

**Expression of Biotinylated Multivalent Peptide Antigens in Bacteria for
Rapid and Effective Generation of Single Domain Antibodies from Phage-
displayed Antibody Libraries**

By

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ABSTRACT

In the present study, two insulin-like growth factor-binding protein 7 (IGFBP7) C-terminal-peptides were expressed as fusion proteins to bacterial verotoxin pentamerization domain as shown by Western blotting, ELISA and mass spectroscopy. Both *in vivo*-biotinylated recombinant products were purified from bacterial lysates by IMAC and used directly for panning along with the recombinant IGFBP7 protein using the LAC-M *Camelidae* naïve single domain antibody (sdAb) library. Target-specific sdAbs to both parental protein and peptide fusions were identified by phage ELISA. Twelve different clones were isolated by phage-ELISA screening and their sdAb genes were sequenced. Soluble sdAbs and their pentameric formats were expressed in TG1 *E. coli*, purified by IMAC and characterized by ELISA and SPR. Several sdAbs are currently under study, however anti-IGFBP7 (P12/M12) was extensively characterized and exhibited promising anti-tumorigenic effect on PANC-1 cell lines by blocking IGFBP7 promoting activity. This study provides the basis for developing a novel imaging/therapeutic reagent for targeting and treating brain tumor angiogenesis in early stages of tumorigenesis and can also be used as a molecular tool to monitor the degree of angiogenesis in gliomas which may help to improve the clinical management of brain tumors.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
Ab	Antibody
ACh	Acetylcholine
Amp	Ampicillin
bp	Base pair
Carb	Carbenicillin
cDNA	Complementary DNA
CDR	Complementarity determining region
cfu	Colony forming unit
CH	Constant domain of heavy chain
CL	Constant domain of light chain
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
F(ab')₂	Bivalent antigen binding fragment of immunoglobulins
Fab	Antigen binding fragment of immunoglobulins
Fc	Crystallizable fragment
fd	Strain of filamentous phage
FR	Framework region
GAM-AP	Goat-anti-mouse antibody conjugated to alkaline phosphatase
GAM-HRP	Goat-anti-mouse antibody conjugated to horseradish peroxidase
GBM	Glioblastoma multiforme
HBS-EP	10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% P-20
HCAb	Heavy-chain antibody from camelid family
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IGFBP7	Insulin-like growth factor-binding protein 7
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside

k_{on}	Association rate constant
KD	Equilibrium dissociation constant
kDa	KiloDalton
k_{off}	Dissociation rate constant
LB	Luria-Bertani media / lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5)
M13	Strain of filamentous phage
mAb	Monoclonal antibody
MPBS	Milk phosphate buffered saline
MW	Molecular weight
o/n	Overnight; 12-16 h
OD	Optical density
PBS	Phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4)
PBST	Phosphate buffered saline with 0.05% Tween 20
PCR	Polymerase chain reaction
PLA2	Phospholipase A2
Rmax	Maximum response defined as saturation of surface plasmon resonance
RT	Room temperature
RU	Response unit (in surface plasmon resonance)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sd Abs	Single domain antibodies
SOC medium	Super optimal catabolite repression medium (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO ₄ , 10 mM MgCl ₂ , 20 mM glucose, pH 7.0)
SPR	Surface plasmon resonance
TEA	Triethylamine
TMB	3,3',5,5'-tetramethyl benzidine
VH	Variable region of heavy chain of a conventional antibody
V_HH	Variable region of a heavy-chain antibody
VL	Variable region of light chain of a conventional antibody
2xYT	Terrific Broth with no salts

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1. GENERAL OVERVIEW AND RESEARCH OBJECTIVES

1.1. Research objectives

The aim of this project is to display peptide/protein antigens in a unique form that provides useful *in vivo*-folded and biotinylated antigenic reagents which can be used for panning of antibody libraries to isolate single domain antibodies (sdAbs) targeting therapeutically important tumor biomarkers. Isolation, characterization and multimerization of high affinity binders to Insulin-like growth factor-binding protein, IGFBP7-peptides and recombinant human IGFBP7 (rh-IGFBP7), a protein therapeutically important cancer target, will be performed to develop diagnostic and, potentially, therapeutic antibody-based reagents in the future. To investigate this, the main objectives of this research are:

- i.* To establish a bacterial antigen (peptide/protein) display platform technology for the panning of single domain antibody libraries.
- ii.* To isolate sdAbs effective against peptides derived from IGFBP7 and the recombinant IGFBP7 protein by panning of a naïve single domain library.

2. LITERATURE REVIEW

2.1. Immunoglobulin

2.2.1. Basic structure of immunoglobulin G (IgG)

Immunoglobulin G (IgG) is the most prominent class of antibody in the serum of healthy humans, accounting for between 70-75 % of total Igs (Male D. et al., 2006). Conventional IgG antibodies are glycoproteins composed of four polypeptide chains: two large or heavy (H) chains (50 – 77 kDa) and two small or light (L) chains (~22 kDa), each with their own variable domain (termed VH and VL, respectively) and constant domains (termed CH1, CH2 and CH3 and CL, respectively). The heavy chains and light chains are linked together by a number of disulfide bonds, which varies among IgG subclasses, to form a tetrameric molecule of about 150 kDa. The antigen binding properties of antibodies were preliminarily assigned to the Fab domain (fragment for antigen binding which includes VH-CH1 and VL-CL domains). However, it was later shown that antigen binding is limited to the VH and VL domains (also termed variable fragment Fv). The Fc region, which corresponds to paired CH2 and CH3 domains, interacts with other effector molecules and cells of the immune system. The Fc region is connected via a flexible linker, referred to as the hinge region, to the Fab domain, which contains a number of cysteine residues to enable homodimerization of heavy chains. The hinge region allows independent movement of Fab arms and allows binding of both arms of an antibody molecule to multiple epitopes on the antigenic protein surface. Moreover, the flexibility of the hinge region enables the interaction of the Fc region with other antibody-binding proteins that mediate immune effector mechanisms (Figure 1) (Janeway, Travers et al. 6th edition, 2005; Maynard J. et al., 2000; Roitt, 2001; Joosten et al., 2003).

2.2.2. Immunoglobulin fold

The IgG domains are composed of two β -pleated sheets each with four and five β -strands in the variable domains (VH and VL) and three and four strands in the constant domains (CH1, CH2 and CH3). The β -pleated sheets are held together with a single and very well conserved disulfide bond which stabilizes the domain structure. The overall topology of the antibody domain roughly resembles a barrel structure. This type of fold is known as the immunoglobulin fold and the antigen binding loops are located at one end of the barrel-shaped structure (Padlan E. et al., 1994). The nine β -strands within the β -sheet frameworks are connected by eight loops, of which three are called hypervariable loops (H1, H2 and H3 for the VH domain and L1, L2 and L3 for the VL domain) due to their amino acid variability in every antibody molecule produced by individual B lymphocytes (Chothia et al., 1989). The origin of this hypervariability lies in the genetic mechanism involved in the generation of antibody molecules, namely, mini-gene (V-D-J in VH domain and V-J in VL domain) assembly, imprecise joining of the mini-genes, and somatic hypermutation concentrated in loop regions (Early P. et al., 1980). The hypervariable regions were defined when large numbers of immunoglobulin sequences were pooled and analyzed (Kabat et al. 1992). As a result, three hypervariable regions were identified and named complementarity determining regions or CDR1 (residues H31-H35; with a possibility of insertion at 35a, b in VH and residues L24-L34 in VL), CDR2 (residues H50-H65 in VH and residues L50-L56 in VL) and CDR3 (residues H95-H102 in VH and residues L89-L97 in VL). Subsequent structural studies showed that the H1, H2 and H3 loops are positioned between residues H26-H32 (with a possible insertion at 31 a, b), H52-56 (with a possible insertion at 52 a, b) and H95-102 (with a possible insertion at 100 a, b, c, d, e, f, g, h, i, j, k), respectively (Chothia et al.,

1989). Similarly, L1, L2 and L3 loops are also positioned between residues L26-32, L50-56 and L89-97, respectively (Chothia et al., 1989). The amino acid residue composition of β -strand regions, known as framework regions and termed FR1, FR2, FR3, and FR4, show low variability in both VH and VL domains. The VH and VL families in immunoglobulins are established based on the sequence variations in the framework regions and members of the same VH or VL family share at least 80 % amino acid identity (Yang et al., 2003).

2.2.3. Antibody engineering and recombinant antibody fragment

Advances in DNA recombinant technology and the discovery of immunoglobulin gene clusters in mammals, including humans and mice in the 1980s, made it possible to clone antibody genes and express them in prokaryotes, preliminarily in *Escherichia coli* (*E. coli*). Mammalian recombinant antibody fragments were fused to bacterial signal sequences and, therefore, directed to the less oxidizing environment of the bacterial periplasm where they can, fold properly with the help of bacterial chaperones (Skerra and Pluckthun, 1988). Subsequent development in this area of research allowed for the expression of whole IgGs in mammalian cell lines (Trill et al., 1995; Rouet et al., 2012). These achievements opened a whole world of engineering opportunities for IgG or fragments thereof, including humanization of rodent antibodies by domain or CDR grafting (Jones et al., 1986; Lo Benny, 2003), improving their biophysicochemical properties such as affinity, specificity, stability, and expression level (Jung et al., 1997; Jung et al., 1999; Willuda et al., 1999; Ionescu et al., 2008) and, last but not least, the possibility of arming antibodies with effector molecules such as drugs, toxins, or enzymes (Payne G, 2003; Wu et al., 2005). Recombinant antibody fragments have several advantages compared to their parent counterparts including smaller size, ease of expression and genetic manipulation in simple prokaryotic systems, and

bypassing undesired effector functions related to the Fc region where only antigen binding, for example, for the purpose of inactivation/neutralization of target molecule, is required (Holliger and Hudson, 2005). Additionally, in some applications such as cancer therapy, small antibody fragments armed with toxic substances are of great value where a deep antibody penetration into solid tumor tissue is desired. However, small antibody fragments below 60 kDa are quickly cleared from circulation (Muller et al., 2007). Conjugating antibody fragments to a serum protein, such as albumin, has been applied to increase *in vivo* half-life (Smith et al., 2001).

