ENGINEERING SINGLE-DOMAIN ANTIBODIES FOR BIOACTIVE PAPER

APPLICATIONS

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

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In partial fulfillment of requirements

for the degree of

Master of Science

March, 2011

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ABSTRACT

ENGINEERING SINGLE-DOMAIN ANTIBODIES FOR BIOACTIVE PAPER APPLICATIONS

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University of Guelph, 2011

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This thesis is an investigation of the fusion of a carbohydrate-binding module (CBM) to a high-affinity single-domain antibody (sdAb) which binds to bacteriophage M13. This fusion protein was applied to paper filters for detection of M13 phage. A CBM-sdAb fusion protein with nanomolar affinity for immobilized M13 phage was successfully expressed in *E. coli*. The CBM-sdAb fusion protein was effective in binding M13 phage in water to a cellulose filter paper. However, the sdAb and the CBM-sdAb fusion protein were ineffective in enhancing filter capture of M13 phage particles from water or air, respectively. This research demonstrates that a CBM-sdAb fusion protein will bind simultaneously to cellulose and a model virus. In the future, CBM-sdAb fusion proteins may be useful in the development of ‘bioactive’ paper products capable of detecting and/or inactivating pathogens which are a threat to public health.
Acknowledgements

I am grateful to my supervisors, Dr. Roger MacKenzie (NRC-IBS) and Dr. Chris Hall (University of Guelph), for their guidance, patience, intelligence, and sense of humor.

I thank Dr. Jamshid Tanha for his constructive feedback. I thank Yonghong Guan, Henk van Faassen, Tomoko Hirama, Dr. Mehdi Arbabi-Ghahroudi, Rebecca To, Yan Luo, Shenghua Li, Shannon Ryan, and the other members of the NRC-IBS Antibody Engineering group for technical assistance, discussions, and good times!

I am grateful to Dr. Dick Kerekes and Dr. Jingliang Mao (University of British Columbia) for supplying the NBHK filter. I am grateful to Ahlstrom (Helsinki, Finland) for supplying filter media.

I thank Dr. Warren Finlay and Dr. Biljana Grgic (University of Alberta) for providing the filter-testing apparatus.

I am grateful to Sonia Leclerc, Tom Devecseri, and Al Ruppel (NRC-IBS) for technical assistance.

I thank Linda Veldhuis and the Hall lab (University of Guelph) for sharing their expertise.

I am grateful to SENTINEL for their innovation and support of bioactive paper research.

I thank my parents for the motivation to complete my thesis!
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Figure 49. M13 phage plaques in a lawn of E. coli observed after overnight incubation at 37°C. Square A contained CBM2a-VH fusion protein and M13 phage. Square B contained M13 phage. Photo: Tom Devecseri (NRC-IBS, Ottawa, Ontario, Canada)... 104

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Figure 51. Effect of a detection layer, which was and was not treated with CBM2a-VH, on capture of M13 phage. A detection layer, which was either non-treated (A) or treated with CBM2a-VH (B), was used in combination with an NBHK pulp filter or an N95 3M 8210 Particulate Respirator during filtration of an aerosol containing 2.4 x 10^8 pfu M13 phage. As positive controls, i.e. no NBHK or N95 filter was used, a non-treated detection layer (A) and a detection layer treated with CBM2a-VH (B) were each exposed to 2.4 x 10^8 pfu aerosolized M13 phage. As a negative control, i.e. no NBHK or N95 filter was used, a non-treated detection layer (A) was exposed to aerosolized sterile water which did not contain M13 phage. The legend is arranged in the same order as the data on the figure (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com)........................................................................ 110
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBM</td>
<td>cellulose-binding module</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
</tr>
<tr>
<td>c-Myc</td>
<td>peptide used for protein purification via immunoprecipitation</td>
</tr>
<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fab</td>
<td>fragment antigen binding</td>
</tr>
<tr>
<td>Fc</td>
<td>constant portion of an antibody</td>
</tr>
<tr>
<td>FFR</td>
<td>filtering face piece respirator</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast-performance liquid chromatography</td>
</tr>
<tr>
<td>HCAb</td>
<td>heavy-chain antibody</td>
</tr>
<tr>
<td>HEPA</td>
<td>high-efficiency particulate air filter</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>6-histidine peptide tag used for protein purification</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal-ion affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
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</table>
\( k_a \) association rate constant
\( k_d \) dissociation rate constant
\( K_{dp} \) equilibrium dissociation constant
\( k_{Da} \) kilodaltons
\( lacZ \) gene coding for beta-galactosidase
\( LB \) Luria-Bertani
\( LRV \) log reduction value
\( M9 \) minimal nutrient growth medium
\( mAU \) milli-absorbance units
\( MPBS \) milk dissolved in phosphate-buffered saline
\( NBHK \) northern bleached hardwood kraft pulp
\( NIOSH \) National Institute for Occupational Safety and Health
\( OD \) optical density
\( OmpA \) outer membrane protein A
\( PAGE \) polyacrylamide gel electrophoresis
\( PEG \) polyethylene glycol
\( PBS \) phosphate-buffered saline
\( PBST \) PBS with 0.05% v/v Tween 20
\( PCR \) polymerase chain reaction
\( pfu \) plaque-forming units
\( PVDF \) polyvinylidene difluoride
\( RT \) reverse transcriptase polymerase chain reaction
\( RU \) resonance units
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain fragment variable</td>
</tr>
<tr>
<td>sdAb</td>
<td>single-domain antibody</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOC</td>
<td>super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>TB</td>
<td>terrific broth media</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TES</td>
<td>buffer containing tris-HCl, EDTA and sucrose</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5' – tetramethylbenzidine</td>
</tr>
<tr>
<td>TSP</td>
<td>transferable solid-phase</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>$V_{H}$H</td>
<td>variable heavy chain antibody fragment</td>
</tr>
<tr>
<td>YT</td>
<td>yeast extract tryptone media</td>
</tr>
</tbody>
</table>
1 INTRODUCTION, OBJECTIVE, AND LITERATURE REVIEW

1.1 Introduction

Recent outbreaks of severe acute respiratory syndrome (SARS), bovine spongiform encephalopathy (BSE), avian H5N1 influenza, and swine H1N1 influenza have had negative impacts on our health and our economy. The 9/11 terrorist attack has prompted concern about biological warfare agents such as *Francisella tularensis*, *Bacillus anthrasis*, *Yersinis pestis*, botulism toxin, smallpox, and hemorrhagic fever viruses (Russell et al. 2003). In response to the threat of pandemics and bioterrorism, the Canadian Network for the Development and Use of Bioactive Paper (SENTINEL) aims to invent paper products which detect and inactivate pathogenic organisms. Paper is a cheap and abundant medium which may be modified with bioactive material to develop protective filters, masks, and clothing, as well as diagnostic test strips, antimicrobial food packaging, and decontaminating towels and bandages (Russell et al. 2003, Aikio et al. 2006).

There is need for improvement upon current protective face masks. The N95 and N99 filtering face piece respirators (FFRs) are widely used for protection from aerosolized infectious particles (Wiwanitkit 2006). According to NIOSH (National Institute for Occupational Safety and Health) certification, N95 and N99 FFRs must filter at least 95% and 99%, respectively, of aerosolized, uncharged sodium chloride particles of 300 nm in diameter at a flow rate of 85 L/min (Balazy et al. 2006b).

NIOSH testing utilizes particles of 300 nm in diameter because 300 nm is the most penetrating particle size (MPPS) for mechanical filters (Eninger et al. 2008). To enhance filtering efficiency, N99 and N95 FFRs are manufactured with charged fibers.
Due to their ‘electret’ properties, N99 and N95 FFRs have been demonstrated to have a MPPS of less than 100 nm (Eninger et al. 2008, Balazy et al. 2006a). A MPPS of less than 100 nm is significant because infectious virions such as those of SARS and influenza A are typically 80-100 nm in diameter (Eninger et al. 2008). N95 FFRs have a pore size of 300-500 nm in diameter (Wiwanitkit 2006). Thus, a SARS or influenza A virion which is ca. 100 nm in diameter has the potential to penetrate an N95 FFR (Wiwanitkit 2006).

Eninger et al. (2008) challenged N95 and N99 respirators with an aerosol of MS2 bacteriophage containing single virions and virion aggregates ranging from 20-90 nm in diameter at a flow rate of 85 L/min. Peak MS2 bacteriophage penetrations were 4.3% and 4.6% for the two N99 FFRs tested and 4.3% for the N95 FFR (Eninger et al. 2008, Balazy et al. 2006a). At these penetration levels, there is potential for viral infection of the FFR user.

Balazy et al. (2006b, 2006a), demonstrated that a NIOSH-certified N95 FFR had a penetration in excess of 5% when challenged with aerosolized MS2 bacteriophage at a flow rate of 85 L/minute. Common surgical masks, which are not NIOSH certified, offer the user even less protection from viral infection. Balazy et al. (2006a) found that surgical masks had MS2 virion penetration as high as 84.5%.

It is not practical to reduce the pore size of the N95 or N99 FFR to improve virion capture because of the associated increased breathing resistance. Furthermore, existing N95 respirators are not suitable for patients with pulmonary or cardiovascular disease because the user is subjected to hypoxic conditions (Huang and Huang 2007). A method
to improve capture of virus by protective face masks without compromising breathing is required.

Single-domain antibodies (sdAbs) may be used to develop bioactive face masks with improved capture and detection of virus. SdAbs are derived from the antigen-binding domains of conventional antibodies, heavy-chain antibodies, and new receptors of antigens (IgNARs) from cartilaginous fish. Their small size, stability, high antigen affinity and specificity, and ease of expression make sdAbs ideal for bioactive paper applications (Saerens et al. 2008).

SdAbs specific for a viral antigen may be immobilized on cellulose-based air filters to hypothetically develop a protective face mask with improved filtering efficiency of infectious virions. SdAbs may be fused to protein domains known as cellulose-binding modules (CBMs) which allow directional immobilization of sdAbs on cellulose (Saerens et al. 2008). Air filters made from wood pulp have been developed which approach the N95 FFR in terms of filter efficiency of sub-micrometer aerosol particles (Mao et al. 2008). A wood pulp filter modified with antiviral sdAbs could be developed as a compact, portable, low cost protective mask capable of rapid immobilization of low concentrations of virions. Alternatively, an N95 FFR or wood pulp filter may be modified with a removable, cellulose ‘detection layer’ containing antiviral sdAb capable of binding any virions breaching the filter.

1.2 Objective

The objective of this research was to develop a fusion protein consisting of a CBM linked to a high-affinity sdAb which binds to bacteriophage M13. The fusion protein was
applied to a cellulose substrate to create a bioactive ‘detection layer’ capable of detecting M13 phage.

1.3 Bacteriophages

1.3.1 Background

Bacteriophages, or phages, are viruses which use bacteria as a host. Phages do not infect animals; however, animals are capable of producing an antibody response against phages. A phage particle with the capacity to infect bacteria is known as a virion. Each virion is composed of a protein structure which houses genetic material, either DNA or RNA. Bacteriophages are responsible for their self-replication; most other life functions are provided by the host cell (Douglas 1975). Bacteriophages share the same natural habitats as their host bacteria; however, dessication, extreme heat, and antibacterial disinfectants may inactivate phages (Douglas 1975).

Bacteriophages have two main categories of life cycles: lytic and lysogenic. Lytic phages, such as the T4 phage, lyse the host cell to release progeny phage. Cell lysis is achieved by the production of lysozyme, an enzyme which degrades the host cell wall. Lysogenic, or temperate phages, on the other hand, do not lyse the host cell unless exposed to an inducing agent. Examples of inducing agents include ultraviolet irradiation and the antibiotic mitomycin C. Bacteriophage M13 is classified as a symbiotic phage which is non-lytic, although it is not considered to have a lysogenic life cycle (Douglas 1975).
1.3.2 Bacteriophage M13

Bacteriophage ‘Munich 13’ (M13) was discovered in a Munich sewage sample by Peter-Hans Hofschneider in 1963. The M13 phage is a rod-shaped virus which measures approximately 6.5-nm in diameter and 900-nm in length (65Å x 9000Å) (Sambrook and Russell 2001, Russel 1995). Based on its shape, and its single-stranded circular DNA genome, bacteriophage M13 is classified as a filamentous phage of the Inoviridae family (Marvin and Hohn 1969). M13, along with its close relatives the fd and the f1 phages, are strains of Inovirus andreios, a nonlytic species of phage which infects male Escherichia coli cells containing an F plasmid. The F plasmid codes for an F pilus which is involved in conjugation at the E. coli cell surface. The M13, fd, and f1 phages are also known as F-specific filamentous (Ff) viruses because these phages adsorb to the F pili on E. coli cells during infection (Marvin and Hohn 1969).

1.3.2.1 M13 genome and proteins

The wild-type M13 phage genome is composed of 6,407 single-stranded DNA nucleotides arranged in a circle. The related fd and f1 phages share approximately 98% of their DNA with the M13 phage. The M13 genome contains nine open reading frames which code for eleven proteins (Sambrook and Russell 2001, Russel 1995, van Wezenbeek et al. 1980). The eleven proteins manufactured by the M13 phage are used to facilitate replication (pII, pV, pX), to build progeny capsids (pIII, pVI, pVII, pVIII, pIX), and to promote assembly and release of progeny virions (pI, pIV, pXI) (Russel 1995). Each M13 virus particle is composed of five transmembrane proteins. The capsid consists of approximately 2,800 copies of the major coat protein pVIII which overlap like fish scales. At the ‘beginning’ end of the virion, which emerges first from the host cell, there
are five copies of each of the minor coat proteins pVII and pIX. At the other end of the viron, there are five copies of each of the minor coat proteins pIII and pVI (Russel 1995).

1.3.2.2 *M13 infection of a host cell and assembly of M13 progeny*

There are two steps involved in M13 infection of a host *E. coli* cell. First, the minor coat protein pIII at the tip of the M13 virion binds to receptors at the tip of the F pilus on the *E. coli* cell, causing the F pilus to withdraw (Sambrook and Russell 2001, van Wezenbeek et al. 1980, Barbas III et al. 2001, Webster 1991). Next, the viral pIII protein interacts with the TolQ, TolR, and TolA proteins located in the bacterial inner membrane to mediate removal of the viral capsid and penetration of the viral DNA into the bacterial cytoplasm (Russel 1995, Webster 1991).

Once inside the *E. coli* cell, the M13 genome is converted into a double-stranded, circular form which replicates via a rolling-circle mechanism to produce single-stranded DNA for progeny virions. At the same time, the double-stranded parent genome is transcribed and translated to produce viral proteins necessary for replication and progeny assembly. The viral proteins pII, pV, pX mediate viral replication. Each progeny DNA molecule is coated with 1,400 pV dimers to mark it for incorporation into progeny capsids (Russel 1995).

The M13 phage continuously assembles and releases progeny phages through the inner and outer host cell membranes, without lysing the host cell (Russel 1991). Assembly of progeny phage particles requires the phage proteins pI and pIV as well as the host protein thioredoxin. pI is anchored in the inner bacterial membrane and associates with an exposed packaging signal in the single-stranded progeny DNA to initiate phage assembly. pIV forms a gated channel through the outer bacterial membrane
to allow the progeny phages to exit the host cell as they are assembled. pI interacts with pIV to stimulate channel opening. The host protein thioredoxin associates with pI to stabilize the elongation of the progeny phages. Phage assembly begins with the addition of the minor coat proteins pIX and pVII to the progeny DNA. During elongation of the phage capsid, the pV dimers coating the progeny DNA are replaced with pVIII major coat proteins. Phage assembly is terminated with the addition of the minor coat proteins pIII and pVI to the end of the phage capsid. In the first hour after infection, a host E. coli cell can release up to 1,000 M13 progeny which will go on to infect adjacent E. coli cells (Russel 1991). Infected E. coli cells grow and divide at a slower rate than uninfected cells. As a result, infected E. coli cells appear as turbid plaques in a lawn of uninfected, faster-growing cells. Each plaque begins with a single phage infecting a single bacterium. The plaque grows in size as progeny phages infect neighboring cells (Sambrook and Russell 2001).

1.4 Antibodies and antibody fragments

1.4.1 Whole IgG

1.4.1.1 Background

B cells contribute to the humoral immune response by manufacturing antibodies, or immunoglobulins (Joosten et al. 2003). Of the five classes of vertebrate immunoglobulins (IgG, IgM, IgA, IgD, and IgE), immunoglobulin G, i.e. IgG, is the most abundant. A conventional IgG protein is approximately 160-kDa and is composed of a pair of “light”, lower molecular weight polypeptide chains, and a pair of “heavy”, higher molecular weight polypeptide chains. Each heavy chain has one variable domain (VH)
and three constant domains (C_H1, C_H2, and C_H3) while each light chain has one variable domain (V_L) and one constant domain (C_L). The variable domains bind to the antigen while the constant, or Fc portion of the antibody provides a surface to which other target cells and complement proteins may bind (Joosten et al. 2003).

The overall structure of the bivalent IgG molecule consists of three parts: two identical Fabs, i.e. fragments antigen binding, and one Fc region (Padlan 1994). The Fabs are linked to the Fc by a ‘hinge’ which permits the Fabs and the Fc to rotate and bend. Each Fab arm contains a light chain (V_L and C_L), and the first two domains of a heavy chain (V_H and C_H1). The Fab arms of the antibody are linked by disulfide bridges. The Fc region of the IgG protein consists of the C-terminal constant domains of the two heavy chains (C_H2 and C_H3) (Padlan 1994).

1.4.1.2 Variable domains

The variable domains of the heavy and light chains (V_H and V_L) are found at the tips of the Fabs; this is the site of antigen-binding (Padlan 1994). The amino acid sequence of the constant domains of the IgG antibody is well conserved; however, the variable region amino acids may differ up to 30% among different antibodies. This variability is mostly confined to hypervariable regions in the variable domains called complementarity-determining regions (CDRs). The CDRs in each variable domain are separated by four non-hypervariable framework regions. Each V_H and V_L has 3 CDRs, for a total of 6 CDRs at the N-terminus of each Fab. The CDRs form loops which bind antigen. The CDR3 loops of the V_H and V_L have been shown to be the most important for antigen binding, with the CDR3 loop of the V_H demonstrating the greatest variability in amino acid sequence and conformation. The great variety of amino acid sequences and
conformations of the CDR loops allows for a wide variety of antigens to be bound by a vertebrate’s IgG repertoire (Padlan 1994, Muyldermans 2001).

The heavy and light chain variable domains acquire their genetic diversity through recombination of mini-genes (Muyldermans and Lauwereys 1999)(Saerens et al. 2008). Each $V_H$ gene is formed by the joining of three mini-genes: one $V_H$ mini-gene, one $D$ mini-gene, and one $J_H$ mini-gene. Each $V_L$ gene is formed by the joining of two mini-genes: one $V_L$ mini-gene and one $J_L$ mini-gene. There are many copies of each mini-gene in the genome, but only one of each type is selected for V(D)J recombination. During V(D)J recombination, extra non-template nucleotides are added to generate additional diversity. After recombination, each $V_H$ and $V_L$ gene is expressed with a constant region gene. Once expressed, the V genes are prone to somatic mutation which can enhance affinity for antigen (Muyldermans and Lauwereys 1999).

1.4.1.3 IgG fragments

Through proteolysis and genetic engineering, a variety of IgG fragments may be generated which have normal, or enhanced ability to bind antigen (Holliger and Hudson 2005). For example, Fab fragments of ca. 55-kDa may be isolated by digesting whole IgG with the protease papain (Joosten et al. 2003). Using genetic engineering, single-chain fragment variable (scFv) fragments of ca. 28-kDa may be created by connecting a $V_H$ and a $V_L$ domain with a polypeptide linker. When used in therapeutic applications, scFv and Fab fragments have advantages over conventional IgG, including enhanced tissue penetration and freedom from toxic effects mediated by the Fc region. Multiple scFvs or Fabs can be linked together into a multimeric format to enhance avidity, i.e.
increased strength of antibody-antigen interaction due to multiple antibody-antigen interactions (Holliger and Hudson 2005).

Single-domain antibodies (sdAbs), consisting of single V\textsubscript{H} or V\textsubscript{L} domain, may also be isolated from whole IgG (Holliger and Hudson 2005). The small size of sdAbs (ca.15-kDa) allows them to bind to cryptic epitopes which would be otherwise unavailable to the whole IgG molecule. Unfortunately, sdAbs from conventional IgGs are difficult to produce due to their poor solubility and their tendency to aggregate (Holliger and Hudson 2005).

