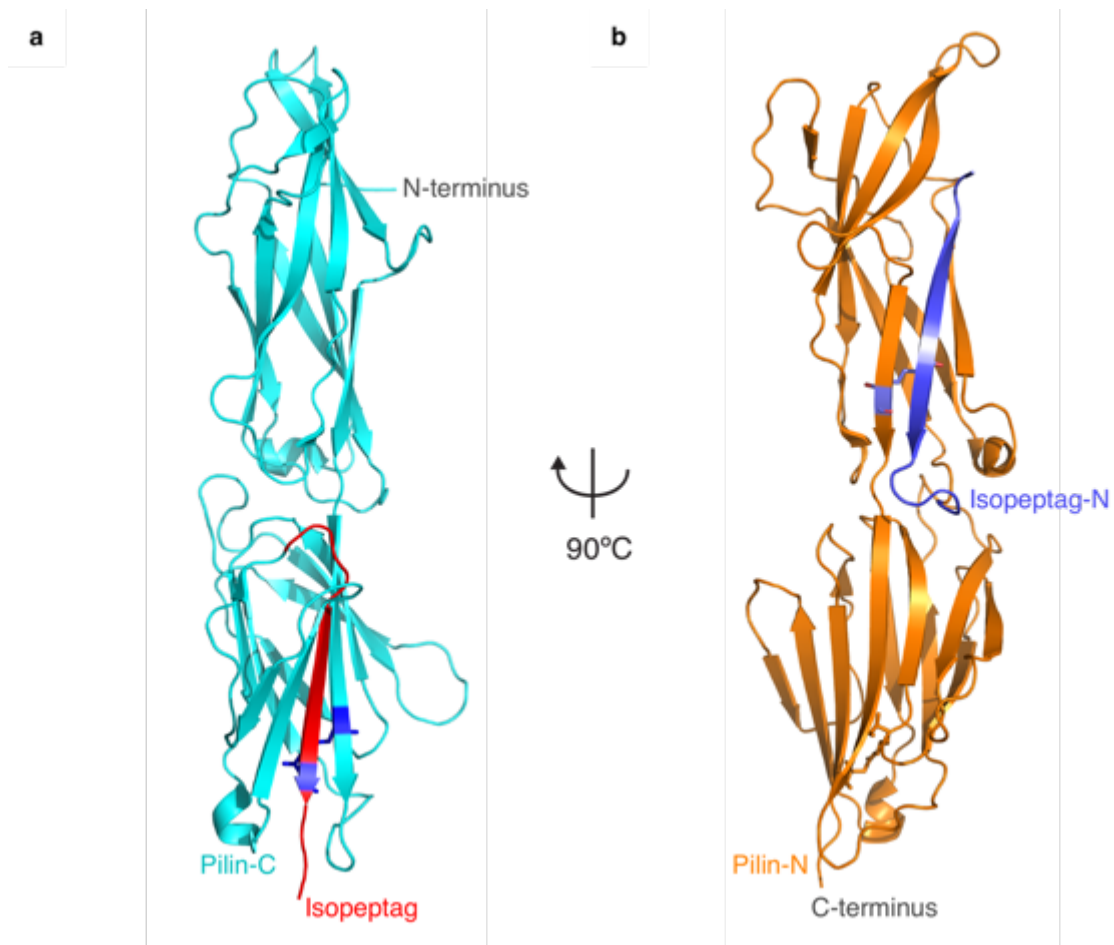


Figure 1.13 Crystal structure of Spy0128 colour-annotated with two different splits. (a) Isopeptag / Pilin-C (b) Isopeptag-N / Pilin-N. (PDB 3B2M) Adapted from (Zakeri and Howarth, 2010)

The protein Spy0128 entails two domains, each an intramolecular isopeptide bond (Kang et al., 2007). Bijan Zakeri split the N-terminal and C-terminal domains of Spy0128, resulting in two peptide-protein pairs capable of forming isopeptide bonds simply upon mixing (Zakeri and Howarth, 2010). C-terminally splitting Spy0128 yielded Isopeptag(-C), containing the reactive Lys, and its Catcher pilin-C bearing the reactive Asn and catalytic Glu residues. Conversely, C-terminally splitting yielded the Isopeptag-C / pilin-C peptide-Catcher pair (Figure 1.13).

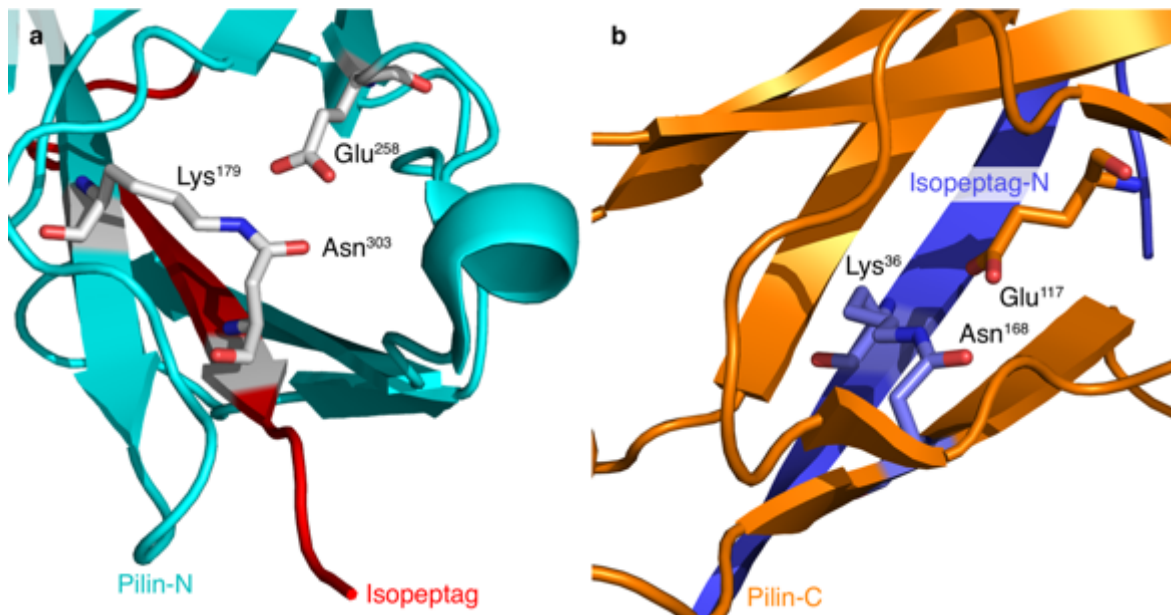


Figure 1.14 Isopeptide triads of Spy0128. Left: Isopeptag / Pilin-C, and right: Isopeptag-N / Pilin-N annotation (PDB 3B2M). Adapted from (Zakeri and Howarth, 2010)

Re-assembly *in vitro* or *in vivo* aligns the reactive triad and spontaneous isopeptide bond formation occurs (Figure 1.14). The reactions proceeded over a temperature range from 4 to 37 °C, within a pH range of at least 5 to 8, in the presence of detergents and wide range of buffers (Zakeri and Howarth, 2010).

The Isopeptag / Pilin-C system has been used for irreversible assembly of protein conjugates, demonstrating spatial and orientational control via Förster resonance energy transfer (FRET) between fluorescent proteins (Abe et al., 2013). The Spy0128 proof-of-principle split laid the foundation for future Catcher / Tag pairs. However, these split proteins suffered from slow reaction kinetics, inability to react to completion and the large size of the protein domains (~35 kDa), stimulating the search for better splits.

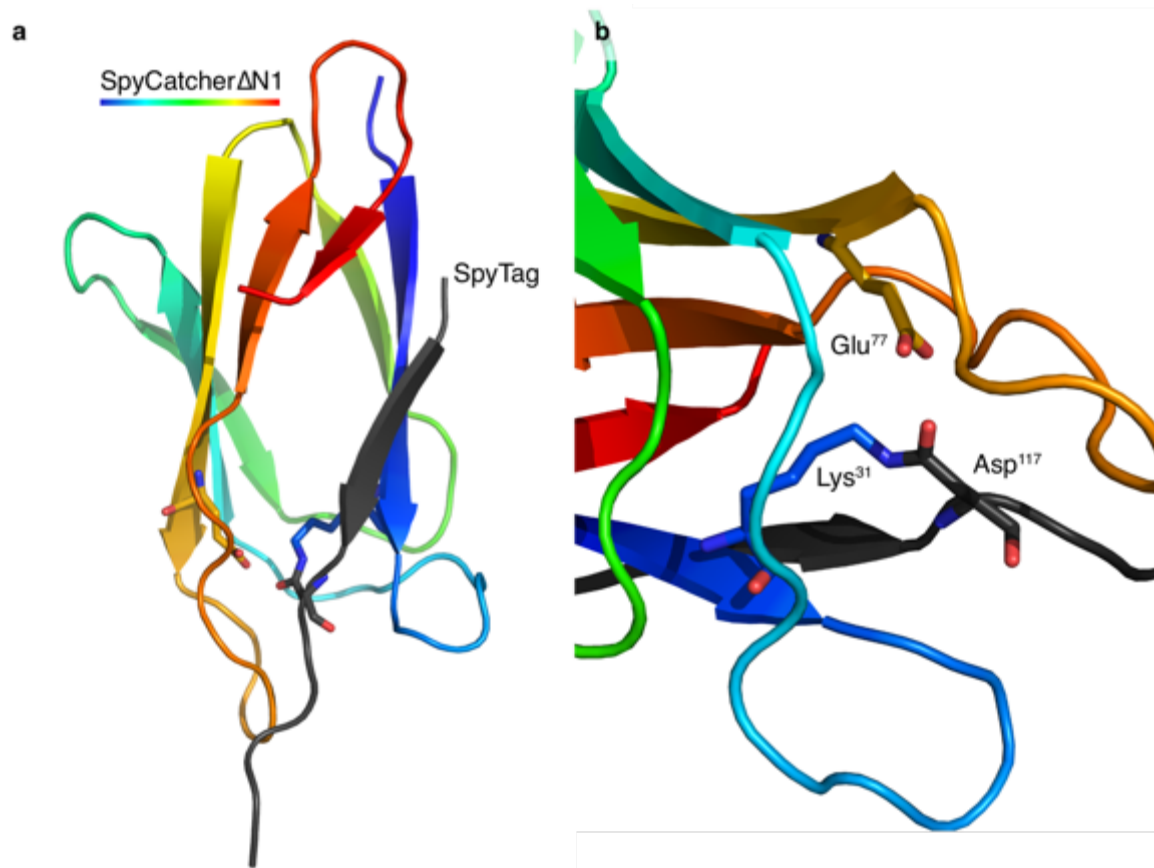
1.2.4.3 SpyTag / SpyCatcher(Δ N1)

Figure 1.15 Structure of SpyCatcher Δ N1 and SpyTag. (a) Crystal structure of SpyCatcher Δ N1 (PDB 4MLS) (b) Isopeptide triad of SpyCatcher(Δ N1). Adapted from (Li et al., 2014)

The CnaB2 domain from fibronectin-binding protein (FbaB) from *Streptococcus pyogenes* contains two reactive residues, Lys31 and Asp117, which form a covalent bond in the presence of the associated Glu77 (Amelung et al., 2011). CnaB2 served as the template for splitting and further engineering efforts that resulted in SpyTag / SpyCatcher, a split into a 13 aa peptide yielded SpyTag, and its 115 aa complementary domain SpyCatcher (Zakeri et al., 2012).

The SpyTag:SpyCatcher amidation occurred in wide range of temperatures, pH values, buffers and also in the presence of detergents (Zakeri et al., 2012).

The SpyTag / SpyCatcher system and its N-terminally truncated variant Δ N1SpyCatcher (Li et al., 2014) has since been used in various applications ranging from bioactive hydrogels for stem cell culture (Sun et al., 2014), biofilm engineering (Nguyen et al., 2014), enzyme cyclization (Schoene et al., 2014, 2016; Wang et al., 2016) and control of macromolecular topology (Zhang et al., 2013).

With particular importance for this thesis, the system has also been successfully used in a vaccinology approach to conjugate a target antigen to a monomeric antibody (anti-DEC205) for DC targeting (Liu et al., 2014). The immune response between SpyCatcher and SpyCatcher Δ N1 was compared (Liu et al., 2014). The Δ N1 truncation was found to be less immunogenic, as it was less effective in inducing antibodies against itself compared to full-length SpyCatcher (Liu et al., 2014). These insights motivated the use of SpyCatcher Δ N1 for display on AP205.

1.2.4.4 SnoopTag / SnoopCatcher

Building on the successful split and engineering of SpyCatcher, the Howarth lab split another isopeptide bond forming protein, yielding the SnoopTag / SnoopCatcher system (Veggiani et al., 2016). The pilus-associated adhesin domain RrgA from *Streptococcus pneumoniae* forms two isopeptide bonds: one between the between Lys191 and Asn695 and the other between Lys742 and Asn854 (Izoré et al., 2010). The D4 domain containing Lys742 and Asn854 is relatively small, devoid of structurally relevant metal-binding sites or Cys residues, which made this a good candidate (Izoré et al., 2010). As the D4 domain utilizes different residues for its isopeptide bond formation compared to the CnaB2 domain from *S.*

pyogenes, splitting D4 resulted in a mutually unreactive system to SpyCatcher / SpyTag, named SnoopTag / SnoopCatcher (Fig. 1.15) (Veggiani et al., 2016).

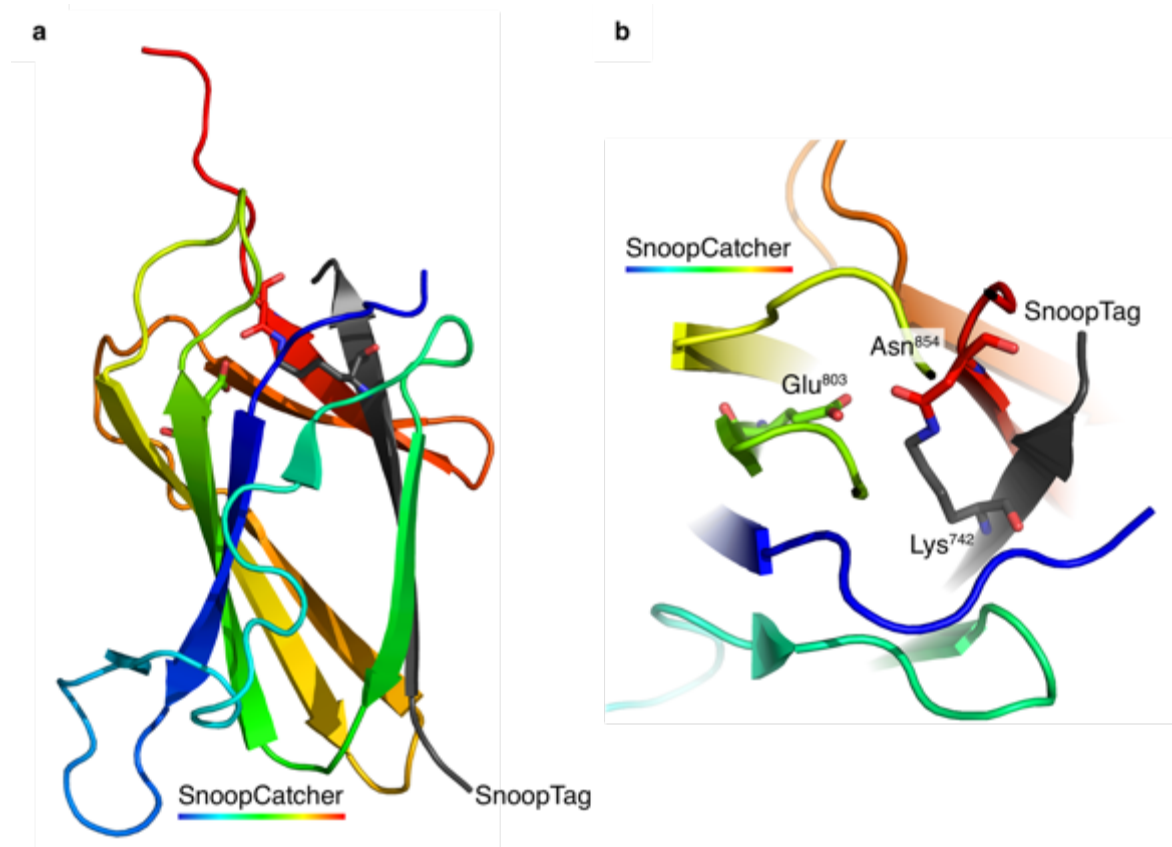


Figure 1.16 Structure of SnoopCatcher and SnoopTag. (a) The split design was modelled onto the structure of RrgA (PDB 2WW8) (b) Close-up view of the isopeptide triad with reactive and catalytic residues annotated. Adapted from (Izoré et al., 2010; Veggiani et al., 2016).

Raphaël Gayet first split the RrgA D4 domain, obtaining RrgACatcher (749-860) and its peptide tag RrgATag (734-748). Tomohiko Nakamura improved on this design, shortening RrgATag by removing its last 3 C-terminal amino acids to yield SnoopTag. The RrgACatcher was enhanced by introducing stabilizing mutations in a β -strand by mutating Gly 842 to Thr and by mutating Asp 848 to Gly (Veggiani et

al., 2016). These modifications resulted in improved stability and reactivity towards SnoopTag (Veggiani et al., 2016).

Reaction of SnoopTag with SnoopCatcher does not proceed as efficiently as the SpyTag / SpyCatcher reaction (Veggiani et al., 2016). Addition of the chemical chaperone trimethylamine *N*-oxide up to 2 M concentration modestly enhanced the reaction (Veggiani et al., 2016). Nonetheless full conjugation can be achieved in monomeric interactions by supplying modest excess (2-4 fold) of the respective partner (Veggiani et al., 2016). The reaction proceeded across a wide range of temperatures (4 - 37 °C), most efficiently at pH 9 but also at lower pH values (as low as ~ pH 5), albeit at markedly reduced rate (Veggiani et al., 2016). Analogously to the previous split isopeptide systems, SnoopCatcher was compatible with a wide range of excipients, such as non-ionic detergents, salts, EDTA or reducing agents (Veggiani et al., 2016).

SnoopTag / SnoopCatcher technology has since been used to build linear and branched chain mixed protein polymers in concert with SpyTag / SpyCatcher (Veggiani et al., 2016), as well as for enzyme cyclization (Schoene et al., 2016).

1.2.5 Thesis objectives

The foremost objective of this thesis was the application and characterization of the SpyCatcher/SpyTag to a VLP scaffold in order to achieve modular, covalent VLP decoration, first using model proteins and then more complex antigens, resilient to classical VLP decoration methods.

The second goal of this thesis was the preparation of immunological grade immunogen formulations and to evaluate the immunological properties of such VLP conjugates in the context of malaria.

Finally I attempted to achieve dual Tag-addressable VLPs and nanoparticles based on Spy-, Snoop-Catcher/Tag technology, in order to display multiple proteins on a single particle in a controlled manner.

Chapter 2 Materials and Methods

2.1 Cloning

2.1.1 Polymerase chain reactions (PCR)

All polymerase chain reactions (PCR) for construction purposes were performed using Q5 high-fidelity DNA polymerase 2× master mix [New England Biolabs, (NEB), M0492S] in C1000 thermal cyclers (Bio-Rad) unless otherwise stated. DNA oligos were purchased either from Integrated DNA Technologies (IDT) or Life Technologies (now Thermo Fisher) desiccated and desalted but without any further purification.

A typical amplification reaction was performed at 25 µL scale with 1 ng template plasmid or 5 ng of genomic DNA. Typical cycling conditions were: initial denaturation step for 30 s at 98°C, 5 cycles of denaturation for 5 s at 98°C, annealing for 15 s at the lowest partial annealing temperature calculated with the NEB Q5 Tm calculator (<http://tmcalculator.neb.com>), elongation at 72°C for 20-30 s/kb, followed by 25 cycles of denaturation for 5 s at 98°C, annealing for 15 s at full (highest) annealing temperature (NEB Q5 Tm calculator), elongation at 72°C for 20-30 s/kb; with a subsequent final elongation step at 68°C for 4× the time required to amplify the desired amplicon length. Samples were stored at room temperature for up to 3 days or at -20 °C for long term storage. Extension temperature was reduced to 68 °C for problematic amplifications of AT rich sequences with compensatory 2-3× increased elongation time (up to 1min/kb) to account for reduced DNA polymerase activity (Su, 1996).

PCR products were analysed by loading 5 µL of amplified reaction on an agarose gel (0.7-2% w/v, depending on the size of the DNA fragment) containing 0.01% (v/v) ethidium bromide (Sigma), and run for ~45 min at 130 V (~8.4V/cm) in TAE (40

mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). Gels were imaged using a Gel Imager Universal Hood II (Bio-Rad) and analysed with Image Lab Software 3.0 (Bio-Rad).

Gel excision where applicable, was performed using 25 μ L input PCR product resolved as above; visualization was achieved by using an XcitaBlue Conversion Screen and Filter (~470nm excitation peak, Bio-Rad) to protect DNA from UV damage using either ethidium bromide at an increased 0.1% (v/v) concentration or SybrSafe (Life Technologies) at 0.5x recommended manufacturer's recommendation. Isolated fragments were either purified using QIAquick Gel Extraction Kit (Qiagen) and GeneJET Plasmid Miniprep Kit (Thermo Fisher) DNA columns or Wizard SV Gel and PCR Clean-Up Kit (Promega).

2.1.2 Isothermal DNA assembly (Gibson assembly)

Gibson assembly was performed as described (Gibson, 2009), with modifications to the enzymes employed. A Phusion-like DNA polymerase expression plasmid (pKB_177) based on patent literature (Wang et al., 2011) was constructed and purified sequentially by heat-denaturation of undesired protein and subsequent IMAC purification, using Ni-NTA agarose (Qiagen, Cat. No. 30210). Titration of enzyme activity was performed against commercial standard (NEB, Phusion high-fidelity DNA polymerase, M0530S) and two generic 500bp amplicons. Likewise a *Taq* DNA ligase expression vector was constructed and purified analogously to the polymerase. Titration was performed against commercially obtained standard (NEB, *Taq* DNA ligase, Cat. No. M0208S). T5 exonuclease was commercially obtained (NEB, Cat. No. M0363S). 2 $\times$ Gibson assembly master mix reactions were

prepared and stored at -20 °C until further usage. For a typical two-part assembly, DNA concentrations after DpnI digest were either inferred from agarose gel analysis or determined spectrophotometrically after purification using either a QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28704) or preferably a SV Gel and PCR Clean-Up (Promega, Cat. No. A9281). Fragments were prepared in equimolar ratio, whilst maximising total DNA input for each 10 µL reaction. Where high purity of PCR products allowed, unpurified fragments were mixed in equivalent volumes and supplied in a final volume of 10 µL to 10 µL of 2× isothermal assembly mix, yielding a 20 µL reaction in total. Reactions were prepared on ice and incubated at 50 °C in a pre-heated C1000 cycler for at least 30 min up to 8 h. For difficult-to-assemble reactions with shorter than ideal overlap (<20 bp), a cycling method was employed. First the mixture was incubated at 50 °C for 10 min to denature any T5 exonuclease, then the reaction was cycled up to 90 times with the following programme (10 sec 40 °C, 10 sec 50 °C, 10 sec 60 °C, repeat). Default ramp rates of the C1000 machine were used. 5 µL were typically transformed into 50 µL aliquots of NEB 5-alpha (NEB, Cat. No. C2987H) homemade chemical competent cells or up to 1 µL in NEB 5-alpha homemade electrocompetent cells.

2.1.3 Splicing by overlap extension (SOE-PCR)

SOE-PCR reactions were performed as described but using Q5 high fidelity PCR polymerase instead (Higuchi et al., 1988; Horton et al., 1990).

2.1.4 Circular polymerase extension cloning (CPEC)

CPEC reactions were performed as described but using Q5 high fidelity PCR polymerase instead (Quan and Tian, 2009).

2.1.5 Desalting of DNA fragments by drop dialysis

Millipore DNA filter paper for dialysis (white mixed cellulose esters, 0.025 μ m Cat. No. VSWP01300 / VSWP02500) was floated onto ~20 mL of MiliQ water. DNA was spotted on top of the floating membrane and incubated for at least 30 min or until no further movement of the membrane across the water surface was observed.

*2.1.6 Transformation of *E. coli**

Initial cloning constructs were transformed into homemade chemically (heat) competent *E. coli* NEB 5 α cells (Hanahan, 1983). For difficult assemblies that did not yield colonies with heat competent cells, homemade electrocompetent *E. coli* MAX Efficiency DH10B (Invitrogen, now Thermo Fisher, Cat. No. 18297010) were employed in 0.1 mm electroporation cuvettes (Bio-Rad) (Dower et al., 1988; Greener and Jerpseth, 2002).

2.1.7 Miniprep of plasmid and BAC DNA

The GeneJET Plasmid Miniprep Kit (Thermo Fisher) and the QIAprep Spin Miniprep Kit (Qiagen) were used interchangeably. For routine isolation of high copy number plasmids (e.g. relaxed pUC origins as found on pGEM1 vector) 1.5 mL of culture was used. For isolation of medium copy number plasmids (e.g. ColE1 *ori* in combination with *rom* and *bom* genes such as pET21 derived plasmids), 5 mL of

culture was used. For isolation of low copy number plasmids (e.g. p15A or pSC101) up to 10-15 mL of saturated overnight culture were employed and buffer volumes doubled to allow efficient lysis and precipitation. For isolation of single copy entities such as bacterial artificial chromosomes (BACs, e.g. miniF or pBAC11) up to 200 mL of culture was used with the genomic DNA isolation procedure as outlined in section 2.1.9.

2.1.8 Maxiprep of plasmid DNA

ZymoPURE Plasmid Maxiprep Kit (Zymo Research, Cat. No. D4203) with minor modifications to the vacuum manifold protocol was used to prepare DNA for mammalian cell transfections. The recommended bacterial input volume was doubled (e.g. 800 mL / per column), as were the recommended buffer volumes used in the subsequent steps. For elution, a sequential 4 step elution procedure used pre-heated elution buffer (10mM Tris HCl, pH8.4 at ~ 60 °C) with 250 µL per fraction. All fractions were pooled in a single LPS free certified mini centrifuge tube.

2.1.9 Genomic DNA isolation from bacteria

Genomic DNA preparations from Gram-positive and -negative bacteria were performed as described (Syn and Swarup, 2000). Briefly 4 mL of dense culture derived pellet was resuspended in 0.75 mL 1% (w/v) NaCl. The mixture was centrifuged at maximum speed for 1 min and the supernatant (SN) was discarded. Washed pellets were resuspended in 0.75 mL TES (10mM Tris-HCl, 10mM EDTA, pH 8.0, 2% SDS) and incubated at 75°C for 15 min. 1mL of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the mixture vortexed

for 30 s at maximum intensity. Subsequent spin for 10 min yielded phase separation and the upper (aqueous) layer was slowly pipetted off into a fresh 2ml tube. Phenol/chloroform/isoamyl alcohol (25:24:1) extraction was repeated with another 1 mL of solution, spun again and the aqueous layer (~1 mL) supplemented with 1 μ L of glycerol, 100 μ L 3M sodium acetate [NaOAc, (pH 5.2)] and 1 mL isopropanol in that order.

The tube was inverted several times and centrifuged at maximum speed for 20 min, 4°C. The SN was discarded and 500 μ L of 70% (v/v) EtOH was added to the pellet incubated for 5 min at RT prior to another spin for 10 min.

Finally the DNA pellet was resuspended in 150 μ L TE (10mM Tris-HCl, 2mM EDTA, pH 8.0) with 1 μ L of 50 μ g/mL Ribonuclease (RNase).

2.1.10 Sequencing

All constructs were verified in their region of interest, typically open reading frame (orf) and, where deemed necessary, in regulatory sequences by Sanger chain-terminating dideoxynucleotide sequencing performed by Source BioScience (Nottingham, UK) or Eurofins Genomics (Ebersberg, Germany).

2.2 Protein expression and purification

2.2.1 Construction of bacterial expression strains

Strain	Source	Reference(s)
C41(DE3)	Anthony Watts (University of Oxford)	(Miroux and Walker, 1996)
BL21 Star (DE3)	Ben Berks (University of Oxford)	(Lopez et al., 1999)
ClearColi BL21(DE3)	Lucigen Corporation	(Mamat et al., 2015)

Table 2.1 Parental strains employed for purposes of protein expression. Strains were obtained either from the Mark Howarth laboratory repository or obtained from the individuals / procured from commercial sources as indicated.

pRIL and pRP were isolated sequentially from BL21-CodonPlus(DE3)-RIPL (Agilent / Stratagene, Cat. No. 230280) with intermediate selection in NEB 5-alpha cells and catalogued as pKB_232 (pRP) and pKB_242 (pRIL). pRIL provides extra tRNA copies for the codons AGG/AGA (Arg), AUA (Ile), and CUA (Leu). pRP provides extra tRNA copies for the codons AGG/AGA (Arg) and CCC (Pro) and thus corrects the codon usage of heterologous genes with different codon frequency compared to host (Rosano and Ceccarelli, 2014).

pFH255, catalogued as pKB_250, was a gift from Gianluca Veggiani. pFH255 is a pLysS derivative containing the gene for sulfhydryl oxidase Erv1p from *S. cerevisiae*, the gene for the mature form of DsbC from *E. coli* (cytosolic mutant) under an arabinose inducible promoter system. Upon induction with arabinose, this vector allows pre-expression of Erv1p and DsbC, resulting in enhanced disulfide bond formation and thus yield of disulfide rich proteins (Nguyen et al., 2011).

pKB_333 was constructed by inserting the tRNA encoding region of pRP into pFH255 (pKB_250) via Gibson assembly to avoid origin of replication and antibiotic marker conflicts of pRP and pFH255.

miniF-lysY was isolated from SHuffle T7 Express *lysY* (NEB, Cat. No. C3030H).

Sequences of plasmids used in this work are supplied in print and electronic format in the appendix.

Strain	Plasmids and modifications
C41(DE3) pFH255	pFH255 (Nguyen et al., 2011) (pKB_250) – pBad- <i>Erv1p-dsbC</i> , pT _{3.5} - <i>lysY</i> [CamR]
C41(DE3) miniF-lysY	miniF-lysY (pKB_233) – pT _{3.5} - <i>lysY</i> [CamR]
C41(DE3) pKB_333	pKB_333 – pBad- <i>Erv1p-dsbC</i> , pT _{3.5} - <i>lysY</i> , pTet-tRNA _{Arg} -tRNA _{Pro} [CamR]
BL21(DE3) pKB_333, pRIL	pKB_333 – pBad- <i>Erv1p-dsbC</i> , pT _{3.5} - <i>lysY</i> , pTet-tRNA _{Arg} -tRNA _{Pro} [CamR] pRIL- tRNA _{Arg} -tRNA _{Ile} -tRNA _{Leu} [StrepR]
BL21(DE3)-RIPL+	pKB_241 - pBBa_J23150- <i>pgl</i> , pTet-tRNA _{Arg} -tRNA _{Pro} [CamR] pRIL- tRNA _{Arg} -tRNA _{Ile} -tRNA _{Leu} [StrepR]
ClearColi BL21(DE3) pFH255, pRIL	pFH255 (Nguyen et al., 2011) (pKB_250) – pBad- <i>Erv1p-dsbC</i> , pT _{3.5} - <i>lysY</i> [CamR] pRIL- tRNA _{Arg} -tRNA _{Ile} -tRNA _{Leu} [StrepR]

Table 2.2 Strains constructed for purposes of protein expression. Existing parental strains were transformed via heat shock with the indicated plasmids or mutated as described below.

2.2.2 Media formulations and incubator parameters

Media were obtained from the Media Glassware Sterilization Laboratory provided by the Department of Biochemistry. Formulation and preparations are described as follows:

LB-Miller (high salt)

10 g tryptone, 5 g yeast extract and 10 g NaCl were dissolved in 1 L of Milli Q water and sterilized by autoclaving for 20 minutes, at 121 °C.

LB-Lennox (low salt)

Formulated as LB-Miller but with lower salt concentration: 5 g/L .

Terrific Broth

12 g tryptone, 24 g yeast extract, 4 mL of 87 % glycerol were dissolved into 900 mL distilled water and autoclaved as above. 100 mL of autoclaved 10x salt solution (23.13 g/L KH_2PO_4 , 123.41 g/L K_2HPO_4) were added aseptically.

2xTY

16 g tryptone, 10 g yeast extract and 5 g NaCl were dissolved into 900 mL Milli Q water, the pH was adjusted to 7.0, and the remainder filled to 1 L with Milli Q water; autoclaved as above.

Standard antibiotic concentrations employed unless otherwise stated:

Ampicillin 100 µg/mL

Chloramphenicol 34 µg/mL

Kanamycin	50 µg/mL
Streptomycin	100 µg/mL
Tetracycline	10 µg/mL

Unless otherwise stated bacterial cultures were grown in an Infors Multitron shaker (Infors UK Ltd, Reigate, UK) with 2.5cm throw at 200 rpm.

2.2.3 Standard E. coli protein expression

Unless otherwise stated, standard *E. coli* expression was performed in BL21-CodonPlus(DE3)-RIPL+ cells. Expression plasmids were transformed and cells selected on chloramphenicol plates supplemented either with ampicillin (pET15 and pET21 derivatives) or kanamycin (pET28 derivatives) overnight (16-24 h). Single colonies were inoculated late in the evening into 10 mL low salt LB, 1% (v/v) glucose and grown overnight (12-16 h) at 37 °C, 180 rpm. Cultures were diluted ~1:100 into fresh medium containing expression plasmid resistance antibiotic only and supplemented with 0.8% (v/v) glucose and grown until A600 0.6-0.8 prior to induction with 0.42 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 30 °C for up to 6h prior to harvest.

2.2.4 Mammalian cell maintenance

Adherent HEK 293T cells were grown in 175cm² angled neck cell culture flasks with vent caps (Corning, Cat. No. 431080). Each flask contained 20 mL Dulbecco's Modified Eagle Medium (DMEM), 10% foetal calf serum (Sigma), 50 U/mL penicillin, 50 µg/ml streptomycin (Sigma) and was incubated in a humidified

chamber at 37 °C, 5 % CO₂, until confluency was reached at ~90%. Suspension HEK293E cells were cultured by Andrew Ishizuka as described in section 2.2.18.1.

2.2.5 Immobilized metal affinity chromatography (IMAC) of monomeric proteins

Unless otherwise described, monomeric His₆-tagged proteins were purified by resuspending cell pellets in Ni-NTA binding buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 7.8) containing 1 mM phenylmethane sulfonyl fluoride (PMSF) and 0.5x manufacturer recommended cOmplete mini EDTA-free protease inhibitor cocktail. Lysozyme was added to 0.1 mg/mL, either benzonase was added to 5-10 U/mL or DNase (Sigma) was added to 10 µg/mL. Resuspended cells were sonicated on ice for four times at 50% pulse for 30 s, with 1 min between each pulse, using a Sonics Vibra-Cell sonicator (Ultrasonic Processor). For soluble protein, cell debris was spun at 17,000 g, 4°C for 10 min and the supernatant was isolated and loaded onto MilliQ washed and 10 mM imidazole Ni-NTA binding buffer-equilibrated Ni-NTA resin (Qiagen). The mixture was incubated at 4°C for 15-30 min on a side-over-side roller (Stuart Equipment) and then centrifuged at 2,000 g for 1 min to collect the resin. After binding of His₆-tagged proteins, the Ni-NTA resin was washed with a minimum of 10 column volumes (CV) 10mM imidazole Ni-NTA buffer, transferred to a Polyprep column (BioRad) and washed with Ni-NTA buffer containing 15 mM imidazole followed by washing with Ni-NTA binding containing 30 mM imidazole. Protein elution was performed with Ni-NTA binding buffer containing 100 mM imidazole and Ni-NTA binding buffer containing 200 mM imidazole typically collecting 0.5 mL fractions. Protein purity was assessed by SDS-PAGE. Eluate was dialysed through a 3.5 kDa MWCO membrane

against either 1,000-fold excess of phosphate buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 2 mM KH_2PO_4 , pH 7.4) or Ni-NTA buffer, replacing dialysis buffer three times at 4°C.

2.2.6 Amylose agarose chromatography

In several cases MBP protein fusions were additionally purified after IMAC purification to obtain homogenous protein. 100 μL of packed amylose resin (NEB, Cat. No. E8021S) was applied to a polyprep column (Bio-Rad), rinsed with 10 mL MilliQ water and equilibrated with 15 CV of Tris buffered saline (TBS: 50 mM Tris HCl, 150 mM NaCl, pH 8.0). Ni-NTA elutions containing imidazole were directly applied to the resin by gravity flow and washed three times with 15 CV TBS. Elution was with TBS buffer containing 50 mM maltose followed by dialysis as described in the IMAC purification procedures.

2.2.7 Gel filtration chromatography

Unless otherwise indicated, gel filtration, or size exclusion chromatography, was performed on a fast protein liquid chromatography (FPLC) system ÄKTA pure 25 (GE Healthcare) at 4 °C. Unless otherwise stated the following columns were used. For proteins of 3-70 kDa, a HiLoad 16/600 Superdex 75 pg (GE Healthcare) column was employed at a flow-rate of 1 mL/min. For proteins or multimers of 60-600 kDa, a HiLoad 16/600 Superdex 200 pg (GE Healthcare) column was employed at a flow-rate of 1 mL/min. For MDa multimer preparations HiPrep 16/60 Sephacryl S-400 HR and HiPrep 16/60 Sephacryl S-500 HR (GE Healthcare) columns were

employed at a flow-rate of 1 mL/min interchangeably. All columns were calibrated against a commercial molecular weight standard (BioRad, Cat. No. 151-1901).

2.2.8 Expression of SpyCatcher-VLPs

The *E. coli* strain C41(DE3) pFH255 was transformed with pGEM-SpyCatcher-AP205cp3 (pKB_215) and plated on LB-Lennox agar plates supplemented with ampicillin and chloramphenicol. The transformation was incubated at 37 °C for 16–20 h. A total of 4 colonies were entirely transferred into one 2 L strongly-baffled glass flask (Scientific Laboratory Supplies, Cat. No. FLA4198), containing 400 mL LB-Lennox medium with 20 µg/mL ampicillin and chloramphenicol. The flask was incubated standing for 10–14 h at 37 °C. The culture was then back-diluted 1:1 with fresh LB-Lennox media and supplemented with arabinose to 2.5 g/L and ampicillin to 20 µg/mL (assuming that all previous ampicillin was degraded) prior to shaking incubation at 120 rpm at 37 °C for 1 h followed by induction at A600 ~0.9 with 1 mM IPTG for 6–8 h at 37 °C, shaking at 180 rpm. In later experiments supplementation of arabinose and IPTG was omitted as it was not deemed necessary.

2.2.9 Purification of SpyCatcher-VLPs.

PBS-washed bacterial pellets derived from 1 L shaking flask culture were resuspended in 10 mL lysis buffer: 20 mM Tris•HCl, 150 mM NaCl, 0.1% (v/v) Triton X-100, 0.1 % (v/v) Tween 20, pH 7.8, 0.1 mg/mL lysozyme at RT. After 15 min incubation at RT, the mixture was placed in an ice-salt water bath for 10 min and sonicated 4 times for 30 s, with 1 min between each pulse. The lysates were

cleared twice via centrifugation in a JA-17 rotor (Beckman Coulter, Wycombe, UK) at 15,000 *g* for 20 min at 4 °C. The supernatant was first filtered using a 1.2 µm glass filter device (Sartorius AG, Göttingen, Germany) and subsequently through a surfactant-free cellulose acetate filter device with 0.45 µm pore size (Nalgene, Rochester, USA). 250 U benzonase (Sigma-Aldrich Ltd, Gillingham, UK) was added and the mixture incubated for 5 min at RT. NTA elution buffer (50 mM Tris•HCl, 300 mM NaCl, 1 M imidazole, pH 7.8) was added to a final concentration of 75 mM imidazole to favour multimeric protein binding. 1 mL of packed Ni-NTA agarose (Qiagen Ltd, Manchester, UK) was added to the cleared lysate. After 10 min incubation at 4 °C on a rotary shaker, the sample was centrifuged for 2 min at 4,700 *g*, 4 °C. Three washes with 15 CV 100 mM imidazole washing buffer was used to deplete contaminants and non-assembled coat protein-SpyCatcher fusions. Subsequently the resin was once more added to the imidazole-conditioned cleared lysate, the process repeated and finally resuspended in 15 mL 100 mM imidazole NTA-buffer. The sample was split into three 5 mL fractions in 15 mL Falcon tube and each tube was filled up to 15 mL with 100 mM washing buffer containing 0.1% (v/v) Triton X-114 (TX114), to remove pyrogens (Zimmerman et al., 2006). Two more washes with full volume 0.1% TX114, 100 mM imidazole were applied, prior to three ice-cold washes without TX114 to remove the detergent. The sample applied to a polyprep column; washed once more with 100 mM washing buffer containing no TX114, to allow the resin to settle. For elution, the column was capped and 2.5 mL of elution buffer [2 M imidazole, 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.5] was added to the resin. This mixture was incubated for 10 min at RT prior to elution. The eluate was once more cleared using a mini polyprep

column (Bio-Rad) to remove any agarose resin. The flow-through in endotoxin free certified 1.5 mL centrifuge tubes was centrifuged for 10 min at 4 °C at 17,000 *g*, pipetted into a 300 kDa MWCO cellulose ester dialysis tubing (SpectrumLabs, Spectrum Europe B.V., Breda, The Netherlands) and dialysed overnight at 4 °C against 1,000-fold excess of 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.0 for buffer exchange and to deplete capsid protein monomers and contaminants. Dialysis was repeated in fresh buffer for an additional 3 h, followed by collection of the sample and a final centrifugation step at 17,000 *g* for 10 min at 4 °C, to remove any aggregate. Centrifugation was repeated 24 hours later. The protein was stored at 4 °C prior to use and was stable for at least 3 months as assayed by SDS-PAGE, retaining its integrity for at least 1 month when used for immunization purposes.

2.2.10 Improved expression and purification of SpyCatcher(Duo)-VLPs

C41(DE3) was transformed with pGEM-SpyCatcher-AP205cp3 (pKB_215). A total of 4 colonies were entirely transferred into one 2.5 L bottom-baffled polypropylene flask [Thompson UltraYield flasks without AirOtop seals (2.5L, Thomson, Cat. No. 931136-B)], containing 500 mL pre-warmed 2xTY medium with 20 µg/mL ampicillin. The flask was incubated shaking at 30 rpm for 10-14 h at 37 °C. The culture (at this point A₆₀₀ 0.6) was then diluted 1:1 (500 mL) with pre-warmed, fresh 2xTY and supplemented with ampicillin to 20 µg/mL (assuming that all previous ampicillin was degraded). The culture (without prior cooling) was incubated shaking at 100 rpm at 22 °C for up to 2 h until induction at A₆₀₀ ~0.6-0.7 with 0.42 mM IPTG for 18 h at 22 °C, vigorously shaking at 200 rpm. Foaming

was controlled by addition of Antifoam A (Sigma, Cat. No. A5633) just prior to induction, and re-applied when necessary until 2-3 h post induction (i.e. not controlled overnight). Cells were harvested, washed as before and resuspended in lysis buffer (10-15 mL/L cell culture pellet, lysis buffer: 20 mM Tris•HCl, 150 mM NaCl, 0.1% (v/v) Triton X-100, 0.1 % (v/v) Tween 20, pH 7.8, 0.1 mg/mL lysozyme, 25 U/mL benzonase) and stored at -80°C until further processing.

Lysis and clearance were performed as before and protein was bound to 0.5 mL of packed Ni-NTA agarose (per L cell culture pellet) by incubation for 30 min at 4°C on a rolling shaker. The resin was applied to a BioRad polyprep column and the flow-through once more re-applied to the resin. The resin was washed 3x with 15 CV 75 mM imidazole washing buffer. Importantly this buffer contained only 150 mM NaCl and was set to pH 7.8. LPS depletion with 0.1% (v/v) Triton X-114 (TX114), where applicable, was also performed with reduced salt concentrations throughout and on the polyprep column. Elution, dialysis and storage were performed as before (section 2.2.9).

2.2.11 Size-exclusion chromatography of SpyCatcher-VLPs for size determination

SpyCatcher-VLPs (5 mL at A280 7) derived from IMAC purification were spun for 30 min at 17,000 g and the supernatant applied to a HiPrep 16/60 Sephacryl S-500 HR (GE Healthcare) on a FPLC system (Purifier 10, GE Healthcare). The mobile-phase was 20mM Tris-HCl, 1M NaCl, pH 8.5. The applied flow-rate was 1.0 mL/min and the run was performed at 4 °C. Elution was monitored at 260 and 280nm. A high molecular weight gel filtration standard (Bio-Rad, Cat. No. 151-1901) was used for size approximation.

2.2.12 Transmission electron microscopy (TEM)

A freshly glow-discharged carbon 200 mesh copper grids was incubated with 10 μ L SpyCatcher(Duo)-VLPs (0.2 mg/mL) for 2 min, blotted with filter paper, and stained with 2% uranyl acetate (w/v) for 10 s, followed by blotting and air drying. Loaded grids were imaged in a FEI Tecnai T12 transmission electron microscope at 120 kV using a Gatan US1000 CCD camera. Particle diameter for each group was determined by manually measuring diameters for hand-picked particles (n=100) and processed with FIJI (ImageJ, version 2.0.0) with 2 nm bin size and plotting in GraphPad Prism (version 6.0, GraphPad Software).

2.2.13 Calculation of maximum numbers of Catcher residues in cross section view

The number of possible residues over the circumference was calculated as described below and rounded up to the next full integer. TEM measurements served as the basis for size estimation of SpyCatcher-/ AP205 carrier VLPs (see section 3.1.3), X-ray data [PDB 4MLS, (Li et al., 2014)] and maximal theoretical length of the 3x GSG linker [0.38nm per residue, (Berg, 2002)] were taken into account.

20	nm	observed radius
4.5	nm	SpyCatcher Δ N1 length
2.9	nm	SpyCatcher Δ N1width
0.4	nm	length of single linker residue
3.4	nm	theoretical length of GSGGSGGSG linker

12	nm	AP205 VLP radius derived by subtraction of Catcher and linker
76	nm	AP205 carrier VLP circumference
1834	nm ²	AP205 carrier VLP surface area

For presumed 120-mer (T=1)

30.6	nm ²	available per Catcher-residue
8.4	nm ²	required per Catcher residue
3.9	nm	assumed square with presumed length
19	#	Theoretical squares / Catcher equivalents over circumference

For presumed 180-mer (T=3)

10.2	nm	available per Catcher-residue
8.4	nm ²	required per Catcher residue
3.2	nm	assumed square with presumed length
24	#	Theoretical squares / Catcher equivalents over circumference

2.2.14 Expression of model proteins SpyTag (DA)-MBP

SpyTag-MBP and SpyTag DA-MBP were expressed and purified as previously described (Zakeri et al., 2012) with minor modifications; briefly, IMAC purification was followed by a single amylose affinity chromatography step (see section 2.2.6) to remove minor impurities with similar Mw to unconjugated SpyCatcher-VLPs.

2.2.15 Expression of CIDR proteins

ClearColi BL21(DE3) pFH255 pRIL was transformed with pET15b-CIDR(IT4var07), pET15b-SpyTag-CIDR(HB3var03) or pET15b-SpyTag-CIDR(IT4var07) and selected on LB-Miller (10 g/L NaCl), supplemented with chloramphenicol and ampicillin. Single colonies (visible after 36-48h) were inoculated into LB-Miller ampicillin, 20 µg/mL chloramphenicol, 30 µg/mL streptomycin for 15h at 37 °C, 180 rpm shaking. The cultures were diluted 1:75-100 into 750 mL LB in 2L strongly baffled glass flasks and grown to A600 0.6. First accessory proteins were induced with 2.5 g L-arabinose, grown as before until A600 of 0.7-1.0 and the CIDR expression was induced with 1mM IPTG for 12-14 h at 22 °C, shaking at 250rpm prior to harvest. Pellets derived from each culture were washed in 50 mL 4 °C cold PBS (pH 7.4) and spun down for 10 min at 4000 g. The supernatant was discarded and the pellets (typical wet cell weight yield 2.5-5 g per litre of culture) were then stored at -20 °C for up to 4 weeks until further processing.

2.2.16 Alternative expression of CIDR proteins

CIDR proteins (pKB_229, 299) for non-immunological purposes were prepared from soluble protein derived from SHuffle T7 Express *lysY* (NEB, Cat. No. C3030H). A single colony was inoculated into 10 mL LB low salt, overnight 37°C and was diluted 1:100 into 1L of pre-warmed 2xTY media, containing 100 µg/mL ampicillin (Thompson, 2.5L UltraYield flask). Cells were grown to A600 0.8-1 and induced with 0.42 mM IPTG after cooling for 30 min to 22°C and expression for 18h at 200 rpm shaking. Cell harvest as described before (section 2.2.15).