Recombinant antibody fragments are basically classified into three categories: Fab, single chain Fv (scFv) and single domain antibodies (sdAb). In a scFv molecule, the VH and VL domains are covalently linked by a synthetic, flexible peptide with an average length of 15 amino acids (Hudson P. et al., 1998), whereas in a Fab molecule the VH (VH-CH1) and VL chains are linked with a naturally occurring disulfide bond (Maynard and Georgiou, 2000). The idea of single domain antibodies (VH and VL), as the smallest antigen binding units, was originated from the work of Ward et al. (Ward et al. 1989). VH domains derived from libraries of the VH gene repertoire from a mouse immunized against lysozyme and keyhole-limpet haemocyanin (KLH) preserve strong binding affinity (in the nM range) to the target antigen in the absence of its VL counterpart. However, the solubility of the isolated VH domain was seriously compromised most likely due to exposure of hydrophobic regions which are usually masked by VL domain association (see section 2.2.5). It is noteworthy to mention that Utsumi and Karush were the first to dissect the binding contribution of heavy and light chains in binding to a hapten antigen and separated the 7S γ -globulin into its subunits A (heavy chain) and B (light chain) (Utsumi & Karush, 1964). They found that

heavy chains can retain a significant amount of the original antigen affinity in the absence of the light chains.

Further engineering, including using methods to multimerize the antibody fragments, has generated a wide range of other antibody fragments such as diabodies, bivalent and bispecific antibody fragments, minibodies, etc. A detailed discussion on this subject is beyond the scope of this study and can be found in several references mentioned herein (Review reference Padlan E., 1994; Hudson P., 1998; Maynard and Georgiou, 2000; Hudson 2005) (Figure 1).

2.2.4. Heavy-chain Antibodies (HCAbs)

In addition to conventional IgGs, a distinct type of homodimeric antibody that lacks light chains, referred to as heavy-chain antibodies (HCAbs), can be found in the sera of dromedaries (Hamers-Casterman et al., 1993). Unlike conventional IgGs, HCAbs interact with antigens with only one single variable domain in each antibody arm and are known as V_HH (variable domain derived from heavy chain antibodies) (Review from Arbabi Ghahroudi et al. 1997; Riechmann L, Muyldermans S., 1999; Serge Muyldermans, 2001; Saerens A. et al., 2008). Additionally, HCAbs have lower molecular weight compared to the heavy chain of conventional antibodies because of the absence of the first constant domain (CH1). Therefore, each heavy chain arm has one single variable domain, V_HH , a hinge region, and two constant domains: CH2 and CH3. In dromedaries and alpaca, heavy chain antibodies comprise ~75% and 50% of total serum immunoglobulins, respectively, whereas in llama serum the heavy chain IgG proportion is between 30-45% (van der Linden et al., 2000). Detailed amino acid analyses and structural studies demonstrated that in V_HH domains there are a number of distinct, important amino acid substitutions in the framework-

two (FR2) regions that normally interact with the VL and CH1 domains in conventional IgG. These substitutions make the V_HH domain more hydrophilic, increasing their solubility and, therefore, reducing protein aggregation (Muylderman S. et al., 1999; Muyldermans S., 2001). Immunoglobulins similar to camelid heavy chains were also reported in nurse sharks and named 'novel antigen receptors' (Ig-NAR). The homodimeric heavy chain complex lacks the light chains and the variable domains function as a bivalent binding site. Despite its low amino acid identity (about 25%) with other mammalian V_H, the known immunoglobulin fold is well preserved in the IgNAR V domain. However, a large part of FR2-CDR2 is deleted, making the NAR V domain (12 kDa) even smaller than the Camelidae V_HHs (Greenberg et al., 1995; Roux et al., 1998).

2.2.5. Single domain antibodies (V_HHs) or Nanobodies

Following the discovery of heavy chain antibodies in the *Camelidae* family and determination of the amino acid sequence of their variable domains, efforts to construct libraries from immunized dromedary and pan against candidate antigens were intensified. The first report of isolation of V_HH single domain antibodies against lysozyme and tetanus toxoid antigens from immunized animals appeared in 1997 (Arbabi Ghahroudi et al., 1997) and it was shown that the isolated V_HH domains (13-15 kDa in size) are extremely soluble, stable, have affinities in the nM range and can be expressed abundantly in bacteria (Muylderman S. et al., 1998; Marco A, 2011). Further structural studies showed that one such V_HH, cAblys3, is able to penetrate into the active site of lysozyme and inhibit enzymatic activities (Desmyter et al., 1996), a fact that was not previously observed with conventional antibody fragments (Desmyter et al 1996; Arbabi-Ghahroudi et al. 1997). In fact, on average, longer CDR3 sequences (16-18 amino acids) in camels provide a

protruding loop for penetration into enzyme and other protein cavities. The average CDR3 length in human and mouse is 12 and 9 amino acids, respectively (Spinell et. al., 2000). The V_HH-based single domain antibodies has also been referred to as nanobodies (an Ablynx trade name) likely due to their nano size which is 2.5 nm in diameter and about 4 nm in high (Revets H. et al., 2005; Muyldermans et al., 2009; Hassanzadeh-Ghassabeh et al., 2011).

Amino acid alignments of large numbers of V_HHs with the human VH3 family as reference showed that all camelidae V_HHs closely resemble the human VH3 family except in FR2 and CDR regions (Vu et al., 1997). The hallmark of V_HH is a number of amino acid replacements from hydrophobic to hydrophilic at key positions interacting with the former VL interface, which make them very soluble and stable under various physiological conditions. These substitutions are observed almost always in the V_HH and include V37F, G44Q/E, L45R and W47G/F/L (Davies and Riechmann, 1996; Vu et al., 1997; Harmsen et al., 2000). There are other less conserved amino acid substitutions at positions 11 (L11S) and 35 (S/H35A/G). Another important observation is the expansion of variability in the CDR1 region to residues 27 and 29. Therefore, CDR1 is expanded from amino acid positions 31-35 in VHs to 27-35 in V_HHs (Nguyen et al., 2000). Indeed, the roles of hypervariable loops in binding to antigens become very prominent due to the lack of VL domain, and there seems to be a substantial variation in their conformations and length in comparison to their human and mouse counterparts. Due to the lack of a VL domain, there seems to be substantial variation in the conformations and lengths of the CDR3 regions of V_HHs in comparison to their human and mouse counterparts. The increased variability in the loop regions will compensate for the absence of VL and enable V_HH to recognize epitopes which are not accessible to the conventional antibodies. Additionally, in V_HH from dromedaries, though

less so in llamas, there is an extra disulfide bridge between CDR1 and CDR3 which stabilizes the longer CDR3 (Muylderman S. et al., 1994; Vu et al., 1997; Harmsen et al., 2000; Nguyen, 2000).

Identification and characterization of naturally occurring single domain antibodies with superior biophysicochemical properties also revitalized the original idea of Ward et al. (Ward et al., 1989) and opened new windows of opportunity for the establishment and application of single domain antibodies as detection, diagnostic and therapeutic reagents.

Riechmann et al. were the first group who introduced mutations in a human VH3 domain by replacing the hydrophobic residues at position 44, 45 and 47 with *Camelidae* hydrophilic residues (G44E, L45R and W47G), in a process referred to as camelization. They found that aggregation was significantly reduced compared to the non-mutant VH clones (Davies and Riechmann, 1994). In a separate study, the key residues involved in solubilizing V_HH were introduced into a human VH derived from a monoclonal antibody followed by construction of a phage library in which CDR1 and CDR3 residues were randomized (Tanha et al., 2001).

Following the original report of isolation of single domain antibodies, extensive research was initiated in many laboratories worldwide, including the re-investigation of human single domain of VH or VL origin by researchers at MRC, England. There have been numerous reports of isolation of high affinity single domain antibodies including V_HH, VH, VL, and VNAR against various target models including haptens, protein antigens, enzymes, toxins, cancer and inflammatory antigens, phage and bacteria, fungi and protozoan cells with affinity constants ranging from μ M to low pM (Saerens et al. 2004; De Genst et al. 2006; Saerens et al., 2008). The biotechnological application of single domain antibodies, in

particular V_HH, has been expanded rapidly in the past few years and these reagents have been used as immunodetection, purification and co-crystallization chaperones for studying protein aggregation and activity regulation (de Marco, 2011; Saerens et al., 2008; Wesolowski et al. 2009).

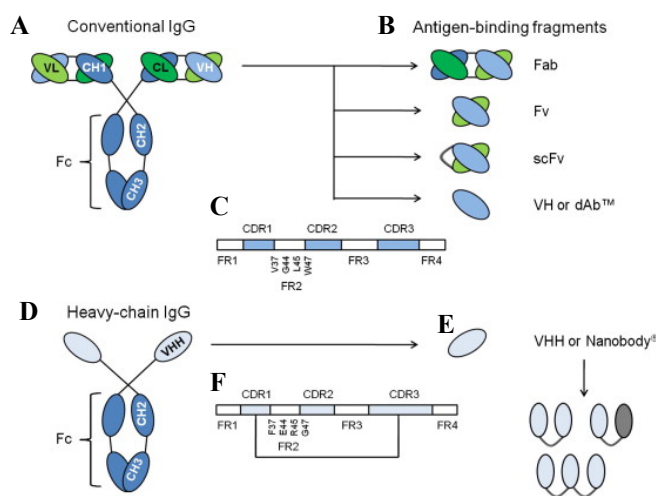


Figure 1. Schematic representation of conventional antibodies and camelid heavy-chain antibodies.