1.4.2 Heavy-chain antibodies

1.4.2.1 Background

In addition to conventional IgG, camelid species (llamas and camels) possess an unusual form of IgG which lacks light chains (Hamers-Casterman et al. 1993). These unique “heavy-chain” antibodies (HCAb) comprise 25-45\% of the total IgG in llama serum (Muyldermans 2001). Camel HCAb have a molecular weight of ca. 90-92-kDa (Muyldermans and Lauwereys 1999) while conventional camel IgG molecules have a molecular weight of ca.160-kDa (Hamers-Casterman et al. 1993). Each HCAb heavy chain consists of a variable domain (V\textsubscript{H}H), a hinge region, and two constant domains: C\textsubscript{H}2 and C\textsubscript{H}3. The C\textsubscript{H}1 domain present in conventional IgG is absent in HCAb (Hamers-Casterman et al. 1993). Similar to the conventional V\textsubscript{H} genes, V\textsubscript{H}H genes are generated through V\textsubscript{H}H-D-J mini-gene recombination (Muyldermans 2001).
1.4.2.2 Advantages of \( V_HH \) fragments

The HCAb variable domain, \( i.e. \ V_HH \), may be isolated to produce a ca.15-kDa antigen-binding fragment (Muyldeermans 2001). \( V_HH \)s have acquired amino acid substitutions in the framework regions which usually interact with the \( V_L \) and \( C_H1 \) domains in conventional \( V_HH \)s. These substitutions enhance the hydrophilicity and solubility of the \( V_HH \). \( V_HH \)s also have excellent thermostability and resilience in the presence of denaturing agents (Muyldeermans 2001). Arbabi-Ghahrouri et al. (1997) found \( V_HH \)s to retain their antigen affinity after a 200-hour incubation at 37°C. \( V_HH \)s heated to 95-100°C have been demonstrated to retain 90% of their antigen-binding capacity (Goldman et al. 2006).

Camelid \( V_HH \)s tend to have longer CDR1 and CDR3 domains compared to conventional \( V_HH \)s, increasing the surface area available to bind antigen (Muyldeermans 2001). The long CDR3 domain enables the \( V_HH \) to access unique epitopes, such as enzyme active sites. Furthermore, the CDRs of camelid \( V_HH \)s may fold into a greater variety of structures compared to conventional \( V_HH \)s. \( V_HH \)s recognize a broad range of antigens with high specificity, including proteins, binding with dissociation constants (\( K_D \)s) in the nanomolar and picomolar ranges (Muyldeermans 2001, Spinelli et al. 2000).

The antigen-binding of \( V_HH \)s can be enhanced through pentamerization, \( i.e. \) by increasing avidity (Zhang et al. 2004). Verotoxin 1 B-subunit (VT1B) is a naturally occurring, monomeric protein which self-assembles into a pentamer, consisting of five VT1B monomers. Five identical \( V_HH \) monomers may be fused to the C-terminus of each of the five VT1B monomers to produce a ‘pentabody’ with increased avidity compared to a single \( V_HH \) monomer. Zhang et al. (2004) developed a \( V_HH \) pentabody with a 3-4
orders of magnitude increase in antigen-binding strength over the monomeric form of the V_{H}H.

1.4.2.3 Expression of recombinant V_{H}H in E. coli

Recombinant V_{H}Hs are easily expressed in E. coli with yields in milligram quantities (Arbabi-Ghahroudi et al. 1997). When E. coli is used as an expression system, the V_{H}Hs are directed to the periplasm, where they fold into their native conformation (Skerra and Plückthun 1988). For example, the signal sequence from the outer membrane protein A (OmpA) may be fused to the N-terminus of the V_{H}H to target it to the periplasm. Once in the periplasm, the OmpA signal sequence is cleaved from the V_{H}H by a signal peptidase, leaving the V_{H}H sequence intact. Expression of the signal peptide-V_{H}H fusion is often controlled by the lac promoter-operator to prevent premature leakage of the V_{H}H into the culture medium. The lac promoter-operator may be induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to the culture medium (Skerra and Plückthun 1988).

Once expression is complete, the V_{H}H may be released from the periplasm via osmotic shock (Skerra and Plückthun 1988). The V_{H}H may be easily purified from the periplasmic fraction using affinity chromatography (Plückthun 1991). For example, a 6-Histidine tag (His_{6}) may be attached to the C-terminus of the V_{H}H, permitting purification by immobilized metal affinity chromatography (IMAC). Alternatively, a C-myc peptide may be linked to the C-terminus of the V_{H}H to allow purification via immunoprecipitation (Plückthun 1991).
1.5 Recombinant single-domain antibody libraries

1.5.1 Background

A recombinant single-domain antibody (sdAb) library is a collection of all sdAb genes that can be cloned from the retrieved B-lymphocytes of an animal and expressed on the surface of bacteriophages (Smith 1985), ribosomes (Mattheakis et al. 1994), or yeast (Boder and Wittrup 1997). Using a procedure called panning, sdAb libraries may be mined for sdAbs which bind to an antigen of interest (Hoogenboom 2005).

SdAb libraries may include V_L, V_H, or V_HH antibody fragments and the libraries may be immune, non-immune, or synthetic (Hoogenboom 2005).

1.5.2 Immune V_HH libraries

Immune libraries are generated by immunizing an animal with the antigen of interest prior to the collection of its B-lymphocytes (Muyldermans 2001). This method of library construction takes advantage of in vivo affinity maturation which biases the B-lymphocyte population toward B-lymphocytes which recognize the target antigen. Arbabi-Ghahroudi et al. (1997) developed an immune camel V_HH library. A camel was immunized with tetanus toxoid and lysozyme and $10^7$ peripheral blood lymphocytes were collected. mRNA was extracted from the lymphocytes and used to make cDNA by RT PCR. The cDNA was PCR-amplified for the V_HH genes. The entire V_HH repertoire of an animal may be cloned using a single set of primers because the V_HH domain is encoded by a single exon, and all V_HH exons belong to the same gene family (Arbabi-Ghahroudi et al. 1997). The V_HH genes were cloned into a phagemid vector and transformed into E. coli, in preparation for phage display of the library. Panning of two libraries, of 500,000
and $10^7$ clones each, yielded $V_H$ which bound to either tetanus toxoid or lysozyme with affinities ranging from $0.2-2 \times 10^8$ $M^{-1}$ (Arbabi-Ghahroudi et al. 1997). When using hyperimmunized animals, usually only small libraries of $10^6-10^7$ clones are required to isolate $V_H$ with nanomolar affinities (Muyldermans 2001).

1.5.3 Non-immune and synthetic $V_H$ and $V_H$ libraries

Non-immune, i.e. naïve libraries, are constructed using the B-lymphocytes of an unimmunized animal (Goldman et al. 2006). Immunization is not possible if the antigen of interest is toxic, transmissible, or non-immunogenic. For example, Goldman et al. (2006) used a non-immune $V_H$ library to identify binders to live vaccinia virus, cholera toxin, ricin, and Staphylococcal enterotoxin B. The disadvantage of non-immune libraries is that there is no in vivo affinity maturation toward the antigens of interest, and thus the sdAbs identified through panning tend to have lower affinity. To overcome this disadvantage, large synthetic non-immune libraries may be generated to yield in vitro affinity maturation. For example, the CDR regions of the $V_H$ genes may be randomized to generate novel $V_H$s. Goldman et al. (2006) converted a $10^6$-clone non-immune $V_H$ library into a $10^9$-clone synthetic $V_H$ library by diversifying the CDR and framework sequences with a error-prone PCR and splicing together the diversified sequences randomly. Yau et al. (2005) devised a method for site-directed in vitro affinity maturation of $V_H$ CDRs. CDR codons, which are natural ‘hotspots’ for mutation in vivo, may be targeted for in vitro mutagenesis to improve $V_H$ affinity (Yau et al. 2005).

$V_H$ and $V_L$ sdAbs are known for their poor solubility and tendency to aggregate; however, naïve human and llama $V_H$ libraries have yielded $V_H$ monomers that do not aggregate and have good solubility and stability (To et al. 2005, Tanha et al. 2002). The
physical properties of $V_H$s may also be improved by ‘camelization’ or ‘llamination’: Camel or llama $V_H$ residues may be incorporated into $V_H$ sequences to improve solubility (Tanha et al. 2001). These $V_H$s may be used as scaffolds for the generation of synthetic libraries by randomizing CDR regions (Tanha et al. 2001).

1.5.4 SdAb library display platforms

1.5.4.1 Background

SdAb libraries may be displayed on ribosomes (Mattheakis et al. 1994), yeast cells (Boder and Wittrup 1997), or filamentous phages (Smith 1985). Once displayed, the sdAb can be selected for specificity to a target antigen through several rounds of panning (Hoogenboom 2005). In ribosome display, a sdAb is expressed in association with its mRNA on the ribosome (Mattheakis et al. 1994). Large, diverse ribosome display libraries may be established without the need for cloning and transformation, and affinity maturation of the sdAb mRNA may be easily achieved in between rounds of panning (Yau et al. 2003). In yeast display, a sdAb may be displayed, selected, and characterized on the surface of a *Saccharomyces cerevisiae* cell (Boder and Wittrup 1997). SdAbs may be attached to *S. cerevisiae* cells via the α-agglutinin yeast adhesion receptor which is present in 10,000-100,000 copies on the surface of each *S. cerevisiae* cell. The main advantage of yeast display is that fluorescence-activated cell sorting (FACS) may be used to affinity select sdAb directly on the yeast cells (Feldhaus and Siegel 2004). Phage display, invented by George Smith (1985) is the most widespread platform used for sdAb library display. Phage-displayed libraries are easy to construct and they may be panned against a wide variety of antigens (Hoogenboom 2005, Hoogenboom et al. 1998). This display platform will be described in more detail.
1.5.4.2 Phage display

1.5.4.2.1 Background

In phage display of a sdAb library, the sdAb phenotype and genotype are associated within the same filamentous phage particle (Paschke 2006). The sdAb molecule is presented on the surface of the phage capsid, while the sdAb genes are located inside the phage capsid. Usually the sdAb is fused to the filamentous phage minor coat protein pIII which binds to the F pilus during infection of E. coli (Bradbury and Marks 2004). In the first phage display system developed for an antibody, a phage vector derived from the bacteriophage fd genome was modified to include a gene for an scFv in between the N-terminus of the pIII gene and the signal sequence directing pIII to the bacterial periplasm (McCafferty et al. 1990). When the recombinant phage vector was expressed in E. coli, progeny fd phage particles were produced which expressed 3-5 copies of the scFv-pIII fusion protein on the phage capsid and contained a single-stranded DNA copy of the recombinant vector within the capsid (Hoogenboom et al. 1998, McCafferty et al. 1990).

1.5.4.2.2 Phagemid vectors

The next innovation in phage display was the development of phagemid vectors (Hoogenboom et al. 1991, Marks et al. 1991). Some of the advantages of phagemids include ease of transformation and the ability to express soluble sdAbs directly from the phagemid (Hoogenboom et al. 1998). High-affinity sdAb may be selected from phagemid-based libraries because there is usually only a single copy of the sdAb expressed on each phage (Bradbury and Marks 2004).
A library phagemid vector contains a phage-derived origin of replication and DNA packaging signal, a plasmid origin of replication, an antibiotic-resistance gene, and a copy of the filamentous phage pIII minor coat protein with N-terminal cloning sites. Each sdAb in a library may be cloned into a phagemid vector and fused to the pIII coat protein (Barbas III et al. 2001). The recombinant phagemids are transformed into F+ *E. coli* cells. During ‘phage rescue’, the transformed cells are superinfected with filamentous helper phage. The helper phage facilitates the replication of both the phagemid and the helper phage genome, and the production of progeny phage particles (Paschke 2006).

The pIII protein is present in 3-5 copies on the filamentous phage capsid (Paschke 2006). Wild-type pIII proteins will be preferentially incorporated into the progeny phage capsids over the sdAb-pIII fusion proteins. A small percentage of the progeny phages will express a single copy of the sdAb-pIII fusion protein, in addition to wild-type pIII proteins. These desired progeny phage may be increased in number by using special strains of helper phages, such as hyperphage. Hyperphage lack the wild-type pIII gene so that during phage rescue, the number of progeny phages expressing the sdAb-pIII fusion protein is increased (Rondot et al. 2001).

1.5.4.2.3 M13K07 helper phage

The progeny phages assembled during phage rescue will contain a single-stranded copy of either the helper phage genome, or the phagemid (Paschke 2006). Only the progeny phages containing the phagemid are useful for selection during panning. To maximize the number of phagemid-containing progeny phages, Vieira and Messing (1987) developed the M13K07 strain of helper phage. The M13K07 genome contains a
mutated version of gene II (coding for a modified version of pII, an important protein in replication), a kanamycin resistance gene, and the origin of replication from plasmid p15A. The p15A and the kanamycin resistance gene are purposely inserted within the origin of replication for the M13K07 genome, making this origin of replication less efficient for the modified pII protein. The modified pII is more efficient at replicating the phagemid DNA (which has a normal origin of replication), and thus, phagemid DNA is preferentially produced and packaged into progeny phages. The p15A origin of replication ensures that the M13K07 genome continues to replicate (without the need of pII) in the presence of the phagemid. The M13K07 genome must continue to replicate so that there are sufficient proteins available to produce and package the progeny phages. The kanamycin-resistance gene provides a selection marker to identify *E. coli* cells containing both the M13K07 genome and the phagemid (Vieira and Messing 1987).

1.6 Panning of phage-displayed sdAb libraries

1.6.1 Background

Panning involves the selection of sdAbs with high affinity for a particular antigen from a library of up to 10 billion different sdAbs (Lou et al. 2001). During panning of a phage-displayed sdAb library, the sdAbs which bind to a particular antigen are selected using four steps (Hoogenboom and Chames 2000). First, the phage-displayed sdAb are incubated with the antigen of interest. The second step is to wash 10-40 times with phosphate buffered saline (PBS) and detergent, *i.e.* Tween-20 to remove phage sdAbs which are non-specific for the antigen (Bradbury and Marks 2004). The third step is to elute the remaining phage sdAbs which bind specifically to the antigen, usually with a pH shock. The fourth, and final step, is to amplify the eluted phage sdAbs in *E. coli* for use in
another round of panning (Hoogenboom and Chames 2000). Usually 2-5 rounds of panning are required to select high-affinity sdAbs (Bradbury and Marks 2004). Affinity maturation may be performed between rounds of panning by isolating the sdAb genes from the phagemid and subjecting them to mutagenesis and diversification (Hoogenboom and Chames 2000, Hawkins et al. 1992).

In the first round of panning, a sample of $10^{12} - 10^{13}$ phages should be incubated with the antigen if the library is $10^8 - 10^{10}$ clones in size. Since less than 10% of the phages in a phagemid library display a sdAb-pIII fusion protein (Clackson and Wells 1994), a sample size of $10^{12} - 10^{13}$ is required to ensure that 10-100 copies of each phage sdAb will be available for selection (Bradbury and Marks 2004, Arbabi-Ghahroudi et al. 2008).

After each round of panning, the phage yield may be calculated by dividing the number of output phages by the number of input phages (Arbabi-Ghahroudi et al. 2008). The enrichment factor refers to the increase in phage yield in subsequent rounds of panning. After four rounds of panning, the enrichment factor should be between $10^3$ and $10^6$ fold. Enrichment may also be monitored by colony PCR, sequencing, restriction enzyme fingerprinting, and phage ELISA of the selected clones (Arbabi-Ghahroudi et al. 2008).

1.6.2 Antigen display

During panning, antigen may be displayed in a variety of formats, including display on solid surfaces, on living cells, or in solution. When purified antigen is available for panning, the antigen is usually immobilized on a solid surface such as a sepharose column (McCafferty et al. 1990), microtiter plate, immunotube, Petri dish (Marks et al. 1991), BIACORE sensorchip (Malmborg et al. 1996), beads (Biard-
Piechaczyk et al. 1999), or transferable solid-phase (TSP) polystyrene pins (Lou et al. 2001). Usually, a minimum of 100-μg of purified antigen is required to complete several rounds of panning (Bradbury and Marks 2004).

Panning against the same antigen using different solid supports may yield different antibodies. Lou et al. (2001) compared selection of an scFv phagemid library against 11 different antigens using both TSP pins and immunotubes for antigen display. The TSP pins had a small surface area which was coated with 10-100 ng of antigen, incubated with a diluted phage library, and subjected to a highly stringent washing procedure. The immunotubes had a larger surface area which was coated with 100-1,000-ng of antigen, incubated with a concentrated phage library and subjected to a less stringent washing procedure. Of the 130 antibodies selected using both selection procedures, less than 10% of the antibodies that were selected by both the TSP pin and the immunotube selection procedures were the same (Lou et al. 2001).

Target antigen may also be displayed on living E. coli cells (Bradbury et al. 1993). The antigen may be fused to an E. coli outer membrane protein such as LamB using recombinant DNA technology. The advantage of this system of antigen display is that the target epitope may be produced in large quantities and it does not need to be additionally processed or purified (Bradbury et al. 1993).

Panning may be conducted in solution when using multivalent display phage vectors to reduce avidity effects which may enhance the binding strength of low affinity antibodies (Hawkins et al. 1992). Multivalent phage-displayed sdAb are incubated with biotinylated antigen in solution, where no avidity effects are present. Streptavidin-conjugated paramagnetic beads are then used to select the phage sdAb bound to
biotinylated antigen (avidity effects may influence this selection process). The paramagnetic beads are collected using a magnet (Hawkins et al. 1992).

1.6.3 Selection strategies: subtractive selection, pathfinder selection, and *in vivo* selection

Sometimes the antigen display formats discussed in Section 1.6.2 are unsuitable for complex antigens; thus, specialized selection strategies including subtractive selection, pathfinder selection, and *in vivo* selection may be employed during panning. Subtractive selection is used when the antigen of interest cannot be purified. For example, a phage sdAb library may be panned against a fixed tissue sample to select binders to a target cell within the tissue (Van Ewijk et al. 1997). The phage sdAb library is preadsorbed with non-target cells present in the tissue to ‘subtract’ the sdAb which are specific for the non-target cells. After the subtraction step, the sdAb library is incubated with the tissue to select sdAb which bind to the target cell.

Subtractive panning may also be used to pan against haptens which are conjugated with a carrier protein (Yau et al. 2003). At the start of each round, the library is incubated with the carrier protein, to subtract any sdAbs specific for the carrier protein. The carrier protein may also be alternated, e.g. between ovalbumin and bovine serum albumin, between rounds of panning (Yau et al. 2003).

Pathfinder selection is utilized when the antigen exists in a complex environment such a cell surface, and there is an available antibody or natural ligand to the target antigen (Osbourn et al. 1998). The previously isolated antibody or ligand is conjugated with horseradish peroxidase (HRP) to act as a ‘pathfinder’ to guide selection of phage sdAbs bound to the target antigen. The phage sdAb library and the HRP-conjugated
pathfinder are incubated with the complex antigen. The addition of a biotin tyramine substrate and hydrogen peroxide cause phage sdAbs bound near the HRP-conjugated pathfinder to be biotinylated. The biotinylated sdAb phage may be isolated using streptavidin-coated magnetic beads which can be collected using a magnet (Osborn et al. 1998).

Phage sdAb libraries may even be selected in vivo against epitopes on the surface of organs. Pasqualini and Ruoslahti (1996) injected phage-displayed peptide libraries intravenously into mice. The kidney and brain were harvested to recover and amplify the phage bound to these organs. The amplified phages were injected into another mouse to initiate the next round of selection (Pasqualini and Ruoslahti 1996).

1.6.4 Minimizing non-specific binding of sdAb to antigen

Non-specific binding of phage sdAbs to antigen during panning may be minimized using several techniques, including blocking agents (Bradbury and Marks 2004) and ‘selectively infective phages’ (Spada and Plückthun 1997). Blocking agents, such as skim milk, gelatin, BSA, or casein, combined with a detergent such as Tween-20, may be incubated with the phage library and the antigen separately, to reduce non-specific adsorption of phages during selection (Bradbury and Marks 2004). Spada and Plückthun (1997) developed the ‘selectively infective phages’ technology where sdAbs in a phage-displayed library are each fused to a pIII coat protein which is missing its N1 and N2 N-terminal domains. Without the N-terminal pIII domains, the phage particles are non-infective. The missing N1 and N2 domains are fused to the target antigen. When the phage sdAb binds to the antigen, the phage acquires the N1 and N2 pIII domains and
regains infectivity. Thus, only phages specific for the target antigen will be able to infect
and proliferate in *E. coli* (Spada and Plückthun 1997).