2.2.17 Purification of CIDR proteins

Each cell pellet was resuspended in 12 mL lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 15mM imidazole, pH 8.0, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mg/mL lysozyme, 2U/mL benzonase), vortexed every 2-3 min. After thawing, this mixture was kept on ice for 30 min and sonicated with 4× 30 s pulses, 1 min rest between each pulse. Lysed cell samples (3x) were then collapsed into one 45 mL polypropylene centrifuge tube and the insoluble fraction spun down at 27,000 *g*, 4 °C using a JA-20 rotor. For pKB_225, 229 and 299, the soluble protein in the supernatant was purified using regular IMAC purification protocols. pKB_228 alone was purified from inclusion bodies as described previously (Lau et al., 2015). Briefly, inclusion bodies were resuspended in 10 mL denaturation buffer (6 M Guanidinium-HCl, 20 mM Tris-HCl, 300 mM NaCl, 15mM imidazole, pH 8.0), β -mercaptoethanol was added to 10 mM final concentration and the inclusion bodies were dissolved overnight at room temperature on a rotary shaker. Protein was then bound to 0.5 mL Ni-NTA resin and sequentially refolded using increasing volumes (2.5 mL per step) of exchange buffer (20 mM Tris, 300 mM NaCl, 15mM imidazole, 3 mM reduced glutathione, 0.3 mM oxidised glutathione, pH 8.0) mixed with decreasing volumes of denaturation buffer. Total washing buffer volume was 25 mL per step, driven by gravity flow in a polyprep column with a total of 11 steps. Protein was eluted with Ni-NTA elution buffer (200 mM imidazole, 20 mM Tris, 300 mM NaCl, pH 8.0). 6xHis-tagged tobacco etch virus protease (TEV) purified in redox buffer (20 mM Tris, 300 mM NaCl, 3 mM reduced glutathione, 0.3 mM oxidised glutathione, pH 8.0) from ClearColi BL21(DE3)-RIPL carrying a MBP-TEV_site-6xHis-TEV_protease was added at 1:100 A280 equivalents (Cabrita et al.,

2007). The digest was performed in the presence of 3mM reduced, 0.3mM oxidized glutathione redox buffer for 4h at 30 °C. The processed eluate was then dialysed twice against 1000-fold excess 20 mM Tris-HCl, 300 mM NaCl, pH 7.8 with a 3.5 kDa MWCO cut-off (Fisher Scientific, Cat. No. BID-050-010F) and applied to 0.5 mL of binding buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.8) equilibrated Ni-NTA resin to capture uncleaved protein, TEV protease and cleaved 6xHis-tag. The flow-through was then concentrated to a final volume of 1 mL using 3.5kDa MWCO cut-off spin filter (GE Healthcare, Cat. No. 28-9322-23) and applied to a HiLoad 16/600 Superdex 75 pg column (GE Healthcare Cat. No. 28-9893-33). The column was equilibrated with 1.5CV column buffer (20 mM HEPES, pH 7.5, 500 mM NaCl) at a flowrate of 1 mL/min. The expected size range was analysed by SDS-PAGE and desirable fractions concentrated to a final concentration of 10-15 mg/mL prior to storage at -80 °C until further usage. Faint extra bands in the pKB_225 and pKB_228, pKB_229 and pKB_299 samples (CIDR samples) visible by SDS-PAGE may either be related to uncleaved N-terminus, post-translational modification by *E. coli* promoted by the 6xHis-tag (Geoghegan et al., 1999), re-folding artefacts or different folding states which however did not affect isopeptide bond formation.

2.2.18 Expression of Pfs25-SpyTag

2.2.18.1 Initial Pfs25-SpyTag expressions for SpyCatcher-VLP experiments

Initial preparations were performed by Andrew Ishizuka. Pfs25 employed in this work was N- and C-terminally truncated (AA 22-193) in order to remove signal sequence and transmembrane respectively, as well as cleared of potential N-linked glycosylation by mutation of asparagines to glutamines (Barr et al., 1991; Goodman

et al., 2011). Plasmid DNA for tPA-Pfs25-SpyTag-Ctag (catalogued as pKB_311) was amplified in Stellar Competent *E. coli* (ClonTech Cat #636763) and purified by QIAGEN Plasmid Plus Maxi kit (QIAGEN Cat #12965). Suspension HEK293E cells were cultured to 2 million cells / mL in Expi293 Expression Medium (Life Technologies Cat #A1435101) containing penicillin/streptomycin at 37°C and 8% CO₂ at 125 rpm. Cells were transiently transfected with 1 µg plasmid complexed to polyethylenimine per million cells. Supernatant was harvested after 72 h.

2.2.18.2 Subsequent expression for Pfs25-SpyTag:SpyCatcher-IMX experiments

Plasmid DNA for tPA-Pfs25-SpyTag-Ctag (catalogued as pKB_311) was obtained from Andrew Ishizuka and propagated in NEB 5-alpha. HEK 293T cells at 1 million cells / mL were complexed with 6.4 µg/mL maxiprep DNA and 4.6 µg/mL polyethylenimine (25 kDa, branched, Sigma) in DMEM containing, 25 mM HEPES, supplemented with 1 % (v/v) penicillin / streptomycin and 4 mM glutamine for 16 h. One equivalent volume of fresh medium containing 2x valproic acid was added to yield a final concentration of 3.8 mM valproic acid prior to further incubation for 84 h. The supernatant was replaced with fresh medium, spent supernatant was spun at 17,000 *g* for 10 min, 0.22 µM syringe-filtered, supplemented with 1mM PMSF, 1x manufacturer recommended cOmplete mini EDTA-free protease inhibitor cocktail (Roche) and stored at -80 °C until further processing. After an additional 8 days of incubation, the 2nd supernatant was collected and processed in the same manner.

2.2.19 Purification of Pfs25-SpyTag

Frozen supernatant (~440 mL) was thawed on ice, centrifuged at 17,000 *g* for 10 min and incubated for 30 min at 4 °C with 2 mL of equilibrated CaptureSelect C-tag affinity matrix (Life Technologies, Cat. No. 193307005) prior to 30CV washes with PBS, pH7.4 at 4 °C. Sample was eluted with PBS containing 2 M MgCl₂, pH7.0, followed by size-exclusion chromatography into PBS, pH 7.4 on a HiLoad 16/600 Superdex 200 (Andrew Ishizuka) or 75 pg column (GE Healthcare). The eluate was analysed by (non)-reducing SDS-PAGE. Fractions containing target protein were pooled and concentrated to ~1 mg / mL prior to centrifugation for 10 min, 17,000 *g* and storage at -80 °C.

2.2.20 Protein gel electrophoresis

2.2.20.1 SDS-PAGE

2.2.20.1.1 Tris-glycine SDS-PAGE

SDS-PAGE was performed using Tris-glycine gels in combination with an XCell SureLock Mini-cell gel chamber (Life Technologies). Samples were mixed with 6× SDS loading buffer (0.23 M Tris HCl pH 6.8, 24% v/v glycerol, 120 µM bromophenol blue, 0.23 M SDS) and heated to 95 °C for 3 min using a C1000 thermal cycler. Tris-glycine gels were typically run at 200 V (~20 V/cm) for 1 h at room temperature in Tris-glycine running buffer (25 mM Tris·HCl, 192 mM glycine, and 0.1% (w/v) SDS. PageRuler (Thermo Fisher, Cat. No. 10066) was used as the standard molecular weight calibration marker.

2.2.20.2 (Native) agarose gel electrophoresis (NAGE)

2.2.20.2.1 NAGE of SpyCatcher-VLPs

An agarose gel [1% (w/v)], prepared with 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0, containing 1× SybrSafe (Life Technologies) according to manufacturer's recommendations was loaded with 20 µL of 15 µM SpyCatcher-VLPs, either conjugated or not in the presence of 30 µM SpyTag-CIDR(IT4var07) or blank buffer in DNA loading buffer [0.15 g/mL Ficoll 400 (Sigma), 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0 supplemented with Orange G (Sigma)]. The samples were run at 120 V for 1h (~7.7 V/cm) in Tris acetate EDTA buffer (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). First the gel was imaged for SybrSafe staining on a UV table equipped with an XcitaBlue™ screen kit for conversion of UV to blue light; e.g. excitation with blue light (~488nm) in combination with a blue light filter on a ChemiDoc XRS+ Imager (Bio-Rad), documented using QuantityOne (version 4.6) software. The gel was then stained with InstantBlue colloidal Coomassie stain and imaged with visible white light illumination using a ChemiDoc XRS imager and documented using Image Lab Software [Bio-Rad, version 3.0 (Mac OS)].

2.2.20.2.2 NAGE of SpyCatcherDuo-VLPs

NAGE for SpyCatcherDuo was performed as in section 2.2.20.2.1 but 30 µL SpyCatcherDuo-VLPs 15 µM (after dialysis and aggregate removal by spinning) were loaded. No SpyTag-CIDR was added.

2.2.20.3 Coomassie blue-staining

SDS-PAGE and native agarose gels were stained for protein either in InstantBlue Coomassie stain (Expedeon) or Quick Coomassie Stain (Generon) interchangeably. Destaining was performed with three successive washes in excess MilliQ water. Imaging was performed on a ChemiDoc XRS+ Imager (Bio-Rad) and analysed with the Image Lab Software [Bio-Rad, version 3.0 or 5.2.1(Mac OS)].

2.2.21 *SpyCatcher-VLP:SpyTag-protein reactivity*

1.5× molar excess of SpyTag interaction partner in a total volume of 150 µL including 15 µL of 10× reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2) was used to react SpyCatcher-VLPs. Reactions that did not fill the set volume of the reaction; e.g. reactions in which SpyCatcher-VLPs and SpyTag-partner did not amount to 150 µL or no partner was added for control purposes, were brought to volume with 20 mM Tris·HCl, 300 mM NaCl, pH 7.8. SpyTag-protein concentrations were: SpyTag-MBP, 82.5 µM; SpyTag DA-MBP, 87 µM; SpyTag-CIDR(HB3var03), 144.3 µM; SpyTag-CIDR(IT4var07, TEV cleaved), 263.5 µM; Pfs25-SpyTag, 59µM. The reaction was performed for 3 h at RT and stopped by boiling for 3 min at 95 °C with 6× SDS-PAGE loading buffer [20% glycerol, 100 mM Tris·HCl, 4% SDS, 0.2% bromophenol blue, 1% (v/v) 2-mercaptoethanol, pH 6.8].

2.2.22 SpyCatcher-VLP:SpyTag-peptide reactivity

2.2.22.1 SpyTag-peptides

SpyTag-peptides were synthesized and validated by HPLC as well as mass spectrometry at >95% purity (Insight Biotechnology). SpyTag-Telo: SpyTag linked to a human telomerase reverse transcriptase mutant epitope implicated with increased risk of various cancers (biotin-GAHIVMVDAYKPTREARPELLTSRLRFIPK) (Kokhaei et al., 2007). Stock solution of SpyTag-Telo was prepared at 100 mg/mL in a 2:1 mixture of H₂O to acetic acid. This stock was diluted 10-fold with VLP buffer (50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.0) prior to conjugation.

EGFRvIII-SpyTag spans the fusion junction epitope of human epidermal growth factor receptor (EGFR) present in many human glioblastoma (LEEKKGNYVVDHGAHIVMVDAYKPTK-biotin) (Gedeon et al., 2013). The peptide was dissolved in a 1:4 mixture of formic acid to H₂O, yielding a concentration of 100 mg/mL. This EGFRvIII-SpyTag stock was 10-fold diluted with 10× reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2) prior to conjugation.

2.2.22.2 SpyCatcher-VLP:SpyTag-peptide reactivity

SpyCatcher-VLPs were expressed and purified as before but washed with NTA imidazole buffer (50 mM Tris•HCl, 300 mM NaCl, pH 7.8, 0.1% v/v Tween 20, 200mM imidazole) and eluted with NTA buffer containing 2 M imidazole with no subsequent dialysis. SpyCatcher-VLPs at ~110µM (2.9 mg/mL) were mixed with a 2.4× molar excess of SpyTag-Telo or a 2.7× molar excess of EGFRvIII-SpyTag (matched to single SpyCatcher moieties). The sample was incubated for 3 h at RT

after addition of 10× reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2). To control for any change in the buffer altering the gel mobility of SpyCatcher-VLPs due to solvent possibly present in peptide preparations; control sample SpyCatcher-VLPs were mixed for 3 h at RT with a solution matching the solvent concentration of the peptide samples (acetic acid for SpyTag-Telo or formic acid for EGFRvIII-SpyTag); no reaction buffer was supplied. Furthermore a solvent and reaction buffer control sample was prepared in the same manner but additionally phosphate-citrate reaction buffer (10×: 400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2) was included. Reactions were stopped as before by adding 6× SDS-PAGE loading buffer and heating for 3 min at 95 °C. Samples were analysed by 14% SDS-PAGE with Coomassie staining.

2.2.23 SpyCatcher-VLP reactivity after heating

SpyCatcher-VLPs were expressed as before but with slight modifications. The expression host was BL21Star (DE3) (Invitrogen, Cat. No. C601003). 5 colonies were picked and inoculated into 500 mL 2xTY, 20 µg/mL ampicillin, 1% (v/v) glucose, 37 °C, 30 RPM overnight (12-16h), in Thompson UltraYield flasks without AirOtop seals (2.5L, Thomson, Cat. No. 931136-B). Cultures were diluted 1:1 with fresh 2xTY medium, containing 40µg/mL ampicillin. Cells were grown shaking at 100 RPM, 37 °C to A600 0.7, cooled down shaking to 22 °C prior to induction with 0.42 mM IPTG and shaking at 200 RPM for 16-18 h. VLP lysis and IMAC washing buffer were as described but only contained 150 mM NaCl throughout. Samples were dialysed, spun and stored at 4 °C until further usage and for control purposes.

200 μ L SpyCatcher-VLPs (25 μ M) were incubated at 65 °C in 2 mL polystyrene screw cap tubes for 16h. Samples were removed from the incubator and allowed to cool to room-temperature for 1 h prior to centrifugation for 30 min, 17,000 g , 4 °C. The supernatant was transferred to new vials and analysed spectrophotometrically in the near UV range for estimation of potential loss of samples. No major differences were observed and concentration was assumed to be that prior the heating step. 10 μ M heat treated SpyCatcher-VLPs and non-heated control SpyCatcher-VLPs stored at 4 °C were incubated with 2 fold molar excess of SpyTag-AffiHer2 (pKB_096) in the presence of 10 $\times$ reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2) in a total volume of 50 μ L for 2 h, RT (24 °C), prior to analysis by reducing SDS-PAGE.

2.2.24 Preparation of SpyCatcher-VLP immunogens

2.2.24.1 SpyCatcher-VLPs:SpyTag-CIDR and control immunogens

SpyCatcher-VLPs (30 μ M) were incubated with 1.5 $\times$ molar excess of SpyTag-CIDR (IT4var07, TEV cleaved to remove His₆-tag) for 3 h at RT with addition of 10 $\times$ reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2). Unreacted SpyTag-CIDR was removed by dialyzing twice against 1,000-fold excess PBS, 0.1% (v/v) Tween 20, through a 300 kDa cut-off membrane (SpectraPor). 20 μ g dose-matched CIDR(IT4var07)-equivalents for the SpyCatcher-VLP + untagged CIDR(IT4var07) group and the CIDR(IT4var07)-alone groups were diluted with sterile endotoxin-free PBS (Life Technologies), 0.1% (v/v) Tween 20 to a total of 50 μ L per dose. Two separate batches prepared within 24 h prior to immunization

were 0.22 µm-filtered using a small retention autoclaved polyvinylidene difluoride (PVDF) syringe filter membrane (Whatman).

2.2.24.2 SpyCatcher-VLPs:Pfs25-SpyTag and control immunogens

SpyCatcher-VLPs (30 µM) were incubated with 1.5× molar excess of Pfs25-SpyTag for 3 h at RT after addition of 10× reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2). The reaction was then dialysed as before to remove unreacted Pfs25-SpyTag. 2.5 µg Pfs25-equivalent dose-matched groups were prepared by dilution with sterile 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20 to a total of 50 µL per dose or 25 µl per dose in groups containing adjuvant. AddaVax (InvivoGen) adjuvanted groups were mixed 1:1 with stock with gentle pipetting and kept on ice until immunization within 2 h. An AddaVax adjuvanted negative control group for microscopy was prepared analogously, replacing Pfs25 with ovalbumin as the immunogen.

2.2.25 Endotoxin quantification and removal

Endotoxin concentrations were quantified with a Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific) following manufacturer's instructions. Values were below 1 Endotoxin Unit/mL (EU/mL) for SpyCatcher-VLP preparations, achieved by on-resin wash with Triton X-114 (Sigma) during IMAC purification (Zimmerman et al., 2006). Control and monomeric SpyTag-protein preparations exceeding 1 EU/mg were LPS-depleted via Triton X-114 (Sigma) phase separation as described (Aida and Pabst, 1990).

2.2.26 SpyCatcher-VLPs immunizations

Animal experiments and procedures were performed by Dr Darren Leneghan according to the UK Animals (Scientific Procedures) Act Project License (PPL 30/2414 and 30/2889) and approved by the Oxford University Local Ethical Review Body. Female, age-matched (~60 days old at first immunization) BALB/c mice (Harlan, UK), were housed in specific-pathogen free environments, injected intramuscularly with immunogens into each rear leg using a protein prime-boost regime. CIDR immunizations were administered on day 0, followed by serum collection at day 13 and boost on day 14 prior to cardiac bleed on day 28. Pfs25 immunogens were administered on day 0, followed by serum collection on day 16, boost on day 17 and cardiac bleed on day 34. Protein samples for injection were prepared as described in section 2.2.24. Blood samples were taken to obtain sera for endpoint ELISA. Sera were obtained from whole blood by leaving samples overnight at 4 °C to clot, followed by 5 min centrifugation at 16,000 g in a benchtop centrifuge at RT. Sera were pipetted into fresh Eppendorf tubes with care not to disturb the pellet. 6 mice were used per condition.

2.2.27 Endpoint ELISA

Endpoint-ELISAs were performed by Dr Darren Leneghan. Nunc-Immuno MaxiSorp plates (Thermo Scientific) were incubated with 100 µL/well of CIDR(IT4var07) or SpyCatcher-VLP at 1 µg/mL in coating buffer (15 mM sodium carbonate with 35 mM sodium bicarbonate) overnight at 4 °C. Plates were washed with PBS/T [PBS, 0.5% (v/v) Tween 20] and blocked for 1 h at RT with 10% skimmed milk in PBS/T. Serum samples were added in duplicate and diluted 3-

fold across the plate, followed by incubation for 2 h at RT and subsequent wash with PBS/T as before. Alkaline phosphatase (AP) conjugated to goat anti-mouse total IgG (Sigma-Aldrich) was added to the plate (1:3,000 dilution in PBS/T) for 1 h at RT. After a final wash with PBS/T, p-nitrophenylphosphate (pNPP) (Sigma-Aldrich) diluted in diethanolamine buffer (1 M diethanolamine, pH 9.8) (Thermo Scientific) was used as AP substrate. A405 was acquired using an ELx800 absorbance microplate reader (Biotek). The endpoint titre was defined as the x-axis intercept of the dilution curve at an absorbance value (± 3 s.d.) greater than the A405 for a serum sample from a mouse immunized with an irrelevant protein control, Pfs25-nanoparticle from a previous experiment.

Endpoint-ELISA against Pfs25 was performed according to a standardized protocol using a reference serum, as described previously (Miura et al., 2008). Briefly, Nunc-Immuno MaxiSorp plates were coated with Pfs25 protein at 0.1 μ g per well in PBS/T overnight at RT, washed with PBS/T and blocked for 1 h with 5% skimmed milk in PBS/T at RT. Test serum samples were diluted in PBS/T and 100 μ L of each were added to the relevant well, followed by incubation for 2 h at RT, and then washing with PBS/T six times at RT. Donkey anti-mouse total IgG conjugated to AP (Jackson ImmunoResearch Laboratories) was added to the plate (1:3,000 dilution in PBS) for 1 h at RT. After a final wash with PBS/T, samples were developed with pNPP and measured as described above. All samples were tested against a serially-diluted standard reference serum with known antibody titre, as described previously (Wu et al., 2006). Absorbance of individual test samples was converted to Pfs25 Antibody Units (AU) using a standard curve generated with this standard reference serum as described (Miura et al., 2008).

2.2.28 Statistical analysis of immunizations

Statistical analyses were performed using GraphPad Prism. Continuous variables between two groups were compared by a two-tailed Mann-Whitney test. Descriptive statistics were calculated as following:

For Figure 3.13	Median Day 13	Median Day 28
CIDR	1	246
SpyCatcher-VLPs + CIDR	1	1
SpyCatcher-VLPs:SpyTag-CIDR	2404	50350

Day 13

CIDR vs. SpyCatcher-VLPs:SpyTag-CIDR

$U=0$, $n_1=n_2=6$, $p= 0.0022$

SpyCatcher-VLPs + CIDR vs. SpyCatcher-VLPs:SpyTag-CIDR

$U=0$, $n_1=n_2=6$, $p= 0.0022$

Day 28

CIDR vs. SpyCatcher-VLPs:SpyTag-CIDR

$U=0$, $n_1=n_2=6$, $p= 0.0022$

SpyCatcher-VLPs + CIDR vs. SpyCatcher-VLPs:SpyTag-CIDR

$U=0$, $n_1=n_2=6$, $p= 0.0022$

Table 2.3 Descriptive statistics for Mann–Whitney U test for Figure 3.13. Tests were performed unpaired and two-tailed. Alpha for all tests 0.05.

For Figure 3.16	Median Day 16	Median Day 34
Pfs25	1	1
Pfs25-SpyTag	1	2049
Pfs25 + AddaVax	1	1
Pfs25 + SpyCatcher-VLPs	1	1
SpyCatcher-VLPs:Pfs25-SpyTag	526	16105
SpyCatcher-VLPs:Pfs25-SpyTag + AddaVax	1395	26849

Day 16

SpyCatcher-VLPs:Pfs25-SpyTag + AddaVax vs. SpyCatcher-VLPs:Pfs25-SpyTag

$U=0$, $n_1=n_2=6$, $p=0.0022$

SpyCatcher-VLPs:Pfs25-SpyTag + AddaVax vs. Pfs25-SpyTag + AddaVax

$U=0$, $n_1=n_2=6$, $p=0.0022$

SpyCatcher-VLPs:Pfs25-SpyTag vs. SpyCatcher-VLPs + Pfs25

$U=0$, $n_1=n_2=6$, $p=0.0022$

Day 34

SpyCatcher-VLPs:Pfs25-SpyTag + AddaVax vs. SpyCatcher-VLPs:Pfs25-SpyTag

$U=5$, $n_1=n_2=6$, $p=0.0411$

SpyCatcher-VLPs:Pfs25-SpyTag + AddaVax vs. Pfs25-SpyTag + AddaVax

$U=0$, $n_1=n_2=6$, $p=0.0022$

SpyCatcher-VLPs:Pfs25-SpyTag + AddaVax vs. Pfs25

$U=0$, $n_1=n_2=6$, $p=0.0022$

SpyCatcher-VLPs:Pfs25-SpyTag vs. Pfs25 + AddaVax

$U=0$, $n_1=n_2=6$, $p=0.0022$

Table 2.4 Descriptive statistics for Mann–Whitney U test for Figure 3.14. Tests were performed unpaired and two-tailed. Alpha for all tests 0.05.

2.2.29 Immunofluorescence (Indirect fluorescent-antibody – IFA)

Immunofluorescence was performed by Iona Brain on ookinetes fixed with paraformaldehyde (PFA) as described (Goodman et al., 2011). Ookinete culture smears of Pfs25DR3 transgenic *Plasmodium berghei* parasite (a chimeric *P. berghei* line which expresses Pfs25 in place of the endogenous Pbs25, provided by Dr Andrew Blagborough, Imperial College London) were fixed for 10 min in PBS with 4% PFA at RT, followed by washing in PBS. Slides were blocked for 1 h in PBS with 3% (w/v) BSA at RT, followed by incubation for 45 min with 2 µg/mL anti-Pfs25 monoclonal antibody 4B7 (Barr et al., 1991) (a kind gift from Kazutoyo Miura, National Institutes of Health, Maryland, USA) or test antiserum (1:100, from mice primed and boosted with AddaVax and SpyCatcher-VLPs:Pfs25-SpyTag or Ovalbumin) in PBS with 3% (w/v) BSA at RT in a humidified chamber. Slides were washed three times in PBS and then incubated with AlexaFluor 488-conjugated goat anti-mouse IgG (1:500) (Thermo Fisher Scientific) in PBS with 3% (w/v) BSA at RT for 45 min. After another three washes with PBS, slides were mounted with mounting medium with DAPI (Vector Laboratories), allowed to set overnight at 4 °C and analysed by wide-field fluorescence microscopy on a DMI3000 B microscope (Leica Microsystems). All samples were stained, imaged and analysed under the same conditions.

2.2.30 Standard membrane feeding assay (SMFA)

SMFA studies were performed by Dr Carole Long's laboratory. Purified IgG was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi*

mosquitoes (n=20 per test group) in SMFA. Midguts were dissected 7 days post-feeding and analysed for oocysts.

2.2.31 SpyCatcherDuo-VLP:SpyTag-protein reactivity

1.5× molar excess of SpyTag-CIDR(IT4var07)(pKB_299, T7SHuffle Express *lysY*) interaction partner per SpyCatcher residue in a total volume of 50 µL including 5 µL of 10× reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2) was used to react SpyCatcherDuo-VLPs. Reactions that did not fill the set volume of the reaction; e.g. reactions in which SpyCatcherDuo -VLPs and SpyTag-partner did not amount to 50 µL or no partner was added for control purposes, were brought to volume with MilliQ water.

0.75× molar excess of Pfs25-SpyTag (pKB_311, HEK) interaction partner per SpyCatcher residue in a total volume of 50 µL including 5 µL of 10× reaction buffer (400 mM Na₂HPO₄, 200 mM sodium citrate, pH 6.2) was used to react SpyCatcherDuo-VLPs. Reactions that did not fill the set volume of the reaction; e.g. reactions in which SpyCatcherDuo -VLPs and SpyTag-partner did not amount to 50 µL or no partner was added for control purposes, were brought to volume with MilliQ water. SpyTag-protein concentrations were: Pfs25-SpyTag 20 µM (in PBS); SpyTag-CIDR(IT4var07) 173 µM (in 20 mM Tris-HCl, 150mM NaCl, pH 8). SpyCatcherDuo-VLPs concentration was 43 µM (in 50 mM glycine, 25 mM sodium citrate, Tween 20 0.1% (v/v), pH 8). The reaction was performed for 3 h at RT and stopped by boiling for 3 min at 95 °C with 6× SDS-PAGE loading buffer [20% glycerol, 100 mM Tris•HCl, 4% SDS, 0.2% bromophenol blue, with or without 1% (v/v) 2-mercaptoethanol, pH 6.8].

2.2.32 Expression of IMX nanoparticles

BL21-CodonPlus(DE3)-RIPL+ was transformed with pET21-SpyCatcher-IMX-SnoopCatcher (pKB_389). One colony was entirely transferred into 50 mL of LB (low salt), with 100 µg/mL ampicillin. The colony was resuspended by inversion and the 50 mL divided into four 50 mL Falcon tubes, which were incubated overnight at 37 °C, 180 rpm. The overnight cultures were diluted 1:200 into 1L pre-warmed 37 °C 2xTY medium in Thompson UltraYield flasks without AirOtop seals (2.5L, Thomson, Cat. No. 931136-B). The cultures, grown at 37 °C until OD 0.6-1 in the presence of 100 µg/mL ampicillin. Induction was performed with 0.42 mM IPTG at 22 °C for 8-16 h prior to harvest. Foaming was controlled by addition of Antifoam A (Sigma, Cat. No. A5633) just prior to induction and re-applied when necessary until 2-3 h post induction (i.e. not controlled overnight). Cells were harvested, washed as before and resuspended in lysis buffer (10-15 mL/L cell culture pellet, lysis buffer: 20 mM Tris•HCl, 300 mM NaCl, 0.1 % (v/v) Tween 20, pH 8, 0.1 mg/mL lysozyme, 5 U/mL benzonase) and stored at -80°C until further processing.

Lysis and clearance were performed by thawing to RT in a water bath, placing the mixture on ice and frequent mixing. Thawed cells were sonicated on ice for four times at 50% pulse for 60 s, with 1 min between each pulse, using a Sonics Vibra-Cell sonicator (Ultrasonic Processor). Cell debris was spun at 17,000 g, 4°C for 30 min. Soluble protein in cleared lysate from 8 L was bound to 1 mL of packed Ni-NTA agarose by incubation for 30 min at 4°C on a rolling shaker in the presence of 50 mM imidazole. The resin was applied to a BioRad polyprep column and the flow-through once more re-applied to the resin. The resin was washed 3x with 15 CV 75 mM imidazole washing buffer. Elution was performed with 2 M imidazole, in

50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.5. Eluate was spun for 30 min, 17,000 g in order to remove aggregates. Dialysis against 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.0 was performed through a 100 kDa MWCO, three times against 1000× excess at 4 °C. The samples was spun at again for 30 min, 17,000 g, 4 °C, quantified and stored at 4 °C until use.

2.2.33 Size-exclusion chromatography of SpyCatcher-IMX-SnoopCatcher for size determination

IMAC eluate from SpyCatcher-IMX-SnoopCatcher (pKB_389) derived from 8 L culture, eluted in 5 mL, was spun for 30 min, 17,000 g to remove aggregate.

The protein was applied to a HiPrep 16/60 Superdex 200pg (GE Healthcare) on a FPLC system (Purifier 10, GE Healthcare). The mobile-phase was 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.0. The applied flow-rate was 1.0 mL/min and the run was performed at 4 °C. Elution was monitored at 215, 230 and 280nm. A high molecular weight gel filtration standard (Bio-Rad, Cat. No. 151-1901) was used for size approximation.

2.2.34 SpyCatcher-IMX-SnoopCatcher:SpyTag-/SnoopTag-protein reactivity

2.2.34.1.1 SpyCatcher-IMX-SnoopCatcher reactivity with AffiHER2-SpyTag and SnoopTag-AffiHER2

2.5× molar excess of SpyTag and SnoopTag interaction partner in a total volume of 50 µL was used to react with SpyCatcher-IMX-SnoopCatcher at 7 µM. Reactions that did not fill the set volume of the reaction; e.g. reactions in which SpyCatcher-

IMX-SnoopCatcher and SpyTag- and / or SnoopTag-partner did not amount to 50 μL or no partner was added for control purposes, were brought to volume with MilliQ water. Stock concentrations were: SpyCatcher-IMX-SnoopCatcher 9 μM in 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.0; AffiHER2-SpyTag 267 μM in PBS (pKB_096); SnoopTag-AffiHER2 192 μM in PBS (pKB_290). The reaction was performed for 22 h at RT, spun for 10 minutes at 17,000 g . 20 μL each were removed without disturbing any possible aggregate pellet and stopped by boiling for 3 min at 95 $^{\circ}\text{C}$ with 6 $\times$ SDS-PAGE loading buffer [20% glycerol, 100 mM Tris $\cdot$ HCl, 4% SDS, 0.2% bromophenol blue, with or without 1% (v/v) 2-mercaptoethanol, pH 6.8].

2.2.34.1.2 SpyCatcher-IMX-SnoopCatcher reactivity with SnoopTag-CIDR and Pfs25-SpyTag

2.5 $\times$ molar excess of SnoopTag-CIDR and 1.5 $\times$ molar excess SpyTag-Pfs25 partner in a total volume of 50 μL) was used to react SpyCatcher-IMX-SnoopCatcher at 5 μM . Reactions that did not fill the set volume of the reaction; e.g. reactions in which SpyCatcher-IMX-SnoopCatcher and SpyTag- and / or SnoopTag-partner did not amount to 50 μL or no partner was added for control purposes, were brought to volume with MilliQ water. Stock concentrations were: SpyCatcher-IMX-SnoopCatcher 9 μM in 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween 20, pH 8.0; Pfs25-SpyTag (pKB_311, HEK) 20 μM in PBS; SnoopTag-CIDR(IT4var07) (pKB_299, T7 SHuffle Express /ysY) 206 μM in PBS. The reaction was performed for 22 h at RT, spun for 10 minutes at 17,000 g . 20 μL each were removed without disturbing any possible aggregate pellet and stopped by

boiling for 3 min at 95 °C with 6× SDS-PAGE loading buffer [20% glycerol, 100 mM Tris•HCl, 4% SDS, 0.2% bromophenol blue, with or without 1% (v/v) 2-mercaptoethanol, pH 6.8].

Chapter 3 Plug-and-Display decoration of Virus-Like Particles

Isopeptide bond-mediated conjugation for modular immunization

3.1 Generation of SpyCatcher-VLPs and their features

3.1.1 Chapter introduction

VLPs are useful tools in immunology and vaccine development due to their self-assembling properties and structural organization which closely resemble those of viruses (De Gregorio and Rappuoli, 2014; Sapsford et al., 2013). Presentation of otherwise poorly immunogenic epitopes on VLPs greatly enhances the immune response to the epitopes (Bachmann and Jennings, 2010; Smith et al., 2013a). Modifying VLPs to display a desired protein or peptide sequence is non-trivial. Genetically fusing an antigen to a VLP by fusion to capsid protein has been successful in numerous examples. Notably the malarial RTS,S/AS01 vaccine is based on fusion of the circumsporozoite protein (CSP) to the Hepatitis B Virus surface antigen (HBsAg) (Rutgers et al., 1988). However genetic fusion remains a time-consuming trial-and-error approach as misfolded or structural constraints on the capsid protein can lead to loss of particulate state. This means that either no VLPs are obtained or co-expression of non-fused native capsid protein may be required to obtain particulates (Brown et al., 2009). Ultimately, co-expression of non-decorated capsid protein reduces the antigen dose per VLP as for example encountered in production of RTS,S for example (Hill et al., 2014; Rutgers et al., 1988). Also problematic are misfolded states of displayed antigen that give rise to ineffective antibodies as encountered in HIV vaccine development (Mascola and Montefiori, 2010; Mateu, 2011; Smith et al., 2013c; Walker et al., 2011). Furthermore the optimal system for antigen expression (cell-free expression, bacterium, yeast, mammalian cell-line, or plant) may have different post-translational modifications (particularly disulfide bond formation and glycosylation)

as compared to the host for VLP carrier production (Sletten and Bertozzi, 2009; Smith et al., 2013c). Hence, strategies involving individual expression of VLP and target antigen are often pursued; subsequently the VLP and target antigen must be joined together. Conjugation through the abundant naturally occurring amino groups (Lys, N-terminus) or carboxyl groups (Asp, Glu, C-terminus) typically yields a heterogeneous mixture, so conjugation through an exposed sulfhydryl group (Cys) on the antigen is the most common route (Jegerlehner et al., 2002b; Sletten and Bertozzi, 2009). Many antigens of pathogenic or human origin contain disulfide bonds, which have major importance protein folding and stabilization. Introducing a new cysteine can be disruptive as non-native disulfide bonds can form (Anelli et al., 2003; Baneyx and Mujacic, 2004). Chemical conjugation thus may provide only limited control over valency and structural orientation, leading to heterogeneous and incomplete VLP coverage with antigen in a potential non-oriented manner (Mascola and Montefiori, 2010; Pattenden et al., 2005). Additional approaches, their strength and drawbacks are discussed in detail in section 1.2.

Therefore, development of a platform approach that separates production of VLP and antigen but allows for predictable, modular decoration under mild conditions as outlined in Figure 3.1 would be of great value for biotechnological applications. To create such a Plug-and-Display platform I employed SpyCatcher:SpyTag technology with a VLP derived from the ssRNA *Acinetobacter* phage AP205. The use of the underlying VLP has been pioneered in groups led by Professor Martin Bachmann (an Oxford Jenner Institute collaborator of the Howarth laboratory), who recommended the usage of said AP205 VLP for genetic and chemical conjugation.

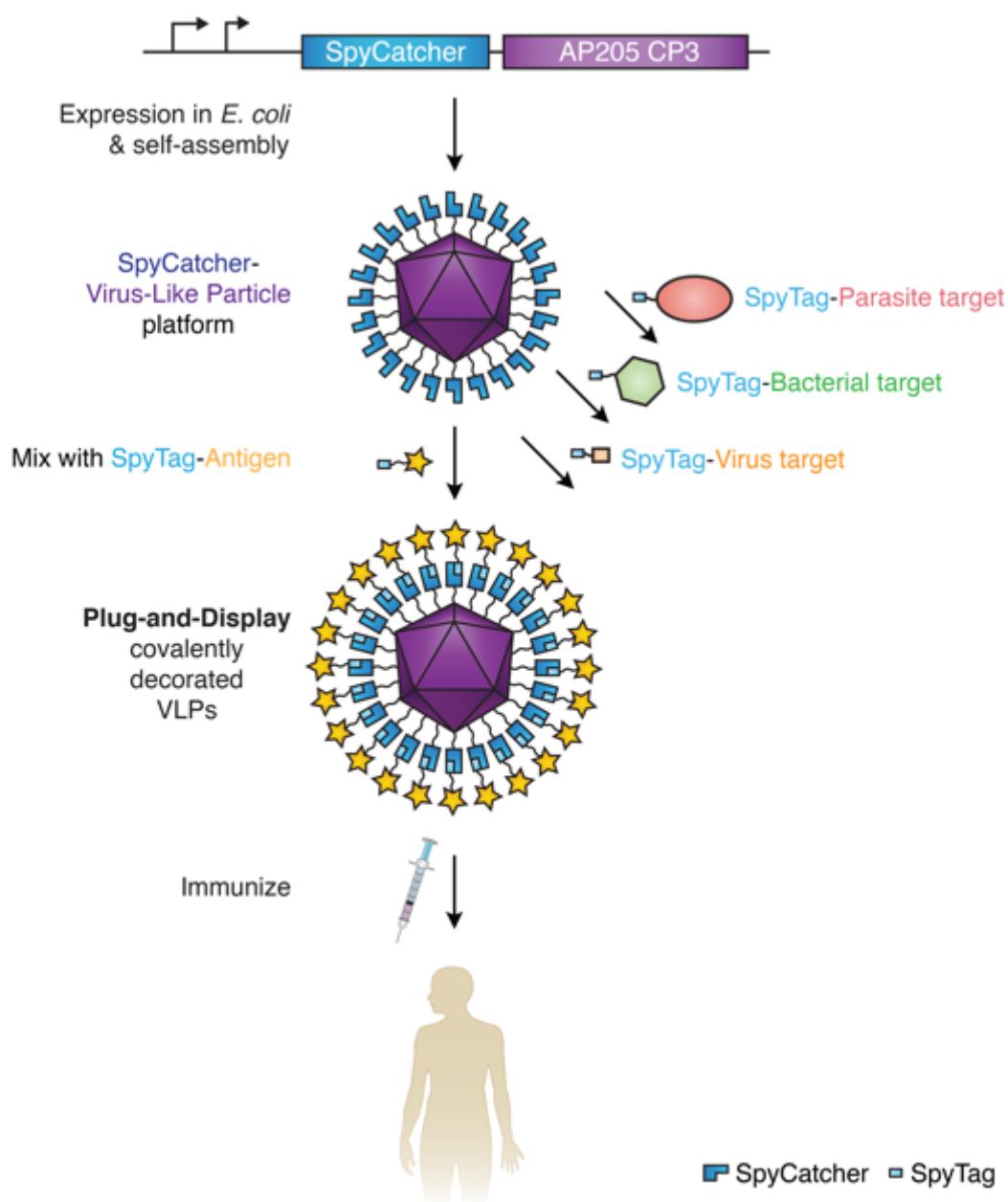


Figure 3.1 Overview of Plug-and-Display VLP assembly. SpyCatcher is genetically fused to the *Acinetobacter* bacteriophage AP205 coat protein (AP205 CP3) and expressed in *E. coli*. Self-assembly of monomers generates SpyCatcher-VLPs. Upon mixing, SpyTag-antigen forms a spontaneous isopeptide bond with SpyCatcher-VLPs, yielding decorated particles for immunization. Cross-section occupancy (24) for SpyCatcher-VLPs was calculated for a single plane across an approximated sphere with the observed compound diameter from transmission electron microscopy studies for an assumed 180mer. Molecular cartoon depictions, e.g. SpyCatcher, SpyTag residue and AP205 carrier VLP are approximately to scale.

3.1.2 Expression studies of SpyCatcher-VLPs

Initial expression studies of full length SpyCatcher-VLPs (pKB_197) in classic T7 expression hosts such as BL21(DE3) or BL21-CodonPlus(DE3)-RIPL proved difficult. Grow up of starter cultures lead to lysis and clumping of overnight cultures, after single colonies from transformations were inoculated and shaken overnight (180 rpm, 37 °C). This indicated intrinsic toxicity of the construct. Thus I screened different expression strains, with diverse genetic backgrounds and various expression conditions in order to generate biomass (Table 3.1).

Strain	With shaking	Without shaking
BL21(DE3)	+++	+, low OD
BL21(DE3) pLysS	++	+, low OD
BL21Star (DE3)	+	+, low OD
BL21-CodonPlus(DE3)-RIPL	++	+, low OD
C41(DE3)	-	-
C43(DE3)	-	-
JM109	-	-
JM109(DE3)	++	+
Rosetta-gami 2(DE3)	++	+
T7 SHuffle Express <i>lysY</i>	+	-, low OD

Table 3.1 *E. coli* strains and observed clumping or lysis during initial expression screen. Observed phenotype after overnight incubation +/- shaking. +++ strong, ++ medium, + weak or – no lytic clumping observed.

C41(DE3) and C43(DE3) were not affected by lysis and clumping effects as opposed to all other tested T7 expression hosts and resulted in highest OD. Non lysogenised JM109 was also not affected, indicating toxicity due to expression from the T7 promoter. Due to the presence of *OmpT* and *Lon* proteases in this K12 derivative, JM109 was not further pursued because of the risk to protein integrity.

For further experiments C41(DE3) was chosen as the expression host. To reduce potential off-target immunogenicity to the Catcher domain, SpyCatcher-AP205cp3 (Figure 3.2) lacking the 23 N-terminal residues (Δ N1) of full-length SpyCatcher was constructed for further experiments and expressed as before (Li et al., 2014; Liu et al., 2014). Reduced antibiotic concentration (20 μ g/mL ampicillin vs. typically 100 μ g/mL) and standing overnight incubation with 1:1 dilution of the culture with fresh medium the next day further increased bacterial density (Bachmann et al., 2012). Unfortunately the majority of target protein was found in inclusion bodies but some soluble protein was observed and yields were simply increased by scaling the bacterial volume.

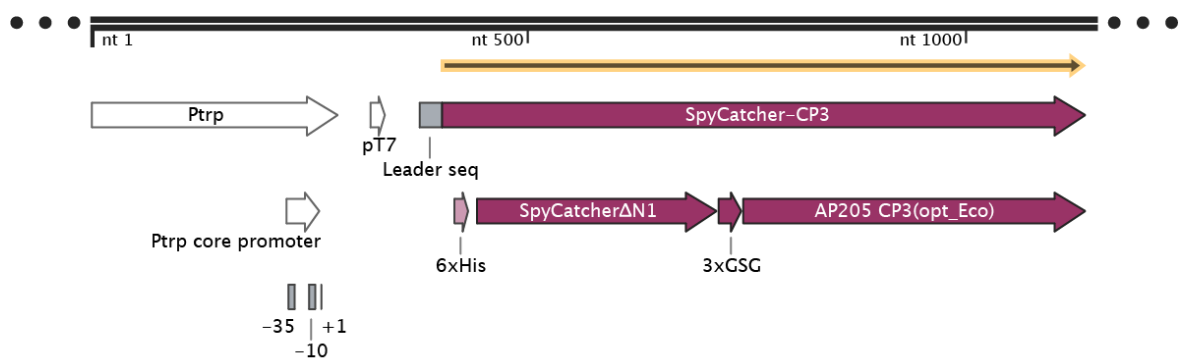


Figure 3.2 Schematic of pGEM-SpyCatcher-AP205cp3 (pKB_215) used to generate first generation SpyCatcher-VLPs. A modified high-copy number pGEM vector was employed (Kozlovskaya et al., 1993). A modified tryptophan promoter (Ptrp) from *E. coli* (Latta et al., 1990) and an additional promoter pT7 from the bacteriophage T7 (Dunn and Studier, 1983) drive the expression of His₆-tagged Δ N1 truncated SpyCatcher (Li et al., 2014; Liu et al., 2014) fused via a flexible 3xGSG to an *E. coli* codon optimized sequence of AP205 coat protein 3 (CP3) (Klovins et al., 2002; Tissot et al., 2010).

SpyCatcher-VLPs were purified to homogeneity from cell-lysate by IMAC with stringent washing (up to 200 mM imidazole) followed by elution with 2 M imidazole and dialysis against a 300 kDa molecular weight cut-off membrane as analysed by

SDS-PAGE (Figure 3.3 and 3.5). Initial preparations eluted and dialysed into NTA buffer quickly aggregated. Hence a screen for improved solubility post elution was performed; an improved buffer made of 50 mM glycine, 25 mM sodium citrate and Tween 20 0.1% (v/v), pH 8.5 was found.

3.1.3 Biochemical and biophysical characterization of SpyCatcher-VLPs

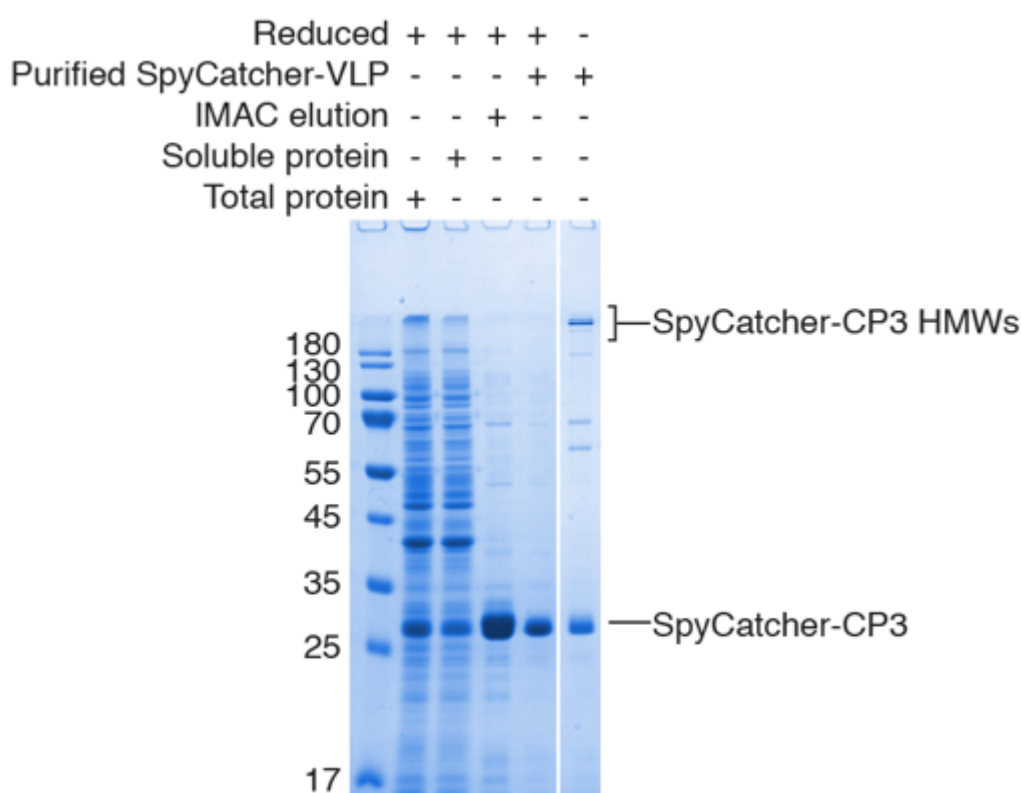


Figure 3.3 SDS-PAGE of SpyCatcher-VLPs. SpyCatcher-VLPs can be produced to high purity. SpyCatcher-VLPs were expressed in C41(DE3) and purified via Immobilized Metal Affinity Chromatography (IMAC). Purified SpyCatcher-VLPs were obtained by dialysis of the IMAC elution through a 300 kDa MWCO membrane and removal of aggregates via centrifugation (17,000 *g*, 30 min). The same volume was applied for IMAC elution and dialysed sample, indicating removal of contaminant and non-assembled SpyCatcher-CP3 species by dialysis, as well as removal of aggregates by spinning. Purified SpyCatcher-CP3 proteins form disulfide bonded multimeric species of high molecular weight (HMWs).

Purity of SpyCatcher-VLPs could be obtained to >95% as analysed by SDS-PAGE (Figure 3.5). The theoretical Mw of 25.8 kDa was in close agreement with the

observed Mw of ~26 kDa as inferred from reducing SDS-PAGE (Figure 3.5). A minor secondary band migrating just above the main band is most likely caused by gluconylation of the His₆-tag preceding the SpyCatcher-CP3 fusion. Gluconylation is typically encountered in *E. coli* B-strain derivatives such as C41(DE3) due to lack of a phosphogluconolactonase (PGL) (Geoghegan et al., 1999; Aon et al., 2008; Zakeri et al., 2012).