(A) Whole conventional IgG antibodies (~150 kDa). (B) Recombinant antibody fragment Fabs (~55 kDa), Fv (~30 kDa), scFvs (~32 kDa) and VH (~15 kDa). (C) The sequence organization of the VH with framework, CDR and hallmark residues. (D) Heavy chain of HCAb lacking the light chain. (E) The recombinant single domain antibody VHH (~15 kDa). (F) The sequence organization of the VHH with framework, CDR and hallmark residues (indicated according to Kabat numbering). Adapted from Vanlandschoot P et al., 2011.

2.2.6. Multivalent and Multispecific dAbs

Using genetic engineering techniques, sdAbs including V_HH, V_H/V_L and VNAR can be either linked covalently to each other via a short linker or alternatively linked to a homopolymerization polypeptide. As a result, bivalent/bispecific or multivalent sdAbs can be generated with high purity in bacterial hosts. The added value includes increasing functional affinity (avidity) and specificity and/or bridging two target antigens located on two different cell types. In the latter case, a bispecific sdAb can, for instance, target a cancer cell and, at the same time, attract a T cytotoxic cell to the tumor environment (Saerens D et al., 2008 c). There are several reports in which the increased potency of bivalent V_HHs against tumor necrosis factor (TNF) for the treatment of rheumatoid arthritis (RA) in mouse models (Coppieters K et al., 2006) or as antithrombotic reagents were demonstrated (www.ablynx.com) (Saerens D. et al., 2008 c).

The antigen-binding of V_HHs can also be improved through multimerization. Two multimerization domains were developed to generate high-avidity single domain antibodies. The avidity effect was achieved by increasing antigen cross-linking. The first single domain antibody to be linked to the naturally occurring monomeric β -subunit of *E. coli* Verotoxin 1 domain (VT1B) to form a homopentamerized fusion molecule was termed “pentabody” (Zhang et al., 2004). It was shown that the recombinant pentabody is able to bind three to four orders of magnitude more strongly than the monomeric form of the V_HH to PTH peptide antigen (Zhang et al., 2004). Alternatively, the V_HH was fused to a coiled-coil peptide derived from cartilage oligomeric matrix protein (COMP48) and the pentameric construct termed combody (Zhu et al., 2010). The functional affinity of the pentameric V_HH-

based combody increased about 10^5 fold when compared with its monomer counterpart $V_{\text{H}}\text{H}$ in binding to HLA-A201/MART-127L complex (Zhu et al., 2010).

2.2.7. Development of Antibody Selection Technologies

The humoral immune response in mammals is a complex process and involves a large number of cells and molecules of the immune system. The nature of an antigen is an important factor in eliciting the appropriate immune response and there are various degrees of involvement and cooperation of B and T lymphocytes encountering foreign antigens or peptides and eliciting the proper immune response (Janeway, Tracers et al 6th edition, 2005). Some common features in the display and production of immunoglobulins by B cells have been imitated *in vitro* and, consequently, recombinant antibody fragments have been isolated and produced in amounts required for diagnostic and therapeutic applications. These features include antibody display by a repertoire of B cells and physical linkage of genotype and phenotype, antibody:antigen interaction and selection of specific antibody clones, amplification of specific B cell clones in spleen and, finally, production and secretion of the desired antibody in large scale by plasma B cells. One of the key elements for the generation of antibodies *in vitro* is repertoire cloning and library construction, by which a pool of matured antibody genes from either non-immunized or immunized hosts are retrieved and cloned into an appropriate vector using standard molecular biology techniques. The screening of the established library is the second important step and requires a vehicle by which a physical linkage can be established between an antibody gene and its phenotypic expression. The pioneering work of George P. Smith (Smith G 1985), in which peptide DNA sequences were fused in-frame to the gene encoding the minor coat protein (gpIII) of filamentous phage (f1), followed by the display of peptide sequences at the filamentous

phage surfaces, was fundamental for the establishment of antibody display and screening technology. The first example of displaying a lysozyme-specific scFv on the surface of fd phage vector was demonstrated by McCafferty and his co-workers in 1990 (McCafferty et al. 1990). Within a year, phage display technology was developed, following a successful application of PCR-based cloning of antibody gene repertoires (Orlandi et al. 1989; Larrick et al 1989) from immunized mice against a phOx hapten antigen, displaying of the library on the surface of fd phage, and isolation of hapten-specific high affinity scFv binders (Clackson et al. 1991). The success of antibody phage display and screening opened a new era in antibody engineering and greatly sped the process of isolation of specific antibodies with the advantage of having direct access to the antibody genes. Phage display antibody technology provides an added value to traditional monoclonal antibody production pioneered by Kohler and Milstein in 1975, by which specific monoclonal IgGs, are screened and produced in mammalian cell lines (Kohler and Milestein 1975).

2.2.8. Antibody Phage display technology

As mentioned above, filamentous phage display was established by Smith in 1985 and a polypeptide fragment encodes a part of EcoRI endonuclease gene were fused to the minor coat protein (gpIII) and displayed on the surface of f1 filamentous phage particles (Cwirla et al 1990; Scott and Smith 1990; Russel M. et al., 2004). This technology established a direct link between genotype (the fused gene) and phenotype (its encoded protein) (Smith G, 1985; Willats W., 2002). Susequently, it was demonstrated that antibody fragment libraries (scFv, Fab), which were constructed by PCR-based cloning of immunoglobulin gene repertoires (Orlandi et al., 1989; Larrick et al, 1989), could be displayed multivalently (3-5 copies of antibody fragments per phage particle) on the phage as fusion to the minor coat protein

(gpIII) and high affinity antigen-specific antibodies against antigen targets were isolated and characterized by a process called ‘panning’ (McCafferty et al., 1990; Clackson et al. 1991, Hoogenboom et al., 1991). The approach was then extended to the monovalent display of antibody fragment on the phage surface using a combination of phagemid-based vector and a filamentous helper phage (Marks et al., 1991; Barbas et al., 1991; Hoogenboom et al., 1991), and high affinity antibody fragments (Fab and scFv) against a number of target antigens were isolated and characterized.

2.1.1.1. Filamentous bacteriophages

The key element in the development of phage display technology was the use of non-lytic F-pilus specific filamentous bacteriophage or Ff family phages including M13, fd and f1, which have 98.5% sequence identity in their genome (Rakonjac J et al., 2011). Due to their small genome size, ability to accommodate foreign DNA and ability to replicate in bacteria, filamentous phages are an excellent platform for recombinant antibody display and screening (Bradbury and Mark 2004). The phage particle is long ($\sim 1 \mu\text{m}$), and its genome is composed of single stranded DNA (6407 base pairs in length) that encodes 9 genes, encoding 11 proteins, five of which account for coat proteins (Figure 2) (Rakonjac J et al., 2011). The filament tube is formed by 2700 copies of major coat protein, pVIII, which encapsulates the phage genome. The distal ends of the phage contain two different pairs of minor coat protein: pVII-pIX which involved in the viral assembly and release and pIII-pVI which are required for properly terminating the phage assembly and contribute to phage particle stability (Carmen S and Jermutus L., 2002). pIII is present in 3-5 copies, is also involved in binding to the F pilus of *E. coli* and is composed of three consecutive N1-, N2- and C-terminal domains. The N-terminal domains are involved in binding and infection while the

C-terminal domain is required for assembly of the virion (Sidhu S, 2001). All of the phage coat proteins have been engineered and utilized to display the fusion protein. However, the pIII coat protein display system is the most efficient and common system since the insertion of larger foreign proteins is tolerated without affecting phage infectivity and stability (Smith and Petrenko, 1997; Russel M. et al., 2004; Willats WGT 2002).

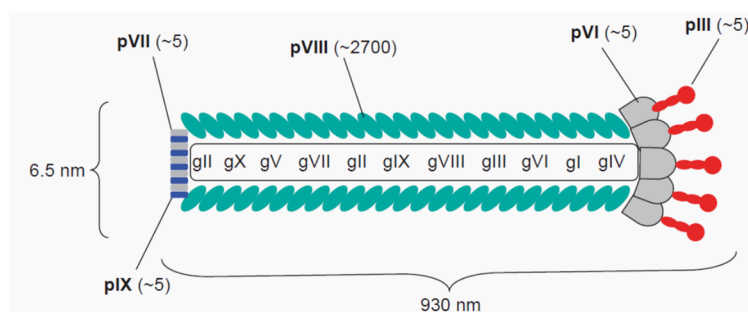


Figure 2. Schematic view of filamentous bacteriophage structure. Viral genome is encased by coat proteins (adapted from Mullen L. et al., 2006).