1.6.5 Elution methods

During panning, there are several methods available to elute phage sdAbs bound
to antigen, including pH shock, enzymatic elution, and competitive elution. pH shock
using acidic or basic solutions is a widely used. Acidic elution solutions such as 100-mM
glycine-HCl pH 3.0 (Ward et al. 1996) may be used to elute bound phage sdAbs,
followed by neutralization with 1-M tris-HCl pH 8.0. Alternatively, basic elution
solutions such as 100-mM triethylamine (Marks et al. 1991) may be used to elute bound
phage sdAbs, followed by neutralization with 1-M tris-HCl, pH 7.4.

Ward et al. (1996) designed a method of enzymatic elution. Within the phagemid,
a recognition sequence for the protease genease I may be inserted between the sdAb gene
and the pIII gene. When phage-displayed sdAbs are bound to antigen, genease I
proteolysis may be used to release the phages from the bound sdAbs. The released phages
are free to re-infect *E. coli*. Elution efficiency with genease I was comparable to
conventional acid elution. Access to a variety of elution methods is important because
different elution methods often retrieve different phages. For example, genease I elution
identified one phage antibody against tetanus toxoid which was not obtained with acid
elution (Ward et al. 1996).

Competitive elution is possible if a pre-existing monoclonal antibody or ligand
with high affinity for the epitope of interest is available (Meulemans et al. 1994). The
phage sdAbs bound to an antigen may be displaced by the previously characterized
antibody or ligand, resulting in elution of the phage sdAbs (Meulemans et al. 1994).
Another elution strategy is to elute using the antigen itself. For example, phage antibodies bound to a hapten-Sepharose column may be eluted with excess soluble hapten (Clackson et al. 1991).

1.6.6 Selecting sdAbs for improved binding kinetics

The dissociation constant \( (K_D) \) of an antibody is the ratio of the dissociation rate constant \( (k_d) \) divided by the association rate constant \( (k_a) \). To optimize the \( K_D \), sdAbs are selected for a slow \( k_d \) and a fast \( k_a \) (Lu et al. 2003). Long wash steps favor clones with a slow \( k_d \), while short antigen-antibody incubation times favor clones with a fast \( k_a \) (Hoogenboom 2005). The stringency of the washing and incubation times may be increased over several rounds of panning.

Lu et al. (2003) selected a human Fab with a dissociation constant \( (K_D= k_d/ k_a) \) of 100-pM from a phage-displayed Fab library by using stringent panning to select Fabs with a slow \( k_d \) and a fast \( k_a \). To select Fabs with a slow \( k_d \), the phages bound to immobilized antigen were washed extensively with either PBS or soluble antigen as a competitor. In the second round, a 0.5-hour wash was performed using 0.1-μM antigen, followed by a 3-hour wash using 2-μM soluble antigen in the third round and a 24-hour wash using 1-μM soluble antigen in the fourth round. To select Fabs with a fast \( k_a \), the number of input phages was reduced from \( 1 \times 10^{12} \) in the first round to \( 1 \times 10^{11} \) in the second round, to \( 2 \times 10^{10} \) in the third and fourth rounds of panning. Also, the incubation time of the phages with the immobilized antigen was reduced from 1-hour in the first round to 0.5-hours in the second and third rounds, to 0.2-hours in the fourth round (Lu et al. 2003).
Phage sdAbs with a slow $k_d$ may also be selected in solution by incubating phage sdAbs bound to biotinylated antigen with excess unlabelled antigen (Hawkins et al. 1992). Over time, phage sdAbs will exchange their bound biotinylated antigen for unlabelled antigen in accordance with their $k_d$. Phage sdAbs with slow $k_d$ rates will remain bound to the biotinylated antigen and may thus be selected using streptavidin-coated paramagnetic beads (Hawkins et al. 1992).

Another method to optimize the $K_D$ of selected sdAb is to decrease the antigen concentration from one round to the next to enhance competition among phage sdAbs. Bradbury and Marks (2004) suggest an antigen concentration of 300-500-nM in solution is appropriate for the first round of selection from a non-immune antibody library. For each subsequent round, the antigen concentration should be five times less than the antigen concentration in the preceding round (Bradbury and Marks 2004). For example, during panning of a phage-displayed Fab library, Lu et al. (2003) decreased the antigen concentration from 10-μg per immunotube in the first round to 2-μg per immunotube in the second round, to 0.4-μg per microtitre well in the third and fourth rounds.

2 DEVELOPMENT OF A CBM-$V_H$ FUSION PROTEIN

2.1 Introduction

2.1.1 Objectives and hypothesis

Given the need for improved protective masks, clothing, and air filters in response to biological threats to modern society, this research addresses the question of whether or not sdAbs may be useful when applied to paper for capture and detection of viruses. As outlined in the objective (Section 1.2), the purpose of this research is to fuse a cellulose-
binding module (CBM) to a high-affinity llama V_{H}H which binds to bacteriophage M13. This fusion protein is to be applied to paper substrates for detection of M13 phage.

The hypothesis tested by this research is:

If a CBM is fused to a llama V_{H}H which binds to bacteriophage M13, then this fusion protein, when applied to paper, will enhance capture of M13 phage.

The introduction to this chapter provides background on the selection of V_{H}Hs which bind to viruses, CBMs, and fusions of V_{H}Hs to CBMs. The body of this chapter describes the development of a bifunctional CBM-V_{H}H fusion protein capable of binding to cellulose and bacteriophage M13. Bioactive paper applications of this fusion protein will be discussed in Chapter 3.

2.1.2 Isolation of V_{H}H sdAbs which bind to a virus

Researchers have been successful in developing V_{H}Hs with nanomolar affinity for a virus. De Haard et al. (2005) constructed an immunized phage display library of llama V_{H}Hs against phage p2. Phage p2 infects Lactococcus lactis, a bacterium used commercially to ferment milk. Nanomolar concentrations of a V_{H}H selected by panning were capable of neutralizing $10^3$-$10^5$ pfu/ml of phage p2 by binding to a tail protein on the phage. Based on the success of these experiments, De Haard et al. (2005) concluded that V_{H}Hs may be selected to neutralize many other viruses.

The M13 phage was chosen as the model virus for the research presented in this thesis because the M13 phage is nonpathogenic to humans and easy to grow. Future research may focus on development of V_{H}H-CBM fusion proteins which bind to pathogenic human viruses. For example, Forsman et al. (2008) developed a llama V_{H}H capable of neutralizing subtypes B and C of the human immunodeficiency virus type 1.
(HIV-1) by binding with picomolar affinity to the HIV-1 envelope spike glycoprotein gp120 which interacts with host cell CD4 receptors during infection. The intended application of this VhH is in HIV-1 microbicide development and vaccine design; however, VhHs specific for human viruses may also be used in CBM fusions (Forsman et al. 2008).

2.1.3 CBM2a from *Cellulomonas fimi* xylanase 10A

*Cellulomonas fimi* expresses xylanase 10A, an extracellular enzyme, which hydrolyzes β-1,4 glycosidic bonds of xylan (McLean et al. 2000). The polysaccharides xylan and cellulose have a similar biochemical structure, and thus, xylanases are also capable of hydrolyzing the β-1,4 bonds of cellulose and hemicellulose in plant cell walls (McLean et al. 2000, Gilkes et al. 1991). Xylanase 10A and other microbial cellulases are composed of two protein domains; a catalytic domain for β-1,4 hydrolysis, and a CBM for binding to the cellulose or xylan substrate. *C. fimi* xylanase 10A contains a family 2a CBM known as CBM2a. CBM2a binds irreversibly to crystalline cellulose; however, CBM2a is still able to diffuse across the cellulose substrate without dissociating (Jervis et al. 1997). A review of CBMs with a focus on family 2a CBMs is found in Hussack (2007). CBM2a is separated from the catalytic domain of xylanase 10A by an extended proline-threonine linker ((PT)$_3$T(PT)$_3$T(PT)$_3$S) which allows for proper functioning of the two protein domains without steric hindrance (Gilkes et al. 1991, Shen et al. 1991). The nucleotide sequence of the *C. fimi* xylanase 10A was reported by O’Neill et al. (1986). The gene encoding the CBM2a polypeptide has been subcloned and expressed in *E. coli* (Ong et al. 1993). The isolated CBM2a polypeptide has the same affinity for β-1,4-glycans as its parent enzyme xylanase 10A (Ong et al. 1993). Using recombinant DNA
technology, a CBM may be fused to another protein domain without reducing the biological activity of either domain (Ramirez et al. 1993). For example, the *Staphylococcus aureus* protein A was fused to the N-terminus of the proline-threonine linker and the CBM2a of *C. fimii*. The protein A-CBM2a fusion was used to bind antibodies to cellulose (Ramirez et al. 1993).

2.1.4 Development of a CBM-VH fusion protein for bioactive paper applications

VHs are ideal for bioactive paper applications due to their intrinsic stability and ease of expression (Saerens et al. 2008). Diverse phage display VH libraries may be constructed and selected against a variety of antigens (see sections 1.5 and 1.6). Furthermore, CBM fusion is an effective method for irreversible immobilization of sdAbs on cellulose for the development of immunosensors (McLean et al. 2000, Lewis et al. 2006). CBM fusion allows the sdAb to be immobilized on the sensor in the correct orientation for binding antigen, minimizing steric hindrance (Hussack et al. 2009).

CBM-VH fusion proteins have been successfully developed for commercial applications. Lewis et al. (2006) fused a llama VH specific for the azo–dye, reactive-red 6 (RR6), to a CBM via a protein linker. Fusion to a CBM reduced the binding affinity of the VH; however, the binding affinity of the VH was still in the nanomolar range. The VH-CBM fusions were used to cross-link RR6-coated particles containing perfume to strands of cotton, suggesting that these VH-CBM fusion proteins may have applications in the manufacture of laundry detergents. The fusion protein retained its ability to bind to RR6 in the presence of components of laundry detergent and under basic conditions (Lewis et al. 2006).
CBM-sdAb fusion proteins may be used in biosensors for detection of pathogens in the environment. Hussack et al. (2009) developed a paper-based immunosensor capable of detecting *Staphylococcus aureus* at a concentration of $10^5$ cfu/ml. The detection agent used was a bispecific pentamer consisting of five anti-*S. aureus* human sdAbs linked to five CBMs via five verotoxin 1 B-subunits (VT1Bs) (Hussack et al. 2009).

### 2.2 Materials and Methods

#### 2.2.1 Selection of $V_{H}H$ monomers

A llama was immunized with bacteriophage M13. Yonghong Guan and Dr. Mehdi Arbabi-Ghahroudi (National Research Council Canada-Institute for Biological Sciences (NRC-IBS), Ottawa, Ontario, Canada) developed an immune $V_{H}H$ library from the immune llama lymphocytes and selected three sdAbs by panning the immune library against M13 phage. The selected sdAbs were identified by Guan and Arbabi-Ghahroudi as 1222-III10f (Figure 1), 1213-16j (Figure 2), and 1222-16e (Figure 3). Guan and Arbabi-Ghahroudi expressed each sdAb separately as both a monomer and a pentamer. Competition ELISAs using the anti-M13 monomers and commercially available antibodies developed against the M13 phage proteins pVIII and pIII suggested that both 1222-III10f and 1222-16e bind to pVIII, while 1213-16j binds to M13 phage protein pIII. This work has not been published and is presented here to identify the source of the $V_{H}H$ used in the $V_{H}H$-CBM fusion.
Figure 1. Amino acid sequence of the 1222-III10f single-domain antibody (EditSeq™6.1, DNASTAR, Inc., Madison, Wisconsin, USA). The underlined sequence represents a portion of the pMED2 expression vector. The pMED2 sequence is followed by a c-Myc and a His6 tag.

EVQLVDSGGGLVQPGGSLRLSCAAGSTTVAMGWYRQAPGKDREFKTVISARNAYNQNY WGGGTQVTSVGQVAGQGSEQQKLISEEDLNNHHHHHH

Figure 2. Amino acid sequence of the 1213-16j single-domain antibody (EditSeq™6.1, DNASTAR, Inc., Madison, Wisconsin, USA). The underlined sequence represents a portion of the pMED2 expression vector. The pMED2 sequence is followed by a c-Myc and a His6 tag.

QVQLVESGGGLVQAGGSLRLSCAAGSTTVAMGWYRQAPGKDREFKTVISARNAYNQNY WGGGTQVTSVGQVAGQGSEQQKLISEEDLNNHHHHHH

Figure 3. Amino acid sequence of the 1222-16e single-domain antibody (EditSeq™6.1, DNASTAR, Inc., Madison, Wisconsin, USA). The underlined sequence represents a portion of the pMED2 expression vector. The pMED2 sequence is followed by a c-Myc and a His6 tag.

QVQLVESGGGLVQAGGSLRLSCAAGSTTVAMGWYRQAPGKDREFKTVISARNAYNQNY WGGGTQVTSVGQVAGQGSEQQKLISEEDLNNHHHHHH

2.2.2 Binding kinetics of the $V_H$ monomers and pentamers to immobilized M13 phage

SPR using a BIACORE 3000 biosensor system (GE Healthcare, Baie d’Urfé, Quebec, Canada) was used to evaluate the binding kinetics of the 1222-III10f, 1213-16j, and 1222-16e monomers and pentamers to immobilized M13 phage. All SPR studies were performed by Tomoko Hirama and Henk van Faassen (NRC-IBS, Ottawa, Ontario, Canada). This work has not been published and is presented here to identify the source of the SPR data used to select a $V_H$ for the CBM-$V_H$ fusion.

M13 phage and a reference protein, i.e. bovine serum albumin (BSA), were immobilized on a C1 sensor chip (GE Healthcare, Baie d’Urfé, Quebec, Canada), and binding of $V_H$ monomers and pentamers were determined following the methods of
Ryan et al. (2009). Briefly, ca. 200 resonance units (RUs) of M13 phage combined with 300 RUs of BSA were immobilized on one flow cell of a C1 sensor chip (GE Healthcare), and 400 RUs of BSA were immobilized on another flow cell of the same C1 sensor chip. Immobilizations were carried out using a 4 x 10^{10} M13 phage/ml solution in 10-mM acetate buffer pH 4.0, and a 50-µg/ml BSA solution in 10-mM acetate buffer pH 4.5. An amine coupling kit (GE Healthcare) was used to immobilize the phage and/or BSA on the sensor chips. Prior to SPR analysis, all V_{H}H monomers and pentamers were purified using a Superdex 75 column (GE Healthcare) to remove possible aggregates. All SPR analyses were performed at 25ºC in running buffer (10-mM HEPES, pH 7.4 containing 150-mM NaCl, 3-mM EDTA and 0.005 % surfactant P20) at a flow rate of 10-µl/min. A 20-µl sample of each V_{H}H monomer or pentamer was applied to the sensor chip and allowed to dissociate over 20-min. In between each sample, the chip was washed thoroughly with running buffer. All data were analyzed with BIAevaluation 4.1 software (GE Healthcare) (Ryan et al. 2009).

2.2.3 BIACORE epitope mapping for selected V_{H}H monomers

SPR using a BIACORE 3000 biosensor system (GE Healthcare, Baie d’Urfé, Quebec, Canada) was used to compare the relative binding sites of the 1222-III10f, 1213-16j, and 1222-16e monomers to immobilized M13 phage. M13 phage was immobilized on a C1 sensor chip as described in section 2.2.2. A V_{H}H monomer was applied to the sensor chip followed by a second V_{H}H monomer. The first monomer was added at a concentration several fold greater than its K_{D} to ensure that the immobilized M13 phage was saturated with V_{H}H prior to addition of the second monomer. Based on the response in RU to each V_{H}H monomer, it was determined whether the binding site of the first V_{H}H
was the same or different than that of the second $V_H$ that was passed over the chip. All SPR studies were performed by Tomoko Hirama and Henk van Faassen (NRC-IBS, Ottawa, Ontario, Canada) as described in section 2.2.2.

2.2.4 Preparation of helper phage M13 KO7

Stocks of M13 phage for use in experiments were prepared according to the three-day protocol outlined below.

Day 1

A 10-ml LB *E. coli* culture was incubated at 37°C until it reached mid-log phase. Serial dilutions of $10^{11}$ pfu/ml M13KO7 stock (New England Biolabs, Ipswich, Massachusetts, USA) were prepared in LB medium. Samples (100-μl) of the *E. coli* culture were infected with 10-μl of selected M13 dilutions ($10^7$, $10^8$, $10^9$ M13 dilutions). A negative control containing only 100-μl *E. coli* culture was also prepared. Each *E. coli* sample infected with M13, as well as the negative control, was mixed with a 3-ml sample of molten top agar at 55°C, and poured onto a pre-warmed minimal media plate. The top agar was allowed to harden and the plates were incubated at 37°C overnight.

Day 2

A 10-ml LB *E. coli* culture was incubated at 37°C until it reached mid-log phase. Using the plates prepared the previous day, an isolated M13 plaque was picked with a sterile Pasteur pipette and transferred to the 10-ml *E. coli* culture. The *E. coli* culture infected with the M13 plaque was grown for two hours at 37°C with shaking at 250-rpm. The two hour culture was added to 500-ml of 2xYT broth in a 2-L flask and grown for 1-hour at 37°C with shaking at 250-rpm. Kanamycin was added to the 2xYT culture to a
final concentration of 50-μg/ml. The 2xYT culture was grown overnight at 30°C with shaking at 250-rpm.

**Day 3**

The 2xYT culture was centrifuged at 4,400-g for 15 min at 4°C to pellet the *E. coli*. The culture supernatant was mixed with 1/5 volume 20% PEG 2.5 M NaCl and incubated on ice for 1-hour. The supernatant was centrifuged at 4,000-g for 30-min to precipitate the M13 phage. The resulting pellet was resuspended in 5-ml 1X PBS. The re-suspended pellet was centrifuged at 18,000-g for 3-min at 4°C to remove any remaining bacterial debris. The supernatant (containing M13 phage) was stored in 50 or 100-μl aliquots at -20°C.

**2.2.5 Titering M13 phage stocks**

This method was used to titer prepared M13 phage stocks as well as to titer M13 phage samples generated during experiments.

A 10-ml LB *E. coli* culture was incubated at 37°C until it reached mid-log phase. Serial dilutions of the M13 sample of unknown titer were prepared in LB medium. Samples (100-μl) of the *E. coli* culture were infected with 10-μl of selected M13 dilutions. A negative control containing only 100-μl *E. coli* culture was also prepared. Each *E. coli* sample infected with M13 phage, as well as the negative control, was mixed with a 3-ml sample of molten top agar at 55°C, and poured onto a pre-warmed minimal media plate. The top agar was allowed to harden and the plates were incubated at 37°C overnight. The next day, the plaques on each plate were counted. The number of plaques per plate was multiplied by the dilution factor to determine the titer of the M13 sample.
2.2.6 ELISA to determine sensitivity of anti-M13 monomers and pentamers for detection of M13 phage

Two ELISAs were performed: one to compare the sensitivity of the anti-M13 $V_{H}H$ monomers for detection of immobilized M13 phage, and another to compare the sensitivity of the anti-M13 $V_{H}H$ pentamers for detection of immobilized M13 phage. For each ELISA, a microtiter plate was coated with 200-μl of a M13 phage dilution/well; each column of wells was coated with a different dilution of M13 (Table 1). For the anti-M13 monomer ELISA, eight columns of the plate were coated with 10-fold dilutions of M13 phage. The concentrations of M13 phage ranged from $3 \times 10^6$ to $3 \times 10^{13}$ pfu/well. For the anti-M13 pentamer ELISA, ten columns of the plate were coated with 10-fold dilutions of M13 phage. The concentrations of M13 phage ranged from $2.8 \times 10^4$ to $2.8 \times 10^{13}$ pfu/well. Since some phages were lost during washing, the actual concentration of phages that remained bound to the wells could not be exactly determined. For the anti-M13 monomer ELISA, the negative control column that was not coated with primary antibody (Table 1) was coated with $3 \times 10^{12}$ pfu M13/well. For the anti-M13 pentamer ELISA, the negative control column that was not coated with primary antibody was coated with $2.8 \times 10^{12}$ pfu M13/well. For both ELISAs, a negative control column of wells was coated with 200-μl of PBS/well instead of M13 (Table 1). The coated plates were covered with parafilm and incubated overnight at 4°C. The next day the wells of the plates were emptied and washed two times with 200-μl/well PBS. The wells were blocked with 200-μl 2% MPBS/well at 37°C for two hours. The wells were emptied and washed five times with 200-μl/well PBS.
Two rows of each plate were coated with each primary antibody being tested (Table 1); each antibody was diluted in 2% MPBS and applied to the plate at 200-μl/well. For the anti-M13 monomer ELISA, the anti-M13 monomers (i.e., 1213-16j, 1222-16e, and 1222-III10f) were applied to the plate at a concentration of 2-μg/well. For the anti-M13 pentamer ELISA, the anti-M13 pentamers (i.e., 1213-16j, 1222-16e, and 1222-III10f) were applied to the plate at a concentration of 80-ng/well.