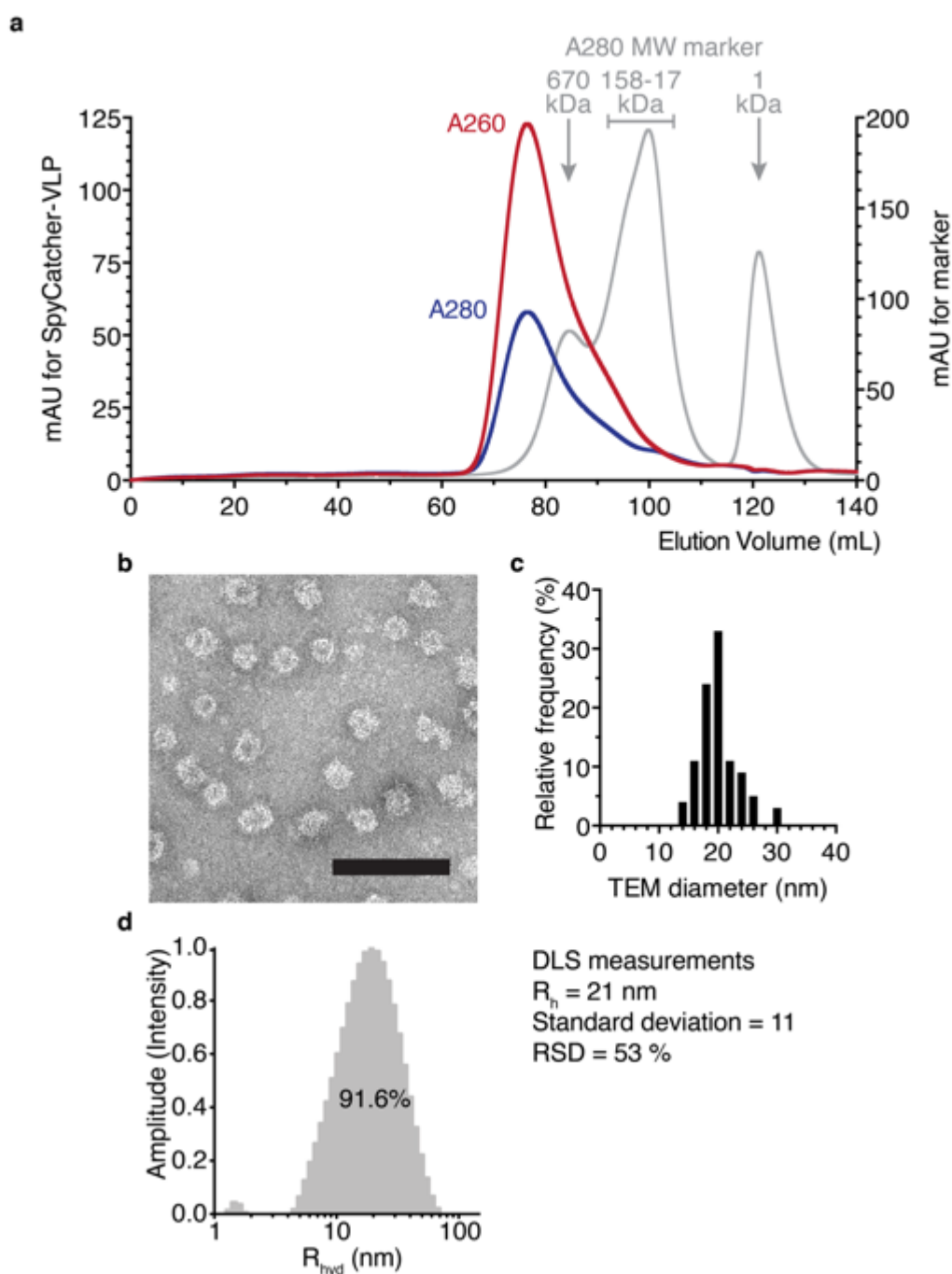


Figure 3.4 Biophysical characterization of SpyCatcher-VLPs. (a) Size-exclusion chromatography (Sephacryl S500) showed SpyCatcher-CP3 assembly into multimers, analysed by absorbance at 260 and 280 nm and compared to Mw standards. (b) Negatively-stained TEM image of IMAC-purified protein shows SpyCatcher-VLPs. Scale bar 100 nm. (c) Size distribution of SpyCatcher-VLPs from TEM (n=100). (d) Size distribution of SpyCatcher-VLPs from dynamic light scattering (DLS) experiments. Parameters obtained are hydrodynamic radius (R_h), standard deviation from the mean relative standard deviation (RSD).

As expected for VLPs, size-exclusion chromatography of IMAC-purified material eluted broadly in the MDa and higher kDa range with simultaneous elution of protein (280 nm absorbance) and nucleic acid species (260 nm absorbance), as known from literature for AP205 VLPs (Figure 3.4a) (Klovins et al., 2002; Spohn et al., 2010; van den Worm et al., 2006). Presence of particles consistent with VLPs was observed in negative-stained transmission electron microscopy (TEM) (Figure 3.4b). Size determination of particle size from TEM by manual measurements gave a diameter of 20 ± 3.2 nm (mean $\pm$ 1 s.d., $n=100$) (Figure 3.4c). Size determination of particles by DLS gave a hydrodynamic radius of 20.9 ± 11.2 nm (mean $\pm$ 1 s.d., RSD 53%) (Figure 3.4d).

3.1.4 SpyCatcher-VLP:SpyTag reaction with model protein MBP

Initial reactivity studies were conducted with an easy-to-produce SpyTag-model protein: SpyTag N-terminally fused to *E.coli* maltose binding protein (MBP, SpyTag-MBP) from *E. coli*. The covalent bond to SpyTag, resistant to boiling in SDS, can be conveniently monitored via SDS-PAGE. As a negative control SpyTag DA-MBP was employed. The reactive aspartic acid residue (D) in SpyTag was mutated to a non-reactive alanine (A). Incubation of SpyTag DA-MBP with SpyCatcher domain does not yield a covalent adduct (Zakeri et al., 2012). Conjugation could be driven to completion by use of excess SpyTag-MBP, the non-reactive SpyTagDA mutant did not yield a covalent adduct as expected (Figure 3.5).

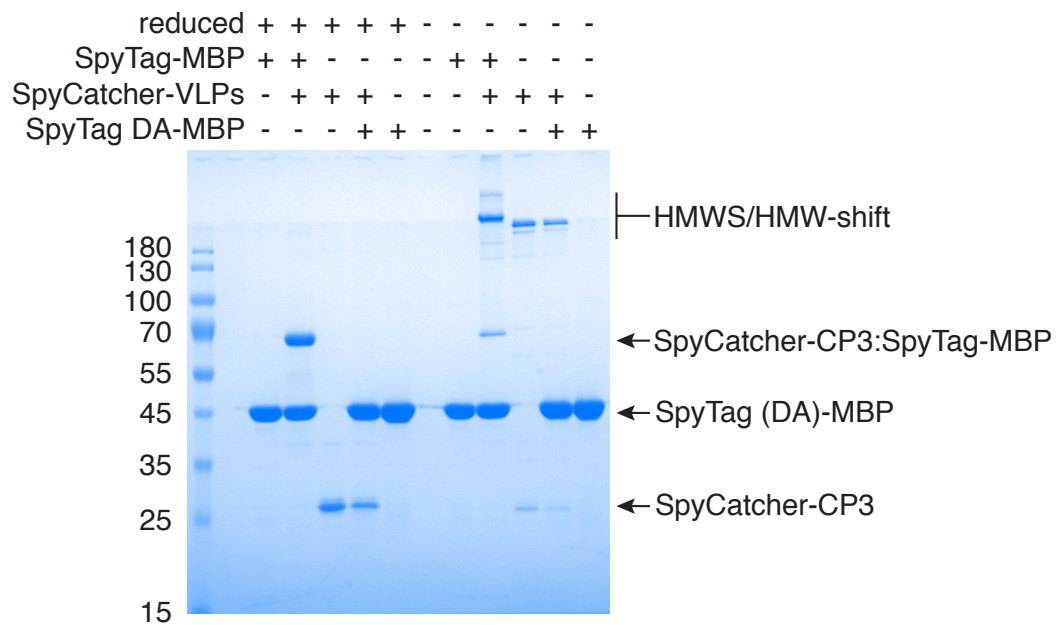


Figure 3.5 SpyCatcher-VLPs:SpyTag reaction with model protein SpyTag-MBP. Complete reaction of SpyCatcher-VLPs with SpyTag. SpyCatcher-VLPs were incubated with SpyTag-MBP or the negative control SpyTag DA-MBP, before boiling in SDS-loading buffer and analysis by reducing SDS-PAGE with Coomassie staining.

3.1.5 SpyCatcher-VLP:SpyTag reaction with complex SpyTag-antigens

After demonstrating full decoration of SpyCatcher-VLPs with SpyTag-MBPs (1:1 stoichiometry), I attempted decoration with more challenging proteins. An antigen that represents a difficult class for traditional conjugation techniques was the cysteine-rich interdomain region (CIDR) from the highly variable *Plasmodium falciparum* erythrocyte membrane protein-1 family (PfEMP1) encoded by the *var* gene family (Lau et al., 2015; Leech et al., 1984). Extracellular PfEMP1 ectodomains comprise 2–10 copies of two *Plasmodium*-specific domain types, the Duffy-binding-like (DBL) and CIDRs (Lau et al., 2015). Individual CIDR and DBL domains can bind to host endothelial surface proteins and carbohydrates, resulting in the sequestration of infected red blood cells in a range of organs (Smith et al., 2013b). One host target is the endothelial protein C receptor (EPCR) and binding by the

malarial parasite is known to correlate with severe malaria (Bernabeu et al., 2016). Binding to EPCR is mediated by CIDR domains. Classification and nomenclature of *var* genes has been performed based on the sequence of *P. falciparum* clones 3D7, HB3 or IT4; with the *var* classifier detailing the assigned number of a *var* variant within a genome (Gardner et al., 2002). As the name implies, CIDRs have a high cysteine content, which would render controlled thiol conjugation extremely challenging if not impossible. As many as 10 cysteine residues per monomer domain were encountered in the CIDR family members employed in this study. Likewise multiple lysine residues are encountered: a total of 36 for the IT4var07 and 33 for the HB3var03 variant (Lau et al., 2015).

The diversity of the *var* gene family is paramount for the immune evasion of *P. falciparum*. Only one *var* gene of a total of ~60 in a single parasite genome is expressed at any given time, a process controlled by transcriptional switching (Miller et al., 2002). Switching of the expressed variant enables the parasite to evade the immune response against the previous variant, as antibodies raised against one do not typically bind another, but function of the variant is often maintained (Lau et al., 2015). Interestingly, some members of the CIDR family can be expressed in *E. coli* and / or refolded in moderate yield at bench scale (Lau et al., 2015).

Another challenging protein tested was Pfs25, a sexual stage antigen of *Plasmodium falciparum*. As a transmission blocking antigen candidate, it is expressed on the surface of zygotes and ookinetes within the mosquito (Vermeulen et al., 1985). Pfs25 contains 22 cysteines, which are thought to form 11 disulfides as observed for its close *P. vivax* homolog Pvs25 (PDB 1Z27) (Saxena et al., 2006).

Pfs25 is a challenging antigen to obtain well folded and correct folding is a prerequisite for generating meaningful antibodies; mispairing of cysteine residues will reduce the capability to raise native Pfs25 recognizing Abs (Barr et al., 1991). I performed reaction studies analogously to the SpyTag-MBP conjugations studies, using 1.5-2.5x molar excess SpyTag antigen protein compared to Catcher residue in order to drive the reaction to completion.

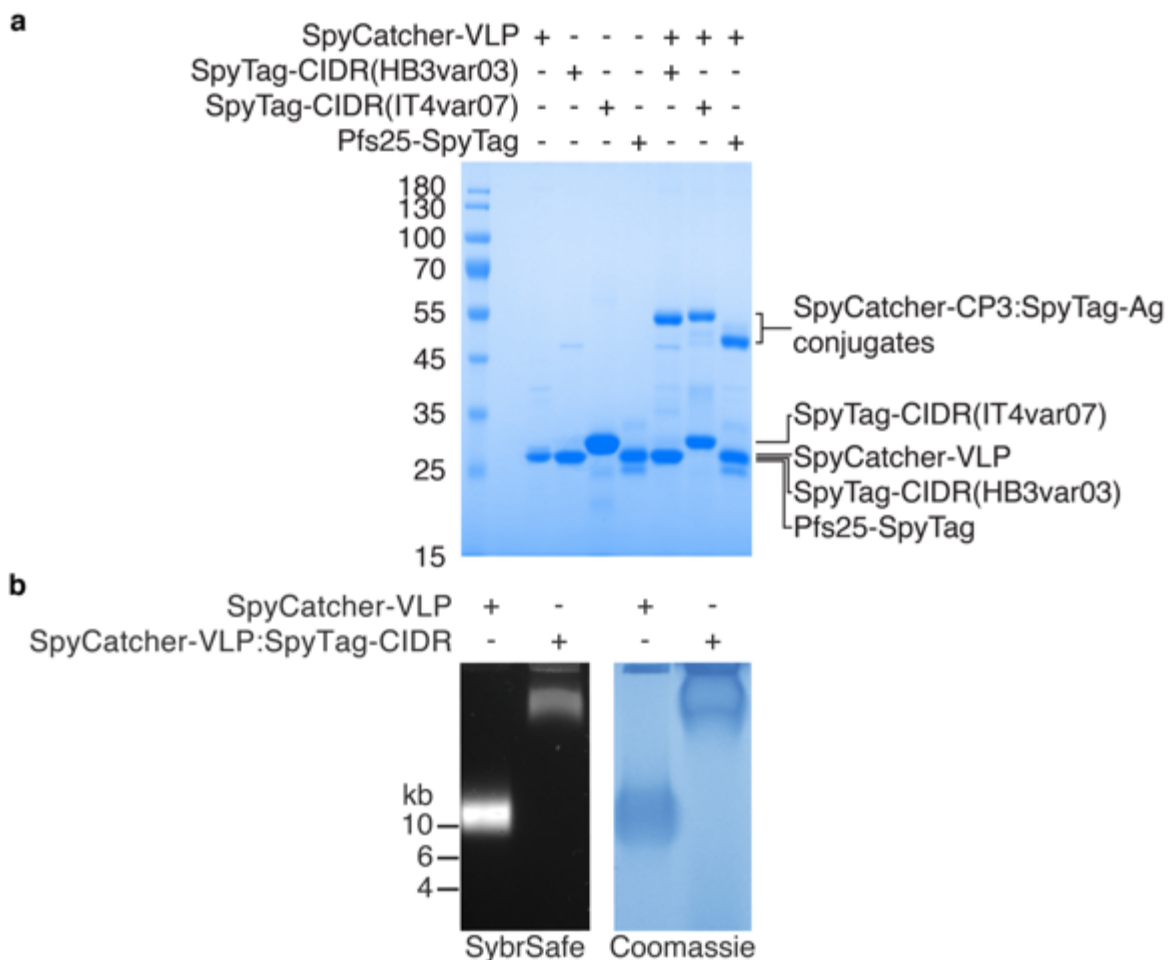


Figure 3.6 SpyCatcher-VLPs reacted with range of malarial protein antigens. (a) SpyCatcher-VLPs were incubated with SpyTag-containing antigens, before boiling in SDS-loading buffer and analysis by reducing SDS-PAGE with Coomassie staining. As in Figure 3.5 but SpyTag-CIDR(HB3var03), SpyTag-CIDR(IT4var07) or Pfs25-SpyTag were supplied in excess to SpyCatcher-VLPs. **(b)** Native agarose gel electrophoresis of SpyCatcher-VLPs. The agarose gel was first stained and imaged with SybrSafe (staining nucleic acid) prior to staining with InstantBlue (a Coomassie dye staining protein), showing unconjugated SpyCatcher-VLPs and SpyCatcher-VLPs conjugated with SpyTag-CIDR(IT4var07).

Efficient conjugation was achieved for all proteins tested, as analysed by reducing SDS-PAGE. Full conjugation of SpyCatcher-VLPs can be observed for SpyCatcher-VLPs:SpyTag-CIDR(IT4var07), as also evidenced by shift of the nucleic acid and protein bands in native agarose gel electrophoresis. Full conjugation of SpyCatcher-VLPs is likely for SpyTag-CIDR(HB3var03) and Pfs25-SpyTag, due to the similar intensity of adduct bands with fixed SpyCatcher-VLP input but cannot be unambiguously confirmed in this figure. Further data for complete reaction of SpyCatcher-VLPs:Pfs25-SpyTag is provided in Figure 3.14 and in Figure 3.11 for SpyCatcher-VLPs:SpyTag-CIDR(IT4var07).

3.1.6 SpyCatcher-VLP:SpyTag reaction with synthesized peptides

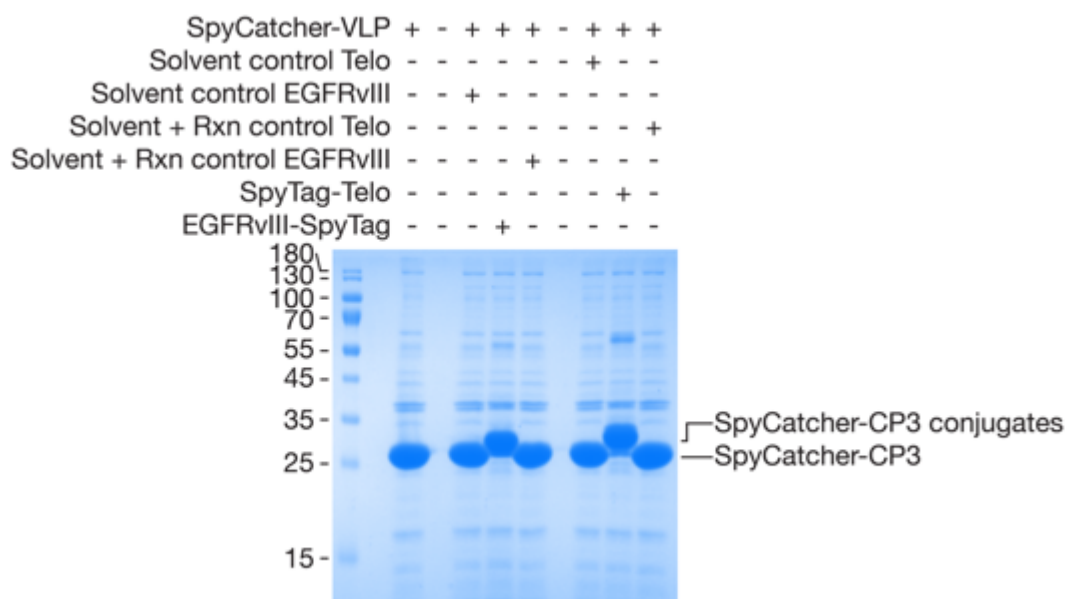


Figure 3.7 SpyCatcher-VLPs:SpyTag-peptide reactivity. SpyTag-Telo or EGFRvIII-SpyTag were supplied in excess to SpyCatcher-VLPs, prior to boiling in SDS-loading buffer and analysis by reducing SDS-PAGE with Coomassie staining. Solvents used to dissolve peptides, as well as VLP reaction buffer were incubated alongside to control for buffer or solvent mediated charge effect that may affect migration of the bands.

Efficient conjugation, as evidenced by gel shifts, was also achieved for all chemically synthesized peptides tested. Analysis by reducing SDS-PAGE showed at least 90% of the SpyCatcher-VLP density shifts upon conjugation with SpyTag enabled peptides; whereas all negative controls did not affect band mobility. Unfortunately, aggregation of conjugates was frequently observed shortly after conjugation.

3.1.7 SpyCatcher-VLP reaction with protein internal SpyTag

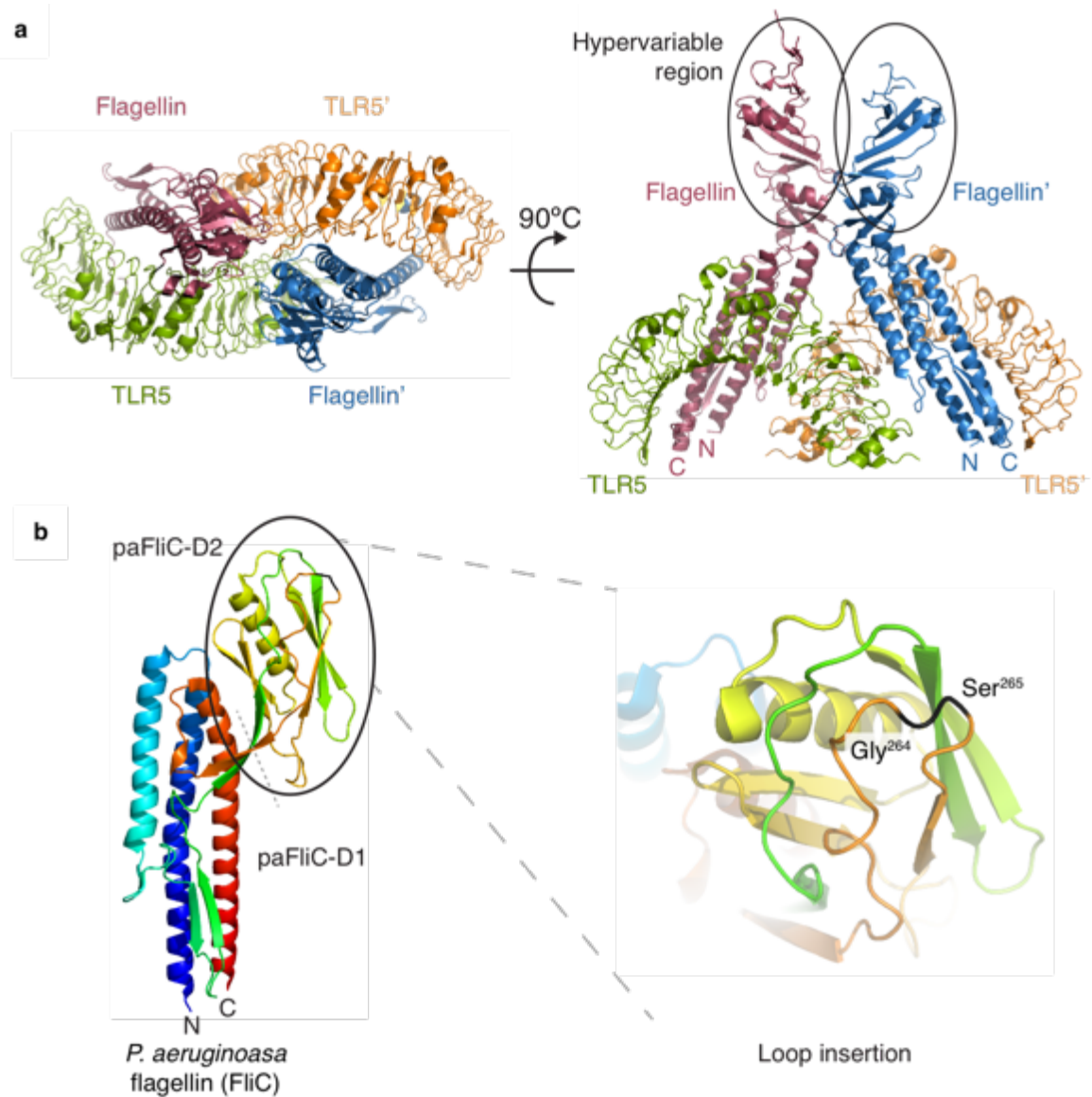


Figure 3.8 Structural basis of TLR5:flagellin recognition and flagellin SpyTag loop-insertion. (a) TLR5 associates with *Salmonella* FliC-ΔD0 in a 2:2 complex structure. Two TLR5 molecules are orientated tail-to-tail with their C-terminal regions facing towards the center of the complex located on the cell membrane. The hypervariable region of flagellin are circled (PDB 3V47). (b) Insertion of SpyTag into the most distal, flexible loop region (black) of *Pseudomonas* A-type flagellin-ΔD0 (PDB 4NX9). Adapted from (Yoon et al., 2012; Song and Yoon, 2014).

Flagellin (FliC) is the main component of bacterial flagella and thus present in large amounts on flagellated bacteria (up to 20,000 molecules / flagellum) (Macnab, 2003). The innate immune system detects pathogen-associated molecular patterns (PAMPs) that are expressed on infectious microbes via multiple mechanisms (Medzhitov, 2001). Toll-like receptors (TLRs) detect PAMPs, such as flagellin (TLR5) typically by dimerization (Figure 3.8a), which in turn stimulates production of cytokines, such as tumour necrosis factor- α (TNF α), by signalling through nuclear factor NF- κ B (Hayashi et al., 2001; Yoon et al., 2012). Genetic fusion of flagellin to otherwise poorly-immunogenic proteins is known to markedly improve immunogenic features, as well as expression and stability. For example flagellin of *Salmonella enterica* (FliC) was N-terminally fused to Pfs25 and enabled efficient production in *E. coli* (Qian et al., 2016), fusion of *Salmonella* FliC to the merozoite surface protein-1 (MSP1₁₉) was shown to increase antibody titres to MSP1₁₉ by 2-4 orders of magnitude (Bargieri et al., 2010) and VLP membrane-anchored flagellin induced enhanced cross-protective heterosubtypic immune responses in influenza (Wang et al., 2008b).

In order to allow easy access of TLR5 to the flagellin PAMP recognition motif on the conserved FliC D1 domain, SpyTag was fused internally into the distal hypervariable D2 domain of A and B-type *Pseudomonas aeruginosa* flagellin. Flagellin could thus serve as a potential immunostimulatory module (ISM) that may be added to SpyCatcher-VLP reactions as well as a model protein for internal SpyTag reactivity. B-type flagellin was fused solely on the basis of a bioinformatics prediction of flexible/disordered regions (Kelley et al., 2015). The structure for A-type flagellin was recently solved and allowed identification of a distal and exposed

loop region in the hypervariable domain of the A-type fliC [PDB 4NX9, (Song and Yoon, 2014)]. An internal SpyTag was introduced between Gly264 and Ser265, flanked by 4 and 6 residue flexible GS linker respectively. (Figure 3.8b).

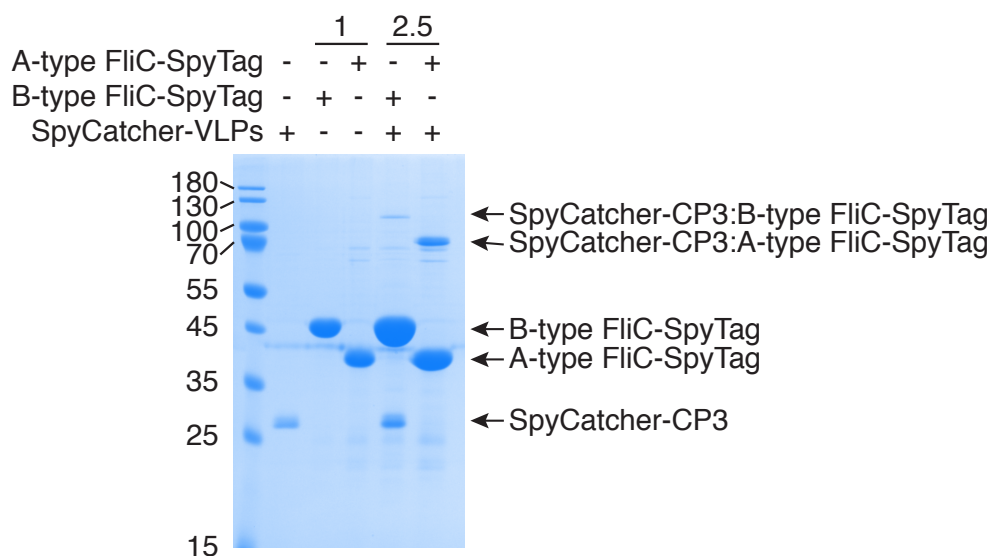


Figure 3.9 SpyCatcher-VLPs can react with an internally fused SpyTag. SpyTag was fused into flexible loops of two variants of *Pseudomonas aeruginosa* flagellin (FliC) yielding tagged A-type (pKB_259) and B-type flagellin (pKB_249). Expressed and purified internal SpyTag-FliC variants reacted with SpyCatcher-VLPs when supplied in 2.5 molar excess in VLP reaction buffer for 5 h at RT. The B-type mutant was a poor substrate, whereas the A-type mutant reacted to completion. The numbers above the gel indicate the molar ratio compared to SpyCatcher-CP3 in the respective lane. The reactions were stopped by boiling in SDS-loading buffer and analysed by reducing SDS-PAGE with Coomassie staining.

Reactivity with SpyCatcher-VLPs was observed for both internally placed SpyTags. Reaction of the B-type internal SpyTag variant was very poor, although supplied in excess. The A-type mutant reacted substantially better compared to the B-type mutant and was able to reach completion, when supplied in excess and incubated for 5 h, instead of the usual 3 h.

3.1.8 Storage of SpyCatcher-VLPs and reactivity

Reactivity after long-term or non-ideal storage of unreacted SpyCatcher is an important criteria. It would be desirable to prepare and stockpile SpyCatcher-VLPs for use at a later time and shipping to other laboratories. Freezing of SpyCatcher-VLPs caused aggregation, so liquid storage was required to avoid loss of protein. Reactivity and immunogenicity after 1 month liquid storage at 4 °C is addressed in section 3.2.2. SpyCatcher-VLPs reacted to completion, retained their integrity and were highly immunogenic with SpyTag-antigen (Figure 3.14 and Figure 3.16).

I assayed reactivity after storage at a higher temperature (60 °C, 12 h), exceeding what would be typically encountered during transport and storage outside of laboratory conditions.

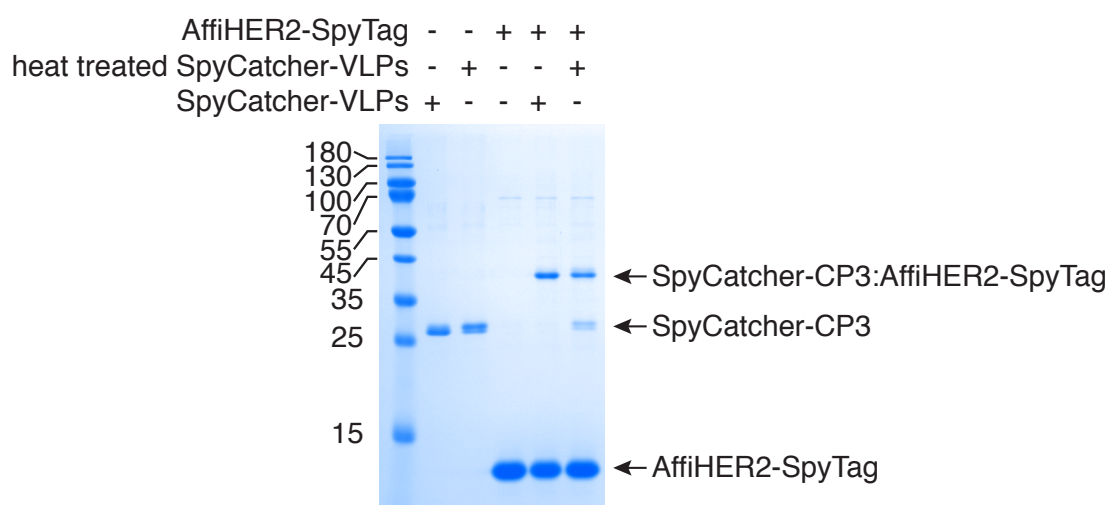


Figure 3.10 SpyCatcher-VLPs reacted after storage at elevated temperature. SpyCatcher-VLPs were incubated at 60 °C for 12 h. Samples were cooled to RT (22 °C) and spun at 17,000 g for 30 min to remove any aggregate. 10 µM SpyCatcher-VLPs were reacted with 2-fold molar excess of AffiHER2-SpyTag in VLP reaction buffer for 3 h. The reactions were stopped by boiling in reducing SDS-loading buffer and analysed by SDS-PAGE with Coomassie staining. Heat treated SpyCatcher-CP3 reacted to 68% with AffiHER2-SpyTag (s.d.=2%, n=3).

Heated SpyCatcher-VLPs reacted to 68% completion with AffiHER2-SpyTag and no significant loss of protein was observed either by spectrophotometric (not shown) or by SDS-PAGE analysis (Figure 3.10). However, a change in protein mobility was observed, with a switch of density in the CP3 from the bottom in the non-treated to the top, in the heated sample.

3.2 Immunogenicity of conjugated SpyCatcher-VLPs

3.2.1 Immunogenicity of SpyTag-CIDR(IT4var07):SpyCatcher-VLPs conjugates

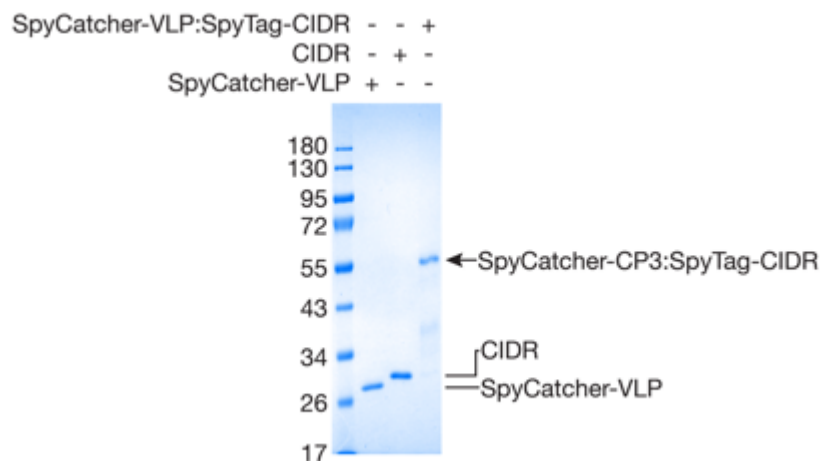


Figure 3.11 SpyCatcher-VLPs:SpyTag-CIDR(IT4var07) immunogens. SpyCatcher-VLPs, CIDR(IT4var07) and the dialysed SpyCatcher-VLP:SpyTag-CIDR(IT4var07) conjugate were boiled in reducing SDS-loading buffer and analysed by SDS-PAGE with Coomassie staining.

After having established modular addressability of the SpyCatcher-VLP platform with a diverse set of proteins from a biochemical perspective, next we wondered if antigen displayed on the VLPs would also be superior immunogens compared to non-conjugated proteins.

Immunogens were prepared as before but great care was taken to remove lipopolysaccharides (LPS) and after removal not to re-introduce endotoxin. The presence of small amounts in injectable formulations can cause severe side effects such as endotoxin shock, tissue injury or even death in humans (Magalhães et al., 2007). In comparison, BALB/c mice are less sensitive to LPS and have been reported to tolerate 75 µg or ~750000 endotoxin units (1 EU = ~0.1-0.2 ng LPS) and higher administrations (5×10^6 EU) without induction of sepsis or lethal effects (Kuo et al., 2005; Thomas et al., 2014). However, LPS tolerance is strain and age

dependent and LPS can induce severe pain in the animal (Tateda et al., 1996). LPS also signals via TLR 4 and can modulate immune responses via tumour necrosis factor-alpha (TNF-alpha), interleukin 1 (IL-1) and IL-6 (Tateda et al., 1996). To avoid obfuscation of immunization results and to reduce pain in mice, it is best practice to remove LPS for immunization studies (Magalhães et al., 2007). Two strategies were pursued to remove LPS, first protein bound to IMAC resin was LPS depleted with several washes of washing buffer containing Triton X-114 (TX114) at a concentration of 0.1% (v/v), 4 °C. TX114 was then removed by washing without the detergent (Zimmerman et al., 2006). Remaining TX114 concentrations were not determined and assumed to be depleted during dialysis. If any was left, TX114 did not hinder reactivity as complete conjugation for SpyCatcher-VLPs was observed; free CIDR was removable to >95% as judged by reducing SDS-PAGE and only minor contaminants were observed (Figure 3.11). To verify LPS depletion or assay any remaining LPS, a chromogenic Limulus Amebocyte Lysate (LAL) assay was used with *E. coli* LPS standard. In case LPS concentrations were still too high (above 10 EU/ml) or increased after storage and handling, possibly by environmental contamination through contaminated plastic consumables, TX114 phase separation depletion was employed and verified as above (Aida and Pabst, 1990). SpyCatcher-VLPs in the CIDR experiment were sufficiently LPS free (below 1 EU/ml), endotoxins in the Pfs25 experiment were removed by repeated phase separation by Dr Darren Leneghan.

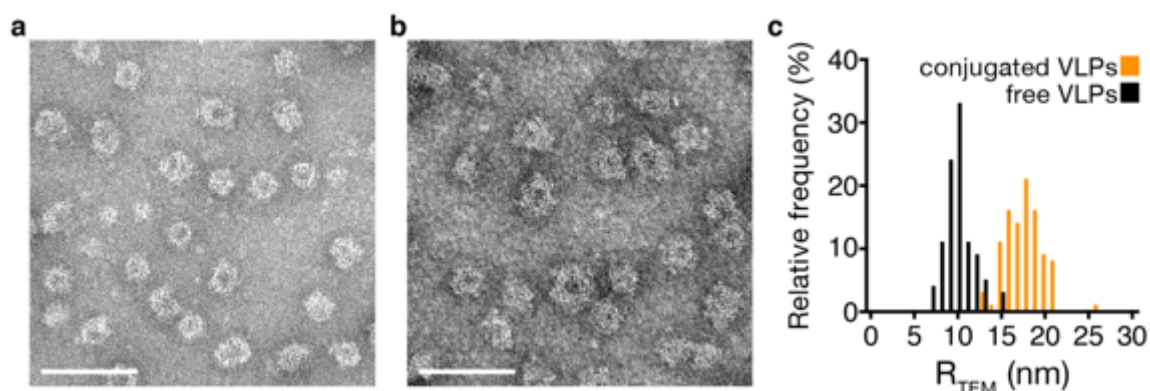


Figure 3.12 Negatively-stained TEM images of SpyCatcher-VLPs. (a) before and **(b)** after conjugation with excess SpyTag-CIDR(IT4var07). Scale bar 100nm. **(c)** Size distribution (radius) of (conjugated) SpyCatcher-VLPs from TEM (n=100).

Furthermore integrity of particles post-conjugation with SpyTag-CIDR(IT4var07) was analysed by native agarose gel which showed concurrent shift of both protein and nucleic acid signal in the conjugated group (Figure 3.6b). Observed VLP dimensions in TEM imaging studies increased substantially from unconjugated mean radius of 10 ± 1.6 nm to a mean radius of 17.7 ± 2.1 nm in the CIDR conjugated group (mean $\pm$ 1 s.d., n=100 per group) (Figure 3.12).

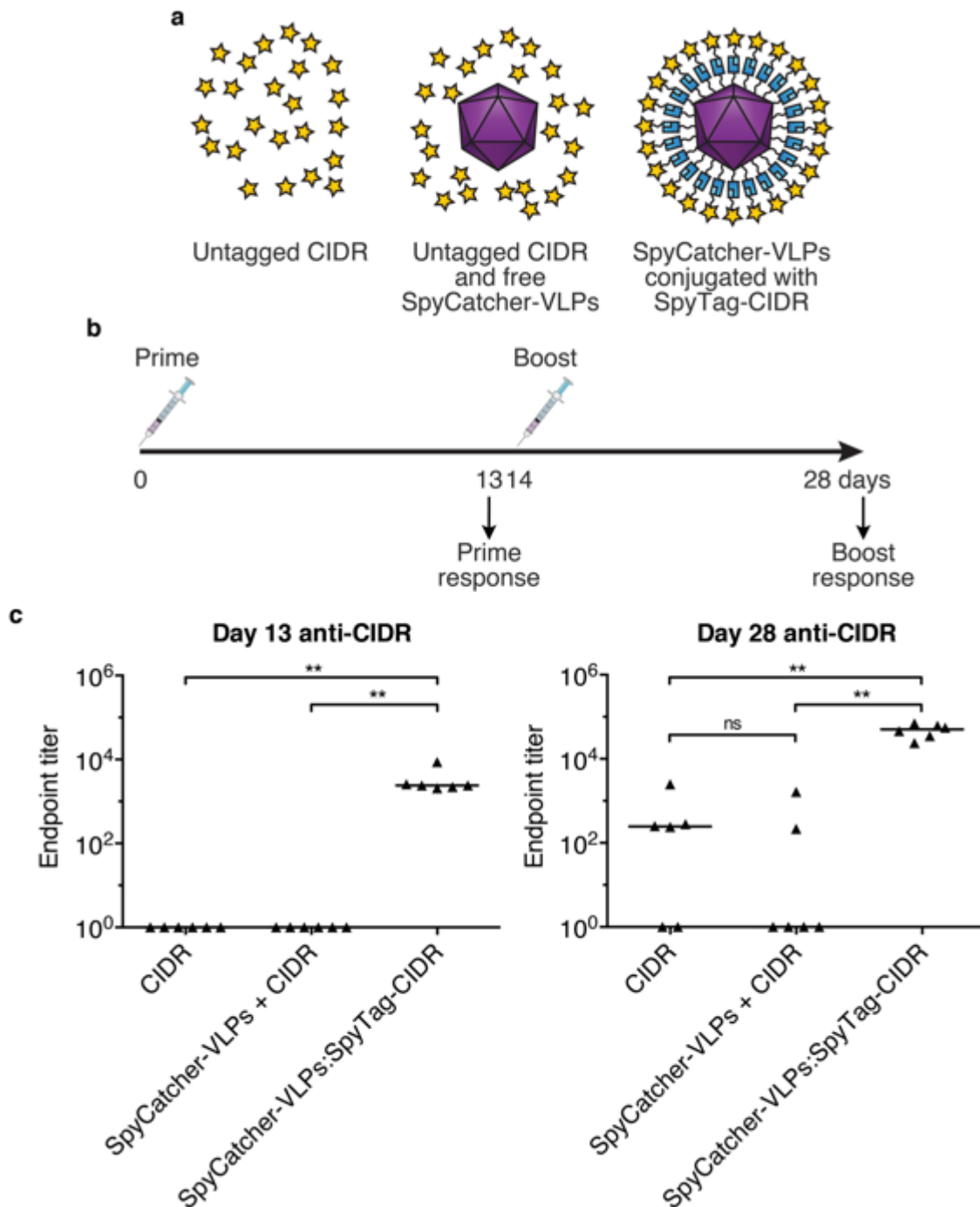


Figure 3.13 Plug-and-Display immunization with CIDR immunogens. (a) Schematic of immunogens. Covalently decorated SpyCatcher-VLPs:SpyTag-CIDR(IT4var07) and the negative controls: SpyCatcher-VLP + un-tagged CIDR (no covalent association) or CIDR alone. (b) Immunization schedule and sampling. (c) Total IgG responses to CIDR after Plug-and-Display immunization. Mice were immunized on days 0 and 14 with CIDR, SpyCatcher-VLPs + untagged CIDR, or SpyCatcher-VLP conjugated to SpyTag-CIDR (n=6 mice per condition). After 13 days (left) or 28 days (right), total IgG anti-CIDR titre was determined by Endpoint Enzyme-Linked Immunosorbent Assay (ELISA). Triangles represent the value for each mouse, while the horizontal bar exemplifies the median. ns not significant, ** $p < 0.01$, determined by Mann-Whitney test (n=6).

After establishing the integrity and reactivity of the CIDR(IT4var07) particles, we explored the potential of SpyCatcher-VLPs to generate an immune response. Dr Darren Leneghan immunized mice intramuscularly with 20 µg CIDR(IT4var07) equivalent, either as SpyCatcher-VLPs:SpyTag-CIDR conjugate or with the negative controls of SpyCatcher-VLPs + untagged CIDR or CIDR alone; all in the absence of adjuvant (Figure 3.13a). A prime boost schedule was employed as depicted (Figure 3.13b). Total IgG titre against CIDR were measured 2 weeks after each immunization (day 13 and 28) via ELISA by Dr Darren Leneghan (Figure 3.13c) and analysed by myself. SpyCatcher-VLPs:SpyTag-CIDR induced a higher anti-CIDR response than either SpyCatcher-VLPs + untagged CIDR or the CIDR-only group at any time-point tested (Figure 3.13c). It was noteworthy that only the SpyCatcher-VLPs:SpyTag-CIDR group showed anti-CIDR antibody generation as early as day 13, significantly outperforming both control groups, SpyCatcher-VLPs + untagged CIDR or the CIDR-only group ($p = 0.002$, $n = 6$, Mann-Whitney test) (Figure 3.13c). Boosting on day 14 resulted in an improved anti-CIDR antibody titre in 6 of 6 mice in the SpyCatcher-VLPs:SpyTag-CIDR group. On the other hand, only 2 out of 6 mice immunized in the SpyCatcher-VLPs + untagged CIDR group and only 4 out of 6 mice immunized in the CIDR-only group responded after the boost. The SpyCatcher-VLPs:SpyTag-CIDR group showed significantly higher responses on day 28 compared to the antibodies raised in either SpyCatcher-VLPs + untagged CIDR group or the CIDR-only group ($p = 0.002$, $n = 6$, Mann-Whitney test) (Figure 3.13c). The presence of unconjugated SpyCatcher-VLPs did not affect the immune response to CIDR (day 28 $p = 0.3$, not significant, $n = 6$, Mann-Whitney test).

3.2.2 Immunogenicity of Pfs25-SpyTag:SpyCatcher-VLPs conjugates

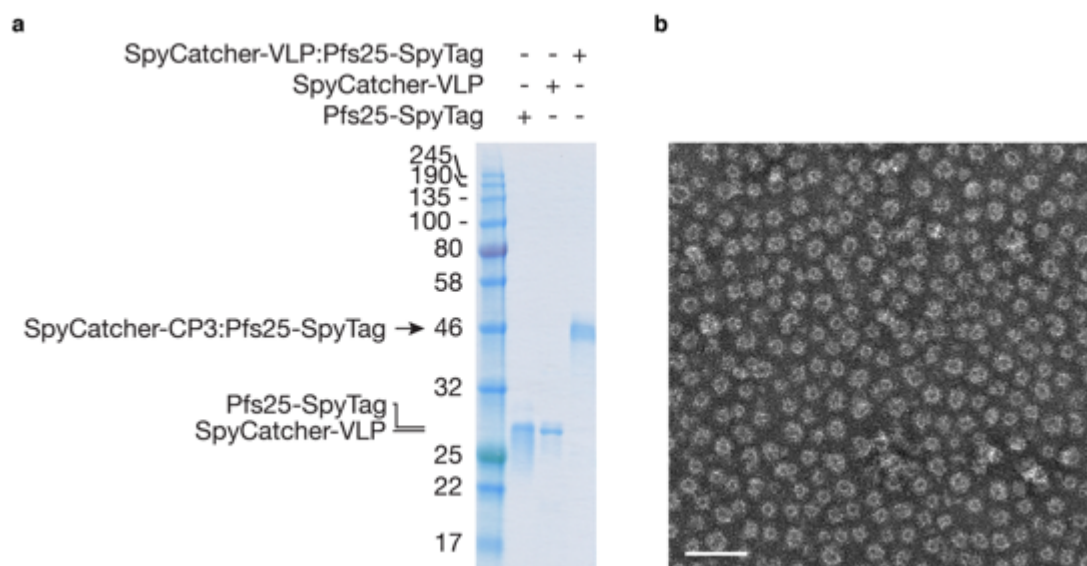


Figure 3.14 SpyCatcher-VLPs:Pfs25-SpyTag immunogens. (a) SpyCatcher-VLPs, Pfs25-SpyTag and the dialysed SpyCatcher-VLP:Pfs25-SpyTag conjugate were boiled in reducing SDS-loading buffer and analysed by SDS-PAGE with Coomassie staining. (b) Negatively-stained TEM images of SpyCatcher-VLPs after conjugation with excess Pfs25-SpyTag and subsequent dialysis. Scale bar 100 nm.

To further support the general applicability of the Plug-and-Display platform, we performed immunization against Pfs25, a promising transmission-blocking antigen (Nikolaeva et al., 2015). Andrew Ishizuka prepared immunization grade Pfs25-SpyTag, I prepared immunization grade (no or low endotoxin) SpyCatcher-VLPs and Dr Darren Leneghan performed the SpyCatcher-VLPs:Pfs25-SpyTag conjugation according to my instructions, followed by immunization of mice. As before, preparation of SpyCatcher-VLPs:SpyTag-Pfs25 yielded fully decorated and intact particles as assessed by SDS-PAGE and TEM (Figure 3.14).