2.1.1.2. Antibody phage libraries

Different formats of the libraries can be constructed from immunoglobulin gene repertoires which may be derived from immune (Barbas et al, 1991; Amersdorfer et al., 2002), naïve (Marks et al., 1991; Vaughan 1996), semi-synthetic (Barbas et al., 1992; Soderlind E et al. 2000) and/or synthetic sources (Hoogenboom H et al., 1992; Griffiths et al., 1994; Knappick et al., 2000). Additionally, depending on the desired display format (monovalent versus multivalent display) phage or phagemid vectors are used (Bradbury and Mark, 2004; Paschke M., 2006). The final outcome of using either vector system is to generate a heterogeneous pool of phage-antibody clones, each of which carries a unique

DNA sequence and as a result express a specific antibody paratope on the phage surface. Upon binding of phage-antibody to the target antigen, the captured phage can be eluted and amplified into identical phage progenies (Smith and Petrenko, 1997). In both phage vectors, the antibody fragment genes are inserted between the natural gp3/gp8 signal sequence or a bacterial origin signal sequence (e.g. *pelB*) and the gene encoding the minor coat protein (gp3) (Hoogenboom et al. 1991) or major coat protein (gp8) under the control of a bacterial promoter such as that of the *lacZ* gene. In addition, the phage/phagemid vectors harbour an antibiotic resistance marker as well as a plasmid and phage-derived origin of replication, and the *fl* intergenic region sequence that promotes the packaging of ssDNA into new phage particles (Carmen S. et. al., 2002; Russel et al., 2004; Kehoe et al., 2005). Phage vectors that are derived from the natural filamentous phage (*fl*, *fd*, and M13) have all of the genes needed for replication and production of new phage particles. In contrast, in phagemid vectors, the necessary genes that are required to produce the mature phage particles are missing and, therefore, these require additional helper phages such as M13 KO7 or VCSM13 (Hoogenboom et al., 1998; Yau et al., 2003; Paschke, 2006). The purpose of adding a helper phage is to supply the phagemid with the proteins and enzymes required to enable the infection, replication, ssDNA production, packaging and encapsidation of proteins. This process is known as “phage rescue” (Winter et al., 1994). A defective origin of replication helper phage and high copy number of phagemid DNA are used in order to reduce the phages containing helper phage genome and to ensure that phagemid genomes are preferentially packaged (Russel et al., 2004; Sidhu, S., 2005; Paschke M., 2006). The true phage display systems generally produce the polyvalent display format. On the other hand, the phagemid-based display systems produce phage particles presenting wild-type coat

protein, pIII, and pIII-protein fusion in monovalent format and therefore avoid avidity effects and allow for the selection of higher affinity clones (Russel et al., 2004).

2.2.9. Panning of phage-displayed antibody libraries

Panning is a procedure that aims to isolate and select Ag-specific antibody fragments from a phage display library based on the specific binding of phage antibodies to an immobilized antigen, an antigen in solution or, if it is available, *in situ*. It is a consecutive process in which antibody sequences from bound phages are isolated and amplified in an F-plus *E. coli* strain (McCafferty et al., 1990; Winter et al., 1994; Willats, 2002; Paschke M., 2006). The principle stages in panning of antibody libraries includes: a) phage production of phage vector or phage rescue of the antibody library; b) exposure of the phage antibodies to the target antigen (on solid surface, in solution, or *in situ*) followed by extensive washing steps to remove the non-specific binders; c) disruption of the antigen-antibody interaction by a change in pH and elution of the antigen-specific binders; and finally, d) infection of F-plus *E. coli* with the eluted phages for enrichment of phage binders (Winter et al., 1994) (Figure 3). Antigen-specific binders with desirable biophysical properties can be enriched after each round of panning by subjecting the amplified phage to increased selection pressure and washing (Brichta J. et al., 2005). Several rounds of panning, usually 3 to 5, result in the isolation of high-affinity binders (Coomber D., 2002). Subsequently, the genes encoding high-affinity clones are amplified by PCR, subcloned into an expression vector, expressed and purified for post-panning specificity analysis using ELISA, Surface Plasmon Resonance (SPR) and appropriate functional assays (Hoogenboom HR, 2005).

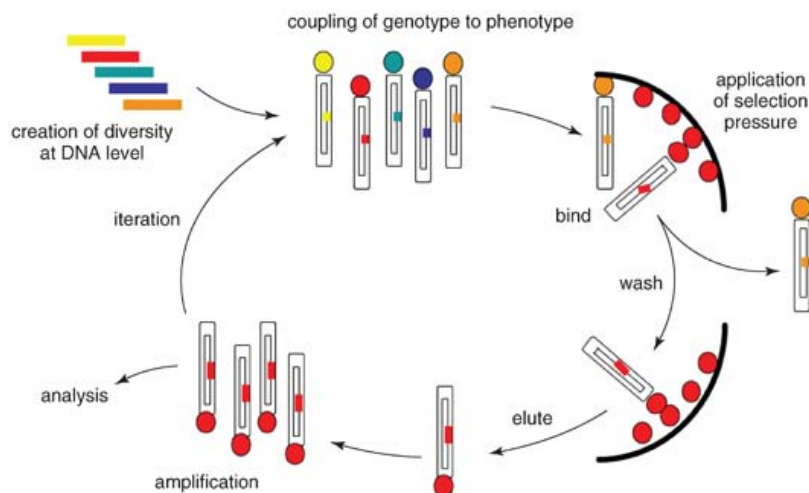


Figure 3. The phage display cycle. (adopted from Bradbury A., 2010)

2.1.1.3. Selection strategies:

Several *in vitro* and *in vivo* selection methods have been developed, although *in vitro* selection has superior advantage since the phage libraries can be screened against biological targets as well as inorganic ones (Whaley et al., 2000). The most commonly used *in vitro* selection method is bio-panning of immobilized antigen coated onto a solid surface. The target antigens are attached to a plastic polystyrene surface (e.g. tube, micro-titer well) through passive adsorption or covalent immobilization, depending on the antigen property, and then subjected to several rounds of affinity selection (Marks et al., 1991; McCafferty and Johnson, 1996; Scholle M. et al., 2004). Even though immobilizing of a target protein on a solid surface is a simple and fast technique, the target antigen may be partially denatured upon immobilization due to the effect of pH and buffers, therefore resulting in no binders or

binders to the denatured protein. In addition, not all of the antigen surfaces will be accessible because the antigen's immobilization may have preferential orientation, making some epitopes inaccessible (Butler J. et al., 1993; Koide A. et al., 2009).

Passage of phage antibody libraries through a column containing an antigen activated matrix is another method for selection of high affinity binders (McCafferty et al., 1990; Hoogenboom H. et al., 1998; Noppe W. et al., 2009). Alternatively, and in order to overcome the limitation associated with immobilization of the antigen to the solid surface, solution-based panning techniques have been used to provide a degree of control during the selection process and to present the antigen in its native form, allowing maximum accessibility of any epitope (Chames P. et al., 2002). In-solution selection is generally performed by using a biotinylated antigen. Biotin (molecular weight 244.3) very strongly interacts with avidin/streptavidin and this has been recognized as one of the strongest binding interactions existing in nature with a K_D of 10^{15} M^{-1} . The avidin/streptavidin:biotin system provides superior detectability with minimal background. The phage library is incubated with biotinylated antigen and the complex is captured by streptavidin-coated magnetic beads (Hawkins R. et al., 1992). This system has the advantage of using very low antigen concentrations during the panning and allows for the setting up of a competitive elution of binders in order to isolate high affinity binders (with the use of non-biotinylated antigen) (McCafferty J, 1996). Additionally, this system provides tools allowing partial or full automation of the panning process using a bead-based selection strategy (Walter G. et al., 2001; Hoogenboom HR, 2005).

Although most of the aforementioned selection methods are performed using pure antigens, these methods, in some cases, are not applicable, especially if the target antigens

are not identified, or if the antigen needs to be in its native environment (such as tumor antigens on a cell surface) or when, for example, tumor-cell-specific markers might easily be lost during the preparation process. As a result, several research groups have investigated the feasibility of cell-based panning/selection approaches (Hoogenboom et al., 1998). Similar to other panning strategies, repertoires of antibody displayed in a phage library are exposed to the target tissue or whole cells followed by several washing and elution steps. In order to reduce non-specific binding, the eluted phages are primarily pre-adsorbed on normal cells or healthy tissue, which eliminates non-specific binders before each round of panning (Cai X. et al., 1995; Watters J. et al., 1997; Radosevic K. et al., 2002; Johns M. et al., 2000; Hoogenboom HR, 2005).

2.1.1.4. Minimizing non-specific binding of phages to antigen

Isolation of specific clones from large phage display libraries is the primary goal of the panning procedure. Several techniques have been described in order to minimize the non-specific binding phages from the phage repertoire, including modulation of washing conditions or alternating the use of blocking buffer. During panning, unbound phages will remain in solution, therefore, washing buffer composition with low pH, high salt concentration and addition of appropriate detergents such as Tween-20 are the principle means of removing a large portion of the non-specific binders. Moreover, the number and stringency of washes can be increased to drive selection towards the specific phage binders with high affinity and to minimize the non-specific binding phages, which are present at much higher levels. Incubating the antigen-coated surface and the phage library, respectively, with proper blocking agents, such as skim milk, gelatin, BSA, or casein, and

alternative switching between them after each round of panning is important to reduce the phage population binding to undesired targets (Coomber W., 2002).

2.1.1.5. Automated and semi-automated Panning by the use of KingFisher System

The process of panning and screening of phage libraries is amenable to be performed in a high-throughput manner. This is important where a large number of antigens need to be panned and screened. For instance, advances in proteomic research provided access to large numbers of protein antigens which require individual characterisation; this demands high-throughput methodologies for panning of large antibody and alternative scaffold libraries. In-solution based panning with the use of biotinylated protein/peptide and magnetic streptavidin beads are particularly amenable to automation. In this regard, a semi-automated system was developed by ThermoFisher Scientific (KingFisher) by which up to 96 biotinylated antigens can be panned through 3-4 cycles of phage library incubation with captured antigens on streptavidin magnetic beads, capturing of bound phages binders, and washing. After each cycle, the eluted phages are amplified in *E. coli* and re-applied to the panning cycle (Figure 4). The fully automated KingFisher system has been developed to be fully robotic including all of the steps starting from robotic colony-pickers, incubation and high-throughput expression, ELISA, high-throughput protein purification, detection devices, PCR machines and sequencing, and a data software system to combine the results from all aforementioned steps (Konthur et al., 2010).