As a positive control, with known sensitivity, two rows of each plate were coated with 80-ng/well of commercially available mouse anti-M13 monoclonal antibody conjugated to HRP (GE Healthcare, Baie d’Urfé, Quebec, Canada) diluted 1:2,500 in 2% MPBS (Table 1). As a negative control, one column of wells of each plate was coated with 200-μl of 2% MPBS instead of primary antibody (Table 1).

The plates were incubated at room temperature for one and a half hours. The plates were washed five times with 200-μl/well PBST. The positive control wells coated with the commercial mouse anti-M13-HRP antibody did not require a secondary antibody; these wells were coated with 200-μl 2% MPBS. All other wells were coated with 200-μl of the secondary antibody, i.e., rabbit anti-6-His conjugated to HRP (Bethyl Laboratories Inc., Montgomery, Texas, USA), diluted 1:5,000 in 2% MPBS. The plates were incubated at room temperature for one hour. The wells of the plates were emptied and the plates were washed five times with 200-μl/well PBST. To develop the plates, the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, Maryland, USA) was used. Equal volumes of TMB Peroxidase Substrate Solution A and Peroxidase Substrate Solution B, contained in the kit, were combined, and 100-μl was added to each well. The plates were incubated at room temperature for 20-min in the dark. The peroxidase
reaction was quenched by adding 100-μl 1M H₃PO₄ to each well. The plates were read with a spectrophotometer at 450-nm.

**Table 1.** Example of an ELISA plate used for testing the sensitivity of the anti-M13 antibodies ‘x’, ‘y’, and ‘z’ when compared to a commercial antibody with a known sensitivity. In this example, the plate was coated with two concentrations of M13 phage (column 1 was coated with concentration ‘i’ and column 2 was coated with concentration ‘ii’). Negative control column 3 was coated with all reagents except for primary antibody. Negative control column 4 was coated with all reagents except for M13 phage. All primary antibodies were applied to the plate at a constant concentration.

<table>
<thead>
<tr>
<th>Primary antibody applied to each row</th>
<th>Column 1: M13 concentration ‘i’</th>
<th>Column 2: M13 concentration ‘ii’</th>
<th>Column 3: Negative Control (no primary antibody)</th>
<th>Column 4: Negative Control (no M13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sdAb ‘x’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sdAb ‘x’</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>sdAb ‘y’</td>
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<tr>
<td>sdAb ‘y’</td>
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<tr>
<td>sdAb ‘z’</td>
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<td>sdAb ‘z’</td>
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<td>Commercial antibody (Positive Control)</td>
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<tr>
<td>Commercial antibody (Positive Control)</td>
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</tbody>
</table>

2.2.7 Agarose gel electrophoresis

All agarose gel electrophoresis was performed using 1% agarose gels. DNA was loaded into the wells with a drop of 6X DNA loading buffer (0.25% bromophenol blue, 40% sucrose). All gels were run at 100-V.
2.2.8 Design of the V_{H}-CBM fusion protein

The fusion protein consisted of 292 amino acids. The protein was designed in two orientations:

1. **CBM2a-10f fusion protein** with CBM2a at the N-terminus and the 1222-III10f anti-M13 V_{H}H at the C-terminus.

2. **10f-CBM2a fusion protein** with the 1222-III10f anti-M13 V_{H}H at the N-terminus and CBM2a at the C-terminus

The molecular weight of the fusion protein (without the OmpA leader sequence) was 28,612 Da (EditSeq™ 6.1, DNASTAR, Inc., Madison, Wisconsin, USA).

2.2.8.1 *Expected amino acid and nucleotide sequence of the CBM2a-10f fusion protein*

The CBM2a-10f protein was designed to include (from N-terminus to C-terminus) an OmpA leader sequence, the CBM2a sequence, a proline-threonine linker sequence, the anti-M13 1222-III10f V_{H}H sequence, a c-Myc tag, and a His_{6} tag. This protein is coded for by 292 amino acids (Figure 4) and 879 nucleotides (Figure 5).

**OmpA leader sequence**  
CBM2a sequence  
MKKTAIAIAVALAGFATVAQASGPAGCQVLWGYNQWNTGFTANVTVDKNTSSAP  
VDGWTLTSFSPGQQVTQAWSSTVTOSGSATVRNAPWNSIPAGGTAQFGFN  
linker sequence  
10f sequence  
GSHTGTNAAPTAFSLNGTPCTVGTPPTPTPTPTPTPTPTPTPTPTPTPTPTQVKLEESGGGLV  
QAGDSLRLSCAASGPTFSTYTMGWRQAPGNEREFFVAAISWVNSATDDYADSVK  
GRFTISRDNKSTAVLYQMNSLKPEDTAHVYCAASRYKNRAVTAYFGWGQGTQ  
c-Myc  
His_{6}  
VTVSAGSEQKLISEDLNHHHHHHHH

**Figure 4.** Expected amino acid sequence of the CBM2a-10f protein.
Figure 5. Expected nucleotide and amino acid sequence of the CBM2a-10f protein (SeqBuilder™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA)).

2.2.8.2 Expected amino acid and nucleotide sequence of the 10f-CBM2a fusion protein

The 10f-CBM2a protein was designed to include (from N-terminus to C-terminus) an OmpA leader sequence, the anti-M13 1222-III10f V₈H sequence, a proline-threonine linker sequence, the CBM2a sequence, a c-Myc tag, and a His₆ tag. This protein is coded for by 292 amino acids (Figure 6) and 879 nucleotides (Figure 7).
**ompA leader sequence**
MKKTAIAIAVALAGFATVAAQAQVKLEESGGGLVQAQDGSRCLAASGPTFSTYT

**10f sequence**
MGWRQAPGNEREFAISWVNSATDYADSVKGRFTISNRNAYKSTAYLQMNLS

**linker sequence**
KPEDTAVVYHCASSRYKYKNAVTAYFGWQQGTQTVTSSPTTPTPTPTPTPTPT

**CBM2a sequence**
PTPTSSGPGACGQVLGWGNWNTGFANTYKVNTSSAPVDGWTTLTFSPFGQV

**c-Myc**

**His**

**TPTCVGGSEQKLISEEDLHHHHHH**

**Figure 6.** Expected amino acid sequence of the 10f-CBM2a fusion protein.

**Figure 7.** Expected nucleotide and amino acid sequence of the 10f-CBM2a protein (SeqBuilder™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA)).
2.2.9 Forward and reverse primers used for DNA assembly of the CBM2a-10f and 10f-CBM2a fusion proteins

Restriction sites for the restriction endonucleases BbsI and BamHI were included in the primer design.

1. The primer pair used to insert a CBM2a sequence at the N-terminus of the CBM2a-10f fusion protein was:

CBM2a_link_N-term forward (5’→3’):

\[
\text{GTG CAT GTT GCG GAA GAC GAC AGG CC AGC GGC CCG GCC GGG TGC}
\]

\text{BbsI CBM2a specific}

CBM2a_link_N-term reverse (5’→3’):

\[
\text{GCT GGT TGG TGT CGG TGT TGG GGT GGT CGG GGT CGG GGT CGG GGT}
\]

\text{Linker specific}

\[
\text{GCT CGG GGT CGG GGT CGG GCC GAC CGT GCA GGG CGT G}
\]

\text{Linker specific CBM2a specific}

2. The primer pair used to insert the 1222-III10f monomer sequence at the C-terminus of the CBM2a-10f fusion protein was:

Link_10f_C-term forward (5’→3’):

\[
\text{CCA ACA CCG ACA CCA ACC AGC CAG GTA AAG CTG GAG GAG TCT G}
\]

\text{Linker specific 10f specific}

Link_10f_C-term reverse (5’→3’):

\[
\text{GAG ACG TTA ATG GGA TCC TGA GGA GAC GGT GAC CTG GGT C}
\]

\text{BamHI 10f specific}
3. The primer pair used to assemble the CBM2a-10f fusion protein was:

CBM2a_link_N-term forward (5’ → 3’):

\[
\text{GTG CAT GTT GCG GAA GAC GAC AGG CC AGC GGC CCG GCC GGG TGC}
\]

\text{BbsI CBM2a specific}

Link_10f_C-term reverse (5’ → 3’):

\[
\text{GAG ACG TTA ATG GGA TCC TGA GGA GAC GGT GAC CTG GGT C}
\]

\text{BamHI 10f specific}

4. The primer pair used to insert the 1222-III10f monomer sequence at the N-terminus of the 10f–CBM2a fusion protein was:

10f_link_N-term forward (5’ → 3’):

\[
\text{CAG GTT CGG GAC GAA GAC GAC AGG CC CAG GTA AAG CTG GAG GAG}
\]

\text{BbsI 10f specific}

\[
\text{TCT G}
\]

10f specific

10f_link_N-term reverse (5’ → 3’):

\[
\text{GCT GGT TGG TGT CGG TGT TGG GGT GGT CGG GGT CGG GGT CGG GGT}
\]

\text{Linker specific}

\[
\text{GGT CGG GGT CGG GGT}
\]

\text{Linker specific 10f specific}

5. The primer pair used to insert a CBM2a sequence at the C-terminus of the 10f-CBM2a fusion protein was:

Link_CBM2a_C-term forward (5’ → 3’):

\[
\text{CCA ACA CCG ACA CCA ACC AGC AGC GGC CCG GCC GGG TGC}
\]

\text{Linker specific CBM2a specific}

Link_CBM2a_C-term reverse (5’ → 3’):

\[
\text{GAG ATC TTC CGA GGA TCC GCC GAC CGT GCA GGG CGT G}
\]

\text{BamHI CBM2a specific}
6. The primer pair used to assemble the 10f-CBM2a fusion protein was:

10f_link_N-term forward (5’ → 3’):

\[
\text{CAG GTT CGG GAC GAA GAC GAC AGG CC CAG GTA AAG CTG GAG GAG TCT G} \\
\text{GAG ATC TTC CGA GGA TCC GCC GAC CGT GCA GGG CGT G} \\
\text{BamHI CBM2a specific}
\]

10f specific

Link_CBM2a-C-term reverse (5’ → 3’):

\[
\text{GAG ATC TTC CGA GGA TCC GCC GAC CGT GCA GGG CGT G} \\
\text{BbsI CBM2a specific}
\]

2.2.10 PCR assembly of the CBM2a-10f fusion protein

2.2.10.1 PCR 1: Amplification of the CBM2a template DNA for use in the CBM2a-10f fusion protein

Reactions were performed in 50-μl volumes containing 1X Expand High Fidelity Buffer with 75-nmol MgCl₂ (Roche Applied Science, Mannheim, Germany), 10-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), 5-pmol forward primer, 5-pmol reverse primer (Invitrogen, Carlsbad, California, USA), 1.5-ng CBM2a template DNA in the pET22 vector (provided by C. Haynes, University of British Columbia), and 3.5-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany). The forward primer, CBM2a_link_N-term forward (5’ GTG CAT GTT GCG GAA GAC GAC AGG CC AGC GGC CCG GCC GGG TGC 3’), and the reverse primer, CBM2a_link_N-term reverse (5’ GCT GGT TGG TGT CGG TGT CGT TGG GGT GGT CGG GGT CGG GGT CGG GGT CGG GGT CGG GCC GAC CGT GCA GGG CGT G 3’), were used to amplify the CBM2a DNA. During amplification, a BbsI restriction site was added to the 5’ end of the CBM2a DNA and a proline-threonine linker, (PT)₃T(PT)₃T(PT)₃S, was added to the 3’ end of the CBM2a
DNA (Figure 8). The PCR product was 413 base pairs in length. The PCR conditions used were 94°C for 5-min, 30 cycles of 94°C for 30-s, 60°C for 30-s, and 72°C for 1-min followed by 72°C for 7-min. Hot start PCR was used (enzyme added after temperature reached 94°C). The amplified CBM2a PCR product was gel purified using a QIAquick® Gel Extraction Kit (QIAGEN Sciences, Germantown, Maryland, USA).

**Figure 8.** Amplification of CBM2a template DNA to attach a *Bbs*I restriction site (5’) and a proline-threonine linker (3’) to the CBM2a DNA, to produce a product of 413 base pairs.

*2.2.10.2 PCR 2: Amplification of the 1222-III10f VH H DNA for use in the CBM2a-10f fusion protein*

Reactions were performed in 50-µl volumes containing 1X Expand High Fidelity Buffer with 75-nmol MgCl₂ (Roche Applied Science, Mannheim, Germany), 10-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), 10-pmol forward primer, 10-pmol reverse primer (Invitrogen, Carlsbad, California, USA), 3-ng 1222-III10f VH H template DNA in the pMED2 vector (provided by Yonghong Guan and Dr. Mehdi Arbabi-Ghahroudi, NRC-IBS, Ottawa, Ontario, Canada), and 3.5-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany). The forward primer, Link_10f_C-term forward (5’CCA ACA CCG ACA CCA ACC AGC CAG GTA
AAG CTG GAG GAG TCT G 3”), and the reverse primer, Link_10f_C-term reverse (5’ GAG ACG TTA ATG GGA TCC TGA GGA GAC GGT GAC CTG GGT C 3’), were used to amplify the 1222-III10f monomer DNA. During amplification, part of the proline-threonine linker sequence, (PT)₃₅, was added to the 5’ end of the 1222-III10f monomer DNA and a BamHI restriction site was added to the 3’ end of the 1222-III10f monomer DNA (Figure 9). The PCR product was 408 base pairs in length. The PCR conditions used were 94°C for 5-min, 30 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. The amplified PCR 2 product was gel purified using a QIAquick® Gel Extraction Kit (QIAGEN Sciences, Germantown, Maryland, USA).

**Figure 9.** Amplification of 1222-III10f monomer DNA to add part of the proline-threonine linker (5’) and a BamHI restriction site (3’) to the 1222-III10f DNA to produce a product of 408 bp.

2.2.10.3 Assembly of PCR 1 product and PCR 2 product to generate DNA template for the CBM2a-10f fusion protein

Reactions were performed in 50-µl volumes containing 1X Expand High Fidelity Buffer with 75-nmol MgCl₂ (Roche Applied Science, Mannheim, Germany), 10-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), equimolar
concentrations of PCR 1 product (5’-BbsI-CBM2a-linker-3’) and PCR 2 product (5’-linker-1222-III10f-BamHI-3’) (0.183-pmoles each), and 3.5-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany). The initial PCR conditions used were 94°C for 5-min, 10 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. This program allowed the double-stranded PCR 1 product and PCR 2 product to denature and then reanneal in the proline-threonine linker region shared by both the PCR 1 product and the PCR 2 product (Figure 10). The PCR program was stopped at 72°C and a second PCR program was started. Five-pmol forward primer and 5-pmol reverse primer were added during the second PCR program (Invitrogen, Carlsbad, California, USA). The PCR conditions used were 94°C for 5-min (primers added to tubes during this time), 30 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. The CBM2a-10f PCR product was amplified with the forward primer, CBM2a_link_N-term forward (5’GTG CAT GTT GCG GAA GAC GAC AGG CC AGC GGC C CG GCC GGG TGC 3’), and the reverse primer, Link_10f_C-term reverse (5’ GAG ACG TTA ATG GGA TCC TGA GGA GAC GGT GAC CTG GGT C 3’). The PCR product was 800 base pairs in length. The CBM2a-10f PCR product was gel purified using a QIAquick® Gel Extraction Kit (QIAGEN Sciences, Germantown, Maryland, USA).
Figure 10. Assembly of PCR 1 product (5’-BbsI-CBM2a-linker-3’) and PCR 2 product (5’-linker-1222-III10f-BamHI-3’) into DNA template for the production of the CBM2a-10f fusion protein (800-bp).

2.2.11 PCR assembly of the 10f-CBM2a fusion protein

2.2.11.1 PCR 3: Amplification of the 1222-III10f V_{H}H DNA for use in the 10f-CBM2a fusion protein

Reactions were performed in 50-µl volumes containing 1X Expand High Fidelity Buffer with 75-nmol MgCl₂ (Roche Applied Science, Mannheim, Germany), 10-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), 10-pmol forward primer, 10-pmol reverse primer (Invitrogen, Carlsbad, California, USA), 3-ng 1222-III10f V_{H}H DNA in the pMED2 vector (provided by Yonghong Guan and Dr. Mehdi Arbabi-Ghahroudi, NRC-IBS, Ottawa, Ontario, Canada), and 3.5-units Expand High
Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany). The forward primer, 10f_link_N-term forward (5’ CAG GTT CGG GAC GAA GAC GAC AGG CC CAG GTA AAG CTG GAG GAG TCT G 3’), and the reverse primer, 10f_link_N-term reverse (5’ GCT GGT TGG TGT CGG TGT TGG GGT GGT CGG GGT CGG GGT CGG GGT CGG TGA GGA GAC GGT GAC CTG GGT C 3’), were used to amplify the 1222-III10f monomer DNA. During amplification, a BbsI restriction site was added to the 5’ end of the 1222-III10f DNA and a proline-threonine linker, (PT)₃(T(PT)₃T(PT)₃S, was added to the 3’ end of the 1222-III10f DNA (Figure 11). The expected size of the PCR product was 458 base pairs. The PCR conditions used were 94°C for 5-min, 30 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. The amplified PCR product was gel purified using a QIAquick® Gel Extraction Kit (QIAGEN Sciences, Germantown, Maryland, USA).

**Figure 11.** Amplification of the 1222-III10f V₉H DNA to add a BbsI restriction site (5’) and a proline-threonine linker (3’) to the 10f DNA to produce a product of 458 base pairs.
2.2.11.2 PCR 4: Amplification of the CBM2a DNA for use in the 10f-CBM2a fusion protein

Reactions were performed in 50-µl volumes containing 1X Expand High Fidelity Buffer with 75-nmol MgCl$_2$ (Roche Applied Science, Mannheim, Germany), 10-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), 5-pmol forward primer, 5-pmol reverse primer (Invitrogen, Carlsbad, California, USA), 1.5-ng CBM2a template DNA in the pET22 vector (provided by C. Haynes, University of British Columbia), and 3.5-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany). The forward primer, Link_CBM2a_C-term forward (5’ CCA ACA CCG ACA CCA ACC AGC AGC GGC CCG GCC GGG TGC 3’), and the reverse primer, Link_CBM2a_C-term reverse (5’ GAG ATC TTC CGA GGA TCC GCC GAC CGT GCA GGG CGT G 3’), were used to amplify the CBM2a DNA. During amplification, part of the proline-threonine linker sequence, (PT)$_3$S, was added to the 5’ end of the CBM2a DNA, and a BamHI restriction site was added to the 3’ end of the CBM2a DNA (Figure 12). The expected size of the PCR product was 363 base pairs. The PCR conditions used were 94°C for 5-min, 30 cycles of 94°C for 30-s, 60°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. A hot start PCR was used (enzyme added after temperature reached 94°C). The amplified CBM2a PCR product was gel purified using a QIAquick® Gel Extraction Kit (QIAGEN Sciences, Germantown, Maryland, USA).
2.2.11.3 Assembly of PCR 3 and PCR 4 product to generate a DNA template for the 10f-CBM2a fusion protein

Reactions were performed in 50-µl volumes containing 1X Expand High Fidelity Buffer with 75-nmol MgCl₂ (Roche Applied Science, Mannheim, Germany), 10-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), equimolar concentrations of PCR 3 product (5’-BbsI-1222-III10f-linker-3’) and PCR 4 product (5’-linker-CBM2a-BamHI-3’) (0.165-pmoles each), and 3.5-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany). The initial PCR conditions used were 94°C for 5-min, 10 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. This program allowed the double-stranded PCR 3 product and PCR 4 product to denature and reanneal in the proline-threonine linker region shared by both the PCR 3 product and the PCR 4 product (Figure 13). The PCR program was stopped at 72°C and a second PCR program was started. Five-pmol forward primer and 5-pmol reverse primer were added during the second PCR program (Invitrogen, Carlsbad, California, USA). The PCR conditions used were 94°C for 5-min (primers added to tubes

Figure 12. Amplification of the CBM2a DNA to add part of a proline-threonine linker (5’) and a BamHI restriction site (3’) to the CBM2a DNA to produce a product of 363 base pairs.
during this time), 30 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min. The 10f N-term assembly PCR product was amplified with the forward primer, 10f_link_N-term forward (5’ CAG GTT CGG GAC GAA GAC GAC AGG CC CAG GTA AAG CTG GAG GAG TCT G 3’), and the reverse primer, Link_CBM2a_C-term reverse (5’ GAG ATC TTC CGA GGA TCC GCC GAC CGT GCA GGG CGT G 3’) (Figure 13). The PCR product was 800 base pairs in length. The 10f-CBM2a PCR product was gel purified using a QIAquick® Gel Extraction Kit (QIAGEN Sciences, Germantown, Maryland, USA).