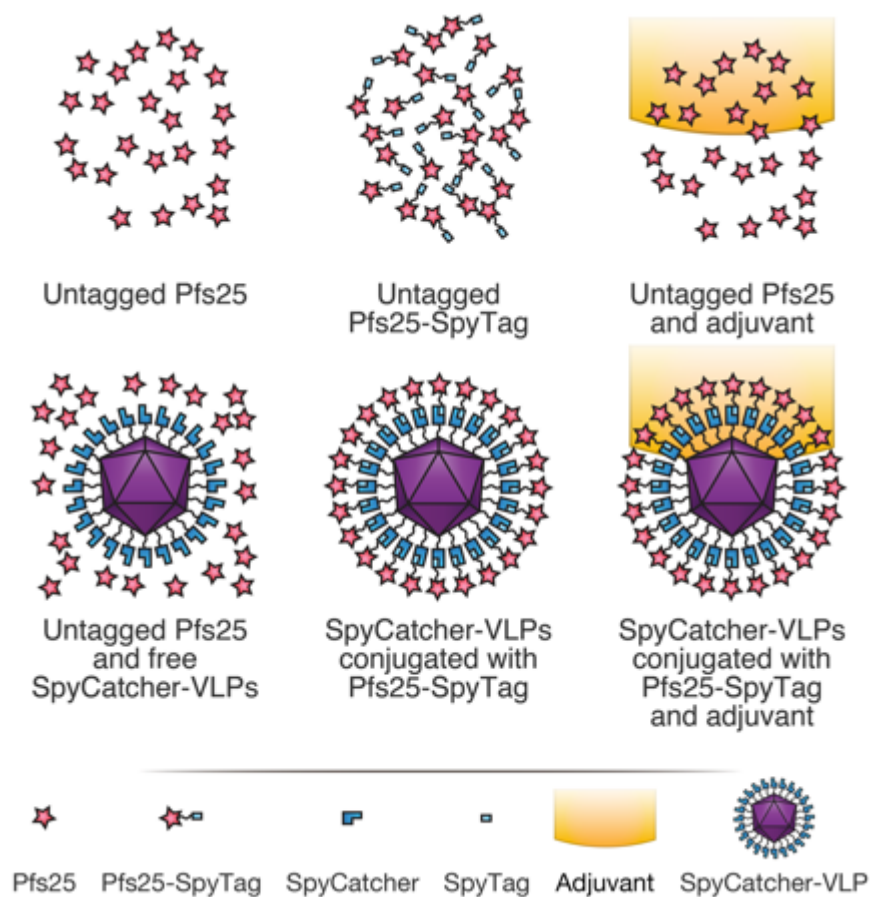


Figure 3.15 Plug-and-Display immunization with Pfs25 immunogens (II). Schematic of immunogens. Covalently decorated SpyCatcher-VLPs:Pfs25-SpyTag with or without adjuvant and the control immunogens: Pfs25 alone, Pfs25-SpyTag alone, adjuvanted Pfs25 alone, SpyCatcher-VLPs + untagged Pfs25 (no covalent association).

To test whether SpyTag itself enhanced the immunogenicity of a linked antigen, Pfs25 and Pfs25-SpyTag were both immunized alone or as before with SpyCatcher-VLPs.

AddaVax, a state of the art adjuvant is based on a squalene oil-in-water nano-emulsion and is similarly formulated to the licensed MF59 adjuvant and may therefore be included in recombinant vaccines for potentiation of cellular (Th1) and humoral (Th2) responses (Calabro et al., 2013). To test how adjuvant would influence the antibody response, AddaVax was complexed to both SpyCatcher-VLP conjugate and monomeric Pfs25 (Figure 3.15).

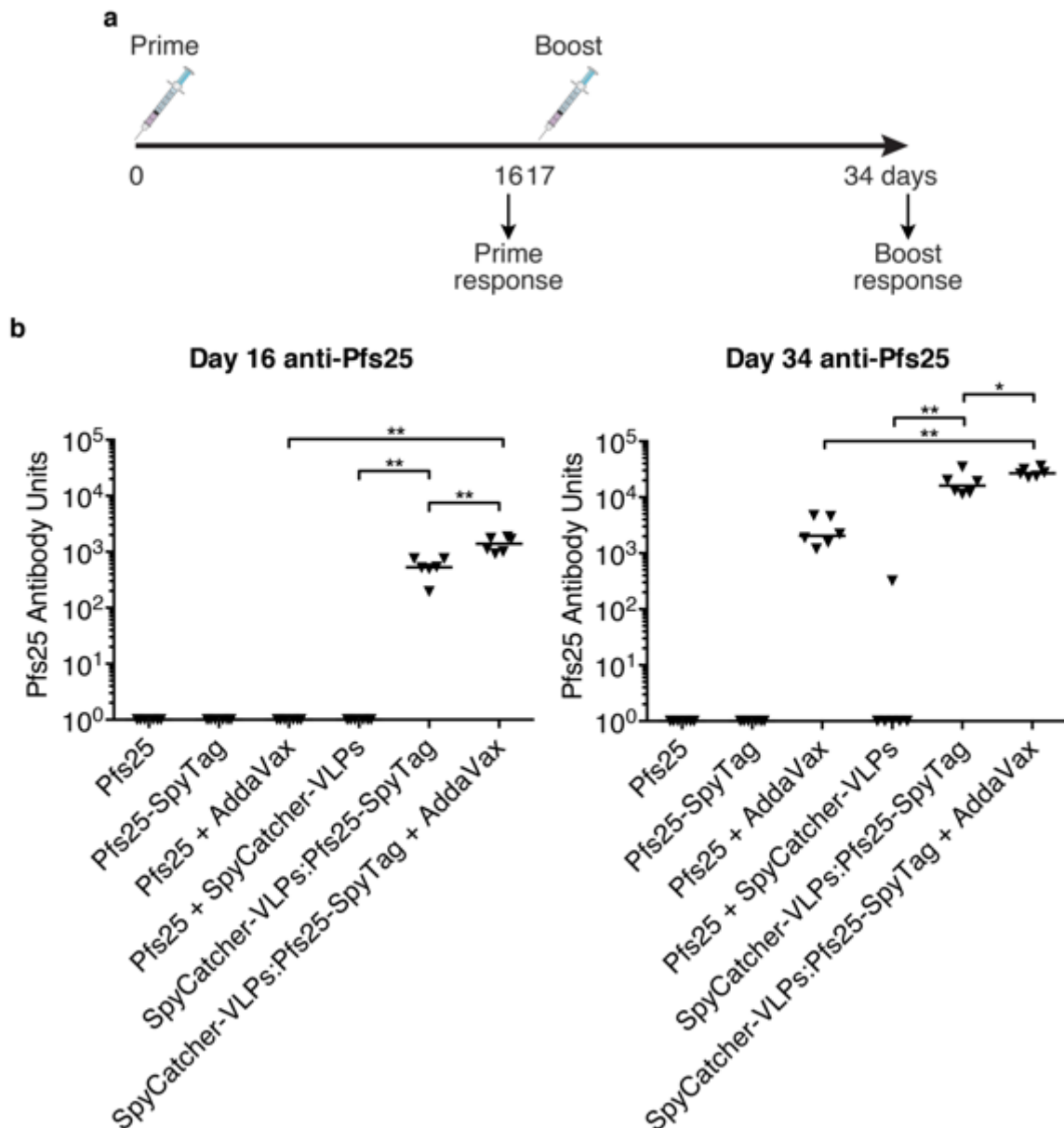
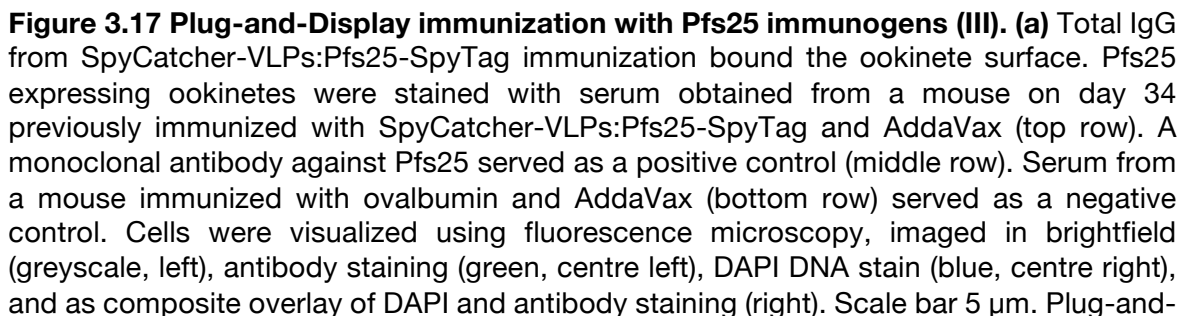


Figure 3.16 Plug-and-Display immunization with Pfs25 immunogens (II). (a) Immunization schedule and sampling. (b) Standardized Pfs25 antibody units after Plug-and-Display immunization. Mice were immunized on days 0 and 17 with Pfs25 alone, Pfs25-SpyTag alone, adjuvanted Pfs25 alone, SpyCatcher-VLPs + untagged Pfs25 (no covalent association), or SpyCatcher-VLPs conjugated to Pfs25-SpyTag with or without the adjuvant AddaVax (n=6 mice per condition). After 16 days (left) or 34 days (right), standardized Pfs25 antibody units (total IgG) were determined by Endpoint-ELISA against a reference serum. Triangles represent the value for each mouse, while the horizontal bar represents the median. ns not significant, * $p < 0.05$, ** $p < 0.01$, determined by Mann-Whitney test (n=6).

Dr Darren Leneghan immunized mice intramuscularly with doses matched to 2.5 μ g Pfs25 per formulation with a prime-boost regimen as outlined in Figure 3.16a

was followed. Dr Darren Leneghan measured antibody titres against Pfs25 on days 16 and 34 via ELISA (Figure 3.16b). Pfs25 fused to SpyTag or on its own did not induce an immune response neither after prime nor boost in the unadjuvanted monomeric state. Pfs25 complexed with AddaVax gave a response after boosting only. SpyCatcher-VLPs conjugated to Pfs25-SpyTag however induced a strong response already after prime, whereas the negative control of SpyCatcher-VLPs + untagged Pfs25 only induced antibodies in one mouse at day 34 and none at day 16 ($p = 0.002$, $n = 6$, Mann-Whitney test). Adjuvanting the SpyCatcher-VLPs:Pfs25-SpyTag conjugate improved both prime ($p = 0.002$, $n = 6$, Mann-Whitney test) and boost response slightly ($p = 0.04$, $n = 6$, Mann-Whitney test) (Figure 3.16b). The adjuvanted but unconjugated Pfs25 protein did not match the response to SpyCatcher-VLPs:Pfs25-SpyTag on either days (day 16 and 34, $p = 0.002$, $n = 6$, Mann-Whitney test).



Display immunization with Pfs25-Spytag yields transmission blocking antibodies. Purified IgG was mixed with *P. falciparum* NF54 cultured gametocytes and fed to *A. stephensi* mosquitoes (n=20 per test group) in SMFA. Control groups were IgG purified from the non-conjugated Pfs25 + SpyCatcher-VLPs immunization and an unrelated protein, ovalbumin with AddaVax (Control). Midguts were dissected 7 days post-feeding. Data points represent the number of oocysts observed in individual mosquitoes.

To further test whether antibodies raised from the SpyCatcher-VLP:Pfs25-SpyTag immunization could recognize native antigen, Iona Brian stained blood smears containing transgenic *P. berghei* ookinetes. These ookinetes express Pfs25 instead of the native Pbs25 on their surface (Mlambo et al., 2008). Secondary, fluorescently labelled antibody staining against mouse antibodies allows detection of Pfs25 recognizing antibodies and Pfs25 on the parasite by fluorescence microscopy (Figure 3.17a). Serum derived from mice boosted with SpyCatcher-VLPs:Pfs25-SpyTag and AddaVax resulted in strong antibody labelling on the surface of the ookinete, similar to the pattern with the validated anti-Pfs25 monoclonal antibody 4B7 (Figure 3.17a). Serum from a mouse immunized with the negative control antigen ovalbumin and AddaVax gave minimal surface staining (Figure 3.17a, bottom row).

Having demonstrated strong antibody responses and native Pfs25 binding, we wondered if the raised antibodies are immunologically relevant in the context of disease. Therefore raised total IgG was assayed for transmission-blocking activity via the standard membrane feeding assay (SMFA) in Dr Carole Long's laboratory. Serum was pooled from each group of vaccinated mice at the end of the vaccination schedule; omitting the single responder in the SpyCatcher-VLP + Pfs25-SpyTag control group. Total IgG was purified using a Protein G column, mixed with *in vitro* cultured *P. falciparum* gametocytes and fed to *A. stephensi*

mosquitoes through a membrane feeder. IgG derived from either negative control groups ovalbumin and AddaVax (control) or untagged Pfs25 + SpyCatcher-VLPs failed to control progression of the ookinete to oocyst stage (Figure 3.17b). Both conjugate immunogens however provided potent transmission blocking activity; the adjuvanted formulation blocking transmission entirely (Figure 3.17b).

3.3 Attempts at improving SpyCatcher-VLP yields

In order to generate a widely usable platform, consistently high yields need to be easily obtainable. Initial preparations of SpyCatcher-VLPs generated by the pGEM vector system in C41(DE3) were low and generally varying in the range of 0.3-1.5 mg/L culture [0.17-0.3 mg/g wet cell weight (wcw)]. Optimization of culture conditions and purification procedures increased yields to 1.5-3 mg/L, with occasionally higher yields of up to 5 mg/L (as determined by bicinchoninic acid assay, BCA). Optimizations were in part performed by Linda Tchatchouang under my guidance. Ms Tchatchouang found that, whereas previously 300 mM NaCl was used to wash the resin, reducing salt concentrations (150 mM NaCl) increased yield. Adoption of 2xTY as the media further increased yields, as did the use of bottom baffled shaking flasks (Thompson UltraYield 2.5L), designed for better aeration; possibly as a function of increased biomass (biomass yields were doubled). 2xTY also provides additional tryptophan, which may have reduced the expression from the Ptrp promoter in pKB_215 and allowed for increased biomass production prior to IPTG induction. To address the yield issue from a molecular perspective, an alternative expression system comprising an pET21 vector with an LacO gated pT7 was employed, which one may expected to increase biomass titres and reduce toxicity, as the LacO reduces leaky transcription from the promoter during the grow up phase (Gilbert and Maxam, 1973). Adoption of the native AP205 CP3 codon usage, which may provide potential packaging signals (PS) motifs for VLP assembly as well as incorporation of the putative RNA hairpin with AP205 CP3 affinity were also explored, in the hope that affinity to its cognate

RNA may stabilize SpyCatcher-CP3 protein *in vivo* and reduce inclusion body formation (Figure 3.18).

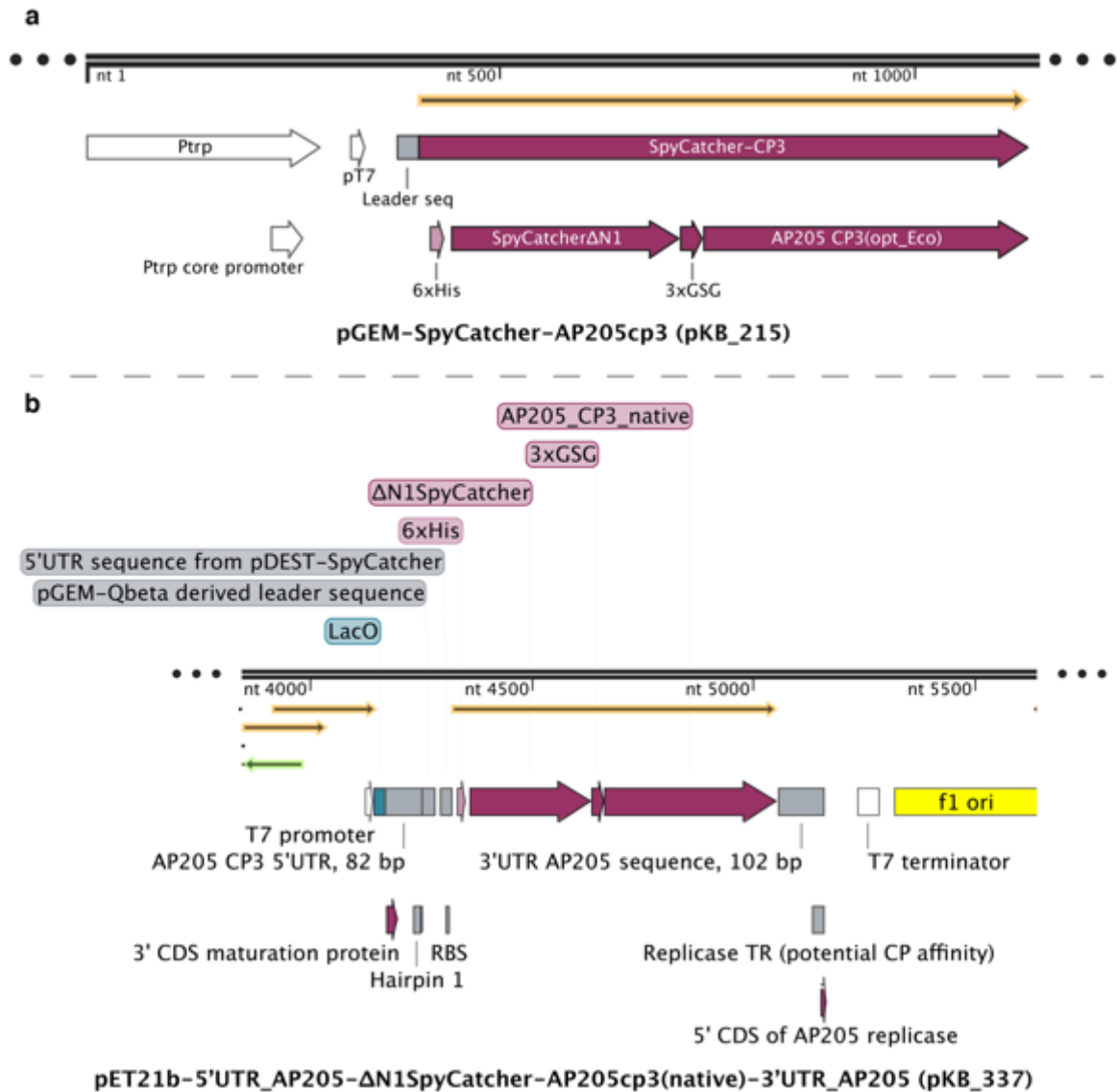


Figure 3.18 Schematic representation of SpyCatcher-CP3 vector inserts. (a) A high-copy number pGEM vector carries a modified tryptophan promoter (P_{trp}) from *E. coli*, an additional promoter pT7 from the bacteriophage T7, both which drive the expression of His₆-tagged ΔN1 truncated SpyCatcher fused via a flexible 3xGSG to an *E. coli* codon optimized sequence of AP205 coat protein 3 (CP3) (pKB_215). Transcription is not terminated in pKB_215 **(b)** A medium copy number ColE1 origin type plasmid, with basis of mobility region from pBR322 (mob) under control of repressor of primer (ROP) serves as the vector backbone. The wildtype codon usage AP205 CP3 is fused to SpyCatcher as above but the CDS is additionally flanked by a 82 bp fragment of the 5'UTR of AP205 as well as an 102 bp fragment 3'UTR region, including the putative 5'UTR replicase hairpin with CP affinity. The predicted amino acid sequences for SpyCatcher-CP3 are identical for both plasmids and both carry an ampicillin resistance gene.

Expression of the pKB_337 plasmid (pET21 system) allowed for increased production of biomass as final fermentation A_{600} was ~ 2 higher compared to pKB_215 [pGEM system (A_{600} 3-4 vs. 7-8)]. However pKB_337 expression directed proteins even more to inclusion bodies, as observed when A_{600} was normalized across samples (Figure 3.19). pKB_337 derived IMAC purified protein also contained substantially more contaminants compared to the pGEM-SpyCatcher-AP205cp3 (pKB_215) system (processed in parallel).

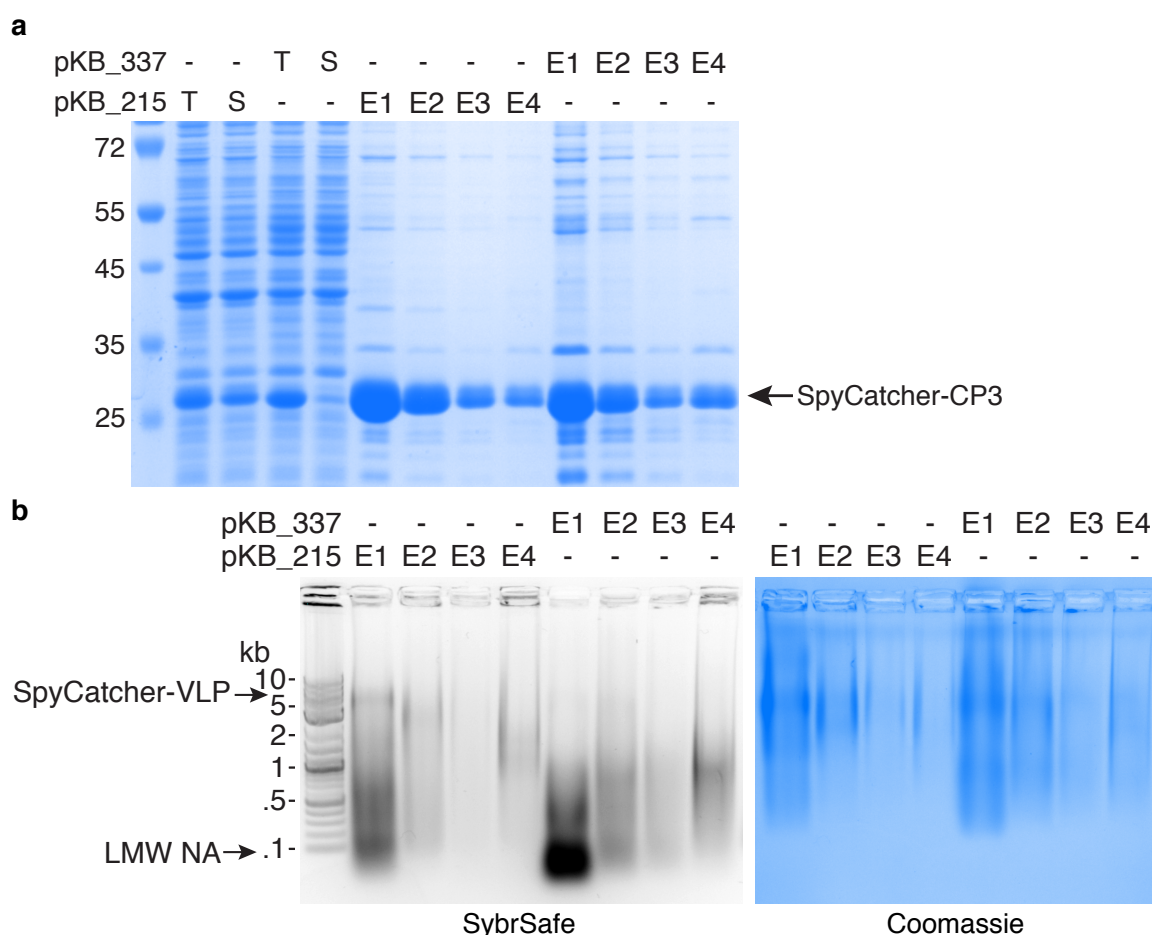


Figure 3.19 IMAC elution profiles of SpyCatcher-VLP expression systems. pET21 expressed target protein but VLP quality was poor, whereas pKB_215 produced SpyCatcher-VLPs. **(a)** Reducing SDS-PAGE of A_{600} normalized induction samples [total protein (T), soluble protein (S)] and IMAC elutions (E_x , 1 mL fractions, 500 μ L packed resin, elution buffer: 2M imidazole, 50 mM glycine, 25 mM sodium citrate, Tween 20 0.1%, pH 8.5). **(b)** Native agarose gel electrophoresis of IMAC elutions. The agarose was first imaged for nucleic acids and then stained for proteins with a Coomassie dye.

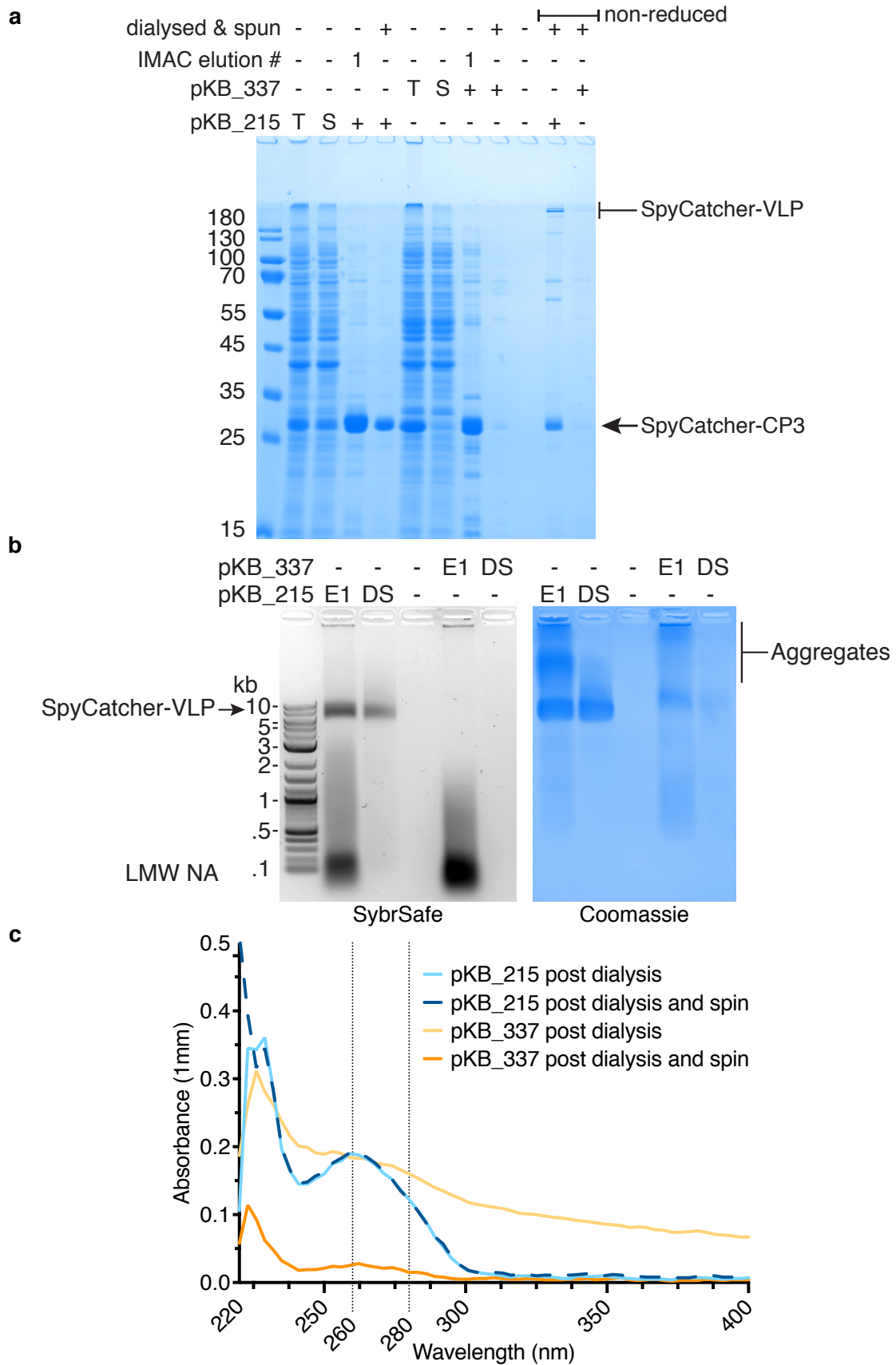


Figure 3.20 Comparison between pKB_215 and pKB_337 derived SpyCatcher-VLPs.
(a) SDS-PAGE of induction, IMAC elution, and fully purified SpyCatcher-VLPs. All samples

except the two final VLP preparation samples to the far right were run in reducing conditions and show disulfide-linked species. IMAC samples were further purified via dialysis through a 300 kDa MWCO membrane and aggregates were removed by spinning for 30 min, 17,000 *g*. The same sample volumes were applied to the gel for the IMAC- and final purified samples. Loss in intensity is a function of removal of contaminant and non-VLP SC-CP3 species, dilution during dialysis and removal of aggregates. **(b)** Native agarose gel electrophoresis of IMAC (E1) and dialysed & spun (DS) SpyCatcher-VLP preparations showing the difference in nucleic acid species (NA) purified. **(c)** UV-vis absorption spectrum for preparations before and after removal of aggregates by centrifugation. LMW – Low molecular weight.

pKB_337 showed exclusively lower MW nucleic acid species which were removed after dialysis and centrifugation, whereas pKB_215 showed higher MW species, which persisted throughout the purification the procedure [Figure 3.19b, 3.20b (pKB_337 <1 kb vs. pKB_215 >4 kb)]. pKB_337 derived protein also showed diffuse co-migration of RNA and protein in native agarose gel electrophoresis post elution / dialysis, and was generally characterized by reduced yield, whereas co-migration was observed for pKB_215 (arrow, Figure 3.19b, 3.20b). pKB_337 derived protein was very susceptible to aggregation shortly after elution from the resin but also after / during dialysis (Figure 3.20c). The pKB_215 preparation shown here had a concentration of 358 ± 10 µg/mL (1 s.d.) protein (BCA) and a nucleic acid content of 95 µg/mL (A260), with a nucleic acid to protein ratio of 1 to 3.8.

3.4 Dual-antigen load: SpyCatcherDuo-VLPs and their features

3.4.1 *The case for increased antigen density*

Antigen density on a single particle is an important consideration for particulate vaccines. Several VLP based vaccine (candidates) require co-expression of the unmodified or truncated coat-protein (CP) in order to assemble. This work-around reduces antigen density however; the prominent example being RTS,S (Heppner et al., 2005; Kaslow and Biernaux, 2015). For *Leviviridae*, reduced antigen density can also be achieved by single-chain fusion of two monomers to each other (Peabody, 1997; Brown et al., 2009). I wondered if it would be possible to increase density beyond the single SpyCatcher residue per CP on AP205, as established for SpyCatcher-VLPs. This may be beneficial as increased antigen density may lead to improved BcR-crosslinking for T-independent responses (Bachmann et al., 1995), but may also provide means for locally increasing T-cell epitope density for the T-dependent immune response (Bullock et al., 2003; Heppner et al., 2005). In an attempt to increase valency on the particle, a single-chain SpyCatcher-SpyCatcher-AP205 CP3 fusion (Figure 3.21) was cloned, expressed and purified similar to SpyCatcher-VLPs, with minor modifications (see section 2.2.10).

3.4.2 SpyCatcherDuo-VLPs and their features

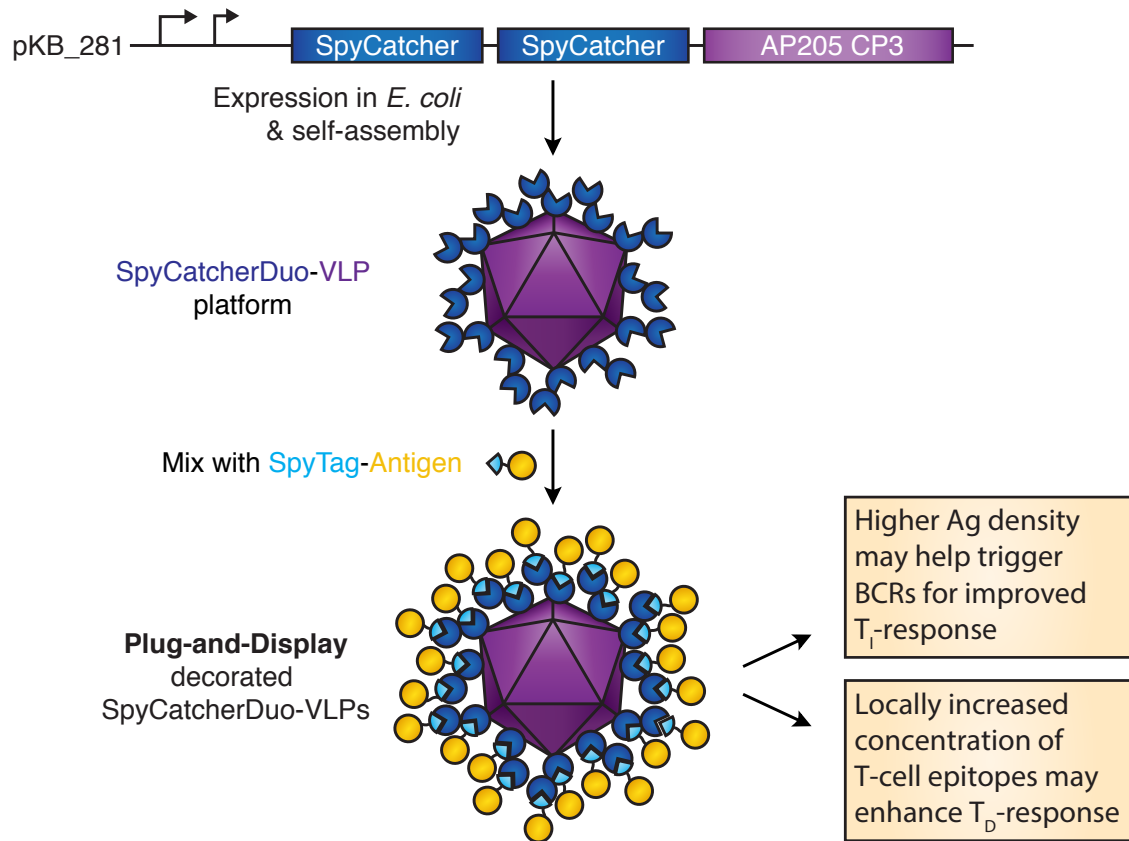


Figure 3.21 SpyCatcherDuo-VLPs for increased antigen display. Two different codon variants of SpyCatcher Δ N1 are fused to each other via a 3xGSG linker and are N-terminally installed on AP205 CP3 through an additional 3xGSG linker. The density of Catcher residue, and thus antigen post conjugation may be doubled compared to 1xSpyCatcher-VLPs. Increased antigen density could allow for increased BcR-crosslinking, therefore improved T-independent (T_i) antibody generation, as well as for local increase of T-cell epitopes, which may allow for dosage reduction.

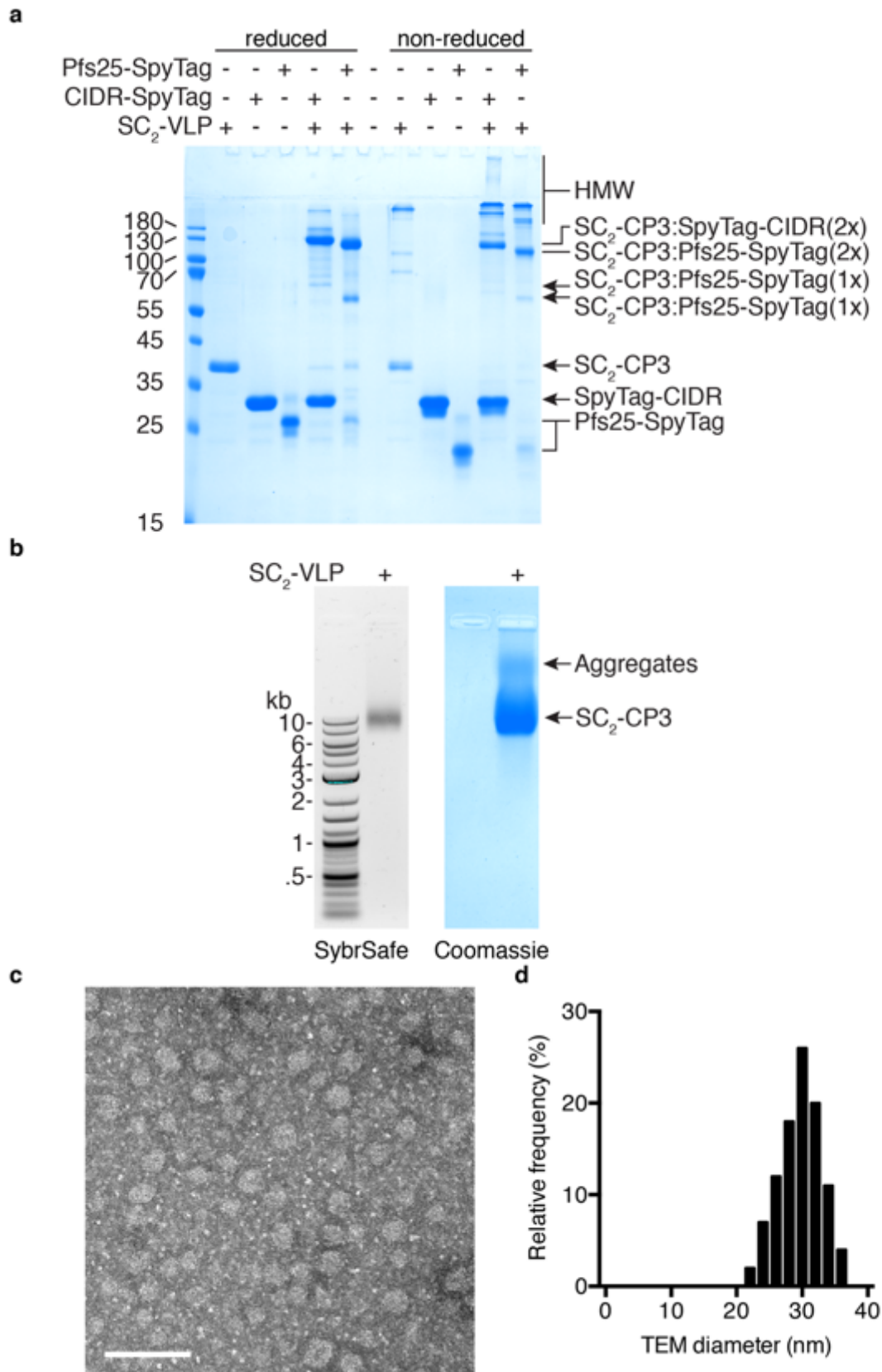


Figure 3.22 SpyCatcherDuo-VLPs. (a) SpyCatcherDuo-VLPs (SC₂-VLPs) were incubated with SpyTag-containing antigens, before boiling in SDS-loading buffer and analysis by

SDS-PAGE with Coomassie staining. SpyTag-CIDR(IT4var07) was supplied in 1.5× molar excess in relation to SpyCatcher moieties, Pfs25-SpyTag was supplied in 0.75× molar ratio and reacted in the presence of 1× reaction buffer for 3 h at RT. **(b)** Native agarose gel electrophoresis of SpyCatcherDuo-VLPs. The agarose gel was first stained and imaged with SybrSafe (staining nucleic acid) prior to staining with InstantBlue (a Coomassie dye staining protein), showing SpyCatcherDuo-VLPs co-migration of nucleic acid and protein signal. **(c)** Negatively-stained TEM images of SpyCatcherDuo-VLPs after IMAC purification and subsequent dialysis through a 300 kDa MWCO membrane. Scale bar 100 nm. **(d)** Size distribution of SpyCatcher-VLPs from TEM (n=100).

IMAC preparations followed by 300 kDa MWCO dialysis of SpyCatcherDuo-VLPs yielded ~1.3 mg protein per litre culture (BCA). The protein was of high purity as determined by SDS-PAGE and the theoretical Mw of 36 kDa was in close agreement with the observed Mw of ~37 kDa as inferred from reducing SDS-PAGE (Figure 3.22a). Disulfide-linked multimers were observed in non-reducing conditions and could be fully reduced (Figure 3.22a). Again, a minor secondary band migrating just above the main band is most likely caused by gluconylation of the His₆-tag preceding the SpyCatcherSpyCatcher-CP3 fusion (Geoghegan et al., 1999; Aon et al., 2008; Zakeri et al., 2012).

In addition to the presence of spherical particles consistent with VLPs observed in negative-stained transmission electron microscopy (TEM), likely aggregates were also observed, which hindered precise size determination. (Figure 3.22c). Manual measurements of large spherically shaped particles (>10 nm) gave a diameter of 29 ± 3.2 nm (mean $\pm$ 1 s.d., minimal diameter 22 nm, maximal diameter 36 nm, n=100). Small particles were also observed with a diameter < 5 nm (Figure 3.22d). Co-migration of protein and nucleic acid species in native agarose gel electrophoresis was observed, as was migration of protein without apparent nucleic acid density (likely aggregates) (Figure 3.22b).

Conjugation with SpyTag-antigens could be driven close to completion by use of 1.5 molar excess SpyTag-CIDR(IT4var07) in relation to SpyCatcher moieties (Figure 3.22a). When a sub-saturating Pfs25-SpyTag concentration was applied, a Pfs25-SpyTag:SpyCatcher-SpyCatcher-VLP intermediate could be observed (Figure 3.22a).

3.5 Discussion

Expression studies of SpyCatcher-VLPs.

The dual promoter system of pGEM-SpyCatcher-AP205cp3 (pKB_215) in combination with the pUC-like high copy number origin likely imposes high metabolic burden for replication and early, leaky expression of target protein leading to the observed toxicity (Table 3.1) (Lin-Chao et al., 1992; Yanisch-Perron et al., 1985). C41(DE3) and C43(DE3), also commonly referred to as the Walker strains, have reduced basal expression of T7 RNA polymerase (T7 RNAP) compared to their parental BL21(DE3) strain (Miroux and Walker, 1996). Three nucleotide mutations in the -10 and +1 regions of the lacUV5 promoter that drives T7 RNAP expression are responsible for both basal and reduced induced transcript levels of both the protein of interest and T7 RNAP (Kwon et al., 2015; Wagner et al., 2008). Hence one may hypothesize that reduced expression from the ungated (no Lac operator) T7 promoter in the pGEM vector system leads to reduced protein and thus less metabolic stress is exerted on the host strain (Sørensen and Mortensen, 2005). However additional mutations in C41(DE3) and C43(DE3) may contribute to increased resilience to lysis as membrane biosynthesis regulation is altered in these strains; one may speculate that increased membrane production protects cells from lysis for example (Arechaga et al., 2000). Furthermore, it is also known that the Walker strains display improved plasmid retention which may allow for improved SpyCatcher-VLP production as more cells in the population carry the expression vector (Dumon-Seignovert et al., 2004). High expression levels of coat protein are required for high yielding VLP preparations. Dual transcription from the serial Ptrp and pT7 promoter should generate abundant mRNA species encoding

for capsid protein. Efficient AP205 VLP assembly also likely benefits from cytosolic RNA species that provide electrostatic multimerization scaffolds guiding the capsid assembly (Basnak et al., 2010). Overexpression of coat protein of the *Leviviridae* can be toxic and cause cells lysis however, as observed for Q β VLPs expressed in pET28 for example (Brown et al., 2009).

The Δ N1SpyCatcher (92 amino acids) domain with affinity tag and linker amount to 114 amino acid residues that are fused to AP205 CP3. The previously largest reported displayed antigen was 55 aa long (HIV polyepitope containing Nef consensus T-cell epitopes) (Tissot et al., 2010). Thrane and co-workers recently reported the display of full-length SpyCatcher on AP205 (+23 aa) but did not disclose the full sequence or obtained yields (Thrane et al., 2016). The protein displayed by Thrane is likely the longest fusion to AP205 reported in literature. I have previously cloned and expressed full-length SpyCatcher-VLPs and found solubility to be comparable to Δ N1SpyCatcher. The increased protein length itself is unlikely to be toxic, as larger proteins can be produced at g scale without toxic effects. One may speculate that rare tRNAs, or depletion of a particular tRNA subset may cause growth retardation. However, the plasmid (pKB_215) does not carry any rare *E. coli* codons in the SpyCatcher-CP3 coding sequence. Non-rare codons are also harmonised, that is no particular codon is used exclusively or >65% for a specific amino acid. For initial protein preparation, expression levels of SpyCatcher-VLPs were sufficient to establish the principle. I describe attempts to scale production in section 3.3.

Biochemical and biophysical characterization of SpyCatcher-VLPs.

The CP3 monomer of AP205 contains 2 cysteines (Klovins et al., 2002). Hence either one intra-molecular or up to two inter-molecular disulfide bonds are possible. Inter-molecular disulfide bonds are observed on non-reducing SDS-PAGE, showing CP3 multimers with close to theoretical Mw of dimer, trimer and pentamer respectively that can be fully reduced (Figure 3.3). The related phage Q β forms inter-molecular disulfide bonds, likely at the five-fold axis, which contribute significantly to the high thermal stability of Q β (Ashcroft et al., 2005). It is possible that wildtype AP205 also forms these five-fold axis disulfides as the cysteine residues within the monomer are similarly located and linearly spaced within a close distance; e.g. 3 amino acids residue distance for AP205 and 5 residues for Q β [Flexibility prediction with PredictProtein suite, PROFbval server (Beekwilder et al., 1995; Schlessinger and Rost, 2005)]. SpyCatcher itself is devoid of cysteine and is generally a monomeric protein (Zakeri et al., 2012; Schoene et al., 2014). A very small fraction of SpyCatcher can undergo autocatalytic reaction of one Catcher residue to the other and is typically only visible on overloaded SDS-PAGE gels (see dimer band at 55-60 kDa, reducing conditions in Figure 3.7). It is currently unknown where this reaction occurs. Thus in rare instances, fusion of CP3 to Δ N1SpyCatcher may cause artificial association of CPs which then form un-natural disulfide bonds causing observed dimeric species. SpyCatcher fusion may itself impose some structural constraints that inhibit formation of all native AP205 disulfides and some species stay trapped. Also, it is possible that smaller multimer species, such as dimers were not entirely removed during dialysis and are thus not assembled in the VLPs.

Incomplete assemblies are apparent from size exclusion chromatography (Figure 3.4a), as protein elutes over a wide-range whereas uniform VLPs would be expected to be exclusively found in the MDa range. Lower Mw protein eluting may represent capsid multimers trapped in assembly dead ends (Basnak et al., 2010). Though particles of VLP shape were clearly observed, extensive variation of particle integrity was apparent from qualitative and quantitative TEM studies (Figure 3.4b, c), as well as evidenced by a broad size distribution as determined by DLS (Figure 3.4d). Wildtype AP205 phage (T=3) is reportedly 27-30 nm in diameter as determined by TEM (Klovins et al., 2002). Recombinant AP205 wildtype phage may assume ~80-90 % T=3 symmetry, while the remaining ~10-20% have T=1 symmetry (ftp://ftp.esrf.eu/pub/UserReports/28776_K.pdf, accessed 31 Jul 2016). Non-fused AP205-VLPs from *E. coli* can also show different symmetries types (Tissot et al., 2010). SpyCatcher-VLPs would be expected to be bigger due to display of the Δ N1SpyCatcher domain and linker (PDB 4MLS) with the following approximate dimension 4.5 nm length and 2.9 nm width of Δ N1SpyCatcher (PDB 4MLS) and approximately 3.42 nm theoretical length for the GSGGSGGSG linker. Δ N1SpyCatcher fusion may favour T=1 symmetry.

The difference in size as determined by TEM vs. DLS (Figure 3.4) is likely due to the different techniques used. DLS provides information about the hydrodynamic radius of the particle whereas TEM determines the projected area diameter. The hydrodynamic diameter provides information not only about the “solid” core particle but also about any coating material and solvent layer attached to the particle as it moves due to Brownian motion (Berne and Pecora, 2000). Noteworthy my SpyCatcher-VLP preparations contain Tween 20 0.1% (v/v) to avoid

aggregation. When estimating size by TEM, this hydration layer is not observed, hence the particle may appear smaller (Pyrz and Buttrey, 2008).

I focused my efforts on development of a simple purification procedure that would enable modestly equipped laboratories to produce SpyCatcher-VLPs, including developing and emerging countries. Simultaneously Thrane et al. independently developed an alternative expression system and purification procedure for SpyCatcher-VLPs, more similar to published preparations of wildtype AP205 VLPs. These preparations, utilizing full length SpyCatcher and gradient ultracentrifugation, appear to yield more uniform particles as analysed by TEM, however neither high resolution images nor descriptive statistics have been made available that would allow direct comparison (Thrane et al., 2016). The DLS measurements are in close agreement to each other e.g. 43 nm (full length SpyCatcher and with PS80 instead of Tween 20 as detergent) vs. 42 nm. Thrane and co-workers report a narrower particle distribution, indicating higher particle purity and homogeneity achieved by the ultracentrifugation step. Purification via IMAC on the other hand may co-purify intermediate states that assemble into less homogeneous multimeric structures, despite stringent washing and dialysis through a 300 kDa MWCO membrane. Larger pore 1 MDa MWCO membrane has recently become available and might provide more uniform particles. The particulate state was deemed sufficient to justify immunization experiments and, as shown in section 3.2, absolute uniformity is not required to induce potent immune response.

SpyCatcher-VLP:SpyTag reaction with model protein MBP, SpyCatcher-VLP:SpyTag reaction with complex SpyTag-antigens and SpyCatcher-VLP:SpyTag reaction with synthesized peptides.

Reaction with SpyTag-MBP proceeded to completion as judged by SDS-PAGE and may be expected for a highly soluble globular protein (Figure 3.5). An important observation is that gel shifts in the high Mw species are also observed in the non-reducing lanes representing decoration of multimeric, disulfide linked capsid monomers.