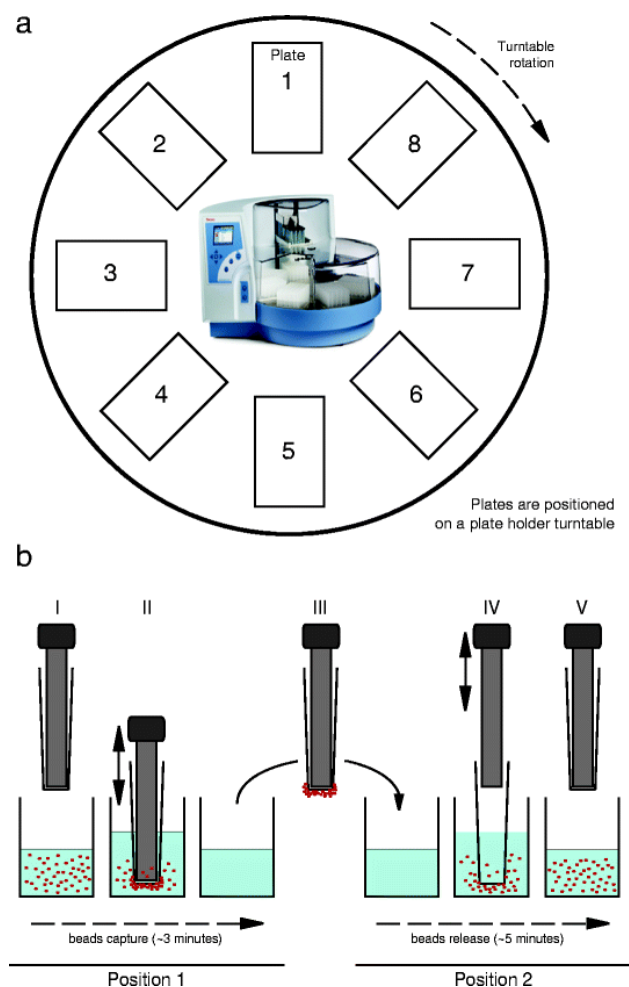


Figure 4. Principle of the magnetic particle processor.

(a) Overview of the Kingfisher Flex instrument, Thermo Scientific rotating table. The magnetic-head, which is installed at a fixed loading position, transfers the beads from one plate to another according to the setting program by rotating the plate holder clockwise.

(b) The basic principle of the magnetic particle process. (I) The plastic cap that covered the magnet moves into the solution containing magnetic beads. (II) The tips move up and down to attract the beads in solution. (III) The attracted beads are transferred to a next plate. (IV) The magnet is removed and the beads are suspended into the solution. (V) The magnet head and plastic cap return to their starting position. (adapted from Konthur et al., 2010).

2.1.1.6. Alternative display technologies

Various antibody display technologies have been applied to screen and isolate recombinant antibodies from large libraries (Li, 2000). The common feature of all the display technologies is the direct physical link between phenotype, the expressed antibody, and genotype, the gene encoding the antibody fragment. This linkage provides direct access to the encoding gene for further engineering to enhance its affinity, specificity and stability (Georgiou et al., 2000). Different *in vivo* and *in vitro* antibody display systems have been established. Antibodies can be displayed on the microbial cell surface (bacteria and yeast) (Boder and Wittrup, 1997, Hoogenboom H., 2005). Bacterial cell wall surfaces are used for displaying antibody fragment libraries, full-length antibodies and peptide libraries (Georgiou et al, 2000). A number of studies have isolated fully functional antibodies with excellent specificity and high affinity for fluorescently-labelled hapten (Daugherty PS. et al., 1998, 1999). Different anchoring motifs or carrier proteins are used to display the protein of interest on the bacterial cell surface (Bloois E. V. et al., 2011). Daugherty et al. have generated a library of scFv fragments displayed on the surface of Gram-negative bacteria (*E. coli*) by fusing the antibody library to a chimeric surface lipoprotein and Outer Membrane Protein A. The target-binding bacteria were isolated by fluorescence-activated cell sorting (FACS) (Daugherty PS. et al., 1998). Another group have anchored the antibody fragment to the periplasmic face of the inner membrane of *E. coli* (Harvey B. at al., 2004). However, *E. coli* has some limitations since the surface anchoring motifs must be fused with protein that needs to be exported through the complex cell envelope structure to the outer membrane (Lee S. et al., 2003). This limitation in Gram-negative bacteria can be bypassed by various gene fusion strategies and choosing an appropriate leader sequence (Yau K. et al., 2003). For

example, fusion to peptidoglycan chaperones was used to transport and anchor single chain antibody fragments to the cell surface without affecting cell viability or cell growth (Fuchs et al., 1991, Dhillon JK et al., 1999). In addition, Gram-positive bacteria such as *Staphylococcus xylosus* and *Staphylococcus carnosus* were used to display single-chain Fv antibodies (Löfblom John, 2011). Gram-positive bacteria possess superior advantages over Gram-negative bacteria for several reasons. First, Gram-positive bacteria have a single layer cell membrane which facilitates the transportation of the recombinant antibody fragments to the cell surface. Second, because of their thick cell wall, Gram-positive bacteria tolerate harsh conditions during affinity selection. Third, the single layer membrane better facilitates the display of large fusion antibody fragments. Finally, there is a higher yield of secreted antibody fragments due to the absence of extracellular proteolytic activity (Yau K. et al., 2003; Lee S. et al., 2003; Löfblom John, 2011).

Yeast displaying antibodies on their cell wall through cell surface α -agglutinin adhesion receptor have also been reported, offering several advantages over bacterial display (Feldhaus M. et al., 2004; Sergeeva A. et al., 2006). Yeast generally shows stable maintenance of surface-displayed proteins resulting from the thick cell wall structure (Georgiou et al., 1997). In addition, unlike the bacterial system, yeast possesses efficient protein folding machinery due to the existence of post-translational modification mechanisms. This closely imitates mammalian cell biochemistry and, therefore, provides an excellent display system of mammalian proteins (Boder and Wittrup, 1997; A. Kondo, M. Ueda, 2004). However, yeast expresses high copy numbers of heterologous recombinant protein (10,000 – 100,000 copies) linked to the surface, which may affect the discrimination of high affinity clones due to the avidity effect (Nakamura et al. 2002; Yau et al., 2003).

Despite yeast display system advantages, yeast libraries are relatively smaller in size compared to their phage or bacterial counterparts. Generally the yeast library size ranges between (10^7 – 10^9) colonies due to the limitations in yeast transformation efficiency (Kontermann R. 2010).

Ribosomal display has been developed as an alternative means for *in vitro* display technologies. In the ribosomal display system, the antibody phenotype is also directly linked to the genotype (the mRNA) and remains connected to the ribosome and to its encoding mRNA forming an antibody–ribosome–mRNA (ARM) complex (He M. and Taussig, 1997; Yau et al., 2003). The ARM complex is subjected to affinity selection and the genes coding for high affinity binders can be isolated and amplified by RT-PCR (Lipovsek D., 2004). Ribosome display offers a number of advantages since it is a cell-free system and libraries with potential sizes of up to 10^{15} different clones can be generated and screened (Roberts R., 1999; Gerotgiou G., 2000; He M. and Khan F., 2005). Furthermore, in the cell-free system unstable, proteolytically sensitive and toxic proteins are produced and consequently there is no bias or over/under-representation of clones in the library due to host restriction mechanisms (Yau et al., 2003; He M. et al., 2004). However, this system cannot be applied in whole cell panning due to the activity of the RNase on the cell surface that leads to rapid RNA degradation. Therefore, this system must remain in an RNase-free environment in order to stabilize the ARM complex (Rothe et al., 2006).

2.2. Recombinant Protein and peptides as antigen and immunogen

2.2.1. Recombinant antigens

Recombinant proteins have widely been applied as reagents in various diagnostic assays and have been important tools in basic and applied research since the first successful production of recombinant insulin in 1983 (Johnson I., 1983). Recombinant proteins are produced through recombinant DNA technology by cloning the exogenous DNA encoding the protein of interest in an appropriate expression vector. The expression vectors contain all of the essential genetic elements that permit the expression of the exogenous protein independently of the host cell DNA machinery. Depending on the protein complexity and post-modification requirements, the appropriate host cell is selected for expression. For smaller proteins (generally below 50 kDa) with no requirement of post-translational modifications, bacterial cells are the optimum choice. However, large and more complex proteins which also require post-translation modifications are expressed in alternative host systems such as yeasts, mammalian cell lines, plants or insect cells. In these systems, DNA encoding recombinant proteins is cloned into an appropriate vector which either can exist as an episomal vector or be integrated into the host cell genome and, therefore, transient or constitutive expression of the protein products are results, respectively. Alternatively, transgenic plant lines or animals with the ability to produce a protein of interest in their natural products including milk, blood and urine have been established (Demain et al., 2009). There are a number of different factors such as protein quality, functionality, production speed and yield which should be considered for the production of recombinant protein in various host systems (Fox et al., 1989). Although prokaryotes provide a rapid, inexpensive expression system for the expression of quite a number of mammalian proteins in a relatively short time period, recombinant proteins expressed in bacterial systems tend to

form inclusion bodies which require additional treatments, such as re-folding to retain their conformations and functions. Furthermore, some antibodies isolated against bacterially-expressed recombinant proteins are unable to recognize mammalian proteins because no post-translational modifications such as glycosylation and phosphorylation are allowed in the bacterial system (Jenkins et al., 1994; Demain et al., 2009). One of the main areas of application of recombinant proteins is in the field of monoclonal antibody generation. There are several advantages in the use of recombinant proteins as immunogens including purity, large scale production and little or no batch-to-batch variation, which are the key elements in raising specific immune response, screening and characterization of monoclonal antibodies, and also in bio-panning of phage antibody libraries and characterization of antibody fragments. They also provide an excellent tool to perform epitope mapping of isolated antibodies (Angeletti, 1999). For instance, 100 mg of p41 HIV envelope protein antigen have been produced consistently using bacterial fermentor systems, free from any other HIV antigen. The flexibility of this technology allows numerous modifications such as insertion or deletion and fusion to other molecules in order to improve the immunogenicity of the recombinant protein (Kieny et al., 1988; Fox et al., 1989). It should be noted that not all naturally occurring proteins *in vivo* can be expressed recombinantly and, indeed, some mammalian proteins, including membrane proteins, are rarely expressed in sufficient quantities for immunization and screening. Additionally, others may be expressed incorrectly folded, for example, by having incorrect disulfide bond pairing, or even being toxic to the host cells (Angeletti, 1999).