Figure 13. Assembly of PCR 3 product (5’-BbsI-1222-III10f-linker-3’) and PCR 4 product (5’-linker-CBM2a-BamHI-3’) into a DNA template for 10f-CBM2a fusion protein (800-bp).
2.2.12 Cloning the CBM2a-10f and the 10f-CBM2a PCR product into the pSJF2H vector

The pSJF2H vector was designed for periplasmic expression of sdAbs in *E. coli* (Tanha et al. 2003) (Figure 14). The sdAb cloning site is located within the *lacZ* gene to allow for IPTG-induced expression of the recombinant protein. The CBM2a-10f PCR product and the 10f-CBM2a PCR product were each cloned into a pSJF2H vector between the *Bbs*I and the *Bam*HI restriction sites (Figure 14). Within each pSJF2H vector, the PCR product was flanked by an N-terminal *ompA* gene and C-terminal *c-myc* and *his*<sub>6</sub> sequences (Figure 14). The OmpA signal peptide was included to direct the transport of the fusion protein-c-Myc-*His*<sub>6</sub> to the periplasm. In the periplasm, the OmpA signal peptide is cleaved by proteases to allow the fusion protein-c-Myc-*His*<sub>6</sub> to fold into its native conformation. The *His*<sub>6</sub> tag was included to facilitate purification and detection of the fusion protein.

![Figure 14](image.png)

*Figure 14.* Map of the pSJF2H expression vector cloning site courtesy of Tanha et al. (2003).

### 2.2.12.1 *Bam*HI digestion of the CBM2a-10f and the 10f-CBM2a PCR product

The CBM2a-10f PCR product and the 10f-CBM2a PCR product were digested with the *Bam*HI restriction endonuclease. Each reaction was performed in a 50-μl volume containing 1X NEBuffer 3, 1X BSA, 40-units of *Bam*HI (New England Biolabs,
Pickering, Ontario, Canada), and all of the PCR product (i.e., 348-ng CBM2a-10f PCR product and 988.4-ng 10f-CBM2a PCR product). The reactions were incubated at 37°C overnight. The digested PCR product was purified using a QIAquick® PCR purification kit (QIAGEN Sciences, Germantown, Maryland, USA).

2.2.12.2 BpiI digestion of the CBM2a-10f and 10f-CBM2a PCR product

The restriction endonuclease BpiI has the same restriction site as BbsI. BpiI was used instead of BbsI due to the enhanced stability of BpiI. The CBM2a-10f PCR product and the 10f-CBM2a PCR product were digested with BpiI. Each reaction was performed in a 50-µl volume containing 1X Buffer G (with BSA), 20-units of BpiI (Fermentas, Burlington, Ontario, Canada), and all of the BamHI-digested PCR product (i.e., 177-ng CBM2a-10f PCR product and 688.8-ng 10f-CBM2a PCR product). The reactions were incubated at 37°C overnight. The digested assembly PCR product was purified using a QIAquick® PCR purification kit (QIAGEN Sciences, Germantown, Maryland, USA).

2.2.12.3 BamHI and BpiI digestion of pSJF2H vector

The pSJF2H vector was digested with BamHI and BpiI as described for the 10f-CBM2a and CBM2a-10f PCR products (Sections 2.2.12.1 and 2.2.12.2). The digested pSJF2H vector was prepared by Shenghua Li (NRC-IBS, Ottawa, Ontario). The BamHI and BpiI-digested pSJF2H vector was dephosphorylated using 1-µg alkaline phosphatase (Roche Applied Science, Mannheim, Germany) for 1-hour at 37°C. The dephosphorylated pSJF2H vector was purified using a QIAquick® PCR purification kit (QIAGEN Sciences, Germantown, Maryland, USA).
2.2.12.4 Ligation of the CBM2a-10f and the 10f-CBM2a PCR product with the pSJF2H vector

The BamHI and BpiI-digested CBM2a-10f and 10f-CBM2a PCR products were ligated with the BamHI and BpiI-digested and dephosphorylated pSJF2H vector. Each 20-μl reaction volume contained 1X DNA ligase reaction buffer, 1-unit T4 DNA ligase (Invitrogen, Carlsbad, California, USA), 26-ng pSJF2H vector, and 45-ng CBM2a-10f or 10f-CBM2a PCR product. The reactions were incubated for 1-hour at room temperature.

2.2.12.5 Transformation of E. coli with recombinant pSJF2H Vector

After ligation of the CBM2a-10f and the 10f-CBM2a PCR product with the pSJF2H vector, the recombinant pSJF2H vector was transformed into E. coli. Electrocompetent TG1 E. coli cells were prepared by Yan Luo (NRC-IBS, Ottawa, Ontario, Canada). The CBM2a-10f and the 10f-CBM2a ligation products (20-μl each) were diluted to 100-μl each with sterile water. Two-μl of each diluted ligation product was added to 50-μl of electrocompetent TG1 E. coli cells. The TG1 E. coli cells were transformed with the recombinant pSJF2H vectors by electroporation. The transformed cells were added to 1-ml SOC media and incubated for 1-hour at 37°C with shaking at 200-rpm. The pSJF2H vector (Tanha et al. 2003), which is derived from the pUCE8 vector (Narang et al. 1987), contains an ampicillin resistance gene. Each 1-ml of transformed cells in SOC was plated on 3 preheated LB ampicillin plates containing 100-μg/ml ampicillin to select for clones having the pSJF2H vector (2 plates with 200-μl SOC/plate and 1 plate with 600-μl SOC/plate). The plates were incubated overnight at 32°C.
2.2.13 Colony PCR of the CBM2a-10f and the 10f-CBM2a transformed clones

Colony PCR was performed on the CBM2a-10f and the 10f-CBM2a recombinant E. coli clones isolated from the LB ampicillin plates to verify that the clones contained an insert of the correct size. Primers (Invitrogen, Carlsbad, California, USA) which bind to the lacZ gene of the pSJF2H cloning site (Figure 14) were used to amplify the CBM2a-10f and the 10f-CBM2a DNA inserts. The sequence of the forward primer was 5’ G CGG ATA ACA ATT TCA CAC AGG AA 3’. The sequence of the reverse primer was 5’ CG CCA GGG TTT TCC CAG TCA CGA C 3’. The expected size of the colony PCR product was 1,002 base pairs consisting of (from 5’ to 3’): part of the lacZ gene (67-bp), the ompA leader sequence (57-bp), two codons from the BbsI restriction site (6-bp), the CBM2a-10f or 10f-CBM2a sequence (756-bp), one codon from the BamHI restriction site (3-bp), the c-myc and his6 sequence (54-bp), two stop codons (6-bp), and part of the lacZ gene (53-bp). Colony PCR was performed on 16 CBM2a-10f recombinant clones and 16 10f-CBM2a recombinant clones. Colony PCR was performed in 15-µl volumes containing 1X Expand High Fidelity Buffer with 22.5-nmol MgCl2 (Roche Applied Science, Mannheim, Germany), 3-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), 1.5-pmol forward primer, 1.5-pmol reverse primer (Invitrogen, Carlsbad, California, USA), 0.525-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany), and 1-µl from a 50-µl sterile water suspension of each recombinant clone. The PCR conditions used were 94°C for 5-min, 30 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min.

A second colony PCR screened 48 CBM2a-10f recombinant clones and 48 10f-CBM2a recombinant clones. Colony PCR reactions were performed in 10-µl volumes.
containing 1X Expand High Fidelity Buffer with 22.5-nmol MgCl₂ (Roche Applied Science, Mannheim, Germany), 2-nmol of each dNTP (New England Biolabs, Pickering, Ontario, Canada), 1-pmol forward primer, 1-pmol reverse primer (Invitrogen, Carlsbad, California, USA), 0.5-units Expand High Fidelity Enzyme mix (Roche Applied Science, Mannheim, Germany), and 1-µl from a 50-µl sterile water suspension of each recombinant clone. The PCR conditions used were 94°C for 5-min, 30 cycles of 94°C for 30-s, 55°C for 30-s, and 72°C for 1-min, followed by 72°C for 7-min.

2.2.14 Screening CBM2a-10f and 10f-CBM2a clones for expression of a His₆-tagged fusion protein using Western blot

The clones which had a band of the expected size (1,002 bp) as determined by colony PCR were further screened for expression of a His₆-tagged protein of the expected size (28.6-kDa). A 2-ml culture of each CBM2a-10f and 10f-CBM2a clone with the insert of expected size was prepared. Two-ml of 2xYT containing 100-µg/ml ampicillin and 10-µl 20% glucose was inoculated with 5-µl of a 50-µl sterile water suspension of the selected clone and incubated overnight at 37°C. 100-µl of each overnight culture was subcultured in 2-ml 2xYT containing 100-µg/ml ampicillin. The subcultures were incubated for 1.5-hours at 37°C until the OD reached 0.5-0.7. 1-mM IPTG was added to each subculture to induce protein expression. The subcultures were incubated for 4-hours at 37°C. A 20-µl sample of each subculture was used in SDS-PAGE and Western blotting (Section 2.2.15).
2.2.15 SDS-PAGE and Western blotting

The SDS-PAGE gels were composed of a 4% bis-acrylamide stacking gel and a 12.5% bis-acrylamide resolving gel. The 1X SDS-PAGE gel-loading buffer contained 50-mM Tris-HCl (pH 6.8), 100-mM dithiothreitol, 2% (w/v) SDS (electrophoresis grade), 0.1% bromophenol blue, and 10% (w/v) glycerol. A sample of each protein of interest (usually 20-μl) was added to an equal volume of 1X SDS-PAGE gel-loading buffer and incubated for 5-min at 95°C. Ten-μl of each sample was loaded into a well of an SDS-PAGE gel. The gel was electrophoresed at ca.100-V for 90-min. The gel was stained for 45-min in Coomassie Brilliant Blue, destained overnight, and then dried. A similar gel was produced and used in Western blotting.

During Western blotting, the protein electrophoresed in the SDS-PAGE gel was transferred to Immobilon™-P Transfer Membrane (Millipore, Billerica, Massachusetts, USA) made of PVDF microporous membrane. The transfer process was carried out at 100-V for 60-min. After the transfer, the PVDF membrane was blocked with 3% skim milk in 1X TBST for 30-min to overnight. The membrane was probed with mouse anti-6-His IgG (GE Healthcare, Baie d’Urfé, Quebec, Canada) diluted 1:2,500 in equal parts 1X TBST and 3% skim milk for 1-hour, washed 5X with 10-ml 1X TBST (3-min per wash), probed with goat-anti-mouse IgG conjugated to alkaline phosphatase (Cedarlane Laboratories Ltd., Burlington, Ontario, Canada) diluted 1:5,000 in equal parts 1X TBST and 3% skim milk for 30-min, washed 5X with 10-ml 1X TBST (3 min per wash), and developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, California, USA). Once bands appeared on the membrane, the membrane was washed with water and dried.
2.2.16 Sequencing plasmid DNA from CBM2a-10f and 10f-CBM2a clones expressing a His$_6$-tagged protein of expected size

Plasmid DNA was extracted and sequenced from the clones expressing the His$_6$-tagged protein of the expected size (28.6-kDa) on Western blot. The selected CBM2a-10f and 10f-CBM2a clones were incubated overnight at 37°C in 3-ml LB with 100-μg/ml ampicillin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Glycerol stocks of each overnight culture were prepared (0.5-ml 70% glycerol and 0.5-ml overnight culture) and stored at -80°C. Plasmid DNA was extracted from the overnight cultures using a QIAprep® Spin Miniprep Kit (QIAGEN Sciences, Germantown, Maryland, USA) and sequenced. Sequencing was performed by Sonia Leclerc (NRC-IBS, Ottawa, Ontario, Canada). Sequencing reactions were performed in 6-μl volumes containing 4-pmol primer, ca.100-ng plasmid DNA, and sterile water (Millipore, Billerica, Massachusetts, USA) to make the volume up to 6-μl. The same primers (Invitrogen, Carlsbad, California, USA) used for colony PCR were used in the sequencing reactions. Each sample was sequenced two times, once with the forward primer (5’ G CGG ATA ACA ATT TCA CAC AGG AA 3’), and once with the reverse primer (5’ CG CCA GGG TTT TCC CAG TCA CGA C 3’). The sequences were analyzed using the Lasergene 6 software package (DNASTAR, Inc., Madison, Wisconsin, USA).

2.2.17 Periplasmic protein expression of the CBM2a-10f I clone

Based on the plasmid DNA sequence results, the CBM2a-10f I clone was chosen for periplasmic expression. A starter culture containing 100-ml M9 medium with 0.1-mg/ml ampicillin and 4-mg/ml casamino acids was inoculated with a CBM2a-10f I colony and incubated at 25°C overnight with shaking at 250-rpm. After incubation, 30-ml
of the starter culture was transferred to 1-L M9 medium containing 0.1-mg/ml ampicillin and 4-mg/ml casamino acids and incubated at 25°C with shaking at 250-rpm overnight. After incubation, periplasmic protein expression was induced by adding 0.15-mM IPTG and 100-ml 10X TB nutrients. The culture was incubated at 25°C for 48-hours with shaking at 250-rpm.

2.2.18 Periplasmic protein extraction from a 1-L culture using TES Buffer

*E. coli* cells were harvested by centrifuging at 4,000-g for 15-min at 4°C. The supernatant was discarded and the cell pellet was placed on ice. The cell pellet was re-suspended in 20-ml ice-cold TES Buffer (0.2-M tris-HCl pH 8.0, 0.5-mM EDTA, 20% sucrose) and chilled on ice for 30-min. Thirty-ml ice-cold 1/8 TES buffer was added to the cell suspension. The cell suspension was vortexed, chilled on ice for 30-min, and centrifuged at 14,000-g for 30-min at 4°C. The supernatant (containing the periplasmic protein) was retained and dialyzed against 6-L of Buffer A (10-mM HEPES, 500-mM NaCl pH 7.0) overnight. The dialyzed supernatant was sterile filtered through a 0.22-μm filter and stored at 4°C. Ten-μl of the supernatant was used in SDS-PAGE and Western blotting (Section 2.2.15) to verify that the supernatant contained the 28.6-kDa His₆-tagged protein of interest.

2.2.19 Purification of the CBM2a-10f I fusion protein

2.2.19.1 *Loading the IMAC column*

IMAC was used for purification. A 5-ml HiTrap IMAC HP™ column (GE Healthcare, Baie d’Urfé, Quebec, Canada) was charged with Ni(II). Using a peristaltic pump, the IMAC column was washed with 10-ml of water, followed by 25-ml of Buffer
A (10-mM HEPES, 500-mM NaCl pH 7.0). Buffer B (2%; 10-mM HEPES, 500-mM NaCl, 500-mM imidazole, pH 7.0) was added to the CBM2a-10f I solution recovered from the periplasmic protein extraction and the solution was loaded onto the IMAC column at 1-ml/minute using a peristaltic pump. The column was washed with 25-ml of a 98% Buffer A, 2% Buffer B solution.

2.2.19.2 FPLC protein purification

The IMAC column containing the CBM2a-10f I solution was attached to an ÄKTAPure™ (GE Healthcare, Baie d’Urfé, Quebec, Canada) for elution of the CBM2a-10f I His₆-tagged fusion protein. The fusion protein was eluted using a combination of Buffer B and Buffer A at a flow rate of 1-ml/minute. During protein elution, the Buffer B concentration increased from 0% to 100% over a 50-min period while the Buffer A concentration decreased from 100% to 0% over the same time period. The UV absorbance of the buffer passing through the column was monitored at 280-nm to identify the elution volumes containing the His₆-tagged fusion protein. Samples of the eluted fractions that corresponded to a peak in UV absorbance were analyzed by SDS-PAGE.

2.2.20 Estimating concentration and yield of purified CBM2a-10f I protein

Based on the SDS-PAGE results, the eluted fractions containing the highest concentration of CBM2a-10f I fusion protein were combined. The combined fractions were dialyzed overnight against 6-L protein dialysis buffer (0.2-M Na₂HPO₄, 0.2-M NaH₂PO₄·H₂O, 150-mM NaCl, and 0.5-mM EDTA). The following formula was used to estimate the protein concentration (C) of the dialyzed CBM2a-10f I protein:

\[ C = \frac{A_{280}}{\Sigma} \times MW \]
where \( A_{280} \) is the absorbance of the protein at 280-nm, \( \sum \) is the extinction coefficient of the protein at 280-nm, and \( MW \) is the formula molecular weight of the protein. The absorbance of the dialyzed CBM2a-10f I protein was measured at 280-nm using the dialysis buffer as a blank. Since the \( A_{280} \) of the dialyzed CBM2-10f I protein was greater than 1, the CBM2a-10f I protein sample was diluted 1:1 in dialysis buffer and the \( A_{280} \) was measured again. The extinction coefficient of the CBM2a-10f I protein at 280-nm was computed using the program ProtParam on the ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/protparam.html). The molecular weight of the CBM2a-10f I protein was computed using EditSeq™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA). To prevent microbial contamination, 0.02% NaN\(_3\) was added to the dialyzed CBM2a-10f I protein and the protein was stored at 4ºC.

**2.2.21 Binding kinetics of the 1222-III10f V\(_{13}\)H and the CBM2a-10f I protein to immobilized M13 phage**

SPR performed with BIACORE 3000 biosensor system (GE Healthcare, Baie d’Urfé, Quebec, Canada) was used to evaluate and compare the binding kinetics of the 1222-III10f V\(_{13}\)H and the CBM2a-10f I protein to immobilized M13 phage. All SPR studies were performed by Tomoko Hirama and Henk van Faassen (NRC-IBS, Ottawa, Ontario, Canada) as described in Section 2.2.2.
2.3 Results

2.3.1 Binding kinetics of the V_{H}H monomers and pentamers to immobilized M13 phage

BIACORE 3000 sensorgrams were generated for the 1213-16j, 1222-16e, and 1222-III10f V_{H}H monomers and pentamers binding to immobilized M13 phage (Figure 15A, Figure 16A, Figure 17A, and Figure 18). Nonspecific binding of each monomer or pentamer to BSA on the reference flow cell was subtracted from the observed binding of the monomer or pentamer to the M13 phage. Table 2 summarizes the data obtained from the monomer sensorgrams. The binding was quantified using R_{max}, the dissociation constant (K_{D}), the dissociation rate constant (k_d), and the association rate constant (k_a). R_{max} is the response when all available sites on the BIACORE flow cell are occupied by antibody. R_{max} was measured in resonance units (RU) which are a measure of the surface capacity (1-RU = 1-pg/mm²). K_{D} is a measure of the affinity or the strength of binding of an antibody to its antigen. K_{D} is defined as the concentration of antibody required to achieve ½ R_{max}. All K_{DS} were determined from steady state analysis, i.e. the response when equilibrium is achieved between bound and unbound antibody (Figure 15B, Figure 16B, and Figure 17B). k_d is an estimate of how fast an antibody dissociates from its antigen, i.e. the dissociation kinetics. k_a is a measure of how fast an antibody binds to its antigen, i.e. the association kinetics. The k_a was calculated from k_d/K_{D}. Complex interactions between a V_{H}H and M13 prevented the calculation of k_d and k_a for some monomers. Complex interactions may be the result of steric hindrance among the bound antibody molecules. Alternatively, aggregate formation within the V_{H}H sample may result in complex interactions. Purification of the anti-M13 V_{H}Hs with a Superdex 75 column (GE Healthcare, Baie d’Urfé, Quebec, Canada) prior to SPR analyses ensured
that aggregate contamination was avoided. As shown in the sensorgram for the 1213-16j monomer (Figure 15A), the four highest concentrations of 1213-16j monomer (0.74-μM to 6-μM) unexpectedly did not demonstrate exponential decay during dissociation from the M13 phage. 1213-16j was hypothesized to bind to pIII on the M13 phage. Perhaps at high concentrations of 1213-16j, stacking of the 1213-16j monomers on the pIII epitopes trapped some of the bound 1213-16j monomers and prevented their dissociation, resulting in non-exponential decay (Figure 15A).