SpyTag-MBP's Mw is approximately 44.9 kDa, twice as big as the more complex CIDR variants or Pfs25, which also reacted to completion (Figure 3.6, 3.11 and 3.14a). Surprisingly, the Salanti group was not able to reach full decoration of SpyCatcher residues on their SpyCatcher-VLP preparations, even with proteins being significantly smaller than 25kDa. The highest achieved conjugation was 88% for CTLA-4-SpyTag (15 kDa), followed by 45% for PD-L1-SpyTag (27 kDa) and only 34% for SpyTag-VAR2CSA after overnight incubation at 4 °C with 1.5x molar excess of SpyTag protein (Thrane et al., 2016). This difference may be explained by different linker lengths employed. A 5 amino acid residue flexible GS-rich linker was employed by the Salanti group to space the Catcher away from the VLP, whereas my $\Delta N1$ SpyCatcher is linked via a 9 amino acid residue flexible GS-rich linker. Assuming a perfect sphere as an approximation of a VLP, small linker length increases may significantly relax space constraints as the surface area of a sphere increases to the second power and the spherical volume increases to the third power. Another major difference were the buffer systems employed, the Salanti group used PBS containing 0.02 % Polysorbate 80 (PS80, also known as Tween

80), pH 7.2 for storage of their VLPs, whereas my SpyCatcher-VLP stock preparations are buffered in 50 mM glycine, 25 mM sodium citrate, Tween 20 0.1% (v/v), pH 8. Glycine's striking solubilizing character and natural occurrence in mammals make it a frequently encountered macromolecule stabilizer in pharmaceutical formulations (Parkins and Lashmar, 2000). Being a zwitterion, glycine has the ability to interact via both its negative and positive charged groups. Due to its small size, glycine is not sterically hindered from binding to multiple parts of the protein, including the peptide backbone (Platts and Falconer, 2015). Glycine may help with solubility of SpyCatcher-VLPs and accessibility of the unsatisfied immunoglobulin-like beta barrel-fold that is non-reacted SpyCatcher. Glycine may also improve solubility of the SpyTag-protein partner to be conjugated, improving its availability and thus reactivity. Another potential major factor is the pH during the conjugation. As SpyCatcher is known to be optimally reactive at pH values close to its theoretical pI, my reactions were supplemented with a strong citrate buffer that reduces the pH of the solution to at least pH 6.9 and thus improves kinetics of the reaction (Fierer et al., 2014; Zakeri et al., 2012).

My results showed that SpyCatcher-VLPs accept protein antigens linked to SpyTag at either terminus. Furthermore the origin of the SpyTag-antigen did not matter as antigen could be either expressed in bacteria or eukaryotic cells or chemically synthesized entirely. The tendency of peptide-labelled particles to precipitate was likely caused by hydrophobic residues of the peptides employed that are required for the intended binding to MHC class I for priming of T-cell responses. Additionally for practical purposes biotin was also included on the peptide, which adds to the hydrophobic character of the peptide (Livnah et al.,

1993). The sequence for SpyTag-Telo was biotin-GAHIVMVDAYKPTREAR**PALL**TSRLR**F**IPK (7 hydrophobic residues in displayed peptide, SpyTag underlined, hydrophobic residues in displayed part in bold) and **L**EEKKGNY**V**WTDHGAHIVMVDAYKPT**K**-biotin (4). Chemical labelling of VLPs with peptides at high density is known to cause solubility problems, which are typically addressed by reducing peptide load (Ruedl et al., 2002; Win et al., 2011). Alternatively, charged residues may be appended to improve solubility. A truncated version of SpyTag (minimal sequence 8 amino acids) still has efficient reactivity towards SpyCatcher. Therefore a shorter Tag might allow more charge or multiple T-cell epitopes to be included in the attached peptide (Zakeri et al., 2012). For this thesis, peptide conjugations were not further pursued and the focus was directed to difficult full-length proteins.

Having demonstrated reactivity with antigens and peptides with terminally located SpyTags, next I wondered if it would be possible to fuse SpyTag internally into protein antigens, in order to rotate them in relation to SpyCatcher-VLPs. Internal fusion of SpyTag can be desirable when N- or C-terminal fusion would obstruct accessibility to T-independent B-cell epitopes. Likewise immunomodulatory protein ligands, such as flagellin, require a certain orientation to allow sterical access to their receptors (Lu and Swartz, 2016; Lu et al., 2013). After minor optimization a flagellin containing an internal SpyTag could be displayed on the SpyCatcher-VLPs, albeit large excess and increased reaction times were required (Figure 3.9). Structures of proteins to be displayed with internal SpyTag are extremely helpful. Otherwise engineering will be mostly a trial and error approach when completion is desired. For immunostimulatory proteins, however, 100%

completion is probably not required; only minute amounts of ligand are typically required to trigger TLRs, for example. TLR5 is activated by only two flagellin molecules through dimerization (Yoon et al., 2012). Kinetics of SpyCatcher-VLP decoration may also be controlled by utilizing shorter SpyTag variants to reduce reactivity or longer linkers to improve accessibility to the Tag and increase reactivity (Zakeri et al., 2012).

Storage of SpyCatcher-VLPs and reactivity

Stability of VLPs is a crucial factor for practical reasons. SpyCatcher-VLPs were prone to aggregation after freezing and thawing. Therefore storage at 4°C was assessed. SpyCatcher-VLPs were structurally stable for at least 1 month storage at 4 °C and retained full reactivity with SpyTag-Pfs25 (Figure 3.14). Immunization with stored and Pfs25-SpyTag reacted particles also elicited potent antibody responses against Pfs25 (Figure 3.16 and 3.17), but no head to head comparison with freshly prepared particles was performed.

Heating of SpyCatcher-VLPs at 65°C for 12 h did not result in major loss of protein (Figure 3.10). Heating did induce a shift in migration for some of the SpyCatcher-CP3 monomers (Figure 3.10). This is striking as samples were boiled and resolved in the presence of SDS, therefore the cause for the shift may be of covalent nature or due chemical modification and warrants further investigations. Particle integrity by TEM was not assessed and is another crucial parameter. Reactivity with the model protein AffiHer2-SpyTag was retained, albeit only to 68% completion (Figure 3.10). Reduced reactivity with SpyTag antigen may not be a major concern. Thrane and co-workers have demonstrated that under-saturated particles are still highly

immunogenic. Coupling efficiency as low as 34% coverage was shown to be significantly more immunogenic compared to monomeric protein as determined by Ab titre (Thrane et al., 2016). It will be interesting to assess how reduced or increased antigen density influences the antibody response to SpyCatcher-VLP conjugated antigens quantitatively.

Immunogenicity of SpyTag-CIDR(IT4var07):SpyCatcher-VLPs conjugates

After having demonstrated reactivity with a range of proteins, immunization experiments with CIDR(IT4var07) were performed. I prepared immunogens as before and validated VLP integrity post-conjugation (Figure 3.11, 3.12b, c). Triton X-114 (TX114) was used to remove lipopolysaccharides (LPS) from the protein preparations, whilst bound to the IMAC resin or by phase separation. Increased particle size was observed as would be expected due to conjugation of protein. The CIDR domain is ~5.5 nm in length (PBD 4V3E). The appended SpyTag and flexible linker can further be extended to a maximum of 6.1 nm. Therefore a maximal radial increase of 11.6 nm is possible, which is also the largest radius increase observed by TEM (Figure 3.12c). Immunizing with the conjugated VLPs yielded a high anti-CIDR antibody titre after a single injection and gave the highest antibody titres at any time-point tested compared to control groups (Figure 3.13). Both control groups also failed to generate a detectable antibody response after the prime (Figure 3.13). Not all animals in the control groups responded after the boost and those who did, had lower titres compared to the conjugated group (Figure 3.13). These results demonstrate the capabilities of the platform and highlight the importance of the particular state of the immunogen; it is also likely

that the nucleic acid within the particle contributed to the antibody response via TLR signalling (Bachmann and Jennings, 2010; Hua and Hou, 2013; Smith et al., 2013a; Zabel et al., 2013).

Antibody titres are mainly a quantitative indicator of the adaptive immune response. Experiments that weren't performed but would be interesting are analysis of avidity of the raised IgG to assess the qualitative differences between VLP-raised antibodies compared to antibodies raised in the monomeric control groups (Hedman and Rousseau, 1989). Avidity has been addressed by the Salanti group and was shown to be superior for SpyCatcher-VLPs displaying the VAR2CSA protein compared to control group (AP205 VLP and free SpyTag-VAR2CSA). VAR2CSA contains the partial CIDR domain Id2a (Clausen et al., 2012; Thrane et al., 2016). The same was observed for SpyTag-tagged VLPs in combination with Pfs25-SpyCatcher compared to the control (AP205 VLPs and free Pfs25-SpyCatcher) (Thrane et al., 2016).

It will be interesting to test whether this immunization induces antibodies recognizing distal parts of the domain in relation to the N-terminal SpyTag-anchor. This of interest to PfEMP1 vaccine development as the CIDR domain binds EPCR via its N-terminal-distal regions (Lau et al., 2015). Exposure to one or few members of PfEMP1 proteins is thought to induce IgG that are widely cross-reactive to other members of the group, thereby protecting individuals who have acquired antibodies against repeated attacks of severe disease (Turner et al., 2015). It may be possible to induce cross-reactive IgG by a single vaccination through SpyCatcher-VLPs that display multiple carefully chosen CIDR variants on the same carrier (Schiller and Lowy, 2015). Spatially controlled, simultaneous exposure of

structurally similar CIDR - EPCR binding domains to B-cells may allow for direct cross-linking of broadly recognizing BCRs (Schiller and Lowy, 2015). Such antibodies can be assayed in competitive CIDR-EPCR binding assays: broadly neutralizing antibodies should block binding of heterologous CIDR variants to EPCR (Lau et al., 2015). Other and possibly unknown CIDR ligands exist, but little or no structural information is known about these binding interactions (Dahlbäck et al., 2011; Higgins and Carrington, 2014). Furthermore, by simultaneously providing T-helper epitopes on the VLP, one may be able to expand broadly neutralizing B-cells and establish broadly protective anti-CIDR memory (Fraser et al., 2014; Rosendahl Huber et al., 2014; Weber et al., 2009).

Immunogenicity of Pfs25-SpyTag:SpyCatcher-VLPs conjugates.

Pfs25 is known to be a poor immunogen after protein subunit immunization (Coban et al., 2004; Kaslow, 2002; Nikolaeva et al., 2015; Qian et al., 2007; Wu et al., 2008). Hence for its first clinical phase I trial, Pfs25 was adjuvanted with montanide ISA 51, which in combination led to severe side effects and halted further efforts with this formulation (Wu et al., 2008). Therefore it is not surprising that Pfs25 alone did not induce any detectable antibody response in this work (Figure 3.16b). Therefore it is not surprising that Pfs25 alone did not induce any detectable antibody response in this work (Figure 3.16b). Importantly Pfs25-SpyTag alone also did not induce an immune response, thus excluding the possibility that SpyTag by itself may improve the response, e.g. as a helper epitope for example (Figure 3.16b). Adjuvanting monomeric Pfs25 with AddaVax, a squalene-based oil-in-water nanoemulsion did not results in a detectable immune response after single

immunization, however after boosting a reasonable titre was achieved (Figure 3.16b). AddaVax is thought to promote a balanced Th1/Th2 immune response and is based on the human licensed MF59 adjuvant (Mbow et al., 2010). To this end we wondered if including a clinical trial compatible adjuvant in the conjugated VLP preparation would further increase antibody titres. Indeed compared to the control group consisting of conjugate only, the adjuvanted formulation further potentiated the antibody response against Pfs25 (Figure 3.16b). Efficacy of a Pfs25-based transmission-blocking vaccine will depend on induction of a high and sustained level of functional Abs that effectively blocks parasite development in the mosquito (Hill et al., 2014). Pfs25 is not expressed within the human host lifecycle, therefore the human Ab response will depend entirely on the vaccine alone, as no boosting through the parasite is expected (Barr et al., 1991; Hill et al., 2014; Kapulu et al., 2015; Li et al., 2016; Miura et al., 2007, 2008). The antibody titres obtained in this work are similar in titre compared to leading transmission blocking stage vaccines and can thus be considered state of the art, as compared against other nanoparticle platforms such Pfs25-IMX313 (Li et al., 2016) or strongly adjuvanted monomeric Pfs25 (Miura et al., 2013). Unfortunately antibody titres in the Salanti work were not normalised against the standard reference serum, so a direct comparison can not be made (Miura et al., 2008; Thrane et al., 2016). On the other hand, long-term persistence of antibodies was evaluated in the Salanti work, with responses being significantly longer-lived compared to immunization with monomeric protein alone. In line with VLP-mediated T cell-independent activation of B cells, the anti-Pfs25 response did decline after several months however (Thrane et al., 2016).

Importantly antibodies raised in our Plug-and-Display studies and in the Thrane work were independently able to block transmission in SMFA studies. Difference in transmission-blocking activity between groups in this work could be explained simply by more anti-Pfs25 IgG present in the sera from the SpyCatcher-VLP:Pfs25-SpyTag vaccinated groups, as observed in the ELISA experiments (Figure 3.16b). Another possibility could be that fusion to SpyTag and conjugation to SpyCatcher-VLPs may have induced qualitatively superior anti-Pfs25 antibodies as discussed for the CIDR experiments. That is Plug-and-Display immunization may favour generation of Abs that recognize different Pfs25 epitopes or cause better antibody avidity. These questions may be addressed by normalizing anti-Pfs25 antibody type and concentrations used for the SMFA across the different groups (Li et al., 2015).

Pfs25 lacks efficient helper T-cell epitopes and direct fusion to Pfs28 has been shown to induce longer-lasting immune response. Combining antibodies raised to Pfs25 and Pfs28 alone yielded synergistic effects in transmission-blocking (Duffy and Kaslow, 1997; Gozar et al., 1998). Though Pfs28 is thought to be expressed relatively late in the mosquito, the mechanism by which fusion to Pfs28 improves long-term response may be due to the presence of a helper T-cell epitope (Gozar et al., 1998; Gregory et al., 2012). Thus incorporating either Pfs28 into the immunogen or other helper epitopes again appears to be an attractive possibility.

Attempts at improving SpyCatcher-VLP yields

The major limitation of the platform described in this chapter is the low yield (2-3 mg/L). SpyCatcher itself can be produced at good yields in *E. coli* (>50 mg/L), though expression in basal transcription reduced BL21(DE3) pLysS is preferred (Dovala et al., 2016; Fierer et al., 2014). SpyCatcher is also very soluble to at least 110 mg/mL in Tris buffered saline (Li et al., 2014). The type of affinity purification via His-tag is not likely to cause low yields, as this purification method has been used previously for MS2 for example (Udit et al., 2008). AP205 mosaic VLPs have been described with yields of at least at 8 mg/g wcw in *E. coli* (Cielens et al., 2014), wildtype VLP yield in *Pichia pastoris* was reported to be 1-1.5 mg/g wcw (Freivalds et al., 2014) .

Hence I focused my efforts on optimizing the VLP expression and solubility *in vivo*. The dual promotor setup in pKB_215 (Ptrp and pT7) is unusual compared to typical expression vector. Hence I moved the SpyCatcher-VLP construct into a traditional pET21 system under control of a single, LacO repressed T7 promoter. Adoption of this system resolved the growth defects and doubled biomass but the majority of protein was directed to inclusion bodies (Figure 3.19). This may be due to higher translation levels mediated by the strong ribosome-binding site (RBS) in the pET / pDEST systems.

Leviviridae phage assembly was thought to be primarily mediated by CP-CP interactions and electrostatic interactions to ensure RNA encapsulation. However over-expression of coat proteins also allows non-phage derived RNA to encapsulate into VLPs and potentially act as a scaffold (Pickett and Peabody, 1993; Plevka et al., 2009). A single high affinity association of the CP to a specific RNA signal, most commonly the translational operator (TR), was thought to

mediate nucleation (Klovins et al., 2002). I included the putative TR preceding the replicase gene of AP205, starting 78 bp downstream of the native CP3 stop codon into the pET system. Likewise a 82 bp structural motif of the 3' untranslated region (UTR) of the native CP3 was also included in the design (Olsthorn and van Duin, 1996; Klovins et al., 2002). As the AP205 wildtype RBS was not predicted to convey significant translation initiation rates for the inserted 6xHis-SpyCatcher in *E. coli*, the RBS preceding 6xHis-SpyCatcher from a well-expressing construct (pDEST14-SpyCatcher) was included (Salis et al., 2009; Espah Borujeni et al., 2014; Zakeri et al., 2012). Recently, additional, long-suspected RNA encoded packaging signals have been reported for the *Leviviridae* member MS2 and are likely to exist and mediate assembly of wildtype AP205 and its VLP as well (Stockley et al., 2016). I hypothesized that reverting the codon-optimized CP3 sequence in the initial system to its native codon usage may provide some packaging signals that would guide the SpyCatcher-CP3 proteins to assembly and away from inclusion body formation. In this context, synonymous wobble base modifications to the coat protein encoding RNA sequence can decrease phage titres by 4 orders of magnitude in MS2, highlighting the importance of RNA secondary structures features for the *Leviviridae* (Klovins et al., 1997). All the modifications combined yielded the plasmid pKB_337 (Figure 3.18).

Protein identical to pKB_215 SpyCatcher-VLPs in its amino acid composition could be purified from pKB_337 expressing C41(DE3) via IMAC as analysed by SDS-PAGE (Figure 3.19), However pKB_337 derived protein did not yield stable VLPs (Figure 3.20), highlighting importance of the RNA structures for VLP assembly. The purification yielded prominent low molecular weight nucleic acid

species, which may be attributed to the introduced RNA structural motifs. Transcription in pKB_337 (6300 bp) is terminated by a T7 terminator, starting 180bp downstream of the CP3 stop codon, which would allow for synthesis of a ~1 kb RNA species from the T7 promoter. The majority of nucleic acid species observed pKB_337 are 1 kb or smaller (Figure 3.20b). pKB_215 (3942 bp) on the other hand does not contain a traditional terminator and mFold secondary structure DNA analysis did not reveal major structures in proximity to the CP3 stop codon that may function as a transcriptional terminator (Zuker, 2003). Transcription is therefore not terminated and allows for longer RNA species, which possibly aid VLP formation. It is likely that termination in pKB_337 results in shorter RNA species that may not be as efficient as long RNA species for guiding VLP assembly. However T7 termination is unlikely to be perfect in terminating all transcripts from the strong T7 promoter and therefore some species may be expected to be longer. Minor residual HMW nucleic acid species as well as protein can be observed after high molecular weight dialysis and removal of large particulates by centrifugation (Figure 3.20), which may represent assembled VLPs. Hence removing the terminator in the pET21 system may resolve this issue.

Thrane and co-workers report use of a pET21 system, however they utilize BL21(DE3) Star for expression (Thrane et al., 2016). The genotype for BL21(DE3) Star is rne131, which encodes for a truncated RNase E enzyme lacking the ability to degrade mRNA with an increase in mRNA stability (Lopez et al., 1999). In my hands, expression of pKB_337 in BL21(DE3) Star did not yield drastically improved particles as analysed by NAGE or better protein yields compared to pKB_215 in C41(DE3).

The length of the RNA species is not the only parameter that could be optimized but also its sequence. Equivalently sized non-phage derived RNAs are known to favour aberrant structures *in vitro* for MS2 for example, whereas the phage RNA yields only capsids of the correct size (Borodavka et al., 2012). One could co-transcribe the entire AP205 wildtype RNA sequence with introduced non-sense point mutations and / or frameshifts to avoid actual expression of coat, lysis or maturation proteins. This RNA would be more likely to exhibit suitable collapse for initiation of capsid formation, provide packaging signals and may improve yield and structural features for SpyCatcher-VLPs based on AP205 (Stockley et al., 2016).

Thrane and co-workers also showed that some antigens, including Pfs25, can tolerate SpyCatcher fusion (Thrane et al., 2016). The protein was also expressed soluble in *E. coli*, did not require re-folding and induced potent transmission-blocking antibodies (d 56), comparable to the unadjuvanted response in this work (d 34). This data challenges the assumption that small Tags are always superior for maintaining folding and establish that SpyCatcher can also acts as powerful fusion partner for delicate antigens, stabilizing folding *in vivo* and allowing expression in conventional bacterial hosts.

To this end, it is conceivable that insertion of SpyTag into VLPs scaffolds would cause less issues for the VLP to assemble. AP205 tagged with SpyTag, either N- or C-terminally or at both ends also yielded VLPs in my hand, but yields were also poor. MS2 can tolerate up to 12aa insertions, canonical SpyTag is 13aa but shorter variants are still sufficiently functional (Stockley and Mastico, 2000; Zakeri and Howarth, 2010). MS2 can also be prepared RNA free, which may be desirable for

regulatory purposes (Stonehouse and Stockley, 1993). Depending on the antigen this approach may be more economical in VLP preparation.

Other VLP platforms may be explored to improve yields. Murine polyomavirus VP1 for example allows internal placement of an dihydrofolate reductase (DFHR, 24kDa) enzyme, however some cleavage events occur that facilitate assembly (Gleiter et al., 1999). Flock House virus (FHV) is a non-enveloped, icosahedral ($T = 3$) insect virus of the family *Nodaviridae* and allows insertion of up to 180aa residues, but the termini of the protein to be displayed need to be together (Manayani et al., 2007).

I also explored non-VLP scaffolds with SpyCatcher, e.g. DNA-binding protein of starvation (EcDPS) from *E. coli* (Crichton and Declercq, 2010), *Pyrococcus furiosus* ferritin (PfFTN) (Tatur et al., 2006), lumazine synthase from *Aquifex aeolicus* (AaLS), pyruvate dehydrogenase subunit E2 (E2DISP) from *Geobacillus stearothermophilus* (He et al., 2016). The tested scaffolds did not result in dramatically higher yields, all suffering from inclusion body formation or failure to assemble. The exception was IMX313, based on the complement inhibitor C4-binding protein (C4bp) (Ogun et al., 2008). IMX313 served as the scaffold for a dually addressable nanoparticle in Chapter 4.

Dual-antigen load: SpyCatcherDuo-VLPs and their features

Experiments on fusion tolerability of AP205 resulted in generation of SpyCatcher-SpyCatcher-AP205cp3 (SpyCatcherDuo-VLPs, pKB_281). Expression of the construct in C41(DE3) and purification showed disulfide-linked high molecular

weight species, as observed for SpyCatcher-VLPs and indicative of AP205 VLP formation (Figure 3.22b).

TEM studies showed VLPs in increased size, but at poorer quality compared to SpyCatcher-VLPs (Figure 3.22c, d vs. 3.4b, d). Smaller particles were also observed, which may be incomplete assembly dead-ends (Figure 3.22c). Dead-end assembly states may also occur in SpyCatcher-VLPs but due to the increased size of the SpyCatcherDuo-CP3 monomer (36 kDa), multimers of SpyCatcherDuo-CP3 may not be as efficiently removed by dialysis through the 300 kDa MWCO membrane or are too small to be observed by TEM. Gradient ultracentrifugation should be able to resolve these species as may dialysis through a 1MDa MWCO or size-exclusion chromatography.

The particles encapsulated nucleic acids as shown in native agarose gel electrophoresis (NAGE) in line with VLP formation (Figure 3.22b). NAGE also showed some migrating protein species without association to nucleic acid, which may either be aggregates capable of entering the gel, or dead-end assembly states observed in TEM studies (Figure 3.22b, d). Alternatively, the coat protein fusion monomer is predicted to have a negative charge of 8.25 at pH 8, the pH set for NAGE experiments in this work. Therefore monomeric and non-VLP multimeric protein should also be able to migrate towards the cathode in the absence of nucleic acid and may be observed (Figure 3.22b).

Reactivity with SpyTag-antigens was surprisingly efficient. Reaction with SpyTag-CIDR in excess yielded close-to-complete decoration one at least 1 residue (>92%) and was also efficient on the second, though quantification was not possible. Reaction with sub-saturating Pfs25-SpyTag also proceeded efficiently but not all

Pfs25-SpyTag was consumed (Figure 3.22a). Non-conjugated SpyCatcherDuo-CP3 was observed (<10% remaining), as was the intermediate (<35% remaining). Sub-saturating amounts of Pfs25-SpyTag were employed due to constraints in availability and concentration of the antigen but also to show the intermediate species (Figure 3.22a). Interestingly only one major intermediate is observed, whereas two may be expected as the conjugation to either the first or the second SpyCatcher in the fusion could produce different migration patterns. It is possible that Catchers in a linear fusion are competing for Pfs25-SpyTag and only one of the two (possibly the one being most exposed) is conjugated under sub-saturating SpyTag-Ag conditions. It may therefore be possible to accomplish full conjugation with higher ratios of Pfs25-SpyTag to SpyCatcherDuo-VLPs. However sterical constraints may hinder full conjugation.

Though this is still work in progress, the possibility to increase antigen density will be interesting to test from an immunological point of view. Optimal conjugation density may now be quickly determined using the SpyCatcher- and SpyCatcherDuo-VLPs, as only one antigen needs to be prepared.

The dual fusion to a single coat-protein could also show that two separate antigens can be fused together on one SpyCatcher-SpyCatcher-CP3 monomer and are therefore delivered on the same VLP. Titration of two SpyTag-antigens on single SpyCatcher-VLPs is also possible (unreported work by Linda Tchatchouang), but no conclusion could be made about the proximity of two separate antigens. The SpyCatcherDuo-VLP approach solves this problem but only partially, as overall distribution of dually conjugated monomers across individual VLPs cannot be

controlled. Therefore molecularly controlled dual-protein particle display was attempted in the next chapter.

Chapter 4 Plug-and-Display: Dually addressable

Nanoparticles

SpyCatcher / SpyTag and SnoopCatcher / SnoopTag technology

applied to IMX313 for dual protein display

4.1 Generation of dually addressable Nano-Particles

4.1.1 Chapter introduction

Displaying two proteins on a single particulate scaffold is desirable for various applications. From an immunological point of view, multiple antigens on the same carrier (as a single component vaccine) may induce immunity to both displayed proteins. This is of particular interest to multi-stage malarial vaccine development (Heppner et al., 2005; Hill et al., 2014).

Therefore I sought to develop a platform which would allow easy swapping of protein modules in order to identify viable antigen-antigen or antigen-immunostimulatory protein combinations.

4.1.1.1 SnoopCatcher

The recently reported SnoopCatcher (section 1.2.4.4) would allow such a dual conjugation strategy in concert with SpyCatcher. However SnoopCatcher currently does not tolerate fusion to its C-terminus without substantial reduction in SnoopTag reactivity (Veggiani et al., 2016). Therefore it is necessary to fuse SnoopCatcher to the C-terminus of a multimerization scaffold protein monomer. Extensive attempts at producing Dual-Catcher-VLPs (e.g. AP205 CP3-SpyCatcher-SnoopCatcher or SpyCatcher-AP205CP3-SnoopCatcher with various linkers) did not yield assembled VLPs (data not shown). The N-terminus of AP205 may be more amenable to fusions (Thrane et al., 2016) or the combined SpyCatcher-SnoopCatcher tandem may be too big to display at the C-terminus of AP205 CP3. Therefore other multimerization scaffolds were explored for their potential for dual Catcher display.

4.1.1.2 Chimeric oligomerization domain from *Gallus gallus* complement inhibitor C4b-binding protein (C4bp) – IMX313

Poorly immunogenic antigens can be rendered highly immunogenic through fusion to the oligomerization domain of the complement inhibitor C4-binding protein (C4bp) (Ogun et al., 2008; Li et al., 2016). This domain spontaneously assumes an oligomeric state that is stabilized by inter-chain disulfide bonds (Figure 4.1). The human full-length C4bp protein forms a plasma glycoprotein complex of 570 kDa (Hofmeyer et al., 2013). Mainly produced in the liver, C4bp is the major inhibitor of complement activation (Hofmeyer et al., 2013). Four different isotypes for human C4bp are known: The major isotype comprises 7 α and one β -chain, but less abundant variants also exist as 6 α 1 β , 7 α , or 6 α . It is unclear how the assembly of the hetero-oligomeric complexes proceeds (Christiansen et al., 2000; Blom et al., 2004; Hofmeyer et al., 2013). IMX313 is a chimeric variant of the α -type (truncated) oligomerization domain from *Gallus gallus* (chicken) C4bp (Figure 4.1) (Ogun et al., 2008). The sequence identity of the α -chain from *G. gallus* to *H. sapiens* is ~26% (Hofmeyer et al., 2013). IMX313 was further engineered in order to remove self-antigen structural features and epitopes against human or murine C4bp by extensive truncation and point mutagenesis, whilst retaining its oligomeric features (Ogun et al., 2008). *G. gallus* C4bp as a heptamer is approximately 5.8 nm in diameter and length, with a diagonal length of 6.8 nm (PDB 2YF2). Therefore C4bp may be considered a nanoparticle (Li et al., 2016).

4.1.2 SpyCatcher-IMX-SnoopCatcher design

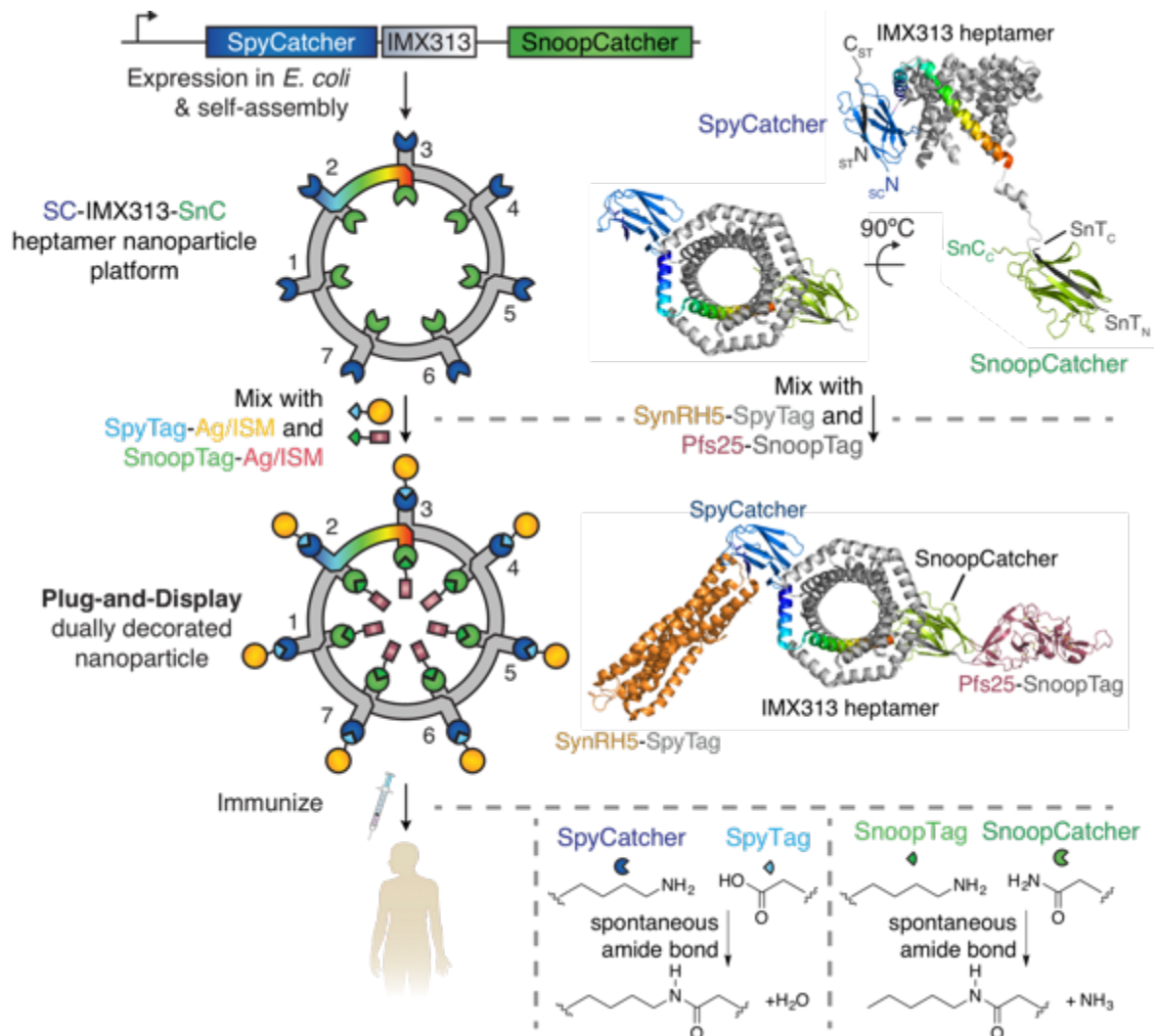


Figure 4.1 Overview of SpyCatcher-IMX-SnoopCatcher dual Plug-and-Display nanoparticle assembly. IMX313 (based on C4bp) is fused at its N-terminus to SpyCatcher (SC) and at its C-terminus to SnoopCatcher (SnC). Expression in *E. coli* and self-assembly of monomers into heptamers yields SC-IMX313-SnC nanoparticles. Upon mixing, SpyTag-antigen (ST-Ag) forms a spontaneous isopeptide bond with the SpyCatcher residue. Likewise, SnoopTag-antigen (SnT-Ag) forms a spontaneous isopeptide bond with the SnoopCatcher residue. Conjugation yields decorated nanoparticles for immunization. The platform is orthogonally addressable and may allow for site-specific conjugation of diverse antigens, such as CIDR, RH5 and Pfs25 onto the same particle. (Left) Schematic depictions are not to scale; (Right) molecular cartoons based on structural data are to scale [PDB: Δ N1SpyCatcher (4MLS), SnoopCatcher (adapted from RrgA, 2WW8), IMX313 (adapted from *G. gallus* C4bp, 2YF2), (Syn-)RH5 (4U0Q), Pfs25 (adapted from Pvs25, 1Z27)]. A single chain SpyCatcher-IMX313-SnoopCatcher fusion is shown coloured. For clarity the SpyCatcher and SnoopCatcher domains of the remaining 6 SpyCatcher-IMX313-SnoopCatcher single chain fusions are not shown.

4.1.3 Purification of SpyCatcher-IMX-SnoopCatcher

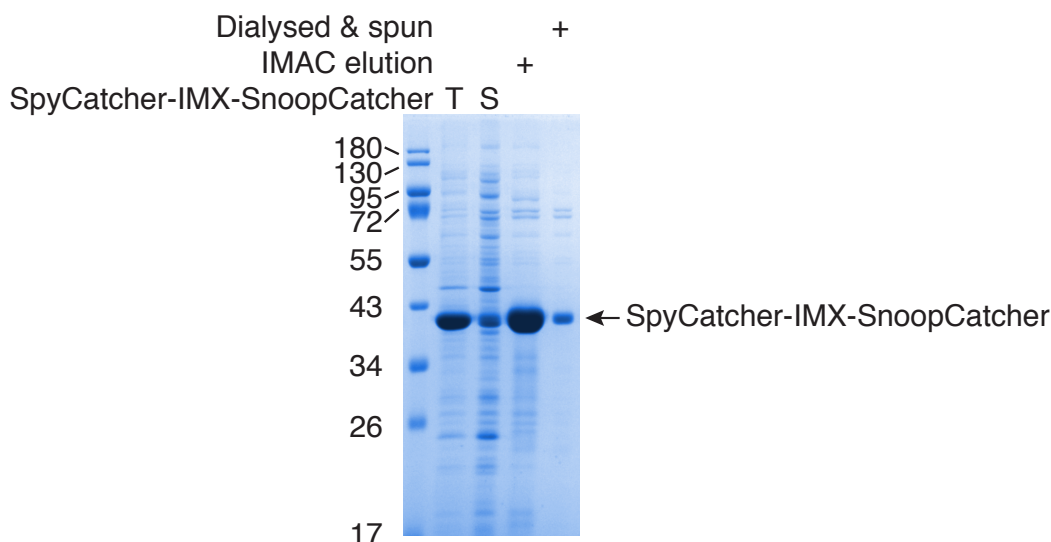


Figure 4.2 SpyCatcher-IMX-SnoopCatcher purification. SpyCatcher-IMX-SnoopCatcher was expressed in *E. coli* BL21-CodonPlus(DE3)-RIPL. Reducing SDS-PAGE showing total protein (T), soluble (S) protein, IMAC elution of soluble protein, and dialysed & spun protein (100 kDa MWCO, 17,000 *g* for 10 min). Equivalent volumes for IMAX elution and dialysed & spun samples were loaded on the gel.

SpyCatcher-IMX-SnoopCatcher was purified from soluble cell-lysate by IMAC with stringent washing (up to 100 mM imidazole), followed by elution with 2 M imidazole (in 50 mM glycine, 25 mM sodium citrate and Tween 20 0.1% (v/v), pH 8.5). The protein was then dialysed through a 100 kDa molecular weight cut-off membrane against VLP buffer (50 mM glycine, 25 mM sodium citrate and Tween 20 0.1% (v/v), pH 8.0) and analysed by SDS-PAGE (Figure 4.2). Two extra bands at ~70 kDa were also observed, as were some lower molecular weight species (Figure 4.2). As initially observed for SpyCatcher-VLPs, eluted protein displayed a tendency to aggregate. The buffer elucidated in Chapter 3 initially allowed higher solubility (up to 20 μ M for up to 12 hours) compared to standard NTA buffer but also showed subsequent aggregation and reduction of the purified protein to ~10-15 μ M (A280).

Yield was poor at 200-300 µg/L culture after size-exclusion chromatography (as determined by BCA; aggregates or species with Mw higher than ~300 kDa were excluded).

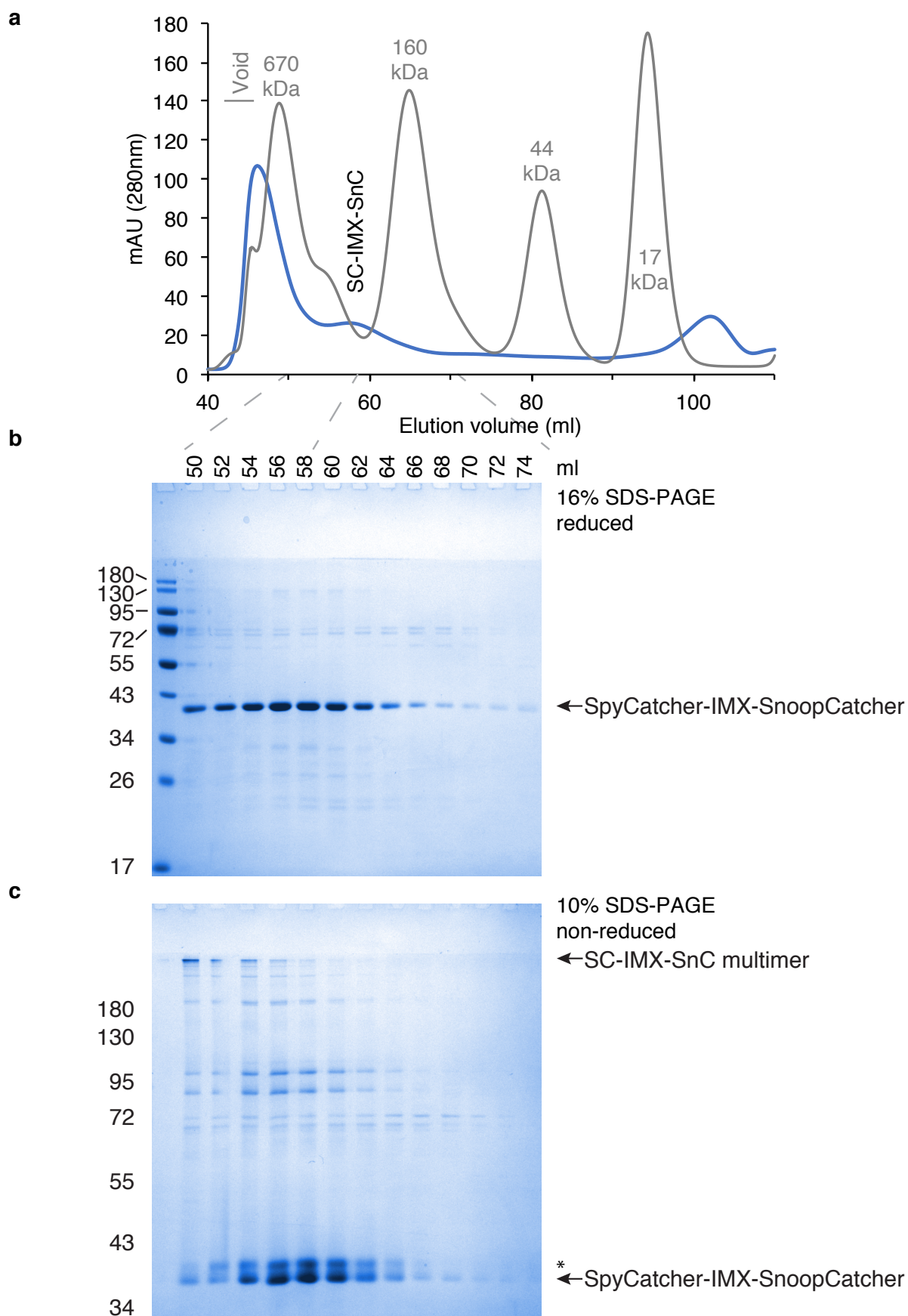


Figure 4.3 SpyCatcher-IMX-SnoopCatcher elution from size-exclusion chromatography (I). **(a)** Size-exclusion chromatography (SEC) of IMAC-purified SpyCatcher-IMX-SnoopCatcher eluate (blue) and SEC calibration marker (grey) on a Superdex 200 16/60pg. **(b)** Reducing SDS-PAGE of SEC elutions showing isolation of target protein monomer in the expected Mw range on SDS-PAGE and also in relation to SEC. **(c)** Non-reducing SDS-PAGE of SEC elutions showing partially disulfide-linked multimers. * Likely post-translationally modified (gluconylation) SpyCatcher-IMX-SnoopCatcher.

SpyCatcher-IMX-SnoopCatcher IMAC eluate was swiftly applied to size-exclusion chromatography, which led to only partial-elution of protein in the expected molecular weight range [$7 \times 35 \text{ kDa} = 245 \text{ kDa}$ (Figure 4.3a)]. Analysis of the expected Mw protein revealed that there was a low degree of disulfide bond formation. Nevertheless, the monomers assembled and migrated at high molecular weight, indicating that multimer assembly did not depend on disulfides (Figure 4.3a, c).

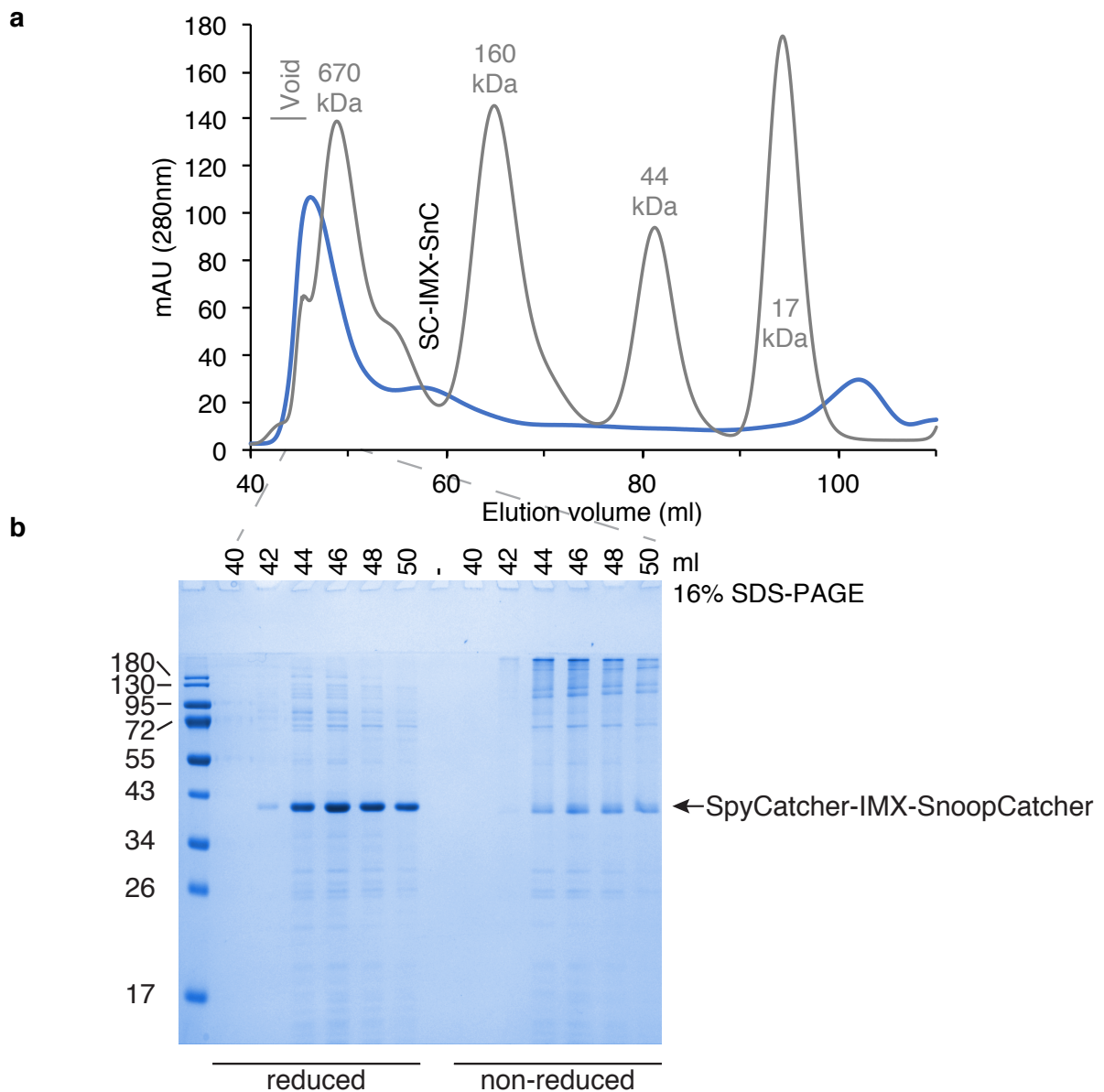


Figure 4.4 Size-exclusion chromatography of SpyCatcher-IMX-SnoopCatcher (II). showed protein eluting close to the void volume.

Analysis of void-eluting (>600 kDa) and near void-eluting protein also showed target protein that was also only partially disulfide-linked (Figure 4.4a, b). Several pronounced contamination bands below and above the target protein were observed, some of which shifted in the non-reducing lanes compared to the reducing lanes. These shifts indicate assembly of non-target protein into a multimeric species by disulfide bonds (Figure 4.4a, b). The higher than expected

Mw protein was able to enter the gel also under non-reducing conditions, as little density was observed in the wells of the gel (Figure 4.4b).

4.1.4 SpyCatcher-IMX-SnoopCatcher reactivity with model proteins

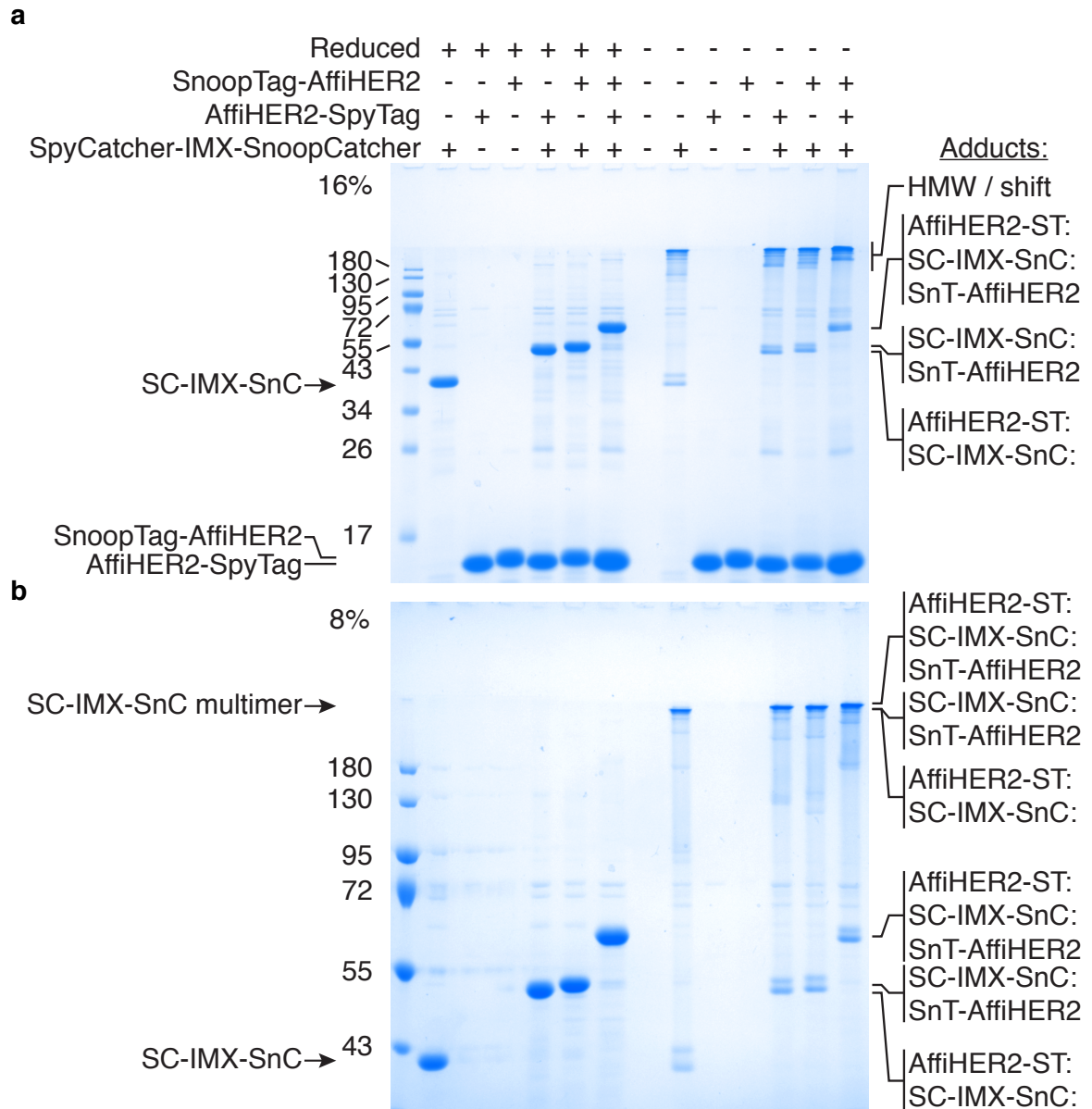


Figure 4.5 SpyCatcher-IMX-SnoopCatcher reaction with Spy/Snoop-model proteins. **(a)** SpyCatcher-IMX-SnoopCatcher (7 μ M) was incubated with 2.5-fold molar excess of AffiHER2-SpyTag (pKB_096) and/or SnoopTag-AffiHER2 (pKB_290) for 22 h at RT. Samples were spun for 10 minutes at 17,000 g to remove aggregates, before boiling in SDS-loading buffer and analysis by 16% SDS-PAGE with Coomassie staining. **(b)** The same samples were run on a 10% SDS-PAGE gel. Disulfide-linked high molecular weight species (HMW) in the non-reduced lanes shifted upon conjugation with Tag-proteins.