2.2.2. Peptide antigen

Peptide antigens have been used successfully as an alternative to conventional recombinant protein antigens where the aim is to generate antibodies against different isoforms or phosphorylated forms of a protein when there is a challenge to express protein antigens in sufficient quantities. Moreover, some protein antigens are bio-hazardous in nature or are toxic in their native state for immunization (Angeletti R, 1999). Several studies have shown that using peptides as antigens/immunogens are as effective as the protein from which they are derived in generating antibodies able to recognize the parental protein (Van Regenmortel, 2001; Grant GA, 2003). In addition, immunizing animals with multiple overlapping peptides can increase the chance of generating antibodies that recognize immunodominant epitopes of a target protein. However, a number of factors should be considered in designing the peptides as immunogens including: selection of the peptide sequence and its location within a protein molecule, peptide synthesis quality and purity, and peptide-carrier protein conjugation. Peptides able to react specifically with the binding site of an antibody vary in their lengths and have different levels of immunogenicity. Generally, peptide lengths between 10-16 amino acids are found to be more immunogenic (Coligan et al., 2001; Algate P et al., 2006). Longer peptides (more than 30 amino acids) are sometimes preferred and have the advantage of forming secondary structures with multiple epitopes that mimic the real folding of native proteins. However, assembling longer peptides is not always possible and there is an upper-size limit (up to 25 amino acids) in the chemistry of peptide synthesis. In terms of the mammalian immune response, peptides are considered to be very small, immunogenic molecules and are cleared quickly by the host (Lee et al., 2010). To circumvent this, peptides, especially those with molecular weight below 3 kDa, are

conjugated to larger carrier proteins such as Keyhole limpet haemocyanin (KLH) or Bovine serum albumin (BSA) in order to remain longer in the blood circulation and to stimulate a stronger immune response (Algate et al., 2006).

Peptide designing processes initially start with correct identification of species and protein sequence; a peptide should have a unique sequence with no cross-reactivity with any other closely related protein. Generally, excellent antigenic epitopes should be hydrophilic, surface orientated, and flexible. It is important to select accessible epitope regions exposed on the surface of the protein since these regions will tend to be more hydrophilic and soluble, unlike the hydrophobic regions buried within the protein core. Hydrophilic and hydrophobic regions can be predicted using antigenicity software (Angeletti, 1999; Lee et al., 2010) and are, therefore, distinguished from the hydrophobic transmembrane regions of intrinsic membrane proteins. Hydrophobic residues in the peptide such as Leu, Val, Ile, Met, Phe, and Trp, would influence the solubility and should be below 50% of total peptide residues. Selecting peptide sequences that are located at either the N- terminal or C-terminal will result in antibodies that recognize the native protein because these terminal ends are unstructured and solvent accessible. If the crystal structure of a target protein has been solved and the three dimensional coordinates are known, choosing linear peptides that occur on long protruding loops will probably increase the chance of isolating antibodies that recognize linear epitopes in a native protein. However, structural data are not available for many proteins and peptide design is mostly dependent on protein sequence. Therefore, by using predictive algorithm programs, secondary structure models can be generated with a relatively high percent of accuracy in predicting α -helix, turns, β -strand and loop structures in a candidate protein (Lee et al., 2010).

2.2.3. Peptide and antigen display

Peptides have been used for panning in order to isolate specific binders from phage display antibody libraries (Duan Z. et al., 2010). The main challenge during the panning process is coating these peptides onto a solid surface which could alter the structure and conformation of the epitopes and make them inaccessible. This issue is important because the peptide could bind in different orientations or conformations, affecting the nature of binding to antibodies and therefore, the selection process (Braitbard et al., 2006). In addition, during the coating process 95% of adsorbed proteins and peptides may not be functional because of passive adsorption that likely denatures the protein (Butler et al., 1992; Davies et al., 1994). Therefore, various techniques have been developed to overcome this issue including: coupling peptides to larger proteins; coupling to amino acid linkers; and peptide biotinylation. In 1989, Oshima et al. used free synthetic peptides encoding the entire human haemoglobin alpha-chain for immunization and determined the binding affinity of antibodies against each peptide. This study used free peptide and peptides conjugated to BSA and concluded that more antibodies were generated against peptide-BSA conjugates than against the same free peptide, suggesting the effectiveness of linking peptides to large carrier proteins as a useful strategy for peptide display (Oshima and Atassin, 1989). Peptide was also displayed by coupling to amino acid linkers such as ϵ -aminocaproic acid that act as a hydrophobic anchor in order to fix the peptide to the solid surface without affecting their conformation (Pyun et al., 1997). This strategy provides optimum peptide presentation and it is very applicable to small synthetic peptides (Pyun et al., 1997; Loomans et al., 1998). Biotinylation is the most successful technique, and has been widely used for peptide display and panning of phage libraries (Chames et al., 2010). Biotin, also known as vitamin H or B7,

has very small molecular weight (244 Da) that has no or little effect on the activity of the target peptide or protein. Biotinylation of target peptides provides a uniform and even distribution on streptavidin coated wells or on the surface of streptavidin coated beads with more than 60% functional molecules (Davies et al., 1994). Additionally, capturing of biotinylated peptides is very efficient since streptavidin has an extremely high affinity constant ($\sim 10^{15} \text{ M}^{-1}$) for biotin (Barat et al., 2007). The streptavidin molecule has a homotetrameric structure, binding four biotinylated molecules simultaneously and, consequently, providing a multivalent display of the target molecules (Diamandis et al., 1991). The use of paramagnetic streptavidin-coated beads with the multivalent display feature offers advantages of a large display surface for the panning of the phage antibody libraries and automation of the panning process (Chames and Baty, 2010).

2.2.4. High throughput screening of surface displayed gene products

Following the completion of human genome sequencing, there was a shift in research interests to study and address the function of the discovered genes. The human genome encodes around 20,000 – 25,000 genes (International Human Genome Sequencing Consortium, 2004) that gives around 90,000 or more distinct proteins (Lander et al., 2001; Venter et al., 2001). Basically, whereas genomics involves the study of the complete gene sequence, functional genomics are more involved in the study of gene expression and the site of expression, as well as interactions amongst related genes (Konthur Z. et al., 2005). Functional genomics and proteomics analysis improves our understanding of abnormal protein expression and function in disease conditions (Wingren C. et al., 2004). Proteomics focus on the study of the protein expression of the genome (El-Aneed et al., 2006) and provide information about which is the most populated functional protein in cell

compartments (Saerens et al., 2008). Therefore, reagents such as antibodies are required to study the localization and quantification of functional proteins within cells. The availability of a large number of gene products demands the improvement of high-throughput technology for antibody library screening and generation of specific binders (Walter G. et al., 2001). Antibody phage display is the most popular and applied methodology to screen and isolate binders with high affinity against multiple protein targets. In addition, phage display technology can be carried out by automated processes to screen different libraries against different target proteins in a high-throughput manner. As an important step in screening, access to purified natural gene products is nearly impossible and therefore tagged, recombinantly expressed proteins are frequently used. As discussed earlier, for large scale proteomics experiments, peptides were used as an alternative solution due to the fact that some recombinant antigens are difficult to express and purify in functional forms (Hust M. et al., 2004). Therefore, high-throughput panning can be performed on a set of properly designed peptides derived from large proteomic data and high-throughput panning can be established. To increase the precision and the throughput, an automated magnetic bead processor was adapted for the selection process in order to isolate binder clones from the large phage antibody libraries originated from various immunoglobulin gene repertoires (Konthur Z. et al., 2002).

2.3. Human Insulin-like growth factor binding protein (IGFBP7) as a model target

2.3.1. General overview of Insulin-like growth factors system (structure/function)

The insulin-like growth factor (IGFs) system consists of insulin-growth factors (IGF-I and IGF-II), insulin-growth factor receptors (IGF-IR and IGF-IIR), insulin receptor (IR), high affinity binding protein (IGFBP1-6), low affinity binding protein (IGFBP-rP1-10) and insulin proteases (Hwa V. et al., 1999; Burger et al., 2005). The insulin growth factor system participates in the regulation of somatic growth and cell proliferation *in vivo* and *in vitro* (Werrerau L. et al., 1999). IGFs are expressed in cells and have several functions: they are important mitogens, chemo-attractants, anti-apoptotic factors and are involved in glucose uptake (Hwa V. et al., 1999; Jones J. et al., 1995; Duan C. 2002). Several studies have linked the formation of new blood vessels, or the promotion of angiogenesis, to IGF-I (Nicosia R. et al., 1994; Sonntag W. et al., 2000; Khandwala H et al., 2000). IGFBPs are the major carrier proteins of IGFs. IGFs bind members of the IGFBP superfamily that includes IGFBPs (1 to 6) and IGFBP related proteins (1 to 10). IGFBP superfamily members are secreted, cysteine-rich factors which share a highly conserved N-terminal cysteine rich domain (IGFBP motif GCGCCXXC). Although IGFBPs have high identity, different binding affinities exist among them. Many studies reported that IGF has high binding affinity to IGFBP (1 to 6) and low binding affinity to IGFBP (7 to 10) (Burger A. et al., 2005). IGF/IGFBP complexes have several functions: they prolong the half-life of IGFs in circulation from a few minutes to several hours by protecting IGFs from degradation, they prevent IGF-induced hypoglycaemia and regulate IGF transport and the bioavailability of free IGF to bind IGFs receptors (Wetterau, L. et al., 1999; Massoner P. et al., 2010).