All three V_{H}H monomers demonstrated affinity for the immobilized M13 phage (Table 2). The 1213-16j and 1222-III10f monomers had K_{D} values in the nanomolar range while monomer 1222-16e had a K_{D} value in the micromolar range. The 1222-III10f monomer had the smallest K_{D} and thus the highest affinity for immobilized M13 phage (Table 2). The binding of the pentamers to immobilized M13 phage was essentially irreversible (Figure 18). During a 600-s time period, none of the pentamers were observed to dissociate from the immobilized M13 phage (Figure 18).

Figure 15. A. BIACORE 3000 sensorgram for the 1213-16j monomer binding to immobilized M13 phage. B. Steady state fitting used to determine the dissociation constant (K_{D}) for the 1213-16j monomer binding to immobilized M13 phage. Samples of 1213-16j monomer at concentrations ranging from 0.1-6-μM were permitted to bind to immobilized M13 phage.
Figure 16. A. BIACORE 3000 sensorgram for the 1222-16e monomer binding to immobilized M13 phage. B. Steady state fitting used to determine the dissociation constant (K_D) for the 1222-16e monomer binding to immobilized M13 phage. Samples of 1222-16e monomer at concentrations ranging from 0.2-10.3-μM were permitted to bind to immobilized M13 phage.

Figure 17. A. BIACORE 3000 sensorgram for the 1222-III10f monomer binding to immobilized M13 phage. B. Steady state fitting used to determine the dissociation constant (K_D) for the 1222-III10f monomer binding to immobilized M13 phage. Samples of 1222-III10f monomer at concentrations ranging from 15.6-500-nM were permitted to bind to immobilized M13 phage.
Figure 18. BIACORE 3000 sensorgram for the 1222-III10f (10-nM), 1222-16e (5-nM), and 1213-16j (1-nM) pentamers binding to immobilized M13 phage.

Table 2. SPR (BIACORE 3000) binding of three llama V_{H}H monomers, 1213-16j, 1222-16e, and 1222-III10f, to immobilized M13 phage. The binding was quantified using the dissociation constant (K_{D}), dissociation rate constant (k_{d}), association rate constant (k_{a}), and R_{max} (RU). Missing values in the table were not computable due to complex antigen-antibody interactions.

<table>
<thead>
<tr>
<th>Llama V_{H}H</th>
<th>K_{D} (M)</th>
<th>k_{d} (s^{-1})</th>
<th>k_{a} (M^{-1}s^{-1})</th>
<th>R_{max} (RU)</th>
</tr>
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<tr>
<td>1213-16j</td>
<td>5 x 10^{-7}</td>
<td>ca. 0.2</td>
<td>-</td>
<td>410</td>
</tr>
<tr>
<td>1222-16e</td>
<td>3 x 10^{-6}</td>
<td>ca. 0.4</td>
<td>ca. 2 x 10^{5}</td>
<td>280</td>
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<tr>
<td>1222-III10f</td>
<td>6 x 10^{-8}</td>
<td>-</td>
<td>-</td>
<td>150</td>
</tr>
</tbody>
</table>

*The k_{d} for 1213-16j was calculated based on the three lowest concentrations of 1213-16j (0.1 μM-0.37-μM) used in the analysis, all of which demonstrated exponential decay during dissociation.

2.3.2 BIACORE epitope mapping for selected V_{H}H monomers

BIACORE epitope mapping was used to determine if the V_{H}H monomers shared similar binding sites on the M13 phage capsid. Two monomers were added sequentially to M13 phage immobilized on a BIACORE sensor chip flow cell. The first monomer was added at a concentration several fold greater than its K_{D} to ensure the immobilized M13 phage was saturated with V_{H}H prior to addition of the second monomer. Monomer pairs which gave two distinct response signals were assumed to have non-overlapping
epitopes. Alternatively, monomer pairs which did not produce distinct response signals were assumed to have overlapping epitopes, or epitopes which were in close proximity on the phage capsid.

To generate an epitope map for the 1222-16e and the 1213-16j monomers using SPR, 6-μM 1222-16e was added to immobilized M13 phage followed by the addition of 5-μM 1213-16j (Figure 19). The BIACORE 3000 sensorgram for the 1222-16e and the 1213-16j monomers depicted two distinct and additive response signals, indicating that these two monomers have distinctly different binding sites on the surface of M13 (Figure 19).

![Figure 19. BIACORE 3000 sensorgram for the 1222-16e (6-μM) and the 1213-16j (5-μM) monomers binding to immobilized M13 phage.](image)

To generate an epitope map for the 1222-III10f and the 1213-16j monomers using SPR, 1-μM 1222-III10f was permitted to bind to immobilized phage followed by the addition of 5-μM 1213-16j (Figure 20). The BIACORE 3000 sensorgram for the 1222-III10f and the 1213-16j monomers depicted two response signals, indicating that the 1222-III10f and the 1213-16j monomers bound to different epitopes on the M13 phage.
The slope of the second signal (response to 1213-16j binding) was reduced compared to
the slope of the first signal (response to 1222-III10f binding) suggesting that the binding
of 1222-III10f interfered with the binding of 1213-16j (Figure 20).

![Sensorgram for 1222-III10f and 1213-16j binding](image)

**Figure 20.** BIACORE 3000 sensorgram for the 1222-III10f (1-μM) and the 1213-16j (5-
μM) monomers binding to immobilized M13 phage.

To generate an epitope map for the 1222-III10f and the 1222-16e monomers, 1-μM
1222-III10f was permitted to bind to immobilized phage followed by the addition of 6-
μM 1222-16e (Figure 21). The BIACORE 3000 sensorgram for the 1222-III10f and the
1222-16e monomers displayed two response signals which were barely distinguishable,
due to a limited response by the 1222-16e monomer. The epitopes of 1222-III10f and
1222-16e were overlapping or in close proximity on the M13 phage; thus, steric
hindrance from bound 1222-III10f interfered with and prevented the binding of 1222-16e
(Figure 21).
Figure 21. BIACORE 3000 sensorgram for the 1222-III10f (1-μM) and the 1222-16e (6-μM) monomers binding to immobilized M13 phage.

2.3.3 ELISA to determine sensitivity of anti-M13 monomers for detection of M13 phage

The sensitivity of the anti-M13 V_{H} monomers for detection of immobilized M13 phage was compared using an ELISA (Figure 22). A limit of detection was established for each monomer, indicating the minimum concentration of M13 phage (in pfu) each monomer was capable of detecting. Overall, the 1222-III 10f monomer gave the strongest signal followed by the 1213-16j monomer, and the 1222-16e monomer. The commercially available anti-M13 mouse monoclonal conjugated to HRP (GE Healthcare, Baie d’Urfé, Quebec, Canada) had a similar absorbance to the 1222-III 10f monomer. The limit of detection for all antibodies tested was 3x10^{11} pfu. According to the product information for the commercial mouse anti-M13-HRP, 40-ng of this antibody can detect 2.5 x 10^9 pfu/ml M13 phage; however, in this experiment, the commercial mouse anti-M13-HRP was found to be 100-fold less sensitive. In general, the A_{450} values for these ELISAs were not high (all less than 1.0) and quite variable (large standard error of the
mean). Furthermore, all three monomers exhibited an unexpected increase in absorbance as the M13 pfu/well decreased, prior to the limit of detection. The mean $A_{450}$ value for the control wells without antibody was 0.068. The mean $A_{450}$ value for the control wells without M13 phage was 0.066. Thus, contamination by antibody or M13 was not present (Figure 22).

![Graph](image)

**Figure 22.** Absorbance at 450-nm of three V12H monomers, 1222-III10f, 1213-16j, and 1222-16e, and the commercially available anti-M13 mouse monoclonal conjugated to HRP (GE Healthcare, Baie d’Urfé, Quebec, Canada) when bound to immobilized M13 phage at concentrations ranging from $3 \times 10^{13}$ to $3 \times 10^{6}$ pfu/well. The monomers were applied to the plate at a concentration of 2000-ng/well. The anti-M13 mouse monoclonal conjugated to HRP (GE Healthcare, Baie d’Urfé, Quebec, Canada) was applied to the plate at a concentration of 80-ng/well. Each data point represents the mean of two replicates of this experiment. The error bars indicate the standard error of the mean for each data point. (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, [www.graphpad.com](http://www.graphpad.com)).

2.3.4 ELISA to determine the sensitivity of the anti-M13 pentamers for detection of M13 phage

The sensitivity of the anti-M13 V12H pentamers for detection of immobilized M13 phage was compared using an ELISA (Figure 23). A limit of detection was established
for each pentamer, indicating the minimum concentration of M13 phage (in pfu) each pentamer was capable of detecting. As expected, the absorbance at 450-nm decreased as the concentration of M13 bound to the wells decreased. A slight increase or leveling in absorbance was observed from $2.8 \times 10^{12} - 2.8 \times 10^{11}$ pfu/well. The 1222-III10f pentamer gave the strongest signal followed by the 1213-16j pentamer, the 1222-16e pentamer, and the commercially available anti-M13 mouse monoclonal conjugated to HRP (GE Healthcare, Baie d’Urfé, Quebec, Canada). The largest standard error of the mean based on two replicates was observed for the wells with $2.8 \times 10^{13}$ pfu M13. The mean $A_{450}$ value for the control wells without antibody was 0.0625. The mean $A_{450}$ value for the control wells without M13 phage was 0.0654. Thus, contamination by antibody or M13 was not present. The limit of detection for all antibodies tested was $2.8 \times 10^{11}$ pfu. The experimental detection limit of the commercial mouse anti-M13-HRP was 100-fold less than the detection limit published in the product information (Figure 23).
Figure 23. Absorbance at 450-nm of three pentamers, 1222-III10f, 1213-16j, and 1222-16e, and the commercially available anti-M13 mouse monoclonal conjugated to HRP (GE Healthcare, Baie d’Urfé, Quebec, Canada) when bound to immobilized M13 phage at concentrations ranging from 2.8 x 10^{13} to 2.8 x 10^{4} pfu/well. All antibodies were applied to the plate at a concentration of 80-ng/well. Each data point represents the mean of two replicates of this experiment. The error bars indicate the standard error of the mean for each data point. (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com).

2.3.5 Design of the CBM-V_{H}H fusion protein

2.3.5.1 Amplification of the CBM2a template DNA for use in the CBM2a-10f protein and the 10f-CBM2a protein

The CBM2a template DNA was PCR amplified to add restriction sites for cloning into the pSJF2 expression vector, and a proline-threonine sequence for linking to the 1222-III10f V_{H}H DNA (Figure 24). Several PCR reactions were done to achieve this goal. First, the CBM2a template DNA was amplified to attach a BbsI cut site (5’) and a proline-threonine linker (3’) to the CBM2a DNA for use in CBM2a-10f fusion protein. Next, the CBM2a template DNA was amplified to add part of a proline-threonine linker
(5’) and a BamHI cut site (3’) to the CBM2a DNA for use in 10f-CBM2a fusion protein. Both PCR reactions resulted in a PCR product of expected size (Figure 24).

**Figure 24.** Agarose (1%) gel run at 100-V. Lane 1 contains 2-μl of the 5’-*Bbs*I-CBM2a-linker-3’ PCR product (413 base pairs). Lane 2 contains 2-μl of the 5’-linker-CBM2a-*Bam*HI-3’ PCR product (363 base pairs). Lane 3 contains 1.5-μl Quick-Load 100 base pair DNA ladder (50-μg/ml) (New England Biolabs, Pickering, Ontario, Canada).

2.3.5.2 Amplification of the 1222-III10f \( V_{HH} \) template DNA for use in the CBM2a-10f protein and the 10f-CBM2a protein

The 1222-III10f \( V_{HH} \) template DNA was PCR amplified to add restriction sites for cloning into the pSJF2H expression vector, and a proline-threonine sequence for linking to the CBM2a DNA (Figure 25). First, the 1222-III10f \( V_{HH} \) DNA was amplified to add part of the proline-threonine linker (5’) and a *Bam*HI cut site (3’) to the 1222-III10f DNA for use in the CBM2a-10f fusion protein. Next, the 1222-III10f \( V_{HH} \) DNA
was amplified to add a BbsI cut site (5’) and a proline-threonine linker (3’) to the 1222-III10f DNA for use in the 10f-CBM2a fusion protein. Both PCR reactions resulted in PCR product of the expected size (Figure 25).

**Figure 25.** Agarose (1%) gel run at 100-V. Lane 1 contains 1.5-μl Quick-Load 100 base pair DNA ladder (50-μg/ml) (New England Biolabs, Pickering, Ontario, Canada). Lane 2 contains 2-μl 5’-linker-1222-III10f-BamHI-3’ PCR product (408 base pairs). Lane 3 contains 2-μl 5’-BbsI-1222-III10f -linker-3’ PCR product (458 base pairs).

### 2.3.5.3 PCR assembly of DNA templates for the CBM2a-10f and the 10f-CBM2a proteins

The 5’-BbsI-CBM2a-linker-3’ and the 5’-linker-1222-III10f-BamHI-3’ PCR products were combined into a DNA template for the CBM2a-10f fusion protein, i.e. a product of 800-bp (Figure 26). The 5’-BbsI-1222-III10f -linker-3’ and the 5’-linker-CBM2a-BamHI-3’ PCR products were combined into a DNA template for the 10f-CBM2a fusion protein (800-bp; Figure 26).
Figure 26. Agarose (1%) gel run at 100-V. Lane 1 contains 1.5-μl Quick-Load 100 base pair DNA ladder (50-μg/ml) (New England Biolabs, Pickering, Ontario, Canada). Lane 2 contains 2-μl of the CBM2a-10f DNA template (800 base pairs). Lane 3 contains 2-μl of the 10f-CBM2a DNA template (800 base pairs).

2.3.6 Colony PCR of the CBM2a-10f and the 10f-CBM2a transformed clones

The CBM2a-10f and the 10f-CBM2a DNA templates were digested with the restriction enzymes BamHI and BpiI and ligated into pSJF2H expression vectors. The recombinant pSJF2H expression vectors were transformed into *E. coli*. Colony PCR of the CBM2a-10f and the 10f-CBM2a transformed clones identified clones that contained an insert of the expected size (1002-bp) at the pSJF2H cloning site. Eight of the 16 CBM2a-10f clones evaluated (lanes 4, 8-12, 14, and 15 of Figure 27) had an insert of the expected size. Twelve of the 16 10f-CBM2a clones evaluated (lanes 4-7 and 9-16 of Figure 28) had an insert of the expected size.
Figure 27. Colony PCR product from 16 CBM2a-10f transformed *E. coli* clones run at 100-V on a 1% agarose gel. Lanes 1-16 contained 5-μl CBM2a-10f colony PCR products (1,002-bp). Lane 17 contained 1.5-μl Quick-Load 100-bp DNA ladder (New England Biolabs, Pickering, Ontario, Canada).

Figure 28. Colony PCR product from 16 10f-CBM2a recombinant *E. coli* clones run at 100-V on a 1% agarose gel. Lanes 1-16 contained 5-μl 10f-CBM2a colony PCR products (1,002-bp). Lane 17 contained 1.5-μl Quick-Load 100-bp DNA ladder (New England Biolabs, Pickering, Ontario, Canada).

2.3.7 Screening of CBM2a-10f and 10f-CBM2a clones for expression of a His$_6$-tagged fusion protein using Western blot

The CBM2a-10f and the 10f-CBM2a clones, which had a band of the expected size (1,002-bp) on colony PCR, were further screened for expression of a His$_6$-tagged protein of the expected size using SDS-PAGE and Western blot. A purified His$_6$-tagged protein was run on each SDS-PAGE gel as a positive control. After cleavage of the OmpA leader sequence in the periplasm, the expected molecular weight of the CBM2a-10f and the 10f-CBM2a fusion proteins was 28,612.38-Da (EditSeq™6.1, DNASTAR, Inc., Madison, Wisconsin, USA). During initial screening of the CBM2a-10f clones, one
CBM2a-10f clone was identified expressing a His₆-tagged protein of the expected size (28.6-kDa) (lane 1, Figure 29). This clone was labeled CBM2a-10f I. During a subsequent screening of the CBM2a-10f clones, another CBM2a-10f clone was identified expressing a 28.6-kDa His₆-tagged protein (lane 10, Figure 30). This clone was labeled CBM2a-10f 7. During initial screening of the 10f-CBM2a clones, one 10f-CBM2a clone was identified that expressed a His₆-tagged protein of 28.6-kDa (lane 3, Figure 31). This clone was labeled 10f-CBM2a I. During a subsequent screening of the 10f-CBM2a clones, two more 10f-CBM2a clones were identified that expressed a 28.6-kDa His₆-tagged protein (lanes 1 and 5, Figure 32). The clone in lane 1 was labeled 10f-CBM2a 6. The clone in lane 5 was not labeled (Figure 32).

**Figure 29.** Western blot of proteins expressed by CBM2a-10f clones (Lanes 1-5). The blot was probed with mouse anti-6-His IgG (GE Healthcare, Baie d’Urfé, Quebec, Canada) for proteins containing a His₆ tag. Lane 6 contained a positive control. Lane 7 contained 5-μl Prestained SDS-PAGE Broad Range Standard (Bio-Rad Laboratories, Hercules, California, USA).
Figure 30. Western blot of proteins expressed by CBM2a-10f clones (Lanes 4-10). The blot was probed with mouse anti-6-His IgG (GE Healthcare, Baie d’Urfé, Quebec, Canada) for proteins containing a His$_6$ tag. Lanes 2-3 contained positive controls. Lane 1 contained 5-μl Prestained SDS-PAGE Broad Range Standard (Bio-Rad Laboratories, Hercules, California, USA).

Figure 31. Western blot of proteins expressed by a 10f-CBM2a clone (Lane 3). The blot was probed with mouse anti-6-His IgG (GE Healthcare, Baie d’Urfé, Quebec, Canada) for proteins containing a His$_6$ tag. Lane 1 contained a positive control. Lane 2 contained 5-μl Prestained SDS-PAGE Broad Range Standard (Bio-Rad Laboratories, Hercules, California, USA).
2.3.8 Sequencing plasmid DNA from CBM2a-10f and 10f-CBM2a clones expressing a His$_6$-tagged protein of expected size

Plasmid DNA was extracted and sequenced from the CBM2a-10f and 10f-CBM2a clones expressing the His$_6$-tagged protein of the expected size (28.6-kDa) on Western blot. Compared to the expected CBM2a-10f DNA sequence, the CBM2a-10f I plasmid DNA had a G to A substitution at position 205 (Figure 33). This non-synonymous mutation resulted in a valine (V) to isoleucine (I) substitution at position 69 (sequential numbering) of the CBM2a-10f I protein sequence (Figure 34). After periplasmic cleavage of the OmpA leader sequence, the molecular weight of the CBM2a-10f I protein was predicted to be 28,626-Da (EditSeq™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA)). The valine to isoleucine substitution in the CBM2a-10f I protein explains why the molecular weight of the CBM2a-10f I protein was larger than its expected molecular weight of 28,612-Da (EditSeq™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA)).
Sequencing identified the CBM2a-10f 7 clone as having a plasmid DNA sequence and protein sequence identical to the expected CBM2a-10f DNA and protein sequences (Figure 35 and Figure 36). The 10f-CBM2a I plasmid DNA sequence had a G to A substitution at position 498 compared to the expected 10f-CBM2a DNA sequence (Figure 37). This non-synonymous substitution resulted in a tryptophan residue (W) being changed to an amber stop codon (.) at position 180 in the 10f-CBM2a I protein sequence (Figure 38). As a result of this mutation, the 10f-CBM2a I clone was not chosen for protein expression. Sequencing identified the 10f-CBM2a 6 clone as having a plasmid DNA sequence and protein sequence identical to the expected 10f-CBM2a DNA and protein sequences (Figure 39 and Figure 40).
Figure 33. Clustal W alignment of the CBM2a-10f I (labeled ‘CBM2aNtermI’) plasmid DNA sequence with the expected CBM2a-10f I (labeled ‘Expected’) DNA sequence (879 base pairs) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA). Nucleotides that did not match the expected CBM2a-10f I DNA sequence were boxed; in this case, one was substituted for G.
Figure 34. ClustalW alignment of the CBM2a-10f I (labeled ‘CBM2aNtermI’) protein sequence with the expected CBM2a-10f (labeled ‘Expected’) protein sequence (292 amino acids) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA). Amino acids that did not match the expected CBM2a-10f protein sequence were boxed; in this case I was substituted for V.
Figure 35. Clustal W alignment of the CBM2a-10f 7 (labeled ‘CBM2aNterm7’) plasmid DNA sequence with the expected CBM2a-10f (labeled ‘Expected’) DNA sequence (879 base pairs) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA).
Figure 36. ClustalW alignment of the CBM2a-10f (labeled ‘CBM2aNterm7’) protein sequence with the expected CBM2a-10f (labeled ‘Expected’) protein sequence (292 amino acids) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA).
Figure 37. Clustal W alignment of the 10f-CBM2a I (labeled ‘AntiM13Nterm’) plasmid DNA sequence with the expected 10f-CBM2a (labeled ‘Expected’) DNA sequence (879 base pairs) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA). Nucleotides that did not match with the expected 10f-CBM2a DNA sequence were boxed; in this case A was substituted for G.
Figure 38. ClustalW alignment of the 10f-CBM2a I (labeled ‘AntiM13Nterm’) protein sequence with the expected 10f-CBM2a (labeled ‘Expected’) protein sequence (292 amino acids) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA). Amino acids that did not match the expected 10f-CBM2a protein sequence were boxed; in this case an amber stop codon was substituted for W.
Figure 39. Clustal W alignment of the 10f-CBM2a 6 (labeled ‘AntiM13Nterm6’) plasmid DNA sequence with the expected 10f-CBM2a (labeled ‘Expected’) DNA sequence (879 base pairs) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA).
Figure 40. ClustalW alignment of the 10f-CBM2a 6 (labeled ‘AntiM13Nterm6’) protein sequence with the expected 10f-CBM2a protein sequence (labeled ‘Expected’) (292 amino acids) created using MegAlign™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA).