Initial reactivity studies were conducted with SpyTag- and SnoopTag-model proteins: SnoopTag fused N-terminally and SpyTag fused C-terminally to AffiHER2. AffiHER2 is an affibody binding the receptor tyrosine kinase and proto-oncogene HER2 (human epidermal growth factor receptor 2) (Wikman et al., 2004). The construct was cloned and expressed by Can Buldun by modifying SnoopTag-AffiHER2-SpyTag (Veggiani et al., 2016). As before, the covalent bonds of SpyTag:SpyCatcher and SnoopTag:SnoopCatcher were resistant to boiling in SDS and could be monitored via SDS-PAGE. Individual conjugation of Catcher-domains derived from IMAC and dialysis could be driven (close) to completion by use of excess Tag protein, yielding higher Mw adducts (Figure 4.5). Dual conjugation on both Catcher-residues also proceeded to high efficiency but did not complete, possibly due to lack of reactivity of the SnoopCatcher residue. SpyCatcher-IMX-SnoopCatcher on its own showed various disulfide-linked oligomeric states, which also shifted upon conjugation with Tag proteins (Figure 4.5b). A pronounced laddering of protein was observed under non-reducing conditions (Figure 4.5b).

4.1.5 Dual-conjugation of SpyCatcher-IMX-SnoopCatcher with complex antigens

After demonstrating individual and dual decoration of SpyCatcher-IMX-SnoopCatcher with AffiHER2-SpyTag and SnoopTag-AffiHER2, I attempted decoration with more challenging proteins. As before the cysteine-rich interdomain region (CIDR) from the highly variable *Plasmodium falciparum* erythrocyte membrane protein-1 family (PfEMP1) was tested but with an N-terminal SnoopTag instead of the SpyTag (SnoopTag-CIDR, pKB_299) (Lau et al., 2015; Leech et al., 1984). I developed a procedure for purification of soluble SnoopTag-CIDR of up to

1.5 mg/L (BCA) culture from *E. coli* T7SHuffle Express *lysY*. The sexual stage antigen Pfs25 of *Plasmodium falciparum* was also tested without modifications as Pfs25-SpyTag. Furthermore I attempted simultaneous dual conjugation. Both proteins were purified to exclusive monomeric state by SEC.

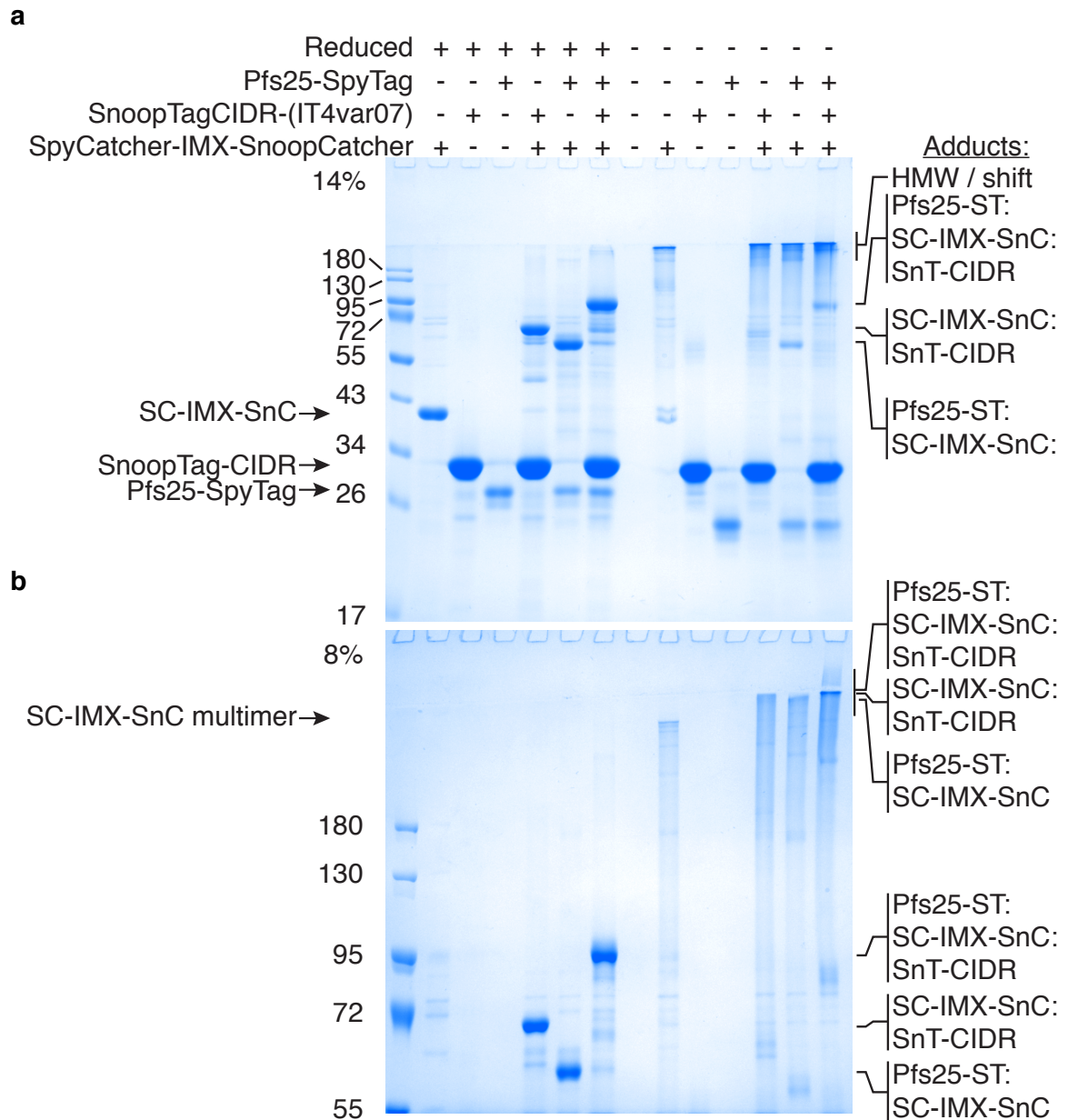


Figure 4.6 SpyCatcher-IMX-SnoopCatcher reacted with the antigens Pfs25-SpyTag and SnoopTag-CIDR. (a) SpyCatcher-IMX-SnoopCatcher (5 μ M) was incubated with 1.5-fold molar excess Pfs25-SpyTag (pKB_311) and/or 2.5-fold excess SnoopTag-CIDR (pKB_299) for 22 h at RT. Samples were spun for 10 minutes at 17,000 g to remove aggregates, before boiling in SDS-loading buffer and analysis by 14% SDS-PAGE with Coomassie staining. **(b)** The same samples were run on a 8% SDS-PAGE gel. Disulfide-linked high molecular weight species (HMW) in the non-reduced lanes shifted upon conjugation with Tag-proteins.

Conjugation of SpyCatcher-IMX-SnoopCatcher proceeded to close to completion for SnoopTag-CIDR and Pfs25-SpyTag individually and yielded the expected Mw

adducts, as observed in reducing SDS-PAGE (Figure 4.6a). However several new smaller bands, possibly adducts of truncated proteins, were also observed for both Tag-proteins (Figure 4.6a). Some distinct new bands were also observed under reducing conditions in the higher molecular weight bands (>100 kDa), especially for the dual conjugation reaction (reduced lanes, Figure 4.6a).

The non-reduced SpyCatcher-IMX-SnoopCatcher bands also shifted Mw upon conjugation with either SnoopTag-CIDR, Pfs25-SpyTag or both in combination (Figure 4.6b). A pronounced laddering effect was observed under non-reducing conditions.

4.1.6 Discussion

Purification of SpyCatcher-IMX-SnoopCatcher

SpyCatcher-IMX-SnoopCatcher has moderate solubility in *E. coli* (Figure 4.2a). IMX expressed by itself is soluble (Del Campo Ascarateil et al., 2014), but fusions expressed in *E. coli* may require re-folding (Ogun et al., 2008). Secreted expression of SpyCatcher-IMX-SnoopCatcher from the yeast *Pichia pastoris* may alleviate this problem in scale-up (Li et al., 2015). Purified, soluble protein had the tendency to aggregate at concentrations >15 μ M, which is likely a consequence of the fusion to SpyCatcher and / or SnoopCatcher, as IMX is otherwise soluble above 100 μ M (Ogun et al., 2008) (personal observation & communication with Can Buldun). To address this problem, an extensive buffer screen may be attempted. For initial studies, *E. coli* was deemed a suitable production host and yield was overcome by scaling bacterial culture.

IMAC was efficient in capturing protein from cleared lysate. After stringent washing with 100 mM imidazole and elution with 2M imidazole, there was a surprising presence of contaminants. Protein at this stage was not or only sparsely disulfide-linked (not shown).

The IMAC eluted material was quickly applied to size-exclusion chromatography, which led to partial-elution of protein in the expected molecular weight range (Figure 4.3a). Analysis of the protein revealed that only a fraction of disulfide bonds had formed, but that the monomers assembled and migrated in a multimeric particle regardless (Figure 4.3a). In addition, aggregates eluting in the void (>600 kDa) were observed and consisted of target protein that was not or only little disulfide-linked (Figure 4.2b, d). Likewise, target protein eluting near-void, but within the resolution of the column and before the expected heptamer, was also not or only little disulfide-linked (Figure 4.2b, d). These “aggregate” species may be multimeric species above the typical heptamer state, as they were able to enter the gel, also in non-reducing conditions. The formation of these “aggregate” species may be promoted by SpyCatcher/SnoopCatcher fusion, and may be effective in multimerizing antigen for immunizations. Alternatively these “aggregate” may be artificially associated non-target proteins, such as chaperones, as they are observed in reducing conditions (Mayer and Bukau, 2005). Removal of these species may be attempted under reducing conditions, use of Mg-ATP, and / or denaturing conditions (Rial and Ceccarelli, 2002; Rohman and Harrison-Lavoie, 2000).

SpyCatcher-IMX-SnoopCatcher reactivity with model proteins

SpyCatcher-IMX-SnoopCatcher was reactive with SpyTag and SnoopTag model proteins (Figure 4.5). The reaction proceeded to completion on the SpyCatcher residue at pH 8, even without addition of VLP reaction buffer. SpyCatcher is most reactive under slightly acidic conditions (pH 5-6) (Zakeri et al., 2012). For SpyCatcher-VLPs, reaction buffer was added to lower the pH from pH 8 to pH 6.9 in order to increase reactivity towards SpyTag-antigen. This strategy was not employed here, as SnoopCatcher reactivity is better at higher pH (optimum pH 8-9) and was determined to be the limiting factor for attempted full conjugation (Veggiani et al., 2016). Nonetheless, SpyCatcher-IMX-SnoopCatcher:AffiHER2-SpyTag reaction proceeded to completion when the reaction was allowed to occur for 22 h (Figure 4.5).

SnoopCatcher reaction may have not reached full conjugation (at least >95 % observed). Trimethylamine N-oxide (TMAO), a naturally occurring osmolyte, has been previously used to improve reactivity for SnoopCatcher (Ma et al., 2014; Veggiani et al., 2016) but TMAO led to aggregation of SpyCatcher-IMX-SnoopCatcher.

Efficient dual conjugation was also observed but stopped short of completion (>90%). The remaining protein is likely unreacted on the SnoopCatcher residue but conjugated on the SpyCatcher part, as all SpyCatcher-IMX-SnoopCatcher is shifted (Figure 4.5).

Importantly, conjugation was also observed for disulfide-multimerized species, which is crucial for the immunological function (Figure 4.5). Ribosomal stalling could have resulted in a fraction of truncated SpyCatcher-IMX-SnoopCatcher, which is purified because of its N-terminal His₆-tag (Keiler, 2015). Several linker

fusions (flexible and helical) on both ends of IMX were also tested, resulting in the variant exhibiting the least truncations, shown here (pKB_389).

Dual-conjugation of SpyCatcher-IMX-SnoopCatcher with complex antigens

Having established conjugation with model Tag-proteins, next I explored if one could simultaneously conjugate SpyCatcher-IMX-SnoopCatcher with two antigens. Conjugation of SpyCatcher-IMX-SnoopCatcher with excess SnoopTag-CIDR or Pfs25-SpyTag reached near-completion (Figure 4.6). Dual conjugation with both SnoopTag-CIDR and Pfs25-SpyTag proceeded efficiently but completion was not achieved (Figure 4.6). This may be due to steric hindrance, imposed by the circular arrangement around the circular IMX core and / or accessibility to the reactive sites within the Catcher domains (Figure 4.1). This hypothesis may be tested by installing longer linker regions between the Tags and antigens. For example, Pfs25-SpyTag (pKB_311) is a direct fusion without any linker between the antigen and the Tag. SnoopTag-CIDR on the other hand is spaced with a 3 residue GGS linker away from the CIDR domain. Longer linkers are typically more prone to proteolysis. Thus optimization needs to be attempted carefully as proteases in the animal model or vaccinee may process the particle before it reaches a lymph node. Longer linkers between IMX and Catcher generally led to increased proteolysis after purification from soluble protein and may require additional modifications (data not shown).

The major limitation for the IMX work is, as for SpyCatcher-VLPs, the low yield (currently 200-300 µg/L). SpyCatcher-IMX expresses substantially better (data not

shown, personal observation and communication with Li Lee). Therefore the drop in yield is likely due to the additional SnoopCatcher.

In the future the poor yield may be addressed with improved Catcher domains that are being developed in the Howarth laboratory. Recently IMX modifications have been described for improved ion-exchange chromatography (IEX), namely introduction of multiple charged Arg residues (Del Campo Ascarateil et al., 2014). It is conceivable that adaption of charged residues could increase solubility for SpyCatcher-IMX-SnoopCatcher (Trevino et al., 2007).

From an immunological point of view, there may be an argument against a single-particle vaccine displaying two or more antigens. Diverse antigens on the same carrier may interfere with efficient BcR-crosslinking and thus reduce the T-independent response towards a single or both antigens (Delpeyroux et al., 1988). On the other hand, helper T-cell epitopes in one antigen, may improve T-dependent and B-cell responses against an otherwise poorly immunogenic but already multimerized antigen (Ding et al., 2009). It may be interesting to investigate how the dually conjugated particles in this chapter perform in immunological experiments.

It will be important to investigate how the dually conjugated particles in this chapter perform in immunological experiments. In particular, it will be valuable to elucidate if a dual-stage, single particle malarial vaccine is beneficial. Here I presented CIDR, a blood-stage antigen, in combination with Pfs25, a transmission-stage antigen. Though our dual display approach requires optimization, using this platform to combine the more promising RH5 blood-stage antigen with Pfs25 will be an

important goal, to confer both individual and herd protection against malaria (Boes et al., 2015; Fine et al., 2011; Hill et al., 2014; Wright et al., 2014).

Chapter 5 Summary, General Discussion and Future Outlook

Recombinant subunit vaccines have emerged as a powerful tool in vaccine development with plenty of success stories and future potential (De Gregorio and Rappuoli, 2014) (see Introduction for examples). The unmatched safety profile of recombinant subunit protein comes at the price of reduced immunogenicity, which often needs to be reengineered into the vaccine (Bachmann and Jennings, 2010). Virus-like particles and nanoparticles are powerful tools to induce potent B-cell responses against displayed antigens and are being developed to induce strong T-cell responses as well (Ruedl et al., 2005; Jennings and Bachmann, 2009; Braun et al., 2012; Zhao et al., 2016). Display of target antigens is a tedious trial and error process, which I have sought to address with this thesis.

In Chapter 3, I first presented SpyCatcher-VLPs, which allowed convenient display of a wide range of complex malarial antigens on AP205 bacteriophage-derived VLPs. High antibody responses after a single immunization were achieved for the blood-stage antigen CIDR(IT4var07), as well as for the leading transmission-blocking antigen Pfs25. Qualitatively meaningful antibodies were generated in the Pfs25-SpyTag:SpyCatcher-VLP immunizations, as shown by standard-membrane feeding assays. Substantial control of malarial transmission through antibodies was established. Furthermore, when SpyCatcher-VLPs:Pfs25-SpyTag was administered in presence of a human-licensed adjuvant, 100% transmission-blocking activity was achieved.

It will be interesting to test this platform with other promising malarial antigens such as RH5 (Wright et al., 2014). RH5 is a complex and difficult protein to handle but preliminary work not described in thesis raises hopes that it is possible to produce SpyCatcher:RH5-SpyTag multimerized particles.

Since our initial publication earlier in 2016, we have shipped the platform to multiple laboratories world-wide, which are making use of the platform presented here also for non-malarial proteins. It will be interesting to see how amenable and robust the platform is in other researchers' hands and if antigens of other diseases can also benefit from this type of multimerization.

The development of SpyCatcher-VLPs was not straightforward and still suffers from low yields. To this end, it is reassuring, that Thrane and co-workers were independently able to develop a very similar system, with results reported shortly after our publication at the beginning of 2016. Thrane's work addresses questions which I had not considered. The number and diversity of different proteins displayed is impressive, as is their immunological work, which extends beyond what is presented here in several aspects (Thrane et al., 2016).

In addition to SpyCatcher-VLPs, I also described prototype development of SpyCatcherDuo-VLPs in Chapter 3. The capacity to double antigen density on AP205 raises interesting mechanistic questions, which will be exciting to answer. The possibility of producing "half-loaded" particles by dimer-fusion, as demonstrated for other *Leviviridae*-based VLPs may render this an exciting opportunity to address VLP-loading and antigen dose-sparing strategies.

In Chapter 4, I describe preliminary work on a nanoparticle based on IMX313 that can be dually and orthogonally addressed using the SpyCatcher/SnoopCatcher system. Though needing further optimization, the feasibility of controlled dual multi-stage conjugation to a single nanoparticle carrier was demonstrated. A single CIDR variant alone is unlikely to provide meaningful protection. In the future, RH5 appears to be a more suitable candidate to attach to this platform. It also remains

to be seen, if a transmission-blocking anti-Pfs25 response can be generated from a dual-decorated particle, and if so, how long such response may last. Potent transmission-blocking Pfs25-IMX313 nanoparticles have been reported previously. Therefore it will be interesting to see if Catcher/Tag technology can contribute further enhancement (Li et al., 2016). One improvement could be the inclusion of Pfs28, a close homologue of Pfs25. Direct fusion of Pfs25 to Pfs28 produces a stronger immune response (Gozar et al., 1998). One may observe synergistic effects for particulate immunogens conjugated dually with Pfs25 and Pfs28.

Independent attachment sites also open possibilities beyond dual antigen display. The secondary moiety may display trapped toxins to the immune system (Manayani et al., 2007). Another opportunity is the inclusion of immunostimulatory molecules, which I have briefly investigated with the construction of a SpyTag-FliC fusion; the modular approach should enable high-throughput manufacturing and testing.

Apart from dually addressable particles, one could make use of the different Catcher systems on different carrier platforms to avoid carrier-induced epitope suppression of antibody responses against the target (Jegerlehner et al., 2010). A target antigen with both SnoopTag and SpyTag could be produced. Single-Catcher VLP or nanoparticle carriers may be substituted during prime and boost. For example, one may prime with SpyCatcher-AP205VLPs:RH5-SpyTag-SnoopTag and boost with IMX-SnoopCatcher:RH5-SpyTag-SnoopTag. This enables heterologous boosting from a single Tag-Tag-antigen purification.

As of now, no information is available on pre-existing antibodies against SpyCatcher or SnoopCatcher. However, also the size of the Catcher domain

remains a concern: it would be desirable if no or only a minimal proteinaceous scar were left after decoration. SpyLigase (Fierer et al., 2014) is a first attempt at such an approach, in which the only scar left consists of the respective tags (<30 aa compared to full length Δ N1SpyCatcher + SpyTag minimal scar of 105aa, plus linker and purification tag). SpyLigase is a tripartite split of CnaB2 into SpyTag, KTag and SpyLigase (Fierer et al., 2014). The covalent bond is formed between Ktag and SpyTag in the presence of SpyLigase but importantly SpyLigase is not part of the covalent complex (Fierer et al., 2014).

Beyond applications in a prophylactic immunological context, another application for multimerized Catcher/Tags could be for immunotherapy, in particular cancer killing. Killing may be achieved by multimerizing death receptor 5 (DR5) (Huet et al., 2014; Veggiani et al., 2016).

It should be possible to load SpyCatcher-VLPs with small molecules, such as cytotoxic drugs or siRNA. Loaded VLPs may be targeted to certain cell-types with the Catcher-Tag system in a modular fashion (Galaway and Stockley, 2013).

Catcher-VLPs may also have potential to tackle chronic diseases such as allergic asthma and rhinitis via therapeutic immunization against self-antigens, such as IgE (Akache et al., 2016). In this context, allergy immunotherapy may be addressed by displaying the respective allergic antigen on VLPs and repeated immunization, which favours a Th1-type microenvironment (e.g., IFN- α / γ , IL-12) (Klimek et al., 2013; Yazdanbakhsh, 2002). However it is not clear if the antigen is really required to alleviate allergy symptoms (Kündig et al., 2014).

Dual Catcher assemblies may have application in enzymatic conversion, in particular for metabolite channelling (Graham et al., 2007; Pröschel et al., 2015; Polka et al., 2016).

Multivalent, interactions are also important for signal transduction triggered by receptor cross-linking (Kiessling et al., 2006). SpyCatcher-IMX-SnoopCatcher may represent a tool to simultaneously heptamerize two different receptor types and could be used to extend the DR5 cross-linking approach to simultaneously target other receptors of the death receptor family (Griffith et al., 1999; Chaudhary et al., 1997).

Reinforcement of low-affinity interaction may be another attractive use of SpyCatcher-multimer scaffolds. Avidity-based screens are commonly employed for identification of cell surface protein-protein interactions (Kerr and Wright, 2012), including MHC-tetramer staining (Altman et al., 1996). Non-covalent interactions are also important for (cancer) cell-capture (Xie et al., 2015). Previously SpyLigase has been employed to generate affibody polymers to enhance the sensitivity of magnetic cancer cell capture (Fierer et al., 2014). However control over polymer length was limited, so defined Catcher/Tag-equipped, multimerizing carriers may offer a tractable solution. In combination with solid-phase linear and branched-chain protein polymer synthesis, this could enable entirely novel architectures (Veggiani et al., 2016).

Bibliography

- Abe, H., Wakabayashi, R., Yonemura, H., Yamada, S., Goto, M., and Kamiya, N. (2013). Split Spy0128 as a Potent Scaffold for Protein Cross-Linking and Immobilization. *Bioconjug. Chem.* 24, 242–250.
- Adams, S.E., Dawson, K.M., Gull, K., Kingsman, S.M., and Kingsman, A.J. (1987). The expression of hybrid HIV:Ty virus-like particles in yeast. *Nature* 329, 68–70.
- Aerni, H.R., Shifman, M.A., Rogulina, S., O'Donoghue, P., and Rinehart, J. (2015). Revealing the amino acid composition of proteins within an expanded genetic code. *Nucleic Acids Res.* 43, e8.
- Aida, Y., and Pabst, M.J. (1990). Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J. Immunol. Methods* 132, 191–195.
- Akache, B., Weeratna, R., Deora, A., Thorn, J., Champion, B., Merson, J., Davis, H., and McCluskie, M. (2016). Anti-IgE Qb-VLP Conjugate Vaccine Self-Adjuvants through Activation of TLR7. *Vaccines* 4, 3.
- Alegre-Cebollada, J., Badilla, C.L., and Fernandez, J.M. (2010). Isopeptide Bonds Block the Mechanical Extension of Pili in Pathogenic *Streptococcus pyogenes*. *J. Biol. Chem.* 285, 11235–11242.
- Algar, W.R., Prasuhn, D.E., Stewart, M.H., Jennings, T.L., Blanco-Canosa, J.B., Dawson, P.E., and Medintz, I.L. (2011). The Controlled Display of Biomolecules on Nanoparticles: A Challenge Suited to Bioorthogonal Chemistry. *Bioconjug. Chem.* 22, 825–858.
- Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94–96.
- Amelung, S., Nerlich, A., Rohde, M., Spellerberg, B., Cole, J.N., Nizet, V., Chhatwal, G.S., and Talay, S.R. (2011). The FbaB-type fibronectin-binding protein of *Streptococcus pyogenes* promotes specific invasion into endothelial cells: Mechanism of *S. pyogenes* endothelial cell invasion. *Cell. Microbiol.* 13, 1200–1211.
- Amit, A.G., Mariuzza, R.A., Phillips, S.E., and Poljak, R.J. (1986). Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science* 233, 747–753.
- Anderson, T., Nair, S., White, M., Cheeseman, I., Nkhoma, S., Bilgic, F., McGready, R., Ashley, E., Pyae Phyo, A., White, N., et al. (2016). Why are there so many independent origins of artemisinin resistance in malaria parasites?

Anelli, T., Alessio, M., Bachi, A., Bergamelli, L., Bertoli, G., Camerini, S., Mezghrani, A., Ruffato, E., Simmen, T., and Sitia, R. (2003). Thiol-mediated protein retention in the endoplasmic reticulum: the role of ERp44. *EMBO J.* 22, 5015–5022.

Antoine, G., Scheifflinger, F., Dorner, F., and Falkner, F.G. (1998). The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* 244, 365–396.

Aon, J.C., Caimi, R.J., Taylor, A.H., Lu, Q., Oluboyede, F., Dally, J., Kessler, M.D., Kerrigan, J.J., Lewis, T.S., Wysocki, L.A., et al. (2008). Suppressing Posttranslational Glucosylation of Heterologous Proteins by Metabolic Engineering of *Escherichia coli*. *Appl. Environ. Microbiol.* 74, 950–958.

Arechaga, I., Miroux, B., Karrasch, S., Huijbregts, R., de Kruijff, B., Runswick, M.J., and Walker, J.E. (2000). Characterisation of new intracellular membranes in *Escherichia coli* accompanying large scale over-production of the b subunit of F₁ F₀ ATP synthase. *FEBS Lett.* 482, 215–219.

Ashcroft, A.E., Lago, H., Macedo, J.M.B., Horn, W.T., Stonehouse, N.J., and Stockley, P.G. (2005). Engineering thermal stability in RNA phage capsids via disulphide bonds. *J. Nanosci. Nanotechnol.* 5, 2034–2041.

Bachmann, M.F., and Jennings, G.T. (2010). Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat. Rev. Immunol.* 10, 787–796.

Bachmann, M.F., and Zabel, F. (2016). Immunology of Virus-Like Particles. In *Viral Nanotechnology*, Y.E. Khudyakov, and P. Pumpens, eds. pp. 121–128.

Bachmann, M.F., and Zinkernagel, R.M. (1997). Neutralizing antiviral B cell responses. *Annu. Rev. Immunol.* 15, 235–270.

Bachmann, M.F., Rohrer, U.H., Kündig, T.M., Bürki, K., Hengartner, H., and Zinkernagel, R.M. (1993). The influence of antigen organization on B cell responsiveness. *Science* 262, 1448–1451.

Bachmann, M.F., Hengartner, H., and Zinkernagel, R.M. (1995). T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: Role of antigen patterns in B cell induction? *Eur. J. Immunol.* 25, 3445–3451.

Bachmann, M.F., Tissot, A., PUMPENS, P.B.R. and S.C., CIELENS, I.B.R. and S.C., and RENHOFA, R.B.R. and S.C. (2012). Molecular antigen arrays using a virus like particle derived from the ap205 coat protein.

Baldwin, A.D., and Kiick, K.L. (2011). Tunable degradation of maleimide-thiol adducts in reducing environments. *Bioconjug. Chem.* 22, 1946–1953.

Ban, H., Gavriluk, J., and Barbas, C.F. (2010). Tyrosine bioconjugation through aqueous ene-type reactions: a click-like reaction for tyrosine. *J. Am. Chem. Soc.* 132, 1523–1525.

Baneyx, F., and Mujacic, M. (2004). Recombinant protein folding and misfolding in *Escherichia coli*. *Nat. Biotechnol.* 22, 1399–1408.

Bargieri, D.Y., Leite, J.A., Lopes, S.C.P., Sbrogio-Almeida, M.E., Braga, C.J.M., Ferreira, L.C.S., Soares, I.S., Costa, F.T.M., and Rodrigues, M.M. (2010). Immunogenic properties of a recombinant fusion protein containing the C-terminal 19kDa of *Plasmodium falciparum* merozoite surface protein-1 and the innate immunity agonist FliC flagellin of *Salmonella Typhimurium*. *Vaccine* 28, 2818–2826.

Barr, P.J., Green, K.M., Gibson, H.L., Bathurst, I.C., Quakyi, I.A., and Kaslow, D.C. (1991). Recombinant Pfs25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals. *J. Exp. Med.* 174, 1203–1208.

Barry, A.E., and Arnott, A. (2014). Strategies for Designing and Monitoring Malaria Vaccines Targeting Diverse Antigens. *Front. Immunol.* 5.

Basnak, G., Morton, V.L., Rolfsson, O., Stonehouse, N.J., Ashcroft, A.E., and Stockley, P.G. (2010). Viral genomic single-stranded RNA directs the pathway toward a T=3 capsid. *J. Mol. Biol.* 395, 924–936.

Bazin, H. (2011). Pasteur and the Birth of Vaccines Made in the Laboratory. In *History of Vaccine Development*, S.A. Plotkin, ed. (Springer New York), pp. 33–45.

Beekwilder, M.J., Nieuwenhuizen, R., and van Duin, J. (1995). Secondary structure model for the last two domains of single-stranded RNA phage Q beta. *J. Mol. Biol.* 247, 903–917.

Berg, J. (2002). *Biochemistry* (New York: W.H. Freeman).

Berg, J.M., Tymoczko, J.L., Gatto, G.J., and Stryer, L. (2015). *Biochemistry* (New York: W.H. Freeman & Company, a Macmillan Education Imprint).

Bernabeu, M., Danziger, S.A., Avril, M., Vaz, M., Babar, P.H., Brazier, A.J., Herricks, T., Maki, J.N., Pereira, L., Mascarenhas, A., et al. (2016). Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass. *Proc. Natl. Acad. Sci.* 113, E3270–E3279.

Berne, B.J., and Pecora, R. (2000). *Dynamic light scattering: with applications to chemistry, biology, and physics* (Mineola, N.Y: Dover Publications).

Berrade, L., and Camarero, J.A. (2009). Expressed protein ligation: a resourceful tool to study protein structure and function. *Cell. Mol. Life Sci.* 66, 3909–3922.

Bessa, J., Zabel, F., Link, A., Jegerlehner, A., Hinton, H.J., Schmitz, N., Bauer, M., Kündig, T.M., Saudan, P., and Bachmann, M.F. (2012). Low-affinity B cells transport viral particles from the lung to the spleen to initiate antibody responses. *Proc. Natl. Acad. Sci.* 109, 20566–20571.

- Billaud, J.-N., Peterson, D., Barr, M., Chen, A., Sallberg, M., Garduno, F., Goldstein, P., McDowell, W., Hughes, J., Jones, J., et al. (2005). Combinatorial Approach to Hepadnavirus-Like Particle Vaccine Design. *J. Virol.* 79, 13656–13666.
- Blokhina, E.A., Kuprianov, V.V., Stepanova, L.A., Tsybalova, L.M., Kiselev, O.I., Ravin, N.V., and Skryabin, K.G. (2013). A molecular assembly system for presentation of antigens on the surface of HBc virus-like particles. *Virology* 435, 293–300.
- Blom, A.M., Villoutreix, B.O., and Dahlbäck, B. (2004). Complement inhibitor C4b-binding protein—friend or foe in the innate immune system? *Mol. Immunol.* 40, 1333–1346.
- Boes, A., Spiegel, H., Voepel, N., Edgue, G., Beiss, V., Kapelski, S., Fendel, R., Scheuermayer, M., Pradel, G., Bolscher, J.M., et al. (2015). Analysis of a Multi-component Multi-stage Malaria Vaccine Candidate--Tackling the Cocktail Challenge. *PLoS One* 10, e0131456.
- Borisova, G.P., Berzins, I., Pushko, P.M., Pumpen, P., Gren, E.J., Tsibinogin, V.V., Loseva, V., Ose, V., Ulrich, R., Siakkou, H., et al. (1989). Recombinant core particles of hepatitis B virus exposing foreign antigenic determinants on their surface. *FEBS Lett.* 259, 121–124.
- Borodavka, A., Tuma, R., and Stockley, P.G. (2012). Evidence that viral RNAs have evolved for efficient, two-stage packaging. *Proc. Natl. Acad. Sci.* 109, 15769–15774.
- van den Bosch, S.M., Rossin, R., Renart Verkerk, P., Ten Hoeve, W., Janssen, H.M., Lub, J., and Robillard, M.S. (2013). Evaluation of strained alkynes for Cu-free click reaction in live mice. *Nucl. Med. Biol.* 40, 415–423.
- Bottazzi, B., Doni, A., Garlanda, C., and Mantovani, A. (2010). An integrated view of humoral innate immunity: pentraxins as a paradigm. *Annu. Rev. Immunol.* 28, 157–183.
- Böttcher, B., Tsuji, N., Takahashi, H., Dyson, M.R., Zhao, S., Crowther, R.A., and Murray, K. (1998). Peptides that block hepatitis B virus assembly: analysis by cryomicroscopy, mutagenesis and transfection. *EMBO J.* 17, 6839–6845.
- Braun, M., Jandus, C., Maurer, P., Hammann-Haenni, A., Schwarz, K., Bachmann, M.F., Speiser, D.E., and Romero, P. (2012). Virus-like particles induce robust human T-helper cell responses: Cellular immune response. *Eur. J. Immunol.* 42, 330–340.
- Brown, S.D., Fiedler, J.D., and Finn, M.G. (2009). Assembly of hybrid bacteriophage Qbeta virus-like particles. *Biochemistry (Mosc.)* 48, 11155–11157.

- Bruckman, M.A., Randolph, L.N., VanMeter, A., Hern, S., Shoffstall, A.J., Taurog, R.E., and Steinmetz, N.F. (2014). Biodistribution, pharmacokinetics, and blood compatibility of native and PEGylated tobacco mosaic virus nano-rods and -spheres in mice. *Virology* 449, 163–173.
- Brune, K.D., Leneghan, D.B., Brian, I.J., Ishizuka, A.S., Bachmann, M.F., Draper, S.J., Biswas, S., and Howarth, M. (2016). Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization. *Sci. Rep.* 6, 19234.
- Budzik, J.M., Marraffini, L.A., Souda, P., Whitelegge, J.P., Faull, K.F., and Schneewind, O. (2008). Amide bonds assemble pili on the surface of bacilli. *Proc. Natl. Acad. Sci.* 105, 10215–10220.
- Bullock, T.N.J., Mullins, D.W., and Engelhard, V.H. (2003). Antigen Density Presented By Dendritic Cells In Vivo Differentially Affects the Number and Avidity of Primary, Memory, and Recall CD8+ T Cells. *J. Immunol.* 170, 1822–1829.
- Bundy, B.C., and Swartz, J.R. (2010a). Direct Attachment of Polypeptides to Virus Like Particles.
- Bundy, B.C., and Swartz, J.R. (2010b). Site-Specific Incorporation of *p* - Propargyloxyphenylalanine in a Cell-Free Environment for Direct Protein–Protein Click Conjugation. *Bioconjug. Chem.* 21, 255–263.
- Bundy, B.C., and Swartz, J.R. (2011). Efficient disulfide bond formation in virus-like particles. *J. Biotechnol.* 154, 230–239.
- Busch, G.K., Tate, E.W., Gaffney, P.R.J., Rosivatz, E., Woscholski, R., and Leatherbarrow, R.J. (2008). Specific N-terminal protein labelling: use of FMDV 3Cprotease and native chemical ligation. *Chem. Commun.* 3369–3371.
- Buynak, E.B., Roehm, R.R., Tytell, A.A., Bertland, A.U., Lampson, G.P., and Hilleman, M.R. (1976). Development and chimpanzee testing of a vaccine against human hepatitis B. *Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. N. Y.* N 151, 694–700.
- Cabrita, L.D., Gilis, D., Robertson, A.L., Dehouck, Y., Rooman, M., and Bottomley, S.P. (2007). Enhancing the stability and solubility of TEV protease using in silico design. *Protein Sci.* 16, 2360–2367.
- Calabro, S., Tritto, E., Pezzotti, A., Taccone, M., Muzzi, A., Bertholet, S., De Gregorio, E., O'Hagan, D.T., Baudner, B., and Seubert, A. (2013). The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect. *Vaccine* 31, 3363–3369.
- Caldeira, J.C., and Peabody, D.S. (2007). Stability and assembly in vitro of bacteriophage PP7 virus-like particles. *J. Nanobiotechnology* 5, 10.

- Caldeira, J.C., and Peabody, D.S. (2011). Thermal stability of RNA phage virus-like particles displaying foreign peptides. *J. Nanobiotechnology* 9, 22.
- Cantin, G.T., Resnick, S., Jin, H., O'Hanlon, R., Espinosa, O., Stevens, A., Payne, J., Glenn, N.R., Rasochova, L., and Allen, J.R. (2011). Comparison of Methods for Chemical Conjugation of an Influenza Peptide to Wild-Type and Cysteine-Mutant Virus-Like Particles Expressed in *Pseudomonas fluorescens*. *Int. J. Pept. Res. Ther.* 17, 217–224.
- Cardinale, D., and Michon, T. (2015). *Enzyme Nanocarriers* (CRC Press).
- Carignan, D., Thérien, A., Rioux, G., Paquet, G., Gagné, M.-È.L., Bolduc, M., Savard, P., and Leclerc, D. (2015). Engineering of the PapMV vaccine platform with a shortened M2e peptide leads to an effective one dose influenza vaccine. *Vaccine* 33, 7245–7253.
- Carrico, Z.M., Romanini, D.W., Mehl, R.A., and Francis, M.B. (2008). Oxidative coupling of peptides to a virus capsid containing unnatural amino acids. *Chem. Commun.* 1205.
- Chackerian, B., Lowy, D.R., and Schiller, J.T. (2001). Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. *J. Clin. Invest.* 108, 415–423.
- Chalker, J.M., Bernardes, G.J.L., Lin, Y.A., and Davis, B.G. (2009). Chemical Modification of Proteins at Cysteine: Opportunities in Chemistry and Biology. *Chem. - Asian J.* 4, 630–640.
- Chang, C.-H., Rossi, E.A., and Goldenberg, D.M. (2007). The Dock and Lock Method: A Novel Platform Technology for Building Multivalent, Multifunctional Structures of Defined Composition with Retained Bioactivity. *Clin. Cancer Res.* 13, 5586s – 5591s.
- Chao, H., Bautista, D.L., Litowski, J., Irvin, R.T., and Hodges, R.S. (1998). Use of a heterodimeric coiled-coil system for biosensor application and affinity purification. *J. Chromatogr. B. Biomed. Sci. App.* 715, 307–329.
- Chatterji, A., Ochoa, W.F., Ueno, T., Lin, T., and Johnson, J.E. (2005). A Virus-Based Nanoblock with Tunable Electrostatic Properties. *Nano Lett.* 5, 597–602.
- Chaudhary, P.M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J., and Hood, L. (1997). Death Receptor 5, a New Member of the TNFR Family, and DR4 Induce FADD-Dependent Apoptosis and Activate the NF- κ B Pathway. *Immunity* 7, 821–830.
- Chen, I., Howarth, M., Lin, W., and Ting, A.Y. (2005). Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. *Nat. Methods* 2, 99–104.

- Chen, Q., Sun, Q., Molino, N.M., Wang, S.-W., Boder, E.T., and Chen, W. (2015). Sortase A-mediated multi-functionalization of protein nanoparticles. *Chem Commun* 51, 12107–12110.
- Cheung, C.L., Chung, S.-W., Chatterji, A., Lin, T., Johnson, J.E., Hok, S., Perkins, J., and De Yoreo, J.J. (2006). Physical Controls on Directed Virus Assembly at Nanoscale Chemical Templates. *J. Am. Chem. Soc.* 128, 10801–10807.
- Christiansen, D., Devaux, P., Réveil, B., Evlashev, A., Horvat, B., Lamy, J., Rabourdin-Combe, C., Cohen, J.H., and Gerlier, D. (2000). Octamerization enables soluble CD46 receptor to neutralize measles virus in vitro and in vivo. *J. Virol.* 74, 4672–4678.
- Cielens, I., Jackevica, L., Strods, A., Kazaks, A., Ose, V., Bogans, J., Pumpens, P., and Renhofa, R. (2014). Mosaic RNA Phage VLPs Carrying Domain III of the West Nile Virus E Protein. *Mol. Biotechnol.* 56, 459–469.
- Clarke, B.E., Newton, S.E., Carroll, A.R., Francis, M.J., Appleyard, G., Syred, A.D., Highfield, P.E., Rowlands, D.J., and Brown, F. (1987). Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* 330, 381–384.
- Clausen, T.M., Christoffersen, S., Dahlback, M., Langkilde, A.E., Jensen, K.E., Resende, M., Agerbaek, M.O., Andersen, D., Berisha, B., Ditlev, S.B., et al. (2012). Structural and Functional Insight into How the Plasmodium falciparum VAR2CSA Protein Mediates Binding to Chondroitin Sulfate A in Placental Malaria. *J. Biol. Chem.* 287, 23332–23345.
- Coban, C., Ishii, K.J., Stowers, A.W., Keister, D.B., Klinman, D.M., and Kumar, N. (2004). Effect of CpG Oligodeoxynucleotides on the Immunogenicity of Pfs25, a Plasmodium falciparum Transmission-Blocking Vaccine Antigen. *Infect. Immun.* 72, 584–588.
- Cohen, S.N., Chang, A.C., Boyer, H.W., and Helling, R.B. (1973). Construction of biologically functional bacterial plasmids in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 70, 3240–3244.
- Conway, J.F., Cheng, N., Zlotnick, A., Stahl, S.J., Wingfield, P.T., Belnap, D.M., Kanngiesser, U., Noah, M., and Steven, A.C. (1998). Hepatitis B virus capsid: localization of the putative immunodominant loop (residues 78 to 83) on the capsid surface, and implications for the distinction between c and e-antigens. *J. Mol. Biol.* 279, 1111–1121.
- Cook, J.C., Joyce, J.G., George, H.A., Schultz, L.D., Hurni, W.M., Jansen, K.U., Hepler, R.W., Ip, C., Lowe, R.S., Keller, P.M., et al. (1999). Purification of virus-like particles of recombinant human papillomavirus type 11 major capsid protein L1 from Saccharomyces cerevisiae. *Protein Expr. Purif.* 17, 477–484.
- Craig, D.B., and Dombkowski, A.A. (2013). Disulfide by Design 2.0: a web-based tool for disulfide engineering in proteins. *BMC Bioinformatics* 14, 346.

- Crichton, R.R., and Declercq, J.-P. (2010). X-ray structures of ferritins and related proteins. *Biochim. Biophys. Acta* 1800, 706–718.
- Crotty, S. (2015). A brief history of T cell help to B cells. *Nat. Rev. Immunol.* 15, 185–189.
- Crowther, R.A., Kiselev, N.A., Böttcher, B., Berriman, J.A., Borisova, G.P., Ose, V., and Pumpens, P. (1994). Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell* 77, 943–950.
- Dahlbäck, M., Jørgensen, L.M., Nielsen, M.A., Clausen, T.M., Ditlev, S.B., Resende, M., Pinto, V.V., Arnot, D.E., Theander, T.G., and Salanti, A. (2011). The chondroitin sulfate A-binding site of the VAR2CSA protein involves multiple N-terminal domains. *J. Biol. Chem.* 286, 15908–15917.
- De Gregorio, E., and Rappuoli, R. (2014). From empiricism to rational design: a personal perspective of the evolution of vaccine development. *Nat. Rev. Immunol.* 14, 505–514.
- Deivanayagam, C.C., Rich, R.L., Carson, M., Owens, R.T., Danthuluri, S., Bice, T., Höök, M., and Narayana, S.V. (2000). Novel fold and assembly of the repetitive B region of the *Staphylococcus aureus* collagen-binding surface protein. *Structure* 8, 67–78.
- Del Campo Ascarateil, J., Turki Hani, I., and Hill, F. (2012). Modified Coiled Coil Type Proteins having Improved Properties. U.S. Patent Application US14570133, December 11, 2012.
- Delpeyroux, F., Peillon, N., Blondel, B., Crainic, R., and Streeck, R.E. (1988). Presentation and immunogenicity of the hepatitis B surface antigen and a poliovirus neutralization antigen on mixed empty envelope particles. *J. Virol.* 62, 1836–1839.
- Didierlaurent, A.M., Collignon, C., Bourguignon, P., Wouters, S., Fierens, K., Fochesato, M., Dendouga, N., Langlet, C., Malissen, B., Lambrecht, B.N., et al. (2014). Enhancement of adaptive immunity by the human vaccine adjuvant AS01 depends on activated dendritic cells. *J. Immunol. Baltim. Md* 1950 193, 1920–1930.
- Ding, F.-X., Wang, F., Lu, Y.-M., Li, K., Wang, K.-H., He, X.-W., and Sun, S.-H. (2009). Multiepitope peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. *Hepatology* 49, 1492–1502.
- Dintzis, H.M., Dintzis, R.Z., and Vogelstein, B. (1976). Molecular determinants of immunogenicity: the immunon model of immune response. *Proc. Natl. Acad. Sci. U. S. A.* 73, 3671–3675.

- Dintzis, R.Z., Middleton, M.H., and Dintzis, H.M. (1983). Studies on the immunogenicity and tolerogenicity of T-independent antigens. *J. Immunol. Baltim. Md 1950* *131*, 2196–2203.
- Dovala, D., Sawyer, W.S., Rath, C.M., and Metzger, L.E. (2016). Rapid analysis of protein expression and solubility with the SpyTag-SpyCatcher system. *Protein Expr. Purif.* *117*, 44–51.
- Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* *16*, 6127–6145.
- Duffy, P.E., and Kaslow, D.C. (1997). A novel malaria protein, Pfs28, and Pfs25 are genetically linked and synergistic as falciparum malaria transmission-blocking vaccines. *Infect. Immun.* *65*, 1109–1113.
- Dumon-Seignovert, L., Cariot, G., and Vuillard, L. (2004). The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). *Protein Expr. Purif.* *37*, 203–206.
- Dunn, J.J., and Studier, F.W. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J. Mol. Biol.* *166*, 477–535.
- Dyson, M.R., and Murray, K. (1995). Selection of peptide inhibitors of interactions involved in complex protein assemblies: association of the core and surface antigens of hepatitis B virus. *Proc. Natl. Acad. Sci. U. S. A.* *92*, 2194–2198.
- Echelman, D.J., Alegre-Cebollada, J., Badilla, C.L., Chang, C., Ton-That, H., and Fernández, J.M. (2016). CnaA domains in bacterial pili are efficient dissipaters of large mechanical shocks. *Proc. Natl. Acad. Sci.* *113*, 2490–2495.
- Eckhardt, M., Anders, M., Muranyi, W., Heilemann, M., Krijnse-Locker, J., and Müller, B. (2011). A SNAP-Tagged Derivative of HIV-1 — A Versatile Tool to Study Virus-Cell Interactions. *PLoS ONE* *6*, e22007.
- Edelman, G.M., and Gall, W.E. (1969). The Antibody Problem. *Annu. Rev. Biochem.* *38*, 415–466.
- Espah Borujeni, A., Channarasappa, A.S., and Salis, H.M. (2014). Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. *Nucleic Acids Res.* *42*, 2646–2659.
- Ewer, K.J., Lambe, T., Rollier, C.S., Spencer, A.J., Hill, A.V., and Dorrell, L. (2016). Viral vectors as vaccine platforms: from immunogenicity to impact. *Curr. Opin. Immunol.* *41*, 47–54.
- Fairhead, M., and Howarth, M. (2015). Site-specific biotinylation of purified proteins using BirA. *Methods Mol. Biol. Clifton NJ* *1266*, 171–184.