2.3.2. IGFBP7

The gene encoding IGFBP7, also known as IGFBP related protein 1 (IGFBP rp1) resides on chromosome 4q12-13 and is the protein that is classified as a low-affinity IGFBP. *igfbp7* was originally cloned by Murphy et al. in 1993, who found the protein expression of IGFBP7 was down regulated in meningioma cell lines, and referred to it as meningioma-associated cDNA 25 (mac25) (Murphy et al., 1993). IGFBP7 was purified and characterized by two independent research groups. Akaogi et al. purified IGFBP7 protein from conditioned medium of human bladder carcinoma cell line EJ-1 and determined it to be a tumor-derived adhesion factor (TAF) (Akaogi et al., 1994). Yamauchi et al. also identified IGFBP7 as a prostacyclin stimulating factor (PSF) (Yamauchi et al., 1994). *igfbp7* gene structure has five exon-coding sequences. Exon one encodes a conserved N-terminal domain and a Kazal-type trypsin inhibitor domain. Exons two to five encode the C-terminal immunoglobulin-like domain.

IGFBP7 is distinct from other low-affinity IGFBP related proteins because it binds insulin with high affinity and modulates insulin binding to its receptor (Yamanaka et al., 1997). IGFBP7 regulates numerous functions including cellular proliferation, adhesion, angiogenesis and the stimulation of prostacyclin synthesis (Burger et al., 2005). IGFBP7 binds heparin and type IV collagen which promote adhesion activity and the accumulation of blood vessels in the basement membrane (Akaogi et al., 1996; Ahmed S. et al., 2006). Akaogi et al. observed the accumulation of IGFBP7 in newly formed blood vessels in some human cancer tissues. This finding suggests that IGFBP7 is a novel angiogenic modulator and it was consequently named angiomodulin (AGM) (Akaogi et al., 1996).

IGFBP7 is expressed widely in endothelial cells and in female reproductive organs such as ovarian blood vessels, the corpus luteum and the uterus (Degeorges A. et al., 2000; Tamura K. et al., 2009). The expression of IGFBP7 is regulated by insulin growth factors and proteolytic cleavage (Ahmed et al., 2003). In neoplastic tissue, *igfbp7* gene expression is down-regulated by methylation. However, transforming growth factor (TGF- β), glucocorticoids and retinoic acid induce IGFBP expression (Burger A. et al., 2005).

2.3.3. IGFBP7 and cancer

Glioblastoma multiforme (GBM) is a highly vascularized brain tumor with a mean survival time of 9–12 months from the time it is diagnosed. It has distinct histo-pathological features, including high proliferation, necrosis and angiogenesis leading to vessels that exhibit morphological abnormalities (Vajkoczy and Menger, 2000). Angiogenesis provides the oxygen and nutritional supply needed for tumor growth and invasion. As a result, the high density of new vessels is a distinctive marker of GBM (Leon et al., 1996). Several studies have demonstrated that IGFBP7 is highly up regulated in tumor blood vessels with little or no expression in normal blood vessels. A study done by Pen et al., a high-density microarray analysis on GBM blood vessels were performed using a combination of laser capture microdissection (LCM) method that preserved the transcriptome profile of the cells, Q-PCR, and immunohistochemistry. The study demonstrated the strong upregulation of IGFBP7 genes in GBM vessels that linked previously to angiogenesis. In addition, the immunostaining study showed IGFBP7 was detected exclusively in the laminin of GBM vessels and not in non-malignant brain blood vessels (Pen A. et al., 2007). Moreover, SPARC gene was also up regulated in GBM vessels and confirmed by Q-PCR. However, the immunoreactivity of SPARC was not detected in GBM endothelial cells and was found in

perivascular cells adjacent to GBM vessels (Pen A. et al., 2007). On the other hand, different targets including vascular endothelial growth factor VEGF and $\alpha_v\beta_3$ have been utilized to assess the degree of angiogenesis and because of their heterogeneous expression and nonspecific targeting to non-tumor vessels these targets has some drawback and limited efficacy in GBM (Iqbal et al., 2010).

3. ANTIGEN DISPLAY, ISOLATION AND GENERATION of LLAMA SINGLE DOMAIN ANTIBODIES AGAINST IGFBP7

3.1. Introduction

sdAbs against IGFBP7 were selected from a naïve phage display library. The antibodies were then functionally and biochemically characterized with respect to affinity, specificity and aggregation states. Their biological effects on tumor cells were determined by using a cell-based soft agar assay.

3.2. Materials and Methods

3.2.1. Vectors construction

3.2.1.1. Construction of pVT2-Human Hinge-Bio expression vectors

The His-tag coding pVT2-Human Hinge-Bio vector was constructed by incorporating a double-stranded oligonucleotide (BioT5R; 5'GATCTGGGCAGCTCGGGTCTTAATGATATTTTTGAAGCTCAGAAGATTGAATGGCATGAAGGAGGTGGGTCCGAAAATCTGTATTTTCAGGGCCATCACCATCACCATCACTAGTGAA-3' and BioT6F; 5'AGCTTTCACTAGTGATGGTGATGGTGATGGCCCTGAAAATACAGATTTTCGGACCCACCTCCTTCATGCCATTCAATCTTCTGAGCTTCAAAAATATCATTAAGACCCGAGCTGCCCA-3') to introduce the biotin signal sequence, GLNDIFETQKIEWHE (Scholle M., 2004), between *Bgl*II and *Hind*III restriction enzyme sites located at the C-terminal region of the bacterial verotoxin (VT) multimerization domain. Ten picomoles of each phosphorylated, synthetic oligonucleotide encoding the biotinylation sequence were boiled at 100 °C for 10 minutes and cooled down at room temperature (RT) to allow annealing of the two complementary strands. The double stranded DNA fragments were ligated with 90 ng of pVT2-Human Hinge vector, previously digested with *Bgl*II and *Hind*III restriction

enzymes, in a total volume of 10 μ L ligation reaction containing 1 U of T4 ligase (Invitrogen, Carlsbad, California, USA) and 1X ligase buffer. After ligation, the recombinant vector DNA was used to transform electrocompetent TG1 *E. coli* cells (Arbabi et al., 2009). The transformed cells were plated on LB-AMP and incubated at 37 °C overnight. Individual colonies were screened for the presence of inserts by standard PCR techniques using M13R reverse and M13F forward primers (Arbabi et al. (a), 2009).

3.2.2. Peptide Antigen display

3.2.2.1. Cloning peptide coding sequence into pVT2-Human Hinge-Bio expression vector

The oligonucleotides encoding the target peptide sequences located at the C-terminal region of IGFBP7 (Table 1) were cloned upstream of the VT multimerization domain of the pVT2-Human Hinge-Bio vector between *Bbs*I and *Apa*I restriction sites. Ten picomoles of phosphorylated synthetic oligonucleotides encoding the peptide sequences were boiled at 100 °C for 10 minutes and allowed to cool down and anneal to their complementary strands at room temperature. The double stranded DNA fragments were incubated with 90 ng of pVT2-Human Hinge-Bio vector digested with *Bbs*I and *Apa*I restriction enzymes (New England Biolabs, Mississauga, ON) in a 10 μ L ligation reaction containing 1 U of T4 ligase and 1X ligase buffer. Proteins expressed from pVT2-Human Hinge-Bio vectors contain a 6XHistag at the C-terminus for purification and detection purposes. The final products were named VT-peptide-3- and VT-peptide-4, respectively.

Table 1. Oligonucleotides encoding the peptide sequences.

<i>Peptide</i>	Oligonucleotides sequences	
Peptide 3	PBio 9 RP	5'CAGGCCGCGCTGCATGAAATTCGGTGAAAAAGGCGAAGGCGCGGAA CTG GGGCC-3'
	PBio 10 FP	5'CCAGTTCGCGCCTTCGCCTTTTTTACCGGAATTCATGCAGCGCGG-3'
Peptide 4	PBio 11 RP	5'CAGGCCCAGGCGAGCGCGAGCGCGAAAAATTACCGTGGTGGATGCGCTG CATGAAATTCGGTGAAA GGGCC-3'
	PBio 12 FP	5'CTTTCACCGGAATTCATGCAGCGCATCCACCACGTAATTTTCGCGCT CGCGCTCGCCTG GG -3'

3.2.2.2. Expression of *in vivo* biotinylated VT-peptide fusion protein

TG1 *E.coli* cells were transformed with the ligated products and transformants identified by colony PCR using M13RP and M13FP primers. DNA isolated from the positive clones was sent for sequencing. TG1 *E. coli* cells harbouring the recombinant plasmids were then co-transformed with pACYC184 (AVB101) (Avidity LLC, Denver, CO) plasmid encoding the *birA* gene. At the same time, AVB 100 strains (Avidity) were also transformed with the recombinant pVT2-HumanHg-Bio plasmids (Warren D. et al., 2005). Single colonies harbouring dual plasmid DNAs related to each peptide were picked and transferred into 10 mL of 2x YT (16 g/L Tryptone, 10 g/L yeast extract and 5 g/L NaCl) starter culture supplemented with 100 µg/mL Amp and 1% (w/v) glucose. Cultures were grown overnight at 37 °C, shaking at 220 rpm. For large-scale expression, 10 mL of starter culture was transferred into 1 L of 2x YT medium supplemented with 1 g/L glucose, 100 µg/mL Ampicillin, 50 mmol/L potassium phosphate (pH 7.2), and 5 mmol/L MgSO₄. Cultures were then grown at 37 °C with continuous shaking to an *A*_{600/cm} of 0.8, and production of VT-peptide fusion and biotin holoenzyme synthetase was induced by the addition of isopropyl β-d thiogalactopyranoside (IPTG) to 1 mM and L-arabinose to 1.5 µM, respectively. The

cultures were also supplemented with 50 μ M d-biotin. Cultures were grown overnight at 28 °C with continuous shaking at 220 rpm. Sixteen hours after induction, the cultures were centrifuged at 6,000 rpm (Beckman J2-21M/E centrifuge, J-10 rotor) for 25 min at 4 °C. The harvested cells were re-suspended in 100 mL of ice-cold lysis buffer (50 mM tris-HCl at pH 8.0, 25 mM NaCl and complete EDTA-free protease inhibitor (Roche, Laval, Quebec) and stored at -20 °C over-night.