2.3.9 Periplasmic protein expression of the CBM2a-10f I clone

The CBM2a-10f I clone was chosen for periplasmic expression despite its valine (V) to isoleucine (I) substitution. The CBM2a-10f 7 and the 10f-CBM2a 6 clones had not yet been identified when the decision was made to proceed with expression of the CBM2a-10f I clone. Western blot confirmed that the periplasmic proteins extracted from the CBM2a-10f I culture contained a His6-tagged protein of the expected size (28.6-kDa) (lane 3, Figure 41).
Figure 41. Western blot of periplasmic proteins expressed by a CBM2a-10f I culture (lane 3). The blot was probed with mouse anti-6-His IgG (GE Healthcare, Baie d’Urfé, Quebec, Canada) for proteins containing a His<sub>6</sub>-tag. Lane 1 contained 5-μl Prestained SDS-PAGE Broad Range Standard (Bio-Rad Laboratories, Hercules, California, USA). Lane 2 contained a positive control. Lanes 3 contained 10-μl of the dialyzed and sterile filtered supernatant from the TES periplasmic protein extraction of a 1-L CBM2a-10f I culture.

2.3.10 FPLC purification of the CBM2a-10f I protein

The CBM2a-10f I solution recovered from the periplasmic protein extraction was loaded onto an IMAC column and purified using FPLC. The elution of the 28.6-kDa CBM2a-10f I His<sub>6</sub>-tagged fusion protein corresponded to a peak that eluted between the elution volumes of 24-30-ml (Figure 42). SDS PAGE of the eluted fractions confirmed that the CBM2a-10f I fusion protein was eluted between fractions 24-30-ml (lanes 9-15, Figure 43).
Figure 42. FPLC elution of the 28.6-kDa CBM2a-10f I His₆-tagged fusion protein from an IMAC column as demonstrated by UV absorbance at 280-nm (milli-absorbance units).

Figure 43. SDS PAGE of eluted fractions collected during FPLC purification of the CBM2a-10f I fusion protein. Lane 1 contained 10-μl fraction 9-ml. Lane 2 contained 5-μl prestained SDS-PAGE Broad Range Standard (Bio-Rad Laboratories, Hercules, California, USA). Lane 3 contained 10-μl fraction 15-ml and lanes 4-15 contained 10-μl each of fractions 19-30-ml.

2.3.11 Estimating concentration and yield of purified CBM2a-10f I fusion protein

Eluted fractions 26-28-ml obtained during FPLC purification of the CBM2a-10f I fusion protein were combined. The total volume of the combined fractions was ca. 3-ml. The combined fractions of CBM2a-10f I fusion protein were dialyzed overnight and the
concentration of the CBM2a-10f I fusion protein was estimated. The absorbance at 280-nm (A280) of the dialyzed CBM2a-10f I fusion protein diluted 1:1 in dialysis buffer was 0.966. The calculated extinction coefficient (\(\varepsilon\)) at 280-nm of the CBM2a-10f I fusion protein was 54,680-M\(^{-1}\)cm\(^{-1}\) (ProtParam, ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/protparam.html)), while the molecular weight (MW) was 28,626-Da (EditSeq\(\textsuperscript{TM}\)6.1 (DNASTAR, Inc., Madison, Wisconsin, USA). Therefore, it was calculated that the concentration (C) of the dialyzed CBM2a-10f I fusion protein diluted 1:1 in dialysis buffer was:

\[
C = \frac{A_{280}}{\varepsilon} \times MW
\]

\[
C = \frac{0.966}{54,680} \times 28,626 = 0.506 \text{ mg/ml}
\]

The less concentrated FPLC eluted fractions 24,25,29,30 were also combined and dialyzed, and the concentration of CBM2a-10f I protein was estimated to be 0.280-mg/ml. The total yield of CBM2a-10f I fusion protein from a 1-L M9 culture was 4.15-mg.

2.3.12 Binding kinetics of the 1222-III10f V\(_h\)H and the CBM2a-10f I protein to immobilized M13 phage

SPR using a BIACORE 3000 biosensor system (GE Healthcare, Baie d’Urfé, Quebec, Canada) was used to evaluate and compare the binding kinetics of the 1222-III10f V\(_h\)H and the CBM2a-10f I protein to immobilized M13 phage (Figure 44, Figure 45). Non-specific binding of the 1222-III10f V\(_h\)H and the CBM2a-10f I protein to BSA on the reference flow cell was subtracted from the observed binding of the proteins to the
immobilized M13 phage. The $K_D$ and sensorgram of the 1222-III10f $V_H$ (Figure 44) were similar to the $K_D$ and sensorgram generated previously for the 1222-III10f $V_H$ (Figure 17). Furthermore, the CBM2a-10f I protein and the 1222-III10f $V_H$ had similar $K_D$ values; both were in the nanomolar range (Table 3). The CBM2a-10f I protein had a greater $R_{\text{max}}$ compared to the 1222-III10f $V_H$ (Table 3). Since the CBM2a-10f I protein has a molecular weight 1.78 times greater than the molecular weight of the 1222-III10f $V_H$, the BIACORE response of the 1222-III10f $V_H$ was multiplied by 1.78 to facilitate comparison to the response of the CBM2a-10f I protein (Figure 46A). The BIACORE responses of the four highest concentrations of both the CBM2a-10f I protein and the 1222-III10f $V_H$ (x 1.78) were normalized to a percent response scale with the response of the highest concentration of each protein set to 100% (Figure 46B). As shown in Figure 46B, the normalized dissociation curves of the CBM2a-10f I protein had slopes which were less steep compared to the normalized dissociation curves of the 1222-III10f $V_H$ (x 1.78), indicating that the CBM2a-10f I protein had a slower dissociation rate compared to the 1222-III10f $V_H$.

![Figure 44. A. BIACORE 3000 sensorgram for the 1222-III10f $V_H$ binding to immobilized M13 phage. B. Steady state fitting used to determine the dissociation constant ($K_D$) for the 1222-III10f $V_H$ binding to immobilized M13 phage. Samples of 1222-III10f $V_H$ at concentrations ranging from 20-800-nM were permitted to bind to immobilized M13 phage.](image)
Figure 45. A. BIACORE 3000 sensorgram for the CBM2a-10f I protein binding to immobilized M13 phage. B. Steady state fitting used to determine the dissociation constant (K_D) for the CBM2a-10f I protein binding to immobilized M13 phage. Samples of CBM2a-10f I protein at concentrations ranging from 5.2-417-nM were permitted to bind to immobilized M13 phage.

Table 3. SPR (BIACORE 3000) binding kinetics of the 1222-III10f V_H and the CBM2a-10f I protein to immobilized M13 phage. The binding was quantified using the dissociation constant (K_D) and R_max (RU) derived from the sensorgrams. All K_Ds were determined from steady state analysis. The molecular weights (MW) of the proteins were determined using EditSeq™6.1 (DNASTAR, Inc., Madison, Wisconsin, USA).

<table>
<thead>
<tr>
<th>Protein</th>
<th>K_D (M)</th>
<th>R_max (RU)</th>
<th>MW (Da)</th>
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<td>16,075</td>
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<tr>
<td>CBM2a-10f I protein</td>
<td>4 x 10^{-8}</td>
<td>1,900</td>
<td>28,626</td>
</tr>
</tbody>
</table>
Figure 46. A. BIACORE 3000 sensorgram of the CBM2a-10f I protein and 1.78 x 1222-III10f V_{H}H binding to immobilized M13 phage. The samples of CBM2a-10f I protein were at concentrations ranging from 5.2-417-nM. The samples of 1222-III10f V_{H}H were at concentrations ranging from 20-800-nM. B. Normalized BIACORE 3000 responses of the four highest concentrations of both the CBM2a-10f I protein (52-417-nM) and the 1.78 x 1222-III10f V_{H}H (100-800-nM) binding to immobilized M13 phage. The response of the highest concentration of each protein was set to 100%. The responses of the other three concentrations of each protein were expressed as a percentage of the response of the highest concentration.
2.4 Discussion

ELISA results indicated that the V\textsubscript{H}H monomers and pentamers evaluated had a limit of detection of ca.10\textsuperscript{11} pfu M13 (Figure 22, Figure 23). During the wash steps of the ELISA, some of the phages may have been displaced from the wells of the microtiter plate. Thus, the true limit of detection of the V\textsubscript{H}H monomers and pentamers may be lower than observed. The discrepancy between the quantity of phages input into the microtiter wells and the quantity of phages which remained bound to the wells at the end of the assay may explain the discrepancy between the published and experimental detection limits of the commercial mouse anti-M13-HRP. Future ELISAs may be improved by capture of the M13 phage using an anti-M13 monoclonal to ensure that a known amount of phage remains bound to the wells. Overall, the ELISA results were not convincing due to large standard error of the mean and unexpected increases in absorbance observed as the M13 pfu/well decreased (Figure 22, Figure 23). Steric hinderance at high concentrations of M13 phage and inconsistent quantities of bound phage may explain the inconsistent results. A limit of detection of 10\textsuperscript{11} pfu would limit the usefulness of a CBM-V\textsubscript{H}H fusion protein when applied to a cellulose detection layer because the concentrations of M13 breaching a filter would likely be much less than 10\textsuperscript{11} pfu.

A 28.6-kDa CBM-V\textsubscript{H}H protein was successfully assembled using PCR and expressed with good yield in \textit{E. coli}. The 1222-III10f V\textsubscript{H}H was included in the CBM-V\textsubscript{H}H protein because the 1222-III10f V\textsubscript{H}H demonstrated nanomolar binding affinity for immobilized M13 phage (Table 2, Figure 17). Compared to other V\textsubscript{H}Hs tested, the 1222-III10f V\textsubscript{H}H gave the greatest ELISA signal when bound to immobilized M13 phage.
(Figure 22, Figure 23). The 1222-III10f pentamer bound irreversibly to M13 phage (Figure 18); however, the 1222-III10f monomer was selected for CBM fusion. It was hypothesized that once the 1222-III10f monomers were immobilized on cellulose, the monomers would bind to M13 phage irreversibly due to increased avidity through multiple 1222-III10f monomer-M13 interactions.

The CBM2a-10f I clone was selected for periplasmic expression, despite its valine to isoleucine substitution (Figure 34). The substitution occurred in the scaffold region of the CBM2a domain, which was not a critical region for cellulose-binding. Both valine and isoleucine are hydrophobic amino acids which were expected to have similar behaviour in the CBM2a domain. The CBM2a-10f 7 and the 10f-CBM2a 6 clones, which each have a plasmid DNA sequence identical to their respective expected sequence, should be considered for future periplasmic expression (Figure 35, Figure 39). FPLC purification of the CBM2a-10f I periplasmic proteins resulted in a pure, highly concentrated (1.01-mg/ml) fraction of CBM2a-10f I protein (Figure 42, Figure 43). The purified CBM2a-10f I protein was applied to a cellulose detection layer and used to detect M13 breaching of an air filter, as discussed in Chapter 3. A total yield 4.15-mg CBM2a-10f I protein was obtained from 1-L of M9 culture, suggesting that good yields of fusion protein may be easily obtained from periplasmic protein expression in E. coli.

SPR confirmed that the CBM2a-10f I protein possessed nanomolar affinity for immobilized M13 phage, suggesting that this protein would be effective in capturing M13 phage on a cellulose detection layer. The addition of the CBM2a domain to the 1222-III10f V_{H}H did not significantly alter the binding affinity of the 1222-III10f V_{H}H to immobilized M13 phage. The CBM2a-10f I protein and the 1222-III10f V_{H}H had similar
$K_D$ values in the nanomolar range (Table 3), although the CBM2a-10f I protein had a slower dissociation rate compared to the 1222-III10f $V_H$H (Figure 46B).

### 3 APPLICATION OF A CBM2a-\(V_H\)H FUSION PROTEIN TO PAPER FOR CAPTURE OF M13 PHAGE

#### 3.1 Introduction

A CBM2a-\(V_H\)H fusion protein, i.e. CBM2a-10f I, described in the previous chapter, was applied to cellulose filter paper to determine if the CBM2a-\(V_H\)H fusion protein enhanced capture of M13 phage in water. Furthermore, an anti-M13 $V_H$H was applied to Disruptor™ filter media, i.e. a filter containing a non-specific nanoalumina electroadisorbent (Ahlstrom, Helsinki, Finland), to determine if the $V_H$H enhanced the capture of M13 in water. The CBM2a-\(V_H\)H fusion protein was also tested for capture of aerosolized M13. In particular, the CBM2a-10f I protein was applied to a cellulose ‘detection layer’ to determine if the fusion protein enhanced detection of aerosolized M13 breaching an NBHK pulp filter and an N95 3M 8210 Particulate Respirator. M13 particles captured by the detection layer were identified using a commercial anti-M13 antibody conjugated to horseradish peroxidase which resulted in a color change.

CBM-\(V_H\)H fusion proteins capable of capturing viruses on paper may have applications in the manufacture of protective masks and equipment.
3.2 Materials and Methods

3.2.1 Application of a CBM2a-V_H fusion protein to cellulose for capture of M13 in solution

Two discs (labeled A and B) of ca. 4.7-cm diameter each were cut from a Buxus filter 13/40 LE, composed of 97% pulp and 3% rayon (Ahlstrom, Helsinki, Finland). CBM2a-V_H, i.e., CBM2a-10f I, (85-μg) diluted in 2-ml sterile water was applied to disc A. Disc A was incubated for 1-hour at room temperature. Each disk, i.e. disks A and B (control), was washed with 500-ml PBST, blocked with 3% MPBS for 1-hour, and washed with 500-ml PBST. M13 phage (7.2 x 10^{10} pfu) diluted in 2-ml sterile water was pipetted onto each disc. The discs were incubated for one hour at room temperature. Each disk was washed with 2-L of PBST to remove any unbound M13 phage. Two 1-cm^2 squares were cut from the middle of each disc, i.e. two ‘A’ squares and two ‘B’ squares.

A 10-ml culture of TG1 E. coli was incubated at 37ºC until it reached mid-log phase. Three samples (100-μl) of the E. coli culture were added to each of three, three-ml samples of molten top agar (55ºC). The top agar was added to each of three pre-warmed minimal media plates, hereafter referred to as plate 1, plate 2, and plate 3. One ‘A’ square and one ‘B’ square were applied to the tacky top agar of plate 1. Plate 2 also received one ‘A’ square and one ‘B’ square. Plate 3 was designated as a negative control and did not receive any paper squares. The top agar was allowed to harden and the plates were incubated at 37ºC overnight. The next day, the plates were examined for M13 plaques.
3.2.2 Application of an anti-M13 V\text{H} to Disruptor\textsuperscript{TM} nanoalumina filter media for capture of M13 in water

The experimental apparatus consisted of a peristaltic pump and a filter holder (Figure 47). Three circular discs, each of ca. 2.7-cm diameter, were cut from Disruptor\textsuperscript{TM} filter media to fit the filter holder. In trial 1, the Disruptor\textsuperscript{TM} disc was modified with 1222-III10f V\text{H}. In trial 2, the Disruptor\textsuperscript{TM} disc was wet with sterile water. In trial 3, the Disruptor\textsuperscript{TM} disc was unmodified. All three trials, each disc was used to filter 5-ml of M13 phage in water.

During trial 1, a 1222-III10f V\text{H} solution was diluted 1:4 with sterile water, i.e., 500-μl monomer and 1.5-ml water, and the $A_{280}$ of the diluted V\text{H} solution was measured. Using the experimental apparatus (Figure 47), the V\text{H} solution was applied to the Disruptor\textsuperscript{TM} disc at 1-ml/min. The $A_{280}$ of the V\text{H} effluent was measured to estimate the quantity of V\text{H} retained on the Disruptor\textsuperscript{TM} disc. During trial 2, 2.0-ml of sterile water was applied to the Disruptor\textsuperscript{TM} disc at 1-ml/min. In all three trials, M13 phage (5-ml) was applied to the Disruptor\textsuperscript{TM} disc at 1-ml/min. The phage effluent was collected and titrated after each trial. The experiment was repeated a total of four times (3 trials each). The concentration of phage was increased with each experiment, i.e., $2 \times 10^6$ pfu, $4.45 \times 10^8$ pfu, $8.4 \times 10^9$ pfu, and $1.25 \times 10^{11}$ pfu.

The quantity of 1222-III10f monomer applied to the Disruptor\textsuperscript{TM} disc in trial 1 was determined by calculating the change in concentration of the 1222-III10f V\text{H} solution after application to the Disruptor\textsuperscript{TM} disc. The concentration (C) of the 1222-III10f V\text{H} solution (2-ml total) prior to and after loading on the Disruptor\textsuperscript{TM} disc was calculated using the following formula:
\[ C = \frac{A_{280}}{\Sigma} \times MW \]

where \( A_{280} \) is the absorbance at 280-nm of the 1222-III10f solution prior to and after application to the Disruptor™ disc, \( \Sigma \) is the extinction coefficient at 280-nm of the 1222-III10f \( \text{V}_{\text{H}} \text{H} \) (27,055-M\(^{-1}\)cm\(^{-1}\); ProtParam, ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/protparam.html)), and MW is the molecular weight of the 1222-III10f \( \text{V}_{\text{H}} \text{H} \) (16,075-Da; EditSeq™6.1, DNASTAR, Inc., Madison, Wisconsin, USA).

**Figure 47.** Apparatus used to filter M13 phage particles through Disruptor™ nanoalumina filter media. Figure labels: A. flask containing solution to be applied to filter, B. peristaltic pump, C. filter holder for Disruptor™ filter media, D. collection flask for filter effluent. Photo: Tom Devecseri (NRC-IBS, Ottawa, Ontario, Canada)
3.2.3 Application of a CBM2a-V_H fusion to cellulose for capture of M13 in air

NBHK and N95 filters, in combination with a detection layer, were exposed to aerosolized M13 phage using an apparatus designed and assembled by Dr. Warren Finlay and Dr. Biljana Grgic of the University of Alberta (Figure 48). The NBHK (Northern Bleached Hardwood Kraft) pulp filter used in this experiment was a wet formed, freeze-dried filter made from beaten NBHK pulp with a base weight of ca. 380-390-g/m² and a thickness of ca. 16-mm (Mao et al. 2008). The filter was prepared by Dr. Dick Kerekes and Dr. Jingliang Mao of the University of British Columbia. The N95 filter was a NIOSH-approved 3M 8210 Particulate Respirator, while the detection layer was made from a Buxus filter 13/40 LE (Ahlstrom, Helsinki, Finland) which was composed of 97% pulp and 3% rayon. The NBHK and N95 filters, as well as the detection layer, were cut to fit the filter holder (4.7-cm dia.; Figure 48).
Figure 48. Apparatus used to expose an NBHK pulp filter and an N95 filter in combination with a detection layer to aerosolized M13 phage particles. The components of the apparatus included: **A.** Air conditioning compressor, the Proneb™ Turbo Compressor (PARI Respiratory Equipment, Inc., Midlothian, Virginia, USA), **B.** PARI LC Plus® Reusable Nebulizer (PARI Respiratory Equipment, Inc., Midlothian, Virginia, USA), **C.** Vent with Respirgard™ II bacterial/viral filter (Vital Signs Colorado, Inc., Englewood, Colorado, USA), **D.** Mixing chamber, **E.** Filter holder, **F.** HHP-103 Hand Held Manometer (OMEGA, Laval, Quebec, Canada), **G.** Collection flask, **H.** HEPA filter, **I.** Thermal Mass Flowmeter model 4043 (TSI, Inc., Shoreview, Minnesota, USA), **J.** Flow control valve (Parker, Schrader Bellows, Richland, Michigan, USA), and **K.** Oil-less vacuum pump model 1531-107B-GSS7X (Gast Manufacturing Co. Ltd., A Unit of IDEX Corporation, Benton Harbor, Michigan, USA). The attachments between the components were sealed with parafilm. Photo: Tom Devecseri (NRC-IBS, Ottawa, Ontario, Canada).