- Fairhead, M., Krndija, D., Lowe, E.D., and Howarth, M. (2014a). Plug-and-Play Pairing via Defined Divalent Streptavidins. *J. Mol. Biol.* *426*, 199–214.
- Fairhead, M., Veggiani, G., Lever, M., Yan, J., Mesner, D., Robinson, C.V., Dushek, O., van der Merwe, P.A., and Howarth, M. (2014b). SpyAvidin Hubs Enable Precise and Ultrastable Orthogonal Nanoassembly. *J. Am. Chem. Soc.* *136*, 12355–12363.
- Falcón, V., García, C., de la Rosa, M.C., Menéndez, I., Seoane, J., and Grillo, J.M. (1999). Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic *Pichia pastoris* yeast when expressing HCV structural proteins (core-E1). *Tissue Cell* *31*, 117–125.
- Fehr, T., Bachmann, M.F., Bucher, E., Kalinke, U., Di Padova, F.E., Lang, A.B., Hengartner, H., and Zinkernagel, R.M. (1997). Role of repetitive antigen patterns for induction of antibodies against antibodies. *J. Exp. Med.* *185*, 1785–1792.
- Fehr, T., Skrastina, D., Pumpens, P., and Zinkernagel, R.M. (1998). T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles. *Proc. Natl. Acad. Sci. U. S. A.* *95*, 9477–9481.
- Fiedler, J.D., Brown, S.D., Lau, J.L., and Finn, M.G. (2010). RNA-Directed Packaging of Enzymes within Virus-like Particles. *Angew. Chem. Int. Ed.* *49*, 9648–9651.
- Fierer, J.O., Veggiani, G., and Howarth, M. (2014). SpyLigase peptide-peptide ligation polymerizes affibodies to enhance magnetic cancer cell capture. *Proc. Natl. Acad. Sci. U. S. A.* *111*, E1176–E1181.
- Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., et al. (1976). Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. *Nature* *260*, 500–507.
- Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G.A., Li, J., Mottram, P.L., McKenzie, I.F.C., and Plebanski, M. (2004). Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. *J. Immunol. Baltim. Md 1950* *173*, 3148–3154.
- Fine, P., Eames, K., and Heymann, D.L. (2011). “Herd Immunity”: A Rough Guide. *Clin. Infect. Dis.* *52*, 911–916.
- Fontaine, S.D., Reid, R., Robinson, L., Ashley, G.W., and Santi, D.V. (2015). Long-Term Stabilization of Maleimide–Thiol Conjugates. *Bioconjug. Chem.* *26*, 145–152.
- Foquet, L., Hermsen, C.C., van Gemert, G.-J., Van Braeckel, E., Weening, K.E., Sauerwein, R., Meuleman, P., and Leroux-Roels, G. (2014). Vaccine-induced monoclonal antibodies targeting circumsporozoite protein prevent *Plasmodium falciparum* infection. *J. Clin. Invest.* *124*, 140–144.

- Fraser, C.C., H. Altreuter, D., Ilyinskii, P., Pittet, L., LaMothe, R.A., Keegan, M., Johnston, L., and Kishimoto, T.K. (2014). Generation of a universal CD4 memory T cell recall peptide effective in humans, mice and non-human primates. *Vaccine* 32, 2896–2903.
- Freivalds, J., Kotelovica, S., Voronkova, T., Ose, V., Tars, K., and Kazaks, A. (2014). Yeast-expressed bacteriophage-like particles for the packaging of nanomaterials. *Mol. Biotechnol.* 56, 102–110.
- Galaway, F.A., and Stockley, P.G. (2013). MS2 Viruslike Particles: A Robust, Semisynthetic Targeted Drug Delivery Platform. *Mol. Pharm.* 10, 59–68.
- Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., et al. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498–511.
- Gautier, A., Juillerat, A., Heinis, C., Corrêa, I.R., Kindermann, M., Beaufils, F., and Johnsson, K. (2008). An Engineered Protein Tag for Multiprotein Labeling in Living Cells. *Chem. Biol.* 15, 128–136.
- Gedeon, P.C., Choi, B.D., Sampson, J.H., and Bigner, D.D. (2013). Rindopepimut. *Drugs Future* 38, 147.
- Geoghegan, K.F., Dixon, H.B., Rosner, P.J., Hoth, L.R., Lanzetti, A.J., Borzilleri, K.A., Marr, E.S., Pezzullo, L.H., Martin, L.B., LeMotte, P.K., et al. (1999). Spontaneous alpha-N-6-phosphogluconoylation of a “His tag” in *Escherichia coli*: the cause of extra mass of 258 or 178 Da in fusion proteins. *Anal. Biochem.* 267, 169–184.
- Germanier, R., and Fiirer, E. (1975). Isolation and Characterization of Gal E Mutant Ty 21a of *Salmonella typhi*: A Candidate Strain for a Live, Oral Typhoid Vaccine. *J. Infect. Dis.* 131, 553–558.
- Gibson, D. (2009). One-step enzymatic assembly of DNA molecules up to several hundred kilobases in size. *Protoc. Exch.*
- Gilbert, W., and Maxam, A. (1973). The nucleotide sequence of the lac operator. *Proc. Natl. Acad. Sci. U. S. A.* 70, 3581–3584.
- Gleiter, S. (2001). Coupling of antibodies via protein Z on modified polyoma virus-like particles. *Protein Sci.* 10, 434–444.
- Gleiter, S., Stubenrauch, K., and Lilie, H. (1999). Changing the surface of a virus shell fusion of an enzyme to polyoma VP1. *Protein Sci. Publ. Protein Soc.* 8, 2562–2569.
- Golmohammadi, R., Valegård, K., Fridborg, K., and Liljas, L. (1993). The refined structure of bacteriophage MS2 at 2.8 Å resolution. *J. Mol. Biol.* 234, 620–639.

- Golmohammadi, R., Fridborg, K., Bundule, M., Valegård, K., and Liljas, L. (1996). The crystal structure of bacteriophage Q beta at 3.5 Å resolution. *Struct. Lond. Engl.* 1993 4, 543–554.
- Goodman, A.L., Blagborough, A.M., Biswas, S., Wu, Y., Hill, A.V., Sinden, R.E., and Draper, S.J. (2011). A viral vectored prime-boost immunization regime targeting the malaria Pfs25 antigen induces transmission-blocking activity. *PloS One* 6, e29428.
- Gozar, M.M., Price, V.L., and Kaslow, D.C. (1998). *Saccharomyces cerevisiae*-secreted fusion proteins Pfs25 and Pfs28 elicit potent *Plasmodium falciparum* transmission-blocking antibodies in mice. *Infect. Immun.* 66, 59–64.
- Graham, J.W.A., Williams, T.C.R., Morgan, M., Fernie, A.R., Ratcliffe, R.G., and Sweetlove, L.J. (2007). Glycolytic Enzymes Associate Dynamically with Mitochondria in Response to Respiratory Demand and Support Substrate Channeling. *PLANT CELL ONLINE* 19, 3723–3738.
- Green, N.M. (1990). Avidin and streptavidin. *Methods Enzymol.* 184, 51–67.
- Greener, A.L., and Jerpseth, B.D. (2002). Method for more efficient electroporation.
- Gregory, J.A., Li, F., Tomosada, L.M., Cox, C.J., Topol, A.B., Vinetz, J.M., and Mayfield, S. (2012). Algae-Produced Pfs25 Elicits Antibodies That Inhibit Malaria Transmission. *PLoS ONE* 7, e37179.
- Griffith, T.S., Rauch, C.T., Smolak, P.J., Waugh, J.Y., Boiani, N., Lynch, D.H., Smith, C.A., Goodwin, R.G., and Kubin, M.Z. (1999). Functional analysis of TRAIL receptors using monoclonal antibodies. *J. Immunol. Baltim. Md 1950* 162, 2597–2605.
- Guy, B., Guirakhoo, F., Barban, V., Higgs, S., Monath, T.P., and Lang, J. (2010). Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine* 28, 632–649.
- Hackeng, T.M., Griffin, J.H., and Dawson, P.E. (1999). Protein synthesis by native chemical ligation: expanded scope by using straightforward methodology. *Proc. Natl. Acad. Sci. U. S. A.* 96, 10068–10073.
- Hagan, R.M., Björnsson, R., McMahon, S.A., Schomburg, B., Braithwaite, V., Bühl, M., Naismith, J.H., and Schwarz-Linek, U. (2010). NMR Spectroscopic and Theoretical Analysis of a Spontaneously Formed Lys-Asp Isopeptide Bond. *Angew. Chem. Int. Ed.* 49, 8421–8425.
- Hallam, T.J., Wold, E., Wahl, A., and Smider, V.V. (2015). Antibody Conjugates with Unnatural Amino Acids. *Mol. Pharm.* 12, 1848–1862.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557–580.

- Hansen, T.H., and Bouvier, M. (2009). MHC class I antigen presentation: learning from viral evasion strategies. *Nat. Rev. Immunol.* 9, 503–513.
- Harrison, M.S., Schmitt, P.T., Pei, Z., and Schmitt, A.P. (2012). Role of Ubiquitin in Parainfluenza Virus 5 Particle Formation. *J. Virol.* 86, 3474–3485.
- Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099–1103.
- Haynes, J.R., Cunningham, J., von Seefried, A., Lennick, M., Garvin, R.T., and Shen, S.-H. (1986). Development of a Genetically–Engineered, Candidate Polio Vaccine Employing the Self–Assembling Properties of the Tobacco Mosaic Virus Coat Protein. *Bio/Technology* 4, 637–641.
- He, L., de Val, N., Morris, C.D., Vora, N., Thinnes, T.C., Kong, L., Azadnia, P., Sok, D., Zhou, B., Burton, D.R., et al. (2016). Presenting native-like trimeric HIV-1 antigens with self-assembling nanoparticles. *Nat. Commun.* 7, 12041.
- Heal, K.G., Hill, H.R., Stockley, P.G., Hollingdale, M.R., and Taylor-Robinson, A.W. (1999). Expression and immunogenicity of a liver stage malaria epitope presented as a foreign peptide on the surface of RNA-free MS2 bacteriophage capsids. *Vaccine* 18, 251–258.
- Hedman, K., and Rousseau, S.A. (1989). Measurement of avidity of specific IgG for verification of recent primary rubella. *J. Med. Virol.* 27, 288–292.
- Heppner, D., Kester, K., Ockenhouse, C., Tornieporth, N., Ofori, O., Lyon, J., Stewart, V., Dubois, P., Lanar, D., and Krzych, U. (2005). Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research. *Vaccine* 23, 2243–2250.
- Hermanson, G.T. (1996). *Bioconjugate techniques* (San Diego: Academic Press).
- Higgins, M.K., and Carrington, M. (2014). Sequence variation and structural conservation allows development of novel function and immune evasion in parasite surface protein families: Parasite Surface Protein Structure and Function. *Protein Sci.* 23, 354–365.
- Higuchi, R., Krummel, B., and Saiki, R. (1988). A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* 16, 7351–7367.
- Hill, A.V., Biswas, S., Draper, S., Rampling, T., and Reyes-Sandoval, A. (2014). Towards a multi-antigen multi-stage malaria vaccine. *Malar. J.* 13, O31.
- Hiltschmann, N. (1969). Die molekularen Grundlagen der Antikörperbildung. *Naturwissenschaften* 56, 195–205.

- Hoesl, M.G., and Budisa, N. (2011). In vivo incorporation of multiple noncanonical amino acids into proteins. *Angew. Chem. Int. Ed Engl.* 50, 2896–2902.
- Hofmeyer, T., Schmelz, S., Degiacomi, M.T., Dal Peraro, M., Daneschdar, M., Scrima, A., van den Heuvel, J., Heinz, D.W., and Kolmar, H. (2013). Arranged Sevenfold: Structural Insights into the C-Terminal Oligomerization Domain of Human C4b-Binding Protein. *J. Mol. Biol.* 425, 1302–1317.
- Horowitz, E.D., Weinberg, M.S., and Asokan, A. (2011). Glycated AAV Vectors: Chemical Redirection of Viral Tissue Tropism. *Bioconjug. Chem.* 22, 529–532.
- Horowitz, E.D., Finn, M.G., and Asokan, A. (2012). Tyrosine cross-linking reveals interfacial dynamics in adeno-associated viral capsids during infection. *ACS Chem. Biol.* 7, 1059–1066.
- Horton, R.M., Cai, Z.L., Ho, S.N., and Pease, L.R. (1990). Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *BioTechniques* 8, 528–535.
- Howarth, M., Chinnapen, D.J.-F., Gerrow, K., Dorrestein, P.C., Grandy, M.R., Kelleher, N.L., El-Husseini, A., and Ting, A.Y. (2006). A monovalent streptavidin with a single femtomolar biotin binding site. *Nat. Methods* 3, 267–273.
- Hua, Z., and Hou, B. (2013). TLR signaling in B-cell development and activation. *Cell. Mol. Immunol.* 10, 103–106.
- Huet, H.A., Growney, J.D., Johnson, J.A., Li, J., Bilic, S., Ostrom, L., Zafari, M., Kowal, C., Yang, G., Royo, A., et al. (2014). Multivalent nanobodies targeting death receptor 5 elicit superior tumor cell killing through efficient caspase induction. *mAbs* 6, 1560–1570.
- Izoré, T., Contreras-Martel, C., El Mortaji, L., Manzano, C., Terrasse, R., Vernet, T., Di Guilmi, A.M., and Dessen, A. (2010). Structural basis of host cell recognition by the pilus adhesin from *Streptococcus pneumoniae*. *Struct. Lond. Engl.* 1993 18, 106–115.
- Jain, N.K., Sahni, N., Kumru, O.S., Joshi, S.B., Volkin, D.B., and Russell Middaugh, C. (2014). Formulation and stabilization of recombinant protein based virus-like particle vaccines. *Adv. Drug Deliv. Rev.* 93, 42–55.
- Janowicz, Z.A., Melber, K., Merckelbach, A., Jacobs, E., Harford, N., Comberbach, M., and Hollenberg, C.P. (1991). Simultaneous expression of the S and L surface antigens of hepatitis B, and formation of mixed particles in the methylotrophic yeast, *Hansenula polymorpha*. *Yeast* 7, 431–443.
- Jegerlehner, A., Storni, T., Lipowsky, G., Schmid, M., Pumpens, P., and Bachmann, M.F. (2002a). Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. *Eur. J. Immunol.* 32, 3305–3314.

- Jegerlehner, A., Tissot, A., Lechner, F., Sebbel, P., Erdmann, I., Kündig, T., Bächli, T., Storni, T., Jennings, G., Pumpens, P., et al. (2002b). A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine* 20, 3104–3112.
- Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H.J., Kopf, M., and Bachmann, M.F. (2007). TLR9 signaling in B cells determines class switch recombination to IgG2a. *J. Immunol. Baltim. Md 1950* 178, 2415–2420.
- Jegerlehner, A., Wiesel, M., Dietmeier, K., Zabel, F., Gatto, D., Saudan, P., and Bachmann, M.F. (2010). Carrier induced epitopic suppression of antibody responses induced by virus-like particles is a dynamic phenomenon caused by carrier-specific antibodies. *Vaccine* 28, 5503–5512.
- Jennings, G.T., and Bachmann, M.F. (2009). Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. *Annu. Rev. Pharmacol. Toxicol.* 49, 303–326.
- Jewett, J.C., and Bertozzi, C.R. (2010). Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev.* 39, 1272–1279.
- Jones, R.M., Chichester, J.A., Mett, V., Jaje, J., Tottey, S., Manceva, S., Casta, L.J., Gibbs, S.K., Musiyshuk, K., Shamloul, M., et al. (2013). A Plant-Produced Pfs25 VLP Malaria Vaccine Candidate Induces Persistent Transmission Blocking Antibodies against *Plasmodium falciparum* in Immunized Mice. *PLoS ONE* 8, e79538.
- Kalia, J., and Raines, R.T. (2007). Catalysis of imido group hydrolysis in a maleimide conjugate. *Bioorg. Med. Chem. Lett.* 17, 6286–6289.
- Kang, H.J., and Baker, E.N. (2011). Intramolecular isopeptide bonds: protein crosslinks built for stress? *Trends Biochem. Sci.* 36, 229–237.
- Kang, H.J., Coulibaly, F., Clow, F., Proft, T., and Baker, E.N. (2007). Stabilizing isopeptide bonds revealed in gram-positive bacterial pilus structure. *Science* 318, 1625–1628.
- Kang, H.J., Paterson, N.G., Gaspar, A.H., Ton-That, H., and Baker, E.N. (2009). The *Corynebacterium diphtheriae* shaft pilin SpaA is built of tandem Ig-like modules with stabilizing isopeptide and disulfide bonds. *Proc. Natl. Acad. Sci.* 106, 16967–16971.
- Kang, S.-M., Yao, Q., Guo, L., and Compans, R.W. (2003). Mucosal Immunization with Virus-Like Particles of Simian Immunodeficiency Virus Conjugated with Cholera Toxin Subunit B. *J. Virol.* 77, 9823–9830.
- Kapulu, M.C., Da, D.F., Miura, K., Li, Y., Blagborough, A.M., Churcher, T.S., Nikolaeva, D., Williams, A.R., Goodman, A.L., Sangare, I., et al. (2015). Comparative Assessment of Transmission-Blocking Vaccine Candidates against *Plasmodium falciparum*. *Sci. Rep.* 5, 11193.

- Kaslow, D.C. (2002). Transmission-Blocking Vaccines. In *Chemical Immunology and Allergy*, P. Perlmann, and M. Troye-Blomberg, eds. (Basel: KARGER), pp. 287–307.
- Kaslow, D.C., and Biernaux, S. (2015). RTS,S: Toward a first landmark on the Malaria Vaccine Technology Roadmap. *Vaccine* 33, 7425–7432.
- Kather, I., Bippes, C.A., and Schmid, F.X. (2005). A stable disulfide-free gene-3-protein of phage fd generated by in vitro evolution. *J. Mol. Biol.* 354, 666–678.
- Kaufmann, S.H.E. (2008). Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nat. Immunol.* 9, 705–712.
- Keiler, K.C. (2015). Mechanisms of ribosome rescue in bacteria. *Nat. Rev. Microbiol.* 13, 285–297.
- Keller, S.A., Bauer, M., Manolova, V., Muntwiler, S., Saudan, P., and Bachmann, M.F. (2010). Cutting Edge: Limited Specialization of Dendritic Cell Subsets for MHC Class II-Associated Presentation of Viral Particles. *J. Immunol.* 184, 26–29.
- Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., and Sternberg, M.J.E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* 10, 845–858.
- Kerr, J.S., and Wright, G.J. (2012). Avidity-based extracellular interaction screening (AVEXIS) for the scalable detection of low-affinity extracellular receptor-ligand interactions. *J. Vis. Exp. JoVE* e3881.
- Khudyakov, Y.E., and Pumpens, P. (2016). Viral nanotechnology.
- Kiessling, L.L., Gestwicki, J.E., and Strong, L.E. (2006). Synthetic multivalent ligands as probes of signal transduction. *Angew. Chem. Int. Ed Engl.* 45, 2348–2368.
- Klaus, G.G., and Humphrey, J.H. (1976). B cell tolerance induced by polymeric antigens. I. Comparison of the dose and epitope density requirements for inactivation of primed and unprimed B cells in vivo. *Eur. J. Immunol.* 5, 361–365.
- Klimek, L., Schendzielorz, P., Mueller, P., Saudan, P., and Willers, J. (2013). Immunotherapy of allergic rhinitis: New therapeutic opportunities with virus-like particles filled with CpG motifs. *Am. J. Rhinol. Allergy* 27, 206–212.
- Klovins, J., Tsareva, N.A., de Smit, M.H., Berzins, V., and van Duin, J. (1997). Rapid evolution of translational control mechanisms in RNA genomes. *J. Mol. Biol.* 265, 372–384.
- Klovins, J., Overbeek, G.P., van den Worm, S.H.E., Ackermann, H.-W., and van Duin, J. (2002). Nucleotide sequence of a ssRNA phage from *Acinetobacter*: kinship to coliphages. *J. Gen. Virol.* 83, 1523–1533.

Köhler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–497.

Koho, T., Ihalainen, T.O., Stark, M., Uusi-Kerttula, H., Wieneke, R., Rahikainen, R., Blazevic, V., Marjomäki, V., Tampé, R., Kulomaa, M.S., et al. (2015). His-tagged norovirus-like particles: A versatile platform for cellular delivery and surface display. *Eur. J. Pharm. Biopharm.* 96, 22–31.

Kokhaei, P., Palma, M., Hansson, L., Osterborg, A., Mellstedt, H., and Choudhury, A. (2007). Telomerase (hTERT 611–626) serves as a tumor antigen in B-cell chronic lymphocytic leukemia and generates spontaneously antileukemic, cytotoxic T cells. *Exp. Hematol.* 35, 297–304.

Koletzki, D., Zankl, A., Gelderblom, H.R., Meisel, H., Dislers, A., Borisova, G., Pumpens, P., Krüger, D.H., and Ulrich, R. (1997). Mosaic hepatitis B virus core particles allow insertion of extended foreign protein segments. *J. Gen. Virol.* 78 (Pt 8), 2049–2053.

Kozlovskaja, T.M., Cielens, I., Dreilina, D., Dislers, A., Baumanis, V., Ose, V., and Pumpens, P. (1993). Recombinant RNA phage Q beta capsid particles synthesized and self-assembled in *Escherichia coli*. *Gene* 137, 133–137.

Kozlovskaja, T.M., Cielens, I., Vasiljeva, I., Strelnikova, A., Kazaks, A., Dislers, A., Dreilina, D., Ose, V., Gusars, I., and Pumpens, P. (1996). RNA phage Q beta coat protein as a carrier for foreign epitopes. *Intervirology* 39, 9–15.

Kozlovskaja, T.M., Pushko, P.M., Stankevich, E.I., Dreimane, A.I., and Sniker, D.I. (1988). [Genetically engineered mutants of the envelope protein of the RNA-containing bacteriophage]. *Mol. Biol. (Mosk.)* 22, 731–740.

Krishnan, V., Gaspar, A.H., Ye, N., Mandlik, A., Ton-That, H., and Narayana, S.V.L. (2007). An IgG-like domain in the minor pilin GBS52 of *Streptococcus agalactiae* mediates lung epithelial cell adhesion. *Struct. Lond. Engl.* 1993 15, 893–903.

Kueng, H.J., Leb, V.M., Haiderer, D., Raposo, G., Thery, C., Derdak, S.V., Schmetterer, K.G., Neunkirchner, A., Sillaber, C., Seed, B., et al. (2007). General strategy for decoration of enveloped viruses with functionally active lipid-modified cytokines. *J. Virol.* 81, 8666–8676.

Kündig, T.M., Klimek, L., Schendzielorz, P., Renner, W.A., Senti, G., and Bachmann, M.F. (2014). Is The Allergen Really Needed in Allergy Immunotherapy? *Curr. Treat. Options Allergy* 2, 72–82.

Kuo, S.-M., Tan, C.-H., Dragan, M., and Wilson, J.X. (2005). Endotoxin increases ascorbate recycling and concentration in mouse liver. *J. Nutr.* 135, 2411–2416.

Kurosaki, T., Kometani, K., and Ise, W. (2015). Memory B cells. *Nat. Rev. Immunol.* 15, 149–159.

- Kushnir, N., Streatfield, S.J., and Yusibov, V. (2012). Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development. *Vaccine* 31, 58–83.
- Kutzler, M.A., and Weiner, D.B. (2008). DNA vaccines: ready for prime time? *Nat. Rev. Genet.* 9, 776–788.
- Kwon, S.-K., Kim, S.K., Lee, D.-H., and Kim, J.F. (2015). Comparative genomics and experimental evolution of *Escherichia coli* BL21(DE3) strains reveal the landscape of toxicity escape from membrane protein overproduction. *Sci. Rep.* 5, 16076.
- Lajoie, M.J., Rovner, A.J., Goodman, D.B., Aerni, H.-R., Haimovich, A.D., Kuznetsov, G., Mercer, J.A., Wang, H.H., Carr, P.A., Mosberg, J.A., et al. (2013). Genomically Recoded Organisms Expand Biological Functions. *Science* 342, 357–360.
- Latham, T., and Galarza, J.M. (2001). Formation of Wild-Type and Chimeric Influenza Virus-Like Particles following Simultaneous Expression of Only Four Structural Proteins. *J. Virol.* 75, 6154–6165.
- Latta, M., Philit, M., Maury, I., Soubrier, F., Denèfle, P., and Mayaux, J.F. (1990). Tryptophan promoter derivatives on multicopy plasmids: a comparative analysis of expression potentials in *Escherichia coli*. *DNA Cell Biol.* 9, 129–137.
- Lau, C.K.Y., Turner, L., Jespersen, J.S., Lowe, E.D., Petersen, B., Wang, C.W., Petersen, J.E.V., Lusingu, J., Theander, T.G., Lavstsen, T., et al. (2015). Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria. *Cell Host Microbe* 17, 118–129.
- LeBien, T.W., and Tedder, T.F. (2008). B lymphocytes: how they develop and function. *Blood* 112, 1570–1580.
- Lechner, F., Jegerlehner, A., Tissot, A.C., Maurer, P., Sebbel, P., Renner, W.A., Jennings, G.T., and Bachmann, M.F. (2003). Virus-Like Particles as a Modular System for Novel Vaccines. *Intervirology* 45, 212–217.
- Lee, K.L., Twyman, R.M., Fiering, S., and Steinmetz, N.F. (2016). Virus-based nanoparticles as platform technologies for modern vaccines: Virus-based nanoparticle vaccines. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 8, 554–578.
- Leech, J.H., Barnwell, J.W., Miller, L.H., and Howard, R.J. (1984). Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. *J. Exp. Med.* 159, 1567–1575.
- Lepow, M.L., Goldschneider, I., Gold, R., Randolph, M., and Gotschlich, E.C. (1977). Persistence of antibody following immunization of children with groups A and C meningococcal polysaccharide vaccines. *Pediatrics* 60, 673–680.

- Leung, A.K.C. (2011). "Variolation" and Vaccination in Late Imperial China, Ca 1570–1911. In *History of Vaccine Development*, S.A. Plotkin, ed. (Springer New York), pp. 5–12.
- Li, L., Fierer, J.O., Rapoport, T.A., and Howarth, M. (2014). Structural analysis and optimization of the covalent association between SpyCatcher and a peptide Tag. *J. Mol. Biol.* 426, 309–317.
- Li, Y., Leneghan, D.B., Miura, K., Nikolaeva, D., Brian, I.J., Dicks, M.D.J., Fyfe, A.J., Zakutansky, S.E., de Cassan, S., Draper, S.J., et al. (2015). Enhancing immunogenicity and transmission-blocking activity of malaria vaccines by fusing Pfs25 to IMX313 multimerization technology. *Sci. Rep. in press*, *SREP-15-24347A*.
- Li, Y., Leneghan, D.B., Miura, K., Nikolaeva, D., Brian, I.J., Dicks, M.D.J., Fyfe, A.J., Zakutansky, S.E., de Cassan, S., Long, C.A., et al. (2016). Enhancing immunogenicity and transmission-blocking activity of malaria vaccines by fusing Pfs25 to IMX313 multimerization technology. *Sci. Rep.* 6, 18848.
- Lilie, H., Richter, S., Bergelt, S., Frost, S., and Gehle, F. (2013). Polyionic and cysteine-containing fusion peptides as versatile protein tags. *Biol. Chem.* 394.
- Liljas, L., Fridborg, K., Valegard, K., Bundule, M., and Pumpens, P. (1994). Crystal Structure of Bacteriophage fr Capsids at 3.5 Å Resolution. *J. Mol. Biol.* 244, 279–290.
- Lin-Chao, S., Chen, W.-T., and Wong, T.-T. (1992). High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Mol. Microbiol.* 6, 3385–3393.
- Link, A., Zabel, F., Schnetzler, Y., Titz, A., Brombacher, F., and Bachmann, M.F. (2012). Innate Immunity Mediates Follicular Transport of Particulate but Not Soluble Protein Antigen. *J. Immunol.* 188, 3724–3733.
- Liu, C.C., and Schultz, P.G. (2010). Adding New Chemistries to the Genetic Code. *Annu. Rev. Biochem.* 79, 413–444.
- Liu, H., and Irvine, D.J. (2015). Guiding Principles in the Design of Molecular Bioconjugates for Vaccine Applications. *Bioconjug. Chem.* 26, 791–801.
- Liu, Z., Zhou, H., Wang, W., Tan, W., Fu, Y.-X., and Zhu, M. (2014). A novel method for synthetic vaccine construction based on protein assembly. *Sci. Rep.* 4, 7266.
- Livnah, O., Bayer, E.A., Wilchek, M., and Sussman, J.L. (1993). Three-dimensional structures of avidin and the avidin-biotin complex. *Proc. Natl. Acad. Sci. U. S. A.* 90, 5076–5080.
- Lopez, P.J., Marchand, I., Joyce, S.A., and Dreyfus, M. (1999). The C-terminal half of RNase E, which organizes the Escherichia coli degradosome, participates in mRNA degradation but not rRNA processing in vivo. *Mol. Microbiol.* 33, 188–199.

- Los, G.V., Encell, L.P., McDougall, M.G., Hartzell, D.D., Karassina, N., Zimprich, C., Wood, M.G., Learish, R., Ohana, R.F., Urh, M., et al. (2008). HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem. Biol.* **3**, 373–382.
- Love, K.R., Swoboda, J.G., Noren, C.J., and Walker, S. (2006). Enabling Glycosyltransferase Evolution: A Facile Substrate-Attachment Strategy for Phage-Display Enzyme Evolution. *ChemBioChem* **7**, 753–756.
- Lu, Y., and Swartz, J.R. (2016). Functional properties of flagellin as a stimulator of innate immunity. *Sci. Rep.* **6**, 18379.
- Lu, W., Qasim, M.A., and Kent, S.B.H. (1996). Comparative Total Syntheses of Turkey Ovomucoid Third Domain by Both Stepwise Solid Phase Peptide Synthesis and Native Chemical Ligation. *J. Am. Chem. Soc.* **118**, 8518–8523.
- Lu, Y., Welsh, J.P., Chan, W., and Swartz, J.R. (2013). Escherichia coli-based cell free production of flagellin and ordered flagellin display on virus-like particles. *Biotechnol. Bioeng.* **110**, 2073–2085.
- Lu, Y., Chan, W., Ko, B.Y., VanLang, C.C., and Swartz, J.R. (2015). Assessing sequence plasticity of a virus-like nanoparticle by evolution toward a versatile scaffold for vaccines and drug delivery. *Proc. Natl. Acad. Sci.* **112**, 12360–12365.
- Ma, J., Pazos, I.M., and Gai, F. (2014). Microscopic insights into the protein-stabilizing effect of trimethylamine N-oxide (TMAO). *Proc. Natl. Acad. Sci.* **111**, 8476–8481.
- Macnab, R.M. (2003). How Bacteria Assemble Flagella. *Annu. Rev. Microbiol.* **57**, 77–100.
- Magalhães, P.O., Lopes, A.M., Mazzola, P.G., Rangel-Yagui, C., Penna, T.C.V., and Pessoa, A. (2007). Methods of endotoxin removal from biological preparations: a review. *J. Pharm. Pharm. Sci. Publ. Can. Soc. Pharm. Sci. Société Can. Sci. Pharm.* **10**, 388–404.
- Malins, L.R., and Payne, R.J. (2014). Recent extensions to native chemical ligation for the chemical synthesis of peptides and proteins. *Curr. Opin. Chem. Biol.* **22**, 70–78.
- Mamat, U., Wilke, K., Bramhill, D., Schromm, A.B., Lindner, B., Kohl, T.A., Corchero, J.L., Villaverde, A., Schaffer, L., Head, S.R., et al. (2015). Detoxifying Escherichia coli for endotoxin-free production of recombinant proteins. *Microb. Cell Factories* **14**.
- Manayani, D.J., Thomas, D., Dryden, K.A., Reddy, V., Siladi, M.E., Marlett, J.M., Rainey, G.J.A., Pique, M.E., Scobie, H.M., Yeager, M., et al. (2007). A Viral Nanoparticle with Dual Function as an Anthrax Antitoxin and Vaccine. *PLoS Pathog.* **3**, e142.

- Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., and Bachmann, M.F. (2008). Nanoparticles target distinct dendritic cell populations according to their size. *Eur. J. Immunol.* 38, 1404–1413.
- Manzenrieder, F., Luxenhofer, R., Retzlaff, M., Jordan, R., and Finn, M.G. (2011). Stabilization of Virus-like Particles with Poly(2-oxazoline)s. *Angew. Chem. Int. Ed.* 50, 2601–2605.
- Mascola, J.R., and Montefiori, D.C. (2010). The Role of Antibodies in HIV Vaccines. *Annu. Rev. Immunol.* 28, 413–444.
- Mastico, R.A., Talbot, S.J., and Stockley, P.G. (1993). Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsid. *J. Gen. Virol.* 74 (Pt 4), 541–548.
- Mateu, M.G. (2011). Virus engineering: functionalization and stabilization. *Protein Eng. Des. Sel.* 24, 53–63.
- Mateu, M.G. (2013). *Structure and physics of viruses: an integrated textbook* (Dordrecht: Springer).
- Mayer, M.P., and Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* 62, 670–684.
- Mbow, M.L., De Gregorio, E., Valiante, N.M., and Rappuoli, R. (2010). New adjuvants for human vaccines. *Curr. Opin. Immunol.* 22, 411–416.
- McCluskie, M.J., Thorn, J., Gervais, D.P., Stead, D.R., Zhang, N., Benoit, M., Cartier, J., Kim, I.-J., Bhattacharya, K., Finneman, J.I., et al. (2015). Anti-nicotine vaccines: Comparison of adjuvanted CRM197 and Qb-VLP conjugate formulations for immunogenicity and function in non-human primates. *Int. Immunopharmacol.* 29, 663–671.
- Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1, 135–145.
- Metzner, C., Mostegl, M.M., Günzburg, W.H., Salmons, B., and Dangerfield, J.A. (2008a). Association of glycosylphosphatidylinositol-anchored protein with retroviral particles. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 22, 2734–2739.
- Metzner, C., Salmons, B., Günzburg, W.H., and Dangerfield, J.A. (2008b). Rafts, anchors and viruses--a role for glycosylphosphatidylinositol anchored proteins in the modification of enveloped viruses and viral vectors. *Virology* 382, 125–131.
- Metzner, C., Kochan, F., and Dangerfield, J.A. (2013). Fluorescence molecular painting of enveloped viruses. *Mol. Biotechnol.* 53, 9–18.
- Miller, J.F., and Mitchell, G.F. (1968). Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice

reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* 128, 801–820.

Miller, L.H., Baruch, D.I., Marsh, K., and Doumbo, O.K. (2002). The pathogenic basis of malaria. *Nature* 415, 673–679.

Miroux, B., and Walker, J.E. (1996). Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* 260, 289–298.

Miura, K., Keister, D.B., Muratova, O.V., Sattabongkot, J., Long, C.A., and Saul, A. (2007). Transmission-blocking activity induced by malaria vaccine candidates Pfs25/Pvs25 is a direct and predictable function of antibody titer. *Malar. J.* 6, 107.

Miura, K., Orcutt, A.C., Muratova, O.V., Miller, L.H., Saul, A., and Long, C.A. (2008). Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine* 26, 193–200.

Miura, K., Takashima, E., Deng, B., Tullo, G., Diouf, A., Moretz, S.E., Nikolaeva, D., Diakite, M., Fairhurst, R.M., Fay, M.P., et al. (2013). Functional Comparison of *Plasmodium falciparum* Transmission-Blocking Vaccine Candidates by the Standard Membrane-Feeding Assay. *Infect. Immun.* 81, 4377–4382.

Mlambo, G., Maciel, J., and Kumar, N. (2008). Murine Model for Assessment of *Plasmodium falciparum* Transmission-Blocking Vaccine Using Transgenic *Plasmodium berghei* Parasites Expressing the Target Antigen Pfs25. *Infect. Immun.* 76, 2018–2024.

Mullard, A. (2014). 2013 FDA drug approvals. *Nat. Rev. Drug Discov.* 13, 85–89.

Nakane, S., Iwamoto, A., and Matsuda, Z. (2015). The V4 and V5 Variable Loops of HIV-1 Envelope Glycoprotein Are Tolerant to Insertion of Green Fluorescent Protein and Are Useful Targets for Labeling. *J. Biol. Chem.* 290, 15279–15291.

Nelson, A.L., Ries, J., Bagnoli, F., Dahlberg, S., Fälker, S., Rounioja, S., Tschöp, J., Morfeldt, E., Ferlenghi, I., Hilleringmann, M., et al. (2007a). RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol. Microbiol.* 66, 329–340.

Nelson, A.L., Ries, J., Bagnoli, F., Dahlberg, S., Fälker, S., Rounioja, S., Tschöp, J., Morfeldt, E., Ferlenghi, I., Hilleringmann, M., et al. (2007b). RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol. Microbiol.* 66, 329–340.

Ng, S., Jafari, M.R., and Derda, R. (2012). Bacteriophages and Viruses as a Support for Organic Synthesis and Combinatorial Chemistry. *ACS Chem. Biol.* 7, 123–138.

Nguyen, P.Q., Botyanszki, Z., Tay, P.K.R., and Joshi, N.S. (2014). Programmable biofilm-based materials from engineered curli nanofibres. *Nat. Commun.* 5, 4945.

- Nguyen, V.D., Hatahet, F., Salo, K.E., Enlund, E., Zhang, C., and Ruddock, L.W. (2011). Pre-expression of a sulfhydryl oxidase significantly increases the yields of eukaryotic disulfide bond containing proteins expressed in the cytoplasm of *E.coli*. *Microb. Cell Factories* 10, 1.
- Nikolaeva, D., Draper, S.J., and Biswas, S. (2015). Toward the development of effective transmission-blocking vaccines for malaria. *Expert Rev. Vaccines* 14, 653–680.
- Nishimura, Y., Shishido, T., Ishii, J., Tanaka, T., Ogino, C., and Kondo, A. (2012). Protein-encapsulated bio-nanocapsules production with ER membrane localization sequences. *J. Biotechnol.* 157, 124–129.
- Noren, C., Anthony-Cahill, S., Griffith, M., and Schultz, P. (1989). A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* 244, 182–188.
- Ogun, S.A., Dumon-Seignovert, L., Marchand, J.-B., Holder, A.A., and Hill, F. (2008). The Oligomerization Domain of C4-Binding Protein (C4bp) Acts as an Adjuvant, and the Fusion Protein Comprised of the 19-Kilodalton Merozoite Surface Protein 1 Fused with the Murine C4bp Domain Protects Mice against Malaria. *Infect. Immun.* 76, 3817–3823.
- Olsthoorn, R.C., and van Duin, J. (1996). Evolutionary reconstruction of a hairpin deleted from the genome of an RNA virus. *Proc. Natl. Acad. Sci. U. S. A.* 93, 12256–12261.
- Ord, R.L., Caldeira, J.C., Rodriguez, M., Noe, A., Chackerian, B., Peabody, D.S., Gutierrez, G., and Lobo, C.A. (2014). A malaria vaccine candidate based on an epitope of the *Plasmodium falciparum* RH5 protein. *Malar. J.* 13, 326.
- Padgett, H.S. (2013). Generation of antigenic virus-like particles through protein-protein linkages.
- Panicali, D., Davis, S.W., Weinberg, R.L., and Paoletti, E. (1983). Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. U. S. A.* 80, 5364–5368.
- Parkins, D.A., and Lashmar, U.T. (2000). The formulation of biopharmaceutical products. *Pharm. Sci. Technol. Today* 3, 129–137.
- Patel, K.G., and Swartz, J.R. (2011). Surface Functionalization of Virus-Like Particles by Direct Conjugation Using Azide–Alkyne Click Chemistry. *Bioconjug. Chem.* 22, 376–387.
- Patel, J.M., Vartabedian, V.F., Kim, M.-C., He, S., Kang, S.-M., and Selvaraj, P. (2015). Influenza virus-like particles engineered by protein transfer with tumor-

associated antigens induces protective antitumor immunity. *Biotechnol. Bioeng.* **112**, 1102–1110.

Pattenden, L.K., Middelberg, A.P.J., Niebert, M., and Lipin, D.I. (2005). Towards the preparative and large-scale precision manufacture of virus-like particles. *Trends Biotechnol.* **23**, 523–529.

Peabody, D.S. (1997). Subunit fusion confers tolerance to peptide insertions in a virus coat protein. *Arch. Biochem. Biophys.* **347**, 85–92.

Pejawar-Gaddy, S., Rajawat, Y., Hilioti, Z., Xue, J., Gaddy, D.F., Finn, O.J., Viscidi, R.P., and Bossis, I. (2010). Generation of a tumor vaccine candidate based on conjugation of a MUC1 peptide to polyionic papillomavirus virus-like particles. *Cancer Immunol. Immunother.* **59**, 1685–1696.

Peyret, H., Gehin, A., Thuenemann, E.C., Blond, D., El Turabi, A., Beales, L., Clarke, D., Gilbert, R.J.C., Fry, E.E., Stuart, D.I., et al. (2015). Tandem fusion of hepatitis B core antigen allows assembly of virus-like particles in bacteria and plants with enhanced capacity to accommodate foreign proteins. *PloS One* **10**, e0120751.

Pickett, G.G., and Peabody, D.S. (1993). Encapsulation of heterologous RNAs by bacteriophage MS2 coat protein. *Nucleic Acids Res.* **21**, 4621–4626.

Pitoiset, F., Vazquez, T., and Bellier, B. (2015). Enveloped virus-like particle platforms: Vaccines of the future? *Expert Rev. Vaccines* **1–3**.

Platts, L., and Falconer, R.J. (2015). Controlling protein stability: Mechanisms revealed using formulations of arginine, glycine and guanidinium HCl with three globular proteins. *Int. J. Pharm.* **486**, 131–135.

Plevka, P., Kazaks, A., Voronkova, T., Kotelovica, S., Dishlers, A., Liljas, L., and Tars, K. (2009). The Structure of Bacteriophage ϕ Cb5 Reveals a Role of the RNA Genome and Metal Ions in Particle Stability and Assembly. *J. Mol. Biol.* **391**, 635–647.

Plotkin, S. (2014). History of vaccination. *Proc. Natl. Acad. Sci.* **111**, 12283–12287.

Plotkin, S.A. (2005). Vaccines: past, present and future. *Nat. Med.* **10**, S5–S11.

Pokorski, J.K., Breitenkamp, K., Liepold, L.O., Qazi, S., and Finn, M.G. (2011). Functional Virus-Based Polymer–Protein Nanoparticles by Atom Transfer Radical Polymerization. *J. Am. Chem. Soc.* **133**, 9242–9245.

Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerley, R.P., and Saul, F. (1973). Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2,8-Å resolution. *Proc. Natl. Acad. Sci. U. S. A.* **70**, 3305–3310.

- Polka, J.K., Hays, S.G., and Silver, P.A. (2016). Building Spatial Synthetic Biology with Compartments, Scaffolds, and Communities. *Cold Spring Harb. Perspect. Biol.* 8, a024018.
- Porter, R.R. (1959). The hydrolysis of rabbit γ -globulin and antibodies with crystalline papain. *Biochem. J.* 73, 119–127.
- Pröschel, M., Detsch, R., Boccaccini, A.R., and Sonnewald, U. (2015). Engineering of Metabolic Pathways by Artificial Enzyme Channels. *Front. Bioeng. Biotechnol.* 3.
- Pumpens, P., and Grens, E. (1999). Hepatitis B core particles as a universal display model: a structure-function basis for development. *FEBS Lett.* 442, 1–6.
- Pumpens, P., and Grens, E. (2001). HBV core particles as a carrier for B cell/T cell epitopes. *Intervirology* 44, 98–114.
- Pyrz, W.D., and Buttrey, D.J. (2008). Particle Size Determination Using TEM: A Discussion of Image Acquisition and Analysis for the Novice Microscopist. *Langmuir* 24, 11350–11360.
- Qian, F., Wu, Y., Muratova, O., Zhou, H., Dobrescu, G., Duggan, P., Lynn, L., Song, G., Zhang, Y., Reiter, K., et al. (2007). Conjugating recombinant proteins to *Pseudomonas aeruginosa* ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. *Vaccine* 25, 3923–3933.
- Qian, F., Li, M., Chen, Y., Jiang, L., and Xu, H. (2016). *Salmonella* flagellin acted as an effective fusion partner for expression of *Plasmodium falciparum* surface protein 25 in *Escherichia coli*. *Hum. Vaccines Immunother.* 1–3.
- Quan, J., and Tian, J. (2009). Circular Polymerase Extension Cloning of Complex Gene Libraries and Pathways. *PLoS ONE* 4, e6441.
- Ramirez, D.C., Mejiba, S.E.G., and Mason, R.P. (2005). Copper-catalyzed Protein Oxidation and Its Modulation by Carbon Dioxide: ENHANCEMENT OF PROTEIN RADICALS IN CELLS. *J. Biol. Chem.* 280, 27402–27411.
- Reddy, S.T., Rehor, A., Schmoekel, H.G., Hubbell, J.A., and Swartz, M.A. (2006). In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. *J. Control. Release Off. J. Control. Release Soc.* 112, 26–34.
- Ren, Y., Wong, S.M., and Lim, L.-Y. (2007). Folic Acid-Conjugated Protein Cages of a Plant Virus: A Novel Delivery Platform for Doxorubicin. *Bioconjug. Chem.* 18, 836–843.
- Rial, D.V., and Ceccarelli, E.A. (2002). Removal of DnaK contamination during fusion protein purifications. *Protein Expr. Purif.* 25, 503–507.

- Richert, L.E., Servid, A.E., Harmsen, A.L., Rynda-Applé, A., Han, S., Wiley, J.A., Douglas, T., and Harmsen, A.G. (2012). A virus-like particle vaccine platform elicits heightened and hastened local lung mucosal antibody production after a single dose. *Vaccine* 30, 3653–3665.
- Rohman, M., and Harrison-Lavoie, K.J. (2000). Separation of Copurifying GroEL from Glutathione-S-Transferase Fusion Proteins. *Protein Expr. Purif.* 20, 45–47.
- Roldão, A., Mellado, M.C.M., Castilho, L.R., Carrondo, M.J.T., and Alves, P.M. (2010). Virus-like particles in vaccine development. *Expert Rev. Vaccines* 9, 1149–1176.
- Rosano, G.L., and Ceccarelli, E.A. (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front. Microbiol.* 5.
- Rosendahl Huber, S., van Beek, J., de Jonge, J., Luytjes, W., and van Baarle, D. (2014). T Cell Responses to Viral Infections - Opportunities for Peptide Vaccination. *Front. Immunol.* 5.
- Rossi, E.A., Goldenberg, D.M., and Chang, C.-H. (2012). Complex and defined biostructures with the dock-and-lock method. *Trends Pharmacol. Sci.* 33, 474–481.
- RTS,S Clinical Trials Partnership (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet* 386, 31–45.
- Ruedl, C., Storni, T., Lechner, F., Bächli, T., and Bachmann, M.F. (2002). Cross-presentation of virus-like particles by skin-derived CD8(-) dendritic cells: a dispensable role for TAP. *Eur. J. Immunol.* 32, 818–825.
- Ruedl, C., Schwarz, K., Jegerlehner, A., Storni, T., Manolova, V., and Bachmann, M.F. (2005). Virus-like particles as carriers for T-cell epitopes: limited inhibition of T-cell priming by carrier-specific antibodies. *J. Virol.* 79, 717–724.
- Rumnieks, J., and Tars, K. (2011). Crystal structure of the read-through domain from bacteriophage Q β A1 protein. *Protein Sci.* 20, 1707–1712.
- Rutgers, T., Gordon, D., Gathoye, A.M., Hollingdale, M., Hockmeyer, W., Rosenberg, M., and Wilde, M.D. (1988). Hepatitis B Surface Antigen as Carrier Matrix for the Repetitive Epitope of the Circumsporozoite Protein of *Plasmodium Falciparum*. *Nat. Biotechnol.* 6, 1065–1070.
- Salis, H.M., Mirsky, E.A., and Voigt, C.A. (2009). Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27, 946–950.
- Samandoulgou, I., Fliss, I., and Jean, J. (2015). Zeta Potential and Aggregation of Virus-Like Particle of Human Norovirus and Feline Calicivirus Under Different Physicochemical Conditions. *Food Environ. Virol.* 7, 249–260.

- Sapsford, K.E., Algar, W.R., Berti, L., Gemmill, K.B., Casey, B.J., Oh, E., Stewart, M.H., and Medintz, I.L. (2013). Functionalizing Nanoparticles with Biological Molecules: Developing Chemistries that Facilitate Nanotechnology. *Chem. Rev.* **113**, 1904–2074.
- Sasmal, P.K., Carregal-Romero, S., Han, A.A., Streu, C.N., Lin, Z., Namikawa, K., Elliott, S.L., Köster, R.W., Parak, W.J., and Meggers, E. (2012). Catalytic azide reduction in biological environments. *Chembiochem* **13**, 1116–1120.
- Sato, Y., and Sato, H. (1999). Development of acellular pertussis vaccines. *Biol. J. Int. Assoc. Biol. Stand.* **27**, 61–69.
- Saxena, A.K., Singh, K., Su, H.-P., Klein, M.M., Stowers, A.W., Saul, A.J., Long, C.A., and Garboczi, D.N. (2006). The essential mosquito-stage P25 and P28 proteins from *Plasmodium* form tile-like triangular prisms. *Nat. Struct. Mol. Biol.* **13**, 90–91.
- Schiller, J.T., and Lowy, D.R. (2015). Raising Expectations For Subunit Vaccine. *J. Infect. Dis.* **211**, 1373–1375.
- Schlessinger, A., and Rost, B. (2005). Protein flexibility and rigidity predicted from sequence. *Proteins* **61**, 115–126.
- Schmitz, N., Dietmeier, K., Bauer, M., Maudrich, M., Utzinger, S., Muntwiler, S., Saudan, P., and Bachmann, M.F. (2009). Displaying Fel d1 on virus-like particles prevents reactogenicity despite greatly enhanced immunogenicity: a novel therapy for cat allergy. *J. Exp. Med.* **206**, 1941–1955.
- Schmitz, N., Beerli, R.R., Bauer, M., Jegerlehner, A., Dietmeier, K., Maudrich, M., Pumpens, P., Saudan, P., and Bachmann, M.F. (2012). Universal vaccine against influenza virus: Linking TLR signaling to anti-viral protection. *Eur. J. Immunol.* **42**, 863–869.
- Schneerson, R., and Robbins, J.B. (1975). Induction of serum *Haemophilus influenzae* type B capsular antibodies in adult volunteers fed cross-reacting *Escherichia coli* 075:K100:H5. *N. Engl. J. Med.* **292**, 1093–1096.
- Schoene, C., Fierer, J.O., Bennett, S.P., and Howarth, M. (2014). SpyTag/SpyCatcher cyclization confers resilience to boiling on a mesophilic enzyme. *Angew. Chem. Int. Ed Engl.* **53**, 6101–6104.
- Schoene, C., Bennett, S.P., and Howarth, M. (2016). SpyRing interrogation: analyzing how enzyme resilience can be achieved with phytase and distinct cyclization chemistries. *Sci. Rep.* **6**, 21151.
- Schwartz, L., Brown, G.V., Genton, B., and Moorthy, V.S. (2012). A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar. J.* **11**, 11.

- Schwarz-Linek, U., and Banfield, M.J. (2014). Yet more intramolecular cross-links in Gram-positive surface proteins. *Proc. Natl. Acad. Sci.* *111*, 1229–1230.
- Sette, A., and Rappuoli, R. (2010). Reverse vaccinology: developing vaccines in the era of genomics. *Immunity* *33*, 530–541.
- Sevier, C.S., and Kaiser, C.A. (2002). Formation and transfer of disulphide bonds in living cells. *Nat. Rev. Mol. Cell Biol.* *3*, 836–847.
- Shapiro, E.D., Berg, A.T., Austrian, R., Schroeder, D., Parcells, V., Margolis, A., Adair, R.K., and Clemens, J.D. (1991). The Protective Efficacy of Polyvalent Pneumococcal Polysaccharide Vaccine. *N. Engl. J. Med.* *325*, 1453–1460.
- Shapiro-Shapin, C.G. (2010). Pearl Kendrick, Grace Eldering, and the Pertussis Vaccine. *Emerg. Infect. Dis.* *16*, 1273–1278.
- Shekhawat, S.S., and Ghosh, I. (2011). Split-protein systems: beyond binary protein–protein interactions. *Curr. Opin. Chem. Biol.* *15*, 789–797.
- Shen, B.-Q., Xu, K., Liu, L., Raab, H., Bhakta, S., Kenrick, M., Parsons-Reponte, K.L., Tien, J., Yu, S.-F., Mai, E., et al. (2012). Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol.* *30*, 184–189.
- Shishovs, M., Rumnieks, J., Diebolder, C., Jaudzems, K., Andreas, L.B., Stanek, J., Kazaks, A., Kotelovica, S., Akopjana, I., Pintacuda, G., et al. (2016). Structure of AP205 Coat Protein Reveals Circular Permutation in ssRNA Bacteriophages. *J. Mol. Biol.* *428*, 4267–4279.
- Skountzou, I., Quan, F.-S., Gangadhara, S., Ye, L., Vzorov, A., Selvaraj, P., Jacob, J., Compans, R.W., and Kang, S.-M. (2007). Incorporation of Glycosylphosphatidylinositol-Anchored Granulocyte-Macrophage Colony-Stimulating Factor or CD40 Ligand Enhances Immunogenicity of Chimeric Simian Immunodeficiency Virus-Like Particles. *J. Virol.* *81*, 1083–1094.
- Sletten, E.M., and Bertozzi, C.R. (2009). Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of Functionality. *Angew. Chem. Int. Ed.* *48*, 6974–6998.
- Smith, K.A. (2011). Edward Jenner and the small pox vaccine. *Front. Immunol.* *2*, 21.
- Smith, D.M., Simon, J.K., and Baker Jr, J.R. (2013a). Applications of nanotechnology for immunology. *Nat. Rev. Immunol.* *13*, 592–605.
- Smith, J.D., Rowe, J.A., Higgins, M.K., and Lavstsen, T. (2013b). Malaria's deadly grip: cytoadhesion of *Plasmodium falciparum* -infected erythrocytes: *Plasmodium falciparum* cytoadhesion and *var* genes. *Cell. Microbiol.* *15*, 1976–1983.

- Smith, M.T., Hawes, A.K., and Bundy, B.C. (2013c). Reengineering viruses and virus-like particles through chemical functionalization strategies. *Curr. Opin. Biotechnol.* 24, 620–626.
- Sominskaya, I., Bichko, V., Pushko, P., Dreimane, A., Snikere, D., and Pumpens, P. (1992). Tetrapeptide QDPR is a minimal immunodominant epitope within the preS2 domain of hepatitis B virus. *Immunol. Lett.* 33, 169–172.
- Song, W.S., and Yoon, S. (2014). Crystal structure of FliC flagellin from *Pseudomonas aeruginosa* and its implication in TLR5 binding and formation of the flagellar filament. *Biochem. Biophys. Res. Commun.* 444, 109–115.
- Sørensen, H.P., and Mortensen, K.K. (2005). Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb. Cell Factories* 4, 1.
- Spicer, C.D., and Davis, B.G. (2014). Selective chemical protein modification. *Nat. Commun.* 5, 4740.
- Spohn, G., Jennings, G.T., Martina, B.E., Keller, I., Beck, M., Pumpens, P., Osterhaus, A.D., and Bachmann, M.F. (2010). A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. *Virol. J.* 7, 146.
- Steinmetz, N.F., Bize, A., Findlay, K.C., Lomonossoff, G.P., Manchester, M., Evans, D.J., and Prangishvili, D. (2008). Site-specific and Spatially Controlled Addressability of a New Viral Nanobuilding Block: *Sulfolobus islandicus* Rod-shaped Virus 2. *Adv. Funct. Mater.* 18, 3478–3486.
- Stephanopoulos, N., and Francis, M.B. (2011). Choosing an effective protein bioconjugation strategy. *Nat. Chem. Biol.* 7, 876–884.
- Stockley, P.G., and Mastico, R.A. (2000). Use of fusions to viral coat proteins as antigenic carriers for vaccine development. *Methods Enzymol.* 326, 551–569.
- Stockley, P.G., Twarock, R., Bakker, S.E., Barker, A.M., Borodavka, A., Dykeman, E., Ford, R.J., Pearson, A.R., Phillips, S.E.V., Ranson, N.A., et al. (2013). Packaging signals in single-stranded RNA viruses: nature’s alternative to a purely electrostatic assembly mechanism. *J. Biol. Phys.* 39, 277–287.
- Stockley, P.G., White, S.J., Dykeman, E., Manfield, I., Rolfsson, O., Patel, N., Bingham, R., Barker, A., Wroblewski, E., Chandler-Bostock, R., et al. (2016). Bacteriophage MS2 genomic RNA encodes an assembly instruction manual for its capsid. *Bacteriophage* 6, e1157666.
- Stonehouse, N.J., and Stockley, P.G. (1993). Effects of amino acid substitution on the thermal stability of MS2 capsids lacking genomic RNA. *FEBS Lett.* 334, 355–359.

- Strable, E., and Finn, M.G. (2009a). Chemical Modification of Viruses and Virus-Like Particles. In *Viruses and Nanotechnology*, M. Manchester, and N.F. Steinmetz, eds. (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 1–21.
- Strable, E., and Finn, M.G. (2009b). Chemical Modification of Viruses and Virus-Like Particles. In *Viruses and Nanotechnology*, M. Manchester, and N.F. Steinmetz, eds. (Springer Berlin Heidelberg), pp. 1–21.
- Strable, E., Prasuhn, D.E., Udit, A.K., Brown, S., Link, A.J., Ngo, J.T., Lander, G., Quispe, J., Potter, C.S., Carragher, B., et al. (2008). Unnatural Amino Acid Incorporation into Virus-Like Particles. *Bioconjug. Chem.* *19*, 866–875.
- Su, X. (1996). Reduced extension temperatures required for PCR amplification of extremely A+T-rich DNA. *Nucleic Acids Res.* *24*, 1574–1575.
- Sun, F., Zhang, W.-B., Mahdavi, A., Arnold, F.H., and Tirrell, D.A. (2014). Synthesis of bioactive protein hydrogels by genetically encoded SpyTag-SpyCatcher chemistry. *Proc. Natl. Acad. Sci.* *111*, 11269–11274.
- Sun, Q., Chen, Q., Blackstock, D., and Chen, W. (2015). Post-Translational Modification of Bionanoparticles as a Modular Platform for Biosensor Assembly. *ACS Nano* 150804145916003.
- Symersky, J., Patti, J.M., Carson, M., House-Pompeo, K., Teale, M., Moore, D., Jin, L., Schneider, A., DeLucas, L.J., Höök, M., et al. (1997). Structure of the collagen-binding domain from a *Staphylococcus aureus* adhesin. *Nat. Struct. Biol.* *4*, 833–838.
- Syn, C.K., and Swarup, S. (2000). A scalable protocol for the isolation of large-sized genomic DNA within an hour from several bacteria. *Anal. Biochem.* *278*, 86–90.
- Tang, S., Xuan, B., Ye, X., Huang, Z., and Qian, Z. (2016). A Modular Vaccine Development Platform Based on Sortase-Mediated Site-Specific Tagging of Antigens onto Virus-Like Particles. *Sci. Rep.* *6*, 25741.
- Tars, K., Fridborg, K., Bundule, M., and Liljas, L. (2000). The Three-Dimensional Structure of Bacteriophage PP7 from *Pseudomonas aeruginosa* at 3.7-Å Resolution. *Virology* *272*, 331–337.
- Tateda, K., Matsumoto, T., Miyazaki, S., and Yamaguchi, K. (1996). Lipopolysaccharide-induced lethality and cytokine production in aged mice. *Infect. Immun.* *64*, 769–774.
- Tatur, J., Hagedoorn, P.-L., Overeijnder, M.L., and Hagen, W.R. (2006). A highly thermostable ferritin from the hyperthermophilic archaeal anaerobe *Pyrococcus furiosus*. *Extremophiles* *10*, 139–148.

- Thomas, R.C., Bath, M.F., Stover, C.M., Lambert, D.G., and Thompson, J.P. (2014). Exploring LPS-induced sepsis in rats and mice as a model to study potential protective effects of the nociceptin/orphanin FQ system. *Peptides* 61, 56–60.
- Thorner, J.W., Emr, S., and Abelson, J. (2000). Applications of chimeric genes and hybrid proteins. Part A, Part A, (San Diego, Calif.: Academic Press).
- Thrane, S., Janitzek, C.M., Agerbæk, M.Ø., Ditlev, S.B., Resende, M., Nielsen, M.A., Theander, T.G., Salanti, A., and Sander, A.F. (2015). A Novel Virus-Like Particle Based Vaccine Platform Displaying the Placental Malaria Antigen VAR2CSA. *PLOS ONE* 10, e0143071.
- Thrane, S., Janitzek, C.M., Matondo, S., Resende, M., Gustavsson, T., de Jongh, W.A., Clemmensen, S., Roeffen, W., van de Vegte-Bolmer, M., van Gemert, G.J., et al. (2016). Bacterial superglue enables easy development of efficient virus-like particle based vaccines. *J. Nanobiotechnology* 14.
- Tissot, A.C., Renhofa, R., Schmitz, N., Cielens, I., Meijerink, E., Ose, V., Jennings, G.T., Saudan, P., Pumpens, P., and Bachmann, M.F. (2010). Versatile virus-like particle carrier for epitope based vaccines. *PloS One* 5, e9809.
- Tolbert, T.J., and Wong, C.-H. (2002). New Methods for Proteomic Research: Preparation of Proteins with N-Terminal Cysteines for Labeling and Conjugation This research was supported by the NIH (R37 GM44154). *Angew. Chem. Int. Ed.* 41, 2171.
- Tong, G.J., Hsiao, S.C., Carrico, Z.M., and Francis, M.B. (2009). Viral Capsid DNA Aptamer Conjugates as Multivalent Cell-Targeting Vehicles. *J. Am. Chem. Soc.* 131, 11174–11178.
- Trevino, S.R., Scholtz, J.M., and Pace, C.N. (2007). Amino Acid Contribution to Protein Solubility: Asp, Glu, and Ser Contribute more Favorably than the other Hydrophilic Amino Acids in RNase Sa. *J. Mol. Biol.* 366, 449–460.
- Tsao, M.-L., Tian, F., and Schultz, P.G. (2005). Selective Staudinger Modification of Proteins Containing p-Azidophenylalanine. *ChemBioChem* 6, 2147–2149.
- Tsukiji, S., and Nagamune, T. (2009). Sortase-Mediated Ligation: A Gift from Gram-Positive Bacteria to Protein Engineering. *ChemBioChem* 10, 787–798.
- Turner, L., Lavstsen, T., Mmbando, B.P., Wang, C.W., Magistrado, P.A., Vestergaard, L.S., Ishengoma, D.S., Minja, D.T.R., Lusingu, J.P., and Theander, T.G. (2015). IgG Antibodies to Endothelial Protein C Receptor-Binding Cysteine-Rich Interdomain Region Domains of *Plasmodium falciparum* Erythrocyte Membrane Protein 1 Are Acquired Early in Life in Individuals Exposed to Malaria. *Infect. Immun.* 83, 3096–3103.

- Udit, A.K., Brown, S., Baksh, M.M., and Finn, M.G. (2008). Immobilization of bacteriophage Q β on metal-derivatized surfaces via polyvalent display of hexahistidine tags. *J. Inorg. Biochem.* *102*, 2142–2146.
- Valegård, K., Liljas, L., Fridborg, K., and Unge, T. (1990). The three-dimensional structure of the bacterial virus MS2. *Nature* *345*, 36–41.
- Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G., and Hall, B.D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* *298*, 347–350.
- Valenzuela, P., Coit, D., Medina-Selby, M.A., Kuo, C.H., Van Nest, G., Lyn Burke, R., Bull, P., Urdea, M.S., and Graves, P.V. (1985). Antigen Engineering in Yeast: Synthesis and Assembly of Hybrid Hepatitis B Surface Antigen-Herpes Simplex 1 gD Particles. *Bio/Technology* *3*, 323–326.
- Veggiani, G., Nakamura, T., Brenner, M.D., Gayet, R.V., Yan, J., Robinson, C.V., and Howarth, M. (2016). Programmable polyproteins built using twin peptide superglues. *Proc. Natl. Acad. Sci. U. S. A.* *113*, 1202–1207.
- Vengadesan, K., and Narayana, S.V.L. (2011). Structural biology of gram-positive bacterial adhesins. *Protein Sci.* *20*, 759–772.
- Venter, P.A., Dirksen, A., Thomas, D., Manchester, M., Dawson, P.E., and Schneemann, A. (2011). Multivalent display of proteins on viral nanoparticles using molecular recognition and chemical ligation strategies. *Biomacromolecules* *12*, 2293–2301.
- Vermeulen, A.N., Ponnudurai, T., Beckers, P.J., Verhave, J.P., Smits, M.A., and Meuwissen, J.H. (1985). Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. *J. Exp. Med.* *162*, 1460–1476.
- Versteegen, R.M., Rossin, R., ten Hoeve, W., Janssen, H.M., and Robillard, M.S. (2013). Click to Release: Instantaneous Doxorubicin Elimination upon Tetrazine Ligation. *Angew. Chem. Int. Ed.* *52*, 14112–14116.
- Vogel, M., Diez, M., Eisfeld, J., and Nassal, M. (2005). In vitro assembly of mosaic hepatitis B virus capsid-like particles (CLPs): rescue into CLPs of assembly-deficient core protein fusions and FRET-suited CLPs. *FEBS Lett.* *579*, 5211–5216.
- Wagner, S., Klepsch, M.M., Schlegel, S., Appel, A., Draheim, R., Tarry, M., Högbohm, M., van Wijk, K.J., Slotboom, D.J., Persson, J.O., et al. (2008). Tuning *Escherichia coli* for membrane protein overexpression. *Proc. Natl. Acad. Sci.* *105*, 14371–14376.
- Walker, A., Skamel, C., and Nassal, M. (2011). SplitCore: An exceptionally versatile viral nanoparticle for native whole protein display regardless of 3D structure. *Sci. Rep.* *1*, 5.

- Wals, K., and Ovaa, H. (2014). Unnatural amino acid incorporation in *E. coli*: current and future applications in the design of therapeutic proteins. *Front. Chem.* 2.
- Wang, A., Winblade Nairn, N., Johnson, R.S., Tirrell, D.A., and Grabstein, K. (2008a). Processing of N-terminal unnatural amino acids in recombinant human interferon-beta in *Escherichia coli*. *Chembiochem Eur. J. Chem. Biol.* 9, 324–330.
- Wang, B.-Z., Quan, F.-S., Kang, S.-M., Bozja, J., Skountzou, I., and Compans, R.W. (2008b). Incorporation of membrane-anchored flagellin into influenza virus-like particles enhances the breadth of immune responses. *J. Virol.* 82, 11813–11823.
- Wang, J., Wang, Y., Wang, X., Zhang, D., Wu, S., and Zhang, G. (2016). Enhanced thermal stability of lichenase from *Bacillus subtilis* 168 by SpyTag/SpyCatcher-mediated spontaneous cyclization. *Biotechnol. Biofuels* 9.
- Wang, L., Zhang, Z., Brock, A., and Schultz, P.G. (2003). Addition of the keto functional group to the genetic code of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 56–61.
- Wang, L., Xie, J., and Schultz, P.G. (2006). Expanding the genetic code. *Annu. Rev. Biophys. Biomol. Struct.* 35, 225–249.
- Wang, Q., Lin, T., Johnson, J.E., and Finn, M.G. (2002a). Natural supramolecular building blocks. Cysteine-added mutants of cowpea mosaic virus. *Chem. Biol.* 9, 813–819.
- Wang, Q., Kaltgrad, E., Lin, T., Johnson, J.E., and Finn, M.G. (2002b). Natural supramolecular building blocks. Wild-type cowpea mosaic virus. *Chem. Biol.* 9, 805–811.
- Wang, Y., Vander, H.P., and Xi, L. (2011). Methods of using improved polymerases.
- Weber, C.A., Mehta, P.J., Ardito, M., Moise, L., Martin, B., and De Groot, A.S. (2009). T cell epitope: Friend or Foe? Immunogenicity of biologics in context☆. *Adv. Drug Deliv. Rev.* 61, 965–976.
- Weber, P.C., Ohlendorf, D.H., Wendoloski, J.J., and Salemme, F.R. (1989). Structural origins of high-affinity biotin binding to streptavidin. *Science* 243, 85–88.
- Weiner, A.M., and Weber, K. (1971). Natural read-through at the UGA termination signal of Q-beta coat protein cistron. *Nature. New Biol.* 234, 206–209.
- Weinstock, M.T., Jacobsen, M.T., and Kay, M.S. (2014). Synthesis and folding of a mirror-image enzyme reveals ambidextrous chaperone activity. *Proc. Natl. Acad. Sci.* 111, 11679–11684.
- Wen, A.M., and Steinmetz, N.F. (2016). Design of virus-based nanomaterials for medicine, biotechnology, and energy. *Chem Soc Rev.*

- Werner, S., Marillonnet, S., Hause, G., Klimyuk, V., and Gleba, Y. (2006). Immunoabsorbent nanoparticles based on a tobamovirus displaying protein A. *Proc. Natl. Acad. Sci. U. S. A.* *103*, 17678–17683.
- White, S.J., Johnson, S., Szymonik, M., Wardingley, R.A., Pye, D., Giles Davies, A., Wälti, C., and Stockley, P.G. (2012). Directed surface attachment of nanomaterials via coiled-coil-driven self-assembly. *Nanotechnology* *23*, 495304.
- Wikman, M., Steffen, A.-C., Gunneriusson, E., Tolmachev, V., Adams, G.P., Carlsson, J., and Stahl, S. (2004). Selection and characterization of HER2/neu-binding affibody ligands. *Protein Eng. Des. Sel.* *17*, 455–462.
- Win, S.J., Ward, V.K., Dunbar, P.R., Young, S.L., and Baird, M.A. (2011). Cross-presentation of epitopes on virus-like particles via the MHC I receptor recycling pathway. *Immunol. Cell Biol.* *89*, 681–688.
- Woolfson, D.N. (2005). The design of coiled-coil structures and assemblies. *Adv. Protein Chem.* *70*, 79–112.
- van den Worm, S.H.E., Koning, R.I., Warmenhoven, H.J., Koerten, H.K., and van Duin, J. (2006). Cryo electron microscopy reconstructions of the Leviviridae unveil the densest icosahedral RNA packing possible. *J. Mol. Biol.* *363*, 858–865.
- Wright, K.E., Hjerrild, K.A., Bartlett, J., Douglas, A.D., Jin, J., Brown, R.E., Illingworth, J.J., Ashfield, R., Clemmensen, S.B., de Jongh, W.A., et al. (2014). Structure of malaria invasion protein RH5 with erythrocyte basigin and blocking antibodies. *Nature* *515*, 427–430.
- Wu, Y., Przysiecki, C., Flanagan, E., Bello-Irizarry, S.N., Ionescu, R., Muratova, O., Dobrescu, G., Lambert, L., Keister, D., Rippeon, Y., et al. (2006). Sustained high-titer antibody responses induced by conjugating a malarial vaccine candidate to outer-membrane protein complex. *Proc. Natl. Acad. Sci.* *103*, 18243–18248.
- Wu, Y., Ellis, R.D., Shaffer, D., Fontes, E., Malkin, E.M., Mahanty, S., Fay, M.P., Narum, D., Rausch, K., Miles, A.P., et al. (2008). Phase 1 Trial of Malaria Transmission Blocking Vaccine Candidates Pfs25 and Pvs25 Formulated with Montanide ISA 51. *PLoS ONE* *3*, e2636.
- Wynne, S.A., Crowther, R.A., and Leslie, A.G. (1999). The crystal structure of the human hepatitis B virus capsid. *Mol. Cell* *3*, 771–780.
- Xie, J., Wang, J., Chen, H., Shen, W., Sinko, P.J., Dong, H., Zhao, R., Lu, Y., Zhu, Y., and Jia, L. (2015). Multivalent Conjugation of Antibody to Dendrimers for the Enhanced Capture and Regulation on Colon Cancer Cells. *Sci. Rep.* *5*, 9445.
- Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* *33*, 103–119.

Yazdanbakhsh, M. (2002). Allergy, Parasites, and the Hygiene Hypothesis. *Science* 296, 490–494.

Yoon, S. -i., Kurnasov, O., Natarajan, V., Hong, M., Gudkov, A.V., Osterman, A.L., and Wilson, I.A. (2012). Structural Basis of TLR5-Flagellin Recognition and Signaling. *Science* 335, 859–864.

Zabel, F., Kündig, T.M., and Bachmann, M.F. (2013). Virus-induced humoral immunity: on how B cell responses are initiated. *Curr. Opin. Virol.* 3, 357–362.

Zakeri, B., and Howarth, M. (2010). Spontaneous Intermolecular Amide Bond Formation between Side Chains for Irreversible Peptide Targeting. *J. Am. Chem. Soc.* 132, 4526–4527.

Zakeri, B., Fierer, J.O., Celik, E., Chittock, E.C., Schwarz-Linek, U., Moy, V.T., and Howarth, M. (2012). Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proc. Natl. Acad. Sci.* 109, E690–E697.

Zhang, W.-B., Sun, F., Tirrell, D.A., and Arnold, F.H. (2013). Controlling Macromolecular Topology with Genetically Encoded SpyTag–SpyCatcher Chemistry. *J. Am. Chem. Soc.* 135, 13988–13997.

Zhao, C., Ao, Z., and Yao, X. (2016). Current Advances in Virus-Like Particles as a Vaccination Approach against HIV Infection. *Vaccines* 4.

Zimmerman, T., Petit Frère, C., Satzger, M., Raba, M., Weisbach, M., Döhn, K., Popp, A., and Donzeau, M. (2006). Simultaneous metal chelate affinity purification and endotoxin clearance of recombinant antibody fragments. *J. Immunol. Methods* 314, 67–73.

Zinkernagel, R.M., and Doherty, P.C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248, 701–702.

Zinkernagel, R.M., and Doherty, P.C. (1979). MHC-Restricted Cytotoxic T Cells: Studies on the Biological Role of Polymorphic Major Transplantation Antigens Determining T-Cell Restriction-Specificity, Function, and Responsiveness. In *Advances in Immunology*, (Elsevier), pp. 51–177.

Zou, Y., Sonderegger, I., Lipowsky, G., Jennings, G.T., Schmitz, N., Landi, M., Kopf, M., and Bachmann, M.F. (2010). Combined vaccination against IL-5 and eotaxin blocks eosinophilia in mice. *Vaccine* 28, 3192–3200.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 31, 3406–3415.

KDB 

Appendix

Plasmid sequences are supplied in electronic GenBank format and can also be obtained from Dr Mark Howarth (mark.howarth@bioch.ox.ac.uk) or Karl Brune (brune.karl@gmail.com). pKB_215 and pKB_337 are also supplied in text format due to the relevance of the DNA sequence to this work.

Amino acid sequences of proteins employed in this work:

pKB_215 (pGEM-SpyCatcher-AP205cp3) (Brune et al., 2016)

MGSSHHHHHHGSGDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQV
KDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDHIGGSGSA
NKPMQPITSTANKIVWSDPTLSTTFSASLLRQRVKVGIAELNNVSGQYVSVYKRPA
PKPEGCADACVIMPNNENQSIRTVISGSAENLATLKAETHKRNVDTLFASGNAGL
GFLDPTAAIVSSDTTA*

pKB_221 [(pET15b-SpyTag-CIDR(HB3var03))] (Lau et al., 2015)

MGAHIVMVDAYKPTKGGSGGSHHHHHHENLYFQGGSGDAKNTEINVINSGDKEG
YIFEKLSEFCTNENNENGKNYEQWKCYDNNKNNKCKMEINIANSKLKNKVTSFDF
EFFDFWVRKLLIDTIKWETELTYCINNTDVTDCNKCNCVCFCDFKWKQKEDEWNTN
IMKLFTNKHDIKPKYYLNINDLFDSFFFQVIYKFNEGEAKWNLKENLKKQIASSKAN
NGTKDSEAAIKVLFNHIKEIATICKDNNTNEGC*

pKB_225 [(pET15b-SpyTag-CIDR(IT4var07))] (Lau et al., 2015)

MGHHHHHHHENLYFQGGSEPAPDVKTTEINVIVSGDEQGIITKKLQDFCMNPNNEN
GTNNQIWKCYKDEKENKCKVETKSGNSTYKEKITSFDEFFDFWVRKLLIDTIKWET
ELTYCINNTTNADCNNECNKNCVCFCDFKWKQKEKEWKNIMDLFTNKHDIKPKYYL
NINDLFNSFFFQVIYKFNEGEAKWNLKENLKKKIESSKKNKGTKDSEAAIKVLFDFHL
KETATICKDNNTNEAC*

pKB_228 [pET15b-SpyTag-CIDR(HB3var03)] (Brune et al., 2016)

MGHHHHHHHENLYFQGGSAHIVMVDAYKPTKGGSGDAKNTEINVINSGDKEGYIFE
KLSEFCTNENNENGKNYEQWKCYDNNKNNKCKMEINIANSKLKNKVTSFDEFF
DFWVRKLLIDTIKWETELTYCINNTDVTDCNKCNCVCFCDFKWKQKEDEWNTNIMK
LFTNKHDIKPKYYLNINDLFDSFFFQVIYKFNEGEAKWNLKENLKKQIASSKANNG
TKDSEAAIKVLFNHIKEIATICKDNNTNEGC*

pKB_229 [pET15b-SpyTag-CIDR(IT4var07)] (Brune et al., 2016)

MGHHHHHHHENLYFQGGSAHIVMVDAYKPTKGGSEPAPDVKTTEINVIVSGDEQGII
TKKLQDFCMNPNNENGTNNQIWKCYYKDEKENKCKVETKSGNSTYKEKITSFDEF
FDFWVRKLLIDTIKWETELTYCINNTTNADCNNECNKNCVCFDKWVKQKEKEWKNI
MDLFTNKHDIPKKYYLNINDLFNSFFFQVIYKFNEGEAKWNKLKENLKKKIESSKKN
KGTKDSEAAIKVLFDHLKETATICKDNNTNEAC*

pKB_263 (pET28a-SpyTag-MBP) (Zakeri et al., 2012)

MGSSHHHHHHSSGLVPRGSHMGAHIVMVDAYKPTKSGSGESGKIEEGKLVWINGD
KGYNGLAEVGGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGY
AQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKT
WEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGGYDIKDVGV
NAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKV
NYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDK
PLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASG
RQTVDEALKDAQTNSSS*

pKB_264 (pET28a-SpyTag DA-MBP) (Zakeri et al., 2012)

MGSSHHHHHHSSGLVPRGSHMGAHIVMVAAYKPTKSGSGESGKIEEGKLVWINGD
KGYNGLAEVGGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGY
AQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKT
WEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGGYDIKDVGV
NAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKV
NYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDK
PLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASG
RQTVDEALKDAQTNSSS*

pKB_269 (pDEST14-ΔN1SpyCatcher)

MSYYHHHHHHHDYDIPTTENLYFQGGSDSATHIKFSKRDEDGKELAGATMELRDSS
GKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQQQVTVNGKAT
KGDAHI*

pKB_281 (pGEM-SpyCatcherDuo-AP205cp3)

MGSSHHHHHHGSGDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQV
KDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQQQVTVNGKATKGDAHIGSGDSAT
HIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPD
GYEVATAITFTVNEQQQVTVNGKATKGDAHIGSGGSGGSGANKPMQPITSTANKIV
WSDPTRLSTTFSASLLRQRVKVGIAELNNVSGQYVSVYKRPAPKPEGCADACVIMP
NENQSIRTVISGSAENLATLKAETHKRNVDTLFAASNAGLGLDPTAAIVSSDTT
A*

pKB_299 [(pET15b-SnoopTag-CIDR(IT4var07))]

MGHHHHHHHENLYFQGGSKLGDIEFIKVNKGGSEPAPDVKTTEINVIVSGDEQGIITK
KLQDFCMNPNNENGTNNQIWKCYYKDEKENKCKVETKSGNSTYKEKITSFDEFFD
FWVRKLLIDTIKWETELTYCINNTTNADCNNECNKNCVCFDKWVKQKEKEWKNIMD
LFTNKHDIPKKYYLNINDLFNSFFFQVIYKFNEGEAKWNKLKENLKKKIESSKKNKGT
KDSEAAIKVLFDHLKETATICKDNNTNEAC*

pKB_311 (pENTR4 LPTOS-tPA-Pfs25-SpyTag-CTag, cloned by Andrew S. Ishizuka) (Brune et al., 2016)

AKVTVDTVCKRGFLIQMSGHLECKCENDLVLVNEETCEEKVLKCDEKTVNKPCGDF
SKCIKIDGNPVSACKCNLGYDMVNNVCIPNECKQVTCGNGKCILDTSNPVKTGVC
SCNIGKVPNVQDQNKCSKDGETKCSLKCLKEQETCKAVDGIYKCDCKDGFIIQES
SICTAHIVMVDAYKPTKEPEA*

pKB_389 (pET21-SpyCatcher-IMX-SnoopCatcher)

MSYYHHHHHHGSGDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQV
KDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDHIGGSGSK
KQGDADVCGEVAYIQSVSDCHVPTAELRTLLEIRKLFLEIQKLKVELQGLSKEGSPA
NLKALEAQKQKEQRQAEEELANAKKLKEQLEKGSHEMKPLRGAVFSLQKQHPDYP
DIYGAIQNGTYQNVRTGEDGKLTFKNLSDGKYRLFENSEPAGYKPVQNKPIVAFQI
VNGEVRDVTIVPQDIPATYEFTNGKHYITNEPIPPK*

pKB_290 (pET28-SnooTag-AffiHER2, cloned by Can Buldun)

MGSSHHHHHHSSGLVPRGSHMGKLGDIKVNKKGSGESGSGASMTGGQQMGR
DPGVDNKFNKEMRNAYWEIALLPNLNNQQKRAFIRSLYDDPSQSANLLAEAKKLN
DAQAPKGLE*

pKB_096 (pET28-SpyTag-AffiHER2, cloned by Can Buldun)

MGSSHHHHHHSSGLVPRGSHMGASMTGGQQMGRDPGVDNKFNKEMRNAYWEI
ALLPNLNNQQKRAFIRSLYDDPSQSANLLAEAKKLNDAQAPKGLEGSSEGSGAHIV
MVDAYKPTK*

pKB_215 (pGEM-SpyCatcher-AP205cp3) (Brune et al., 2016)

tgcagctgtggtgtcatggtcggatgccagggtgccgacgcgcacatctcgactgcacgggtgcaccaatgc
 ttctggcgtcaggcagccatcggaagctgtggtatggctgtgcaggctgtaaatcactgcataattcgtgtcgct
 caaggcgcactcccgttctggataatgtttttgcgccgacatcataacggttctggcaaatttctgaaatgag
 ctgttgacaattaatcatcgaactagttaactagtagcgaagttcacgtaaaaagggtatgccaggaattcga
 gctcgcccctggcttatcgaaattaatacactcactatagggagaccggaattcgagctcgccgggggac
 ctctagattaacccaacgcgtaggagtcaggccatgggcagcagccatcatcatcatcacggcagcgg
 cgatagtgtacctatattaaattctcaaaacgtgatgaggacggcaaagagttagctggtgcaactatggagt
 tgcgtgattcatctggtaaaactattagtagatggatttcagatggacaagtgaagatttctacctgtatccagg
 aaaatatacatttgcgaaaccgcagcaccagacgggtatgaggtagcaactgctattacctttacagttaatga
 gcaaggtcaggttactgtaaacggcaaagcaactaaaggtagcgtcatattggcgggtcaggggtccgcgaa
 caaacgatgcagccgatcacctctaccggaacaaaatcggttggctgacccgacccgtctgtctaccac
 cttctctgcgtctctgctgcgtcagcgtgttaaagttggatcgcggaactgaacaacgtttctggctcagtacgtt
 ctgtttacaacgtccggcgccgaaaccggaaggttgcgcggacgcgtgcgttatcatgccgaacgaaaac
 cagtctatccgtaccgttatctctggttctgcggaaaacctggcgaccctgaaagcgggaatgggaaaccac
 aaacgtaacgttgacaccctgttcgcgtctggtaacgcgggtctgggttccctggacccgaccgcggcgtatc
 gtttcttctgacaccaccgcgtaagcttgattctatagtgacctaataatcgatgtgtatgatacataaggttatg
 tattaattgtagccgcgttctaacgacaatatgtacaagcctaattgtgtagcatctggcttactgaagcagacc
 tatcatctctctcgtaaactgccgtcagagtcgggttgggtggacgaaccttctgagtttctggtaacgccgttcc
 gcaccccggaatggtcaccgaaccaatcagcagggtcacgctagccagatcctctacgccggacgcacat
 cgtggccggcatcaccggcgccacaggtgcggttgcgtggcgccctatatcgccgacatcaccgatggggaa
 gatcgggctcgccacttcgggctcatgagcgttgttcggcggtgggtatggtggcaggccccgtggccggg
 ggactgttgggcgccatctccttgcacgaccattccttgcggcgccgggtgctcaacggcctcaacctactact
 gggctgcttccctaatgcaggagtcgcataaggagagcgctcgatatggtgcactctcagtacaattctgctctg
 atgccgcatagttaagccaactccgctatcgctacgtgactgggtcatggctgcgccccgacacccgccaac
 acccgctgacgcgccctgacgggcttgcgtcctccggcatccgcttacagacaagctgtgaccgtctccgg
 gagctgcatgtgtcagaggtttaccgctcatcaccgaaacgcgcgagggcagcttgaagacgaaagggcct
 cgtgatacgccctattttataggttaattgcatgataataatggtttcttagacgtcaggtggcacttttcggggaaa
 tgtgcgcggaacccctatttttttttaataacattcaaataatgtatccgctcatgagacaataaccctgata
 aatgcttcaataattgaaaaaggaagagtagagattcaacatttccgtgtcgccctattccctttttgcggc
 attttgccttctgttttgcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacg
 agtgggttacatcgaactggatctcaacagcggtaagatccttgagagtttgcggccgaagaacgtttccaa
 tgatgagcacttttaagttctgctatgtggcgcggtattatcccgattgacgccggggaagagcaactcggtc
 gccgatacactattctcagaatgacttgggtgagtactaccagtcacagaaaagcatcttacggatggcatg
 acagtaagagaattatgcagtgtgccataaccatgagtgataaacactgcggccaacttacttctgacaacga
 tcggaggaccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactcgcttgcgttggga
 accggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacg
 ttgcgcaaactattaactggcgaactacttacttagcttcccggaacaattaatagactggatggaggcgga
 taaagttgcaggaccacttctgcgtcggcccttccggctgggttattgctgataaatctggagccgggtga
 gcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgatcgtagttatctacacg
 acggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcat

Appendix

tggttaactgtcagaccaagtttactcatatatacttttagattgatttaaaacttcatttttaatttaaaaggatctaggt
gaagatccttttgataatctcatgaccaaatacccttaacgtgagtttcgttccactgagcgtcagaccccgta
gaaaagatcaaaggatcttcttgagatcctttttctgcgcgtaatctgctgcttgcaaacaaaaaaccaccg
ctaccagcgggtggtttgtttgccggatcaagagctaccaactcttttccgaaggtaactggcttcagcagagc
gcagataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgccta
catacctcgctctgctaatacctgttaccagtggtgctgctgccagtggcgataagtcgtgtcttaccgggttgact
caagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagctt
ggagcgaacgacctacaccgaactgagatacctacagcgcgagcattgagaaagcgccacgcttcccgaa
gggagaaaggcggacaggtatccggttaagcggcagggctcggaacaggagagcgcacgagggagcttcc
agggggaaacgcctggtatctttatagtcctgtcgggttcgccacctctgacttgagcgtcgattttgtgatgct
cgtcaggggggcgaggcctatggaaaaacgccagcaacgcggccttttacggttcttggccttttgctggc
ctttgctcacatgttcttctgctgtatcccctgattctgtggataaccgtattaccgcctttgagtgagctgatac
cgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaata
cgcaaaccgcctctccccgcgcgttggccgattcattaa

pKB_337 (pET21-5UTR-SpyCatcher-CP3(native)-3UTR)

ggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgctttttgcacaacatgggggatcat
 gtaactcgcttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgat
 gcctgcagcaatggcaacaacgttgcgcaaaactattaactggcgaactacttactctagcttcccggcaacaa
 ttaatagactggatggaggcggataaaagttgcaggaccacttctgcgctcggcccttcgggctggctggtttatt
 gctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccc
 tcccgatcgtagtattctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgag
 ataggtgcctcactgattaagcattggtaactgtcagaccaagttactcatatatacttttagattgatttaaaactt
 cattttaatttaaaaggatctaggtgaagatccttttgataatctcatgacaaaaatcccttaacgtgagtttcgt
 tccactgagcgtcagacccgtagaaaagatcaaaggatcttcttgagatcctttttctgcgcgtaatctgctg
 cttgcaaaaaaaaaccaccgctaccagcgggtggtttgtttgcccggatcaagagctaccaactcttttccga
 aggtaactggcttcagcagagcgcagataccaaatactgtccttctagttagccgtagttaggccaccacttc
 aagaactctgtagcaccgcctacatacctcgctctgctaactcgttaccagtggtgctgcccagtgccgataa
 gtcgtgtcttaccgggttgactcaagacgatagttaccggataaggcgcagcggctcgggctgaacggggg
 gttcgtgcacacagcccagcttgagcgaacgacctacaccgaactgagatacctacagcgtgagctatga
 gaaagcggcacgcttcccgaaggagaaaggcggacaggtatccggttaagcggcagggctcgaacagg
 agagcgcacgaggagcttccaggggaaacgcctggtatctttatagtcctgtcgggttcgccacctctga
 cttgagcgtcgtttttgtatgctcgtcaggggggaggagcctatggaaaaacgccagcaacgcggcctttt
 acggttcctggccttttgcgtggccttttgcctcacatgttcttctgcgttatccctgattctgtggataaccgtatta
 ccgcttttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcagtgactgagcggagga
 agcgggaagagcgcctgatgcggtattttctccttacgcactgtgcggtatttcacaccgcataatggtgact
 ctgagtaaatctgctctgatgccgcatagttaagccagtatacactccgctatcgctacgtgactgggtcatgg
 ctgcgccccgacacccgccaacacccgctgacgcgcccgtgacgggctgtctgctcccggcatccgcttac
 agacaagctgtgaccgtctccgggagctgcatgtgtcagagggtttcaccgctatcaccgaaacgcgcgagg
 cagctgcggtaaagctcatcagcgtggtcgtgaagcgattcacagatgtctgcctgttcatccgcgtccagctc
 gttgagtttctccagaagcgttaatgtctggcttctgataaagcgggccatgtaaggcggtttttctgtttggt
 cactgatgcctccgtgtaaggggatttctgttcatgggggtaatgataccgatgaaacgagagaggatgctc
 acgatacgggttactgatgatgaacatgcccggttactggaacggttgtagggtaaacactggcggtatgga
 tgcggcgggaccagagaaaaatcactcagggtaatgccagcgttcgtaatacagatgtaggtgttccac
 agggtagccagcagcatcctgcgatgcagatccggaacataatggtgcagggcgctgacttccgcgtttcca
 gactttacgaaacacggaacccaagaccattcatgttgttgcaggtcgcagacgtttgcagcagcagtc
 gcttcacgttcgctcgcgtatcgggtgattcattctgctaaccagtaaggcaaccccgccagcctagccgggtc
 ctcaacgacaggagcacgatcatgcgacccgctggggccgcatgccggcgataatggcctgcttctcgcc
 gaaacgtttggtggcgggaccagtgacgaaggcttgagcgaagggcgcaagattccgaataccgcaagc
 gacaggccgatcatcgtcgcgtccagcgaaagcggctcctcgccgaaaatgaccagagcgtgcccggc
 acctgtcctacgagttgatgataaagaagacagtcataagtgcggcgacgatagtcacccccgcgccac
 cggaggagctgactgggtgaaggctctcaagggtcaggtcgagatcccgggtgcctaatagtgagcta
 acttacattaattgcgttgcgtcactgcccgttccagtcgggaaacctgtcgtgccagctgcattaatgaat
 cggccaacgcgcggggagaggcggtttgcgtattggcgccagggtggtttttctttaccagtgagacgg
 gcaacagctgattgcccttaccgcctggccctgagagagttgcagcaagcgggtccacgctggtttgccccca
 gcaggcgaaaatcctgtttgatggtggttaacggcgggatataacatgagctgtcttcgggtatcgctgatccca
 ctaccgagatatccgcaccaacgcgcagcccgactcggttaatggcgcgcatgcccagcgcgccatctg
 atcgttggcaaccagcatcgagtggaacgatgccctcattcagcatttgcattggtttgtgaaaaccggaca
 tggcactccagtcgccttcccgttccgctatcggtgaatttgattgcgagtgagatattatgccagccagcca
 gacgcagacgcgccgagacagaacttaatgggcccgcctaacagcgcgatttgcgtggtgacccaatgcgac
 cagatgctccacgcccagtcgcgtaccgttctcatgggagaaaataactgttgatgggtgtctggtcagag
 acatcaagaaataacgccggaacattagtgaggcagcttccacagcaatggcatcctggtcatccagcgg

Appendix

atagttaatgatcagcccactgacgcgttgcgcgagaagattgtgcaccgccgcttacaggcttcgacgccg
cttcgttctaccatcgacaccaccacgctggcaccacagttgatcggcgcgagatttaacgccgcgacaattt
gcgacggcgcggtgcagggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttg
ttgtgccacgcggttggaatgtaattcagctccgccatcgccgcttcacttttcccgcggtttcgagaaac
gtggctggcctggttcaccacgcgggaaacggctctgataagagacaccggcatactctgcacatcgtataa
cgttactggtttcacattcaccaccctgaattgactctctccggcgctatcatgccataccgcgaaagggtttg
cgccattcgatgggtgtccgggatctcgacgctctcccttatgcgactcctgcattaggaagcagcccagtagta
ggttgaggccgttgagcaccgccgcccgaaggaatgggtgatgcaaggagatggcgcccaacagtccccc
ggccacggggcctgccaccatacccacgccgaacaagcgctcatgagcccgaagtggcgagcccgatc
ttccccatcggtgatgtcggcgatataggcgccagcaaccgcacctgtggcgccggtgatgccggccacga
tgcgtccggcgtagaggatcgagatctcgatcccgcgaaattaatacgactcactataggggaattgtgagcg
gataacaattcctctagtttaagatcagtcgagctacttgacaattttctgcgcacccatcccgggtggcgccc
aaagtgagggaaaatcacataaattaaccaacgcgtaggagtcaggcctgtagaataattttgttaacttta
taaggagatataccatgtcgtactaccatcaccatcaccatcacggcagcggcgatagtgtacccatattaa
attctcaaaacgtgatgaggacggcaaagagttagctggtgcaactatggagttgcgtgattcatctggtaaaa
ctattagtagatggatttcagatggacaagtgaaagatttctacctgtatccaggaaaatacatttgcgaaac
cgcagcaccagacggttatgaggtagcaactgctattacctttacagttaatgagcaaggctcaggttactgtaa
acggcaaagcaactaaagggtgacgctcatattggcagtggtggttcagggggtccgggtgcaaataagccaa
tgcaaccgatcacatctacagcaaataaaattgtgtggagtgatccaactcgtttatcaactacattttcagcaa
gtctgttacgccaacgtgttaaagttggtattgccgaactgaataatgtttcaggtcaatatgtatctgtttataagc
gtcctgcacctaaaccggaagggtgtgcagatgcctgtgtcattatgccgaatgaaaccaatccattcgcac
agtgatttcagggtcagccgaaaacttggtaccttaaaagcagaatgggaaactcaciaacgtaacgttga
cacactcttcgcgagcggcaacgcgggtttgggttccttgaccctactgcggctatcgatcgtctgatactac
tgcttaagtgggtattactgtgcctaaaagtcaaaataaacgacaaataagacgcagttcttccgttaattacaa
gaatatcgttaaagcttgaatgatgcaatgctaaacgtaattaataagatccggctgctaacaagcccga
aggaagctgagttggctgctgccaccgctgagcaataactagcataaccccttggggcctctaaacgggtctt
gaggggtttttgctgaaaggaggaactatatccggttgccgaatgggacgcgccctgtagcggcgcatata
gcgcggcggggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccctagcggccgctcctttcg
ctttcttcccttcttctcgcacgctcgcggctttcccgctcaagctctaaatcgggggctccctttagggttc
cgatttagtgctttacggcacctcgaccccaaaaaacttgattaggggtgatgggtcacgtagtgggcatcgcc
ctgatagacgggttttcgccctttgacgttgagtgccacgttcttaatagtggactctgttccaaactggaacaa
cactcaaccctatctcggctctattcttttgattataagggattttgccgatttcggcctattgggtaaaaaatgagct
gatttaacaaaaattaacgcgaattttaacaaaatattaacgtttacaatttcaggtggcacttttcggggaaatg
tgcgcggaacccctatttgttattttctaaatacattcaaatatgtatccgctcatgagacaataacccctgataa
tgcttcaataatattgaaaaaggaagagtatgagtattcaacattccggtgtcgccctattccctttttgcggcat
tttccttctgttttctcaccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgag
tgggttacatcgaactggatctcaacagcggtaagatccttgagagtttcgccccgaagaacgttttcfaatg
atgagcacttttaaagttctgctatgtggcgcggtattatcccgattgacgccgggcaagagcaactcggtcg
ccgcatacactattctcagaatgacttggtgagtactaccagtcacagaaaagcatcttacggatggcatga
cagtaagagaattatgcagtgctgccataaccatgagtgataacactgc