Within the experimental apparatus, an air compressor (A) (Figure 48) was used to generate air flow through a nebulizer (B), which created an aerosol of M13 phage. The M13 aerosol passed to a mixing chamber (D) where it was diluted with filtered air entering the apparatus through a vent (C). The M13 aerosol could be visualized in the mixing chamber. After mixing, the aerosol was passed through a filter holder (E) containing the NBHK filter, N95 filter, NBHK plus detection layer, or N95 plus detection layer. A manometer (F), attached to the filter holder, measured the pressure drop across the filter(s). The stream of air containing M13 phage particles was bubbled into distilled water contained in a collection flask (G). A HEPA filter (H) was used to collect any remaining M13 phage not trapped by the water in the collection flask. A flowmeter (I)
measured the airflow through the apparatus. A vacuum pump (K) fitted with a flow
control valve (J) was used to maintain constant airflow through the apparatus (Figure 48).

The first experiment (experiment 1) was composed of five trials (Trials 1-5 listed
in Table 4). The second experiment (experiment 2) was composed of seven trials (Trials
1-7 listed in Table 4). The contents of the filter holder varied with each trial (Table 4). In
trials 2, 4, and 6, 85-μg of CBM2a-V_{H}, i.e., CBM2a-10f I, diluted in 2-ml of water was
applied to the detection layer (Table 4), and the detection layer was incubated at room
temperature for 1-hour. In all trials, the detection layer was washed with 250-ml PBST.
The detection layer was blocked overnight with 3% milk diluted in PBS and washed
again with 250-ml PBST. All wash steps were performed using a vacuum-operated glass
filter holder assembly (Millipore, Billerica, Massachusetts, USA) consisting of a funnel,
fritted base, stopper, clamp, and a 1-L filtering flask connected to a vacuum pump. The
designated filter and/or detection layer (Table 4) were inserted into the filter holder. A
retention ring was used to hold the filter and/or detection layer in the filter holder.
**Table 4.** Experimental trials to evaluate the performance of a detection layer modified with CBM2a-V_{1H} fusion protein in detecting viral breaching of an NBHK and an N95 filter. Trials used as a negative or positive control are indicated.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Filter holder contents</th>
<th>Input M13 phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unmodified detection layer (Negative control)</td>
<td>0 pfu</td>
</tr>
<tr>
<td>2</td>
<td>NBHK filter plus detection layer modified with CBM2a-V_{1H} fusion protein (85-μg)</td>
<td>Experiment 1: 1.72 x 10^{10} pfu Experiment 2: 2.4 x 10^{8} pfu</td>
</tr>
<tr>
<td>3</td>
<td>NBHK filter plus unmodified detection layer</td>
<td>Experiment 1: 1.72 x 10^{10} pfu Experiment 2: 2.4 x 10^{8} pfu</td>
</tr>
<tr>
<td>4</td>
<td>Detection layer modified with CBM2a-V_{1H} fusion protein (85-μg) (Positive control)</td>
<td>Experiment 1: 1.72 x 10^{10} pfu Experiment 2: 2.4 x 10^{8} pfu</td>
</tr>
<tr>
<td>5</td>
<td>Unmodified detection layer (Positive control)</td>
<td>Experiment 1: 1.72 x 10^{10} pfu Experiment 2: 2.4 x 10^{8} pfu</td>
</tr>
<tr>
<td>6</td>
<td>N95 filter plus detection layer modified with CBM2a-V_{1H} fusion protein (85-μg)</td>
<td>Experiment 2: 2.4 x 10^{8} pfu</td>
</tr>
<tr>
<td>7</td>
<td>N95 filter and unmodified detection layer</td>
<td>Experiment 2: 2.4 x 10^{8} pfu</td>
</tr>
</tbody>
</table>

M13 (2-ml) were aerosolized in each trial, except for trial 1, in which 2-ml of sterile water was aerosolized as a negative control (Table 4). During experiment 1, 1.72 x 10^{10} pfu M13 were added to the nebulizer during each trial. During experiment 2, 2.4 x 10^{8} pfu M13 were added to the nebulizer during each trial (Table 4). After the air compressor and vacuum pump were turned on to initiate the flow of aerosol through the filter holder, the flow control valve on the vacuum pump was adjusted to maintain the flow rate at ca. 8.56-L/min. The pressure drop across the filter holder and the flow rate were monitored using the manometer and the flow meter, respectively. The aerosol was observed in the mixing chamber. After 10-15-min of operation of the apparatus, the
aerosol was no longer visible in the mixing chamber, and the 2-ml of input M13 phage or sterile water was assumed to be completely aerosolized. The air compressor and vacuum pump were turned off. The filter and/or detection layer were removed from the filter holder. The filter was discarded. The detection layer was washed with 500-ml PBST to remove any M13 phages which were not strongly bound to the detection layer by the CBM2a-V_{H}H fusion protein (if present).

The detection layer was incubated with 1.5-ml commercial mouse anti-M13 monoclonal conjugated to horseradish peroxidase (HRP) (GE Healthcare, Baie d’Urfé, Quebec, Canada) diluted 1:5,000 in 3% MPBS for 30-min. The detection layer was washed with 500-ml PBST. The vacuum-operated glass filter holder assembly (Millipore, Billerica, Massachusetts, USA) used in all wash steps caused the edges of the detection layer to remain unwashed. As a result, the unwashed edges of the detection layer were removed prior to being developed in 5-ml TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, Maryland, USA). A sample (100-μl) of substrate was removed from the developing detection layer at one minute intervals for 10-min (10 samples total). Immediately after each 100-μl sample of substrate was taken, 100-μl 1M H_{3}PO_{4} was added to the sample to halt color development. Once sampling was complete, the absorbance of the samples was read at 450-nm.

3.3 Results

3.3.1 Application of a CBM-V_{H}H fusion protein to cellulose for capture of M13 in solution

The objective of this experiment was to ascertain if CBM2a-V_{H}H fusion protein bound M13 in solution to cellulose-based filter paper as determined by M13 plaques in a
lawn of \textit{E. coli}. Square A contained CBM2a-V\textsubscript{H}H protein, i.e. CBM2a-10f I, while Square B did not. Contrary to our expectations, Square B produced a larger area of M13 plaques than square A, even though CBM2a-V\textsubscript{H}H protein was not present on square B (Figure 49). This experiment was repeated on a second minimal media plate. Both plates gave similar results. M13 plaques were not observed on the control plate (not shown).

\textbf{Figure 49.} M13 phage plaques in a lawn of \textit{E. coli} observed after overnight incubation at 37°C. Square A contained CBM2a-V\textsubscript{H}H fusion protein and M13 phage. Square B contained M13 phage. Photo: Tom Devecseri (NRC-IBS, Ottawa, Ontario, Canada).

It was hypothesized that the CBM2a-V\textsubscript{H}H protein on square A bound the M13 phage tightly to the paper, preventing the phage from infecting the surrounding \textit{E. coli}. The M13 phage on square B, however, were free to infect the surrounding \textit{E. coli} because the phage were not bound by fusion protein.
3.3.2 Application of an anti-M13 $V_{H}H$ to Disruptor™ nanoalumina filter media for capture of M13 in solution

The objective of this experiment was to determine if the addition of the anti-M13 1222-III10f $V_{H}H$ to Disruptor™ nanoalumina filter media (Ahlstrom, Helsinki, Finland) enhanced capture of M13 in solution.

The Disruptor™ filter media (Ahlstrom, Helsinki, Finland) is an electropositive non-woven filter with a pore size of 2-μm and a large surface area of ca. 500-m$^2$/g which filters particles by electroadsorption (Tepper and Kaledin 2007). The Disruptor™ filter media is composed of a combination of nanoalumina fibers made of boehmite (AlO(OH)) and microglass fibers. A 0.8-mm-thick layer of Disruptor™ filter media was shown to remove 99.5% of MS2 phage particles (25-nm in size) when 10-ml of a $6 \times 10^5$ pfu/ml MS2 solution at pH 7.2 was passed through the filter media at flow rate of 160-L/m$^2$/min (Tepper and Kaledin 2007).

Since the Disruptor™ filter media does not contain cellulose, a CBM would be ineffective in immobilizing the 1222-III10f $V_{H}H$ on the Disruptor™ filter media. Thus, the 1222-III10f $V_{H}H$, instead of the CBM2a-$V_{H}H$ fusion protein, was applied directly to the Disruptor™ filter media. Biological molecules, such as antibodies, may be adsorbed to the Disruptor™ filter media via electroadhesive forces (Tepper and Kaledin 2007). The 1222-III10f $V_{H}H$ has a theoretical isoelectric point of 6.71 (ProtParam, ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/protparam.html)). It was hypothesized that the $V_{H}H$ would adhere to the electropositive Disruptor™ filter media at pH values greater than or equal
to pH 6.71. The Disruptor™ filter was reported to have optimal performance at pH values ranging from 5-10 (Tepper and Kaledin 2007).

Capture of M13 in solution by Disruptor™ filters that were and were not loaded with 1222-III10f V_HH were compared (Table 5). When non-treated Disruptor™ filters were used, all M13 at concentrations of 2x10^6 pfu, 4.45 x 10^8 pfu, and 8.4 x 10^9 pfu were captured. When Disruptor™ filters treated with 1222-III10f V_HH were used, all M13 at concentrations of 2x10^6 pfu, and 4.45 x 10^8 pfu were captured; however, only 1.0 LRV (91.0%) of 8.4 x 10^9 pfu M13 phage were captured. Furthermore, the non-treated Disruptor™ filter captured 5.2 LRV (99.9%) of 1.25 x 10^{11} pfu M13, while the Disruptor™ filter treated with 1222-III10f V_HH captured only 0.4 LRV (59.2%) of 1.25 x 10^{11} pfu M13. The Disruptor™ disc modified with 2-ml sterile water removed 3.8 LRV (99.9%) of 1.25 x 10^{11} pfu M13 (Table 5). Thus, the addition of the 1222-III10f V_HH, as well as wetting of the Disruptor™ filter, decreased the ability of the Disruptor™ filter to capture M13. The 1222-III10f V_HH may have occupied sites on the Disruptor™ filter which would have otherwise been available for M13 capture. Furthermore, there may not have been sufficient time for the V_HH to bind to M13 during filtering (Table 5).
Table 5. M13 phage filtered through Disruptor™ filters that were and were not modified with 1222-III10f V_{H}H. The filter effluent was titered to determine the pfu (plaque forming units) of M13 phage passing through the filter. Based on the effluent phage titer, the percent of M13 phage captured by the Disruptor™ disc and the LRV (log reduction value) were computed for each trial.

<table>
<thead>
<tr>
<th>Modification of Disruptor™ disc</th>
<th>Input phage titer (pfu)</th>
<th>Effluent phage titer (pfu)</th>
<th>Percent phage capture (%)</th>
<th>LRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1222-III10f V_{H}H (84-μg)</td>
<td>8.4 x 10^9</td>
<td>7.6 x 10^8</td>
<td>91.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2-ml water</td>
<td>8.4 x 10^9</td>
<td>2.5 x 10^4</td>
<td>99.9</td>
<td>5.5</td>
</tr>
<tr>
<td>No modification (dry when M13 solution was added)</td>
<td>8.4 x 10^9</td>
<td>0</td>
<td>100</td>
<td>9.9</td>
</tr>
<tr>
<td>1222-III10f V_{H}H (50-μg)</td>
<td>1.25 x 10^{11}</td>
<td>5.1 x 10^{10}</td>
<td>59.2</td>
<td>0.4</td>
</tr>
<tr>
<td>2-ml water</td>
<td>1.25 x 10^{11}</td>
<td>2.2 x 10^7</td>
<td>99.9</td>
<td>3.8</td>
</tr>
<tr>
<td>No modification (dry when M13 solution was added)</td>
<td>1.25 x 10^{11}</td>
<td>8.8 x 10^3</td>
<td>99.9</td>
<td>5.2</td>
</tr>
</tbody>
</table>

3.3.3 Application of a CBM2a-V_{H}H fusion to cellulose for capture of M13 in air

The objective of this experiment was to determine if the addition of CBM2a-V_{H}H fusion protein, i.e. CBM2a-10f I, to a cellulose detection layer enhanced capture of M13 breaching an NBHk pulp filter and an N95 3M 8210 Particulate Respirator. The detection layer was developed using a commercial anti-M13 monoclonal antibody conjugated to horseradish peroxidase to detect M13 bound to the layer. Figure 50 compares M13 capture of a non-treated detection layer and a detection layer treated with CBM2a-V_{H}H when exposed to 1.72 x 10^{10} pfu aerosolized M13 in combination with an NBHk filter. Both the non-treated detection layer and the treated detection layer had similar absorbance values at 450-nm after development as the negative control which was not exposed to M13 phage. Thus, the NBHk pulp filter captured all of the 1.72 x 10^{10} pfu aerosolized M13 and prevented the phage from reaching the detection layers (Figure 50).
The positive controls used in this experiment, i.e. a non-treated detection layer and a CBM2a-VH fusion protein-treated detection layer each exposed to 1.72 x 10^{10} pfu aerosolized M13 in the absence of an NBHK filter, were more useful for evaluating the ability of the CBM2a-VH fusion protein to capture M13 phage (Figure 50). Since the untreated detection layer had a greater absorbance value at 450-nm after development than the treated detection layer, the untreated detection layer captured a greater quantity of M13 phage, and thus the CBM2a-VH fusion protein did not enhance M13 capture (Figure 50).

![Graph](image-url)

Figure 50. Effect of a detection layer, which was and was not treated with CBM2a-VH, on capture of M13 phage. A detection layer, which was either non-treated (A) or treated with CBM2a-VH (B), was used in combination with an NBHK pulp filter during filtration of an aerosol containing 1.72 x 10^{10} pfu M13 phage. As positive controls, i.e. no NBHK filter was used, a non-treated detection layer (A) and a detection layer treated with CBM2a-VH (B) were each exposed to 1.72 x 10^{10} pfu aerosolized M13 phage. As a negative control, i.e. no NBHK filter was used, a non-treated detection layer (A) was exposed to aerosolized sterile water which did not contain M13 phage. The legend is arranged in the same order as the data on the figure (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, [www.graphpad.com](http://www.graphpad.com)).

Figure 51 compares M13 capture of a non-treated detection layer and a detection layer treated with CBM2a-VH when exposed to 2.4 x 10^8 pfu aerosolized M13 in
combination with an NBHK filter or an N95 filter. Both the non-treated detection layer and the treated detection layer had similar absorbance values at 450-nm after development as the negative control which was not exposed to M13 phage (Figure 51). Thus, the NBHK filter and the N95 filter both captured all of the $2.4 \times 10^8$ pfu aerosolized M13 and prevented the phage from reaching the detection layers. Future experiments should utilize higher phage concentrations or increased flow rates to facilitate M13 breaching of the NBHK and N95 filters, and comparison of M13 capture between the untreated detection layer and the detection layer treated with CBM2a-VH fusion protein. Similar to Figure 50, the positive controls used in this experiment, i.e. a non-treated detection layer and a CBM2a-VH-H-treated detection layer each exposed to $2.4 \times 10^8$ pfu aerosolized M13 in the absence of an NBHK filter or an N95 filter, were more useful for evaluating the ability of the CBM2a-VH fusion protein to capture M13 phage (Figure 51). Once again, the untreated detection layer had a greater absorbance value at 450-nm after development than the treated detection layer, indicating that the CBM2a-VH fusion protein did not enhance M13 capture (Figure 51).
Figure 51. Effect of a detection layer, which was and was not treated with CBM2a-V₁H₁H, on capture of M13 phage. A detection layer, which was either non-treated (A) or treated with CBM2a-V₁H₁H (B), was used in combination with an NBHK pulp filter or an N95 3M 8210 Particulate Respirator during filtration of an aerosol containing $2.4 \times 10^8$ pfu M13 phage. As positive controls, i.e. no NBHK or N95 filter was used, a non-treated detection layer (A) and a detection layer treated with CBM2a-V₁H₁H (B) were each exposed to $2.4 \times 10^8$ pfu aerosolized M13 phage. As a negative control, i.e. no NBHK or N95 filter was used, a non-treated detection layer (A) was exposed to aerosolized sterile water which did not contain M13 phage. The legend is arranged in the same order as the data on the figure (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com).

3.4 Discussion

The CBM2a-V₁H₁H fusion protein, i.e. CBM2a-10f I, was effective in capturing M13 phage from solution on to cellulose filter paper during a one hour incubation period with M13 phage in water (Section 3.2.1). It was hypothesized that the captured M13 were so tightly bound that they were unable to migrate from the paper to infect surrounding *E. coli* (Figure 49). Conversely, the 1222-III10f V₁H₁H did not enhance the Disruptor™ filter media in the capture of M13 phage particles from solution (Section 3.2.2). Similarly, the application of CBM2a-V₁H₁H fusion protein, i.e. CBM2a-10f I, to a cellulose detection layer did not enhance capture of aerosolized M13 (Section 3.3.3). In these experiments
(Sections 3.2.2 and 3.3.3), the 1222-III10f V_{H}H and the CBM2a-10f I fusion protein had a shorter time length, i.e. one second rather one hour, in which to bind M13. SPR data for the 1222-III10f V_{H}H and the CBM2a-10f I protein binding to immobilized M13 phage (Figure 44 and Figure 45) indicated that a period of ca. 150 seconds was required to achieve peak binding response to M13 phage, although significant binding may occur within a few seconds. Thus, the length of time required for stable V_{H}H-antigen interactions may limit the usefulness of sdAb in bioactive paper applications.

4 CONCLUSION AND FUTURE DIRECTIONS

As outlined in the objective (Section 1.2), the purpose of this research was to fuse a cellulose-binding module (CBM) to a high-affinity llama V_{H}H which binds to bacteriophage M13. This fusion protein was then applied to paper substrates for detection of M13 phage.

The hypothesis tested by this research was:

If a CBM is fused to a llama V_{H}H which binds to bacteriophage M13, then this fusion protein, when applied to paper, will enhance capture of M13 phage.

A CBM2a-V_{H}H fusion protein with nanomolar affinity for immobilized M13 phage was successfully expressed in *E. coli*. The CBM2a-V_{H}H fusion protein was effective in binding M13 phage in water to a cellulose filter paper. However, the V_{H}H monomer and the CBM2a-V_{H}H fusion protein were ineffective in enhancing filter capture of M13 phage particles from water or air, respectively. These results do not support the hypothesis tested by this research. These experiments should be repeated using slower flow rates for the peristaltic and vacuum pumps to determine if M13 capture by the the V_{H}H monomer and the CBM2a-V_{H}H fusion protein may be improved.
In all cases, the CBM2a-V_{H}H fusion protein bound successfully to cellulose substrate, supporting the usefulness of CBMs for immobilization of biological molecules on paper. Their stability, ease of expression, and high specificity make sdAbs good candidates for bioactive filter applications. In the future, perhaps sdAbs with faster association rates (k_{a}) may be selected by modifying the panning procedure with shorter antigen-antibody incubation times, and decreasing both the concentration of input phage and antigen over several rounds of panning (see Section 1.6.6.).

The idea for a bioactive ‘detection layer’ capable of detecting M13 phage should still be pursued. A detection layer capable of changing color upon M13 capture, without a separate development step, is envisioned. Although the CBM2a-V_{H}H fusion protein did not enhance filter capture of M13 phage, our work demonstrates that a CBM-sdAb fusion protein will bind simultaneously to cellulose and a model virus. Thus, paper products modified with sdAb still have the potential to detect and/or mitigate emerging infectious viruses which threaten public health. The Canadian Network for the Development and Use of Bioactive Paper (SENTINEL) should continue to explore sdAb as biological indicators for bioactive paper.
5 REFERENCES


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