3.2.2.3. Purification of peptide fusion protein

Verotoxin fusion proteins were extracted by a cell lysis method. The induced cell pellets stored at -20 °C in lysis buffer (refer to section 3.2.2.2.) were thawed at room temperature with occasional shaking. To lyse the cells, 5 mL of freshly prepared lysozyme (at a concentration 100 μ g/mL, Sigma-Aldrich, Oakville, ON) were added to the thawed cells. The suspension was incubated at room temperature for 30-50 minutes with occasional shaking. When the suspension became viscous, 300 μ L of 15 units/ μ L DNase I (Sigma, Sigma-Aldrich, Oakville, ON; 15 units/ μ L stock in 1 M $MgCl_2$) was added and the lysate was incubated at room temperature until the suspension became more fluid. To separate the soluble fractions, the lysate was centrifuged at 12,000 rpm (Beckman J2-21M/E centrifuge, J-17 rotor) for 30 min. at 4 °C. Centrifugation was repeated with the soluble fraction until it became clear. The fraction which contained soluble peptide fusion proteins was filtered through a 0.22 μ m sterile filter (Millipore, Nepean, ON). Soluble VT-peptide fusion proteins were purified by standard immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare). The recombinant proteins were eluted using a linear gradient with 500 mM imidazole buffer. Western blots and SDS-PAGE were done to confirm expression and purification. The tagged VT-peptide fusion proteins

were detected using two different approaches. First, by using streptavidin conjugated to alkaline phosphatase (1:10,000 dilution) (GE Healthcare; UK). Second, anti-5X His monoclonal antibody (Qiagen, Mississauga, ON) was used as primary antibody (1:10,000 dilution) followed by incubation with goat anti-mouse IgG (H+L) mAb (Cedarlane, Burlington, Ontario) conjugated to alkaline phosphatase (GAM-AP) (1:5,000 dilution). Membranes were washed and developed with alkaline phosphatase substrate (AP Conjugate Substrate Kit; Bio-Rad, Mississauga, Ontario). Fractions containing VT-peptide fusion proteins were dialyzed overnight against SPR analysis buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA; HBS-E) with a 3,500 MW cut-off dialysis membrane. The absorbance of the proteins was measured at 280 nm (A₂₈₀) and the Expasy ProtParam tool (<http://ca.expasy.org/tools/protparam.html>) was used to calculate the theoretical extinction coefficient (E) of each VT-peptide fusion. The concentrations of the proteins were then determined using the following formula: $C = A_{260} / E$ where C is concentration in mg/mL, A is absorbance and E is the extinction coefficient. The purified VT-peptide fusion proteins were stored at 4 °C in PBS-3 mM EDTA buffer.

3.2.2.4. Mass spectrometry on fusion protein

In order to confirm that each peptide fusion protein was biotinylated, 50 µg of each peptide fusion protein were dialysed overnight against PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) and sent for analysis to the NRC-Sussex mass spectrometry laboratory (Dr. John Kelly, NRC-IBS, Ottawa, Ontario, Canada). Briefly, the VT-peptide 3 was separated by gradient elution, on an Agilent HP1100 HPLC, at the following conditions: 1-20% buffer B in 2 min, 20-60% buffer B in 20 min (B: Acetonitrile in 0.1% Formic Acid). A pre-column splitter was used to direct 30 µL/min through an Agilent mRP High Recovery

C18 0.5x100mm column and into the electrospray interface (ESI) of a Q-TOF 2 mass spectrometer (Waters, Milford, MA), for real-time monitoring of ion elution profile, in positive-ion mode. The VT-peptide 3 mass spectrum data was acquired and processed using MassLynx 4.1 software, and MaxEnt1, a program included in Masslynx 4.1, was used to obtain the deconvoluted protein mass.

3.2.3. Naïve Library panning

3.2.3.1. Peptide panning using semi-automated KingFisher® technology

An existing naïve llama V_HH phage library (Methods in Molecular Biology, July 2012) was used for the isolation of binders to two overlapping IGFBP7-derived (peptides 3 and 4). Before each round of panning, the rescued phage antibodies were first pre-incubated/pre-adsorbed overnight at 4 °C in 100 µL StartingBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher) containing 10 µg verotoxin domain and 200 µL streptavidin-coated paramagnetic beads (CELLlection™ Biotin Binder Kit, Invitrogen, Burlington, ON) to deplete streptavidin and VT pentameric domain binders. Ten micrograms of VT-peptide 3 and 4 fusion proteins were anchored to 200 µL Streptavidin magnetic beads (CELLlection™ Biotin Binder Kit, Invitrogen, Burlington, ON) for 1 hour at room temperature then incubated overnight at 4 °C with continuous rotation. Next, KingFisher plates were arranged for semi-automated panning (see Table 2). The bound phages were eluted in the first 3 rounds of panning using 100 µL of 100 mM, pH 10 Triethylamine (TEA) and neutralized with 50 µL of 1 M Tris-HCl, pH 7.4. In the fourth round of panning, the elution was done first with 20 µg of recombinant IGFBP7 protein and then with 100 µL of 100 mM, pH 10 triethylamine followed by neutralization with 50 µL of 1 M Tris-HCl, pH 7.4. Eluted phages were used to infect 10 mL of exponentially growing *E. coli* TG1 without shaking for 30 minutes at 37 °C. One hundred

microlitres of exponentially growing TG1 cells were kept to use as a negative control. After infection, a 15 μ L aliquot of infected *E. coli* was used to make a serial dilution (from 10^{-1} to 10^{-3}) in 150 μ L of 2xYT to determine the phage titer (output). One hundred and fifty microlitres of each dilution was spread on 2xYT plates containing 12.5 mg/mL tetracycline. The remaining infected cultures (~10 mL) were centrifuged at 3000 g for 15 min at 4 °C. The pellets were re-suspended in 300 μ L 2xYT spread on 2xYT/tetracycline plates and incubated overnight at 37 °C to amplify the phage. The next morning, the cells were collected from the plates using a plastic loop and 50 mL of 2xYT-Tet. Five millilitres of 2xYT-Tet were inoculated with 5 mL of collected cells and incubated at 30 °C for 5 hours with shaking at 220 rpm. The 2xYT culture was centrifuged at 5000 g for 30 min at 4 °C to pellet the *E. coli*. The culture supernatant was passed through a 0.22 μ m sterile filter (Millipore, Nepean, ON) and mixed with 1/5 the volume 20% (w/v) PEG 8K, 2.5 M NaCl solution and incubated on ice for 1 hour. The phage supernatant was centrifuged at 10,000 g for 15 min at 4 °C to precipitate the phage. The resulting pellet was re-suspended in 200 μ L of 1X PBS. The re-suspended phage was stored at -20 °C.

Table 2. Overview of semi-automated KingFisher® magnetic bead-based panning procedure

<i>Plate no.</i>	<i>Panning round 1</i>	<i>Panning round 2</i>	<i>Panning round 3</i>	<i>Panning round 4</i>
1	Ag-coated bead plate	Ag-coated bead plate	Ag-coated bead plate	Ag-coated bead plate
2	Wash plate buffer 1	Wash plate buffer 1	Wash plate buffer 1	Wash plate buffer 1
3	Blocking buffer starting block	Blocking buffer 1% Casein	Blocking buffer starting block	Blocking buffer 1% Casein
4	Phage library	Phage library	Phage library	Phage library
5	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)
6	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)
7		Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)	Wash plate PBS-T (0.05%)
8	Elution with TEA	Elution with TEA	Elution with TEA	1 st elution with free IGFBP7 protein
9				2 nd Elution with TEA

3.2.3.2. IGFBP7 solid phase panning

For round 1 panning, one well of a NUNC Maxisorb ELISA plate (VWR) was coated with 100 μ L sterile PBS, pH 7.4 and a second well was coated with 50 μ g of recombinant IGFBP7 (rIGFBP7) diluted in 100 μ L sterile PBS, pH 7.4. For rounds 2 and 3 of panning, the coating concentration of rIGFBP7 was decreased to 20 μ g/100 μ L. One hundred microlitres of amplified phage and 100 μ L of StartingBlock™ T20 (PBS) Blocking Buffer were mixed and incubated overnight at 4 °C with continuous rotation. After overnight incubation, wells were washed twice with 200 μ L PBS and blocked with 200 μ L of StartingBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher); the wells were then sealed and incubated at room temperature for two hours. During this time, the pre-incubated phages were centrifuged at 13,000 g for 10 min at 4 °C and transferred to the PBS coated well to adsorb non-specific binders. After the blocking incubation was complete, 100 μ L of phages were transferred to the protein coated well and incubated at room temperature for 1.5 hours. Unbound phages were removed by washing 5x, 7x, 10x and 12x with 300 μ L PBST for panning rounds 1, 2, 3 and 4, respectively, and then washed 5x, 7x, 10x and 12x with 300 μ L PBS for panning rounds 1, 2, 3 and 4, respectively. To elute bound phages, 100 μ L of 100 mM triethylamine (TEA) were added to the well and incubated at room temperature for 10 min. For the last two minutes of this incubation, the content of the wells were stirred by pipetting up and down several times. Eluted phages were transferred to a microcentrifuge tube and vortexed with 50 μ L of 1 M Tris-HCl, pH 7.4 to neutralize the TEA. One hundred and fifty microlitres of eluted phage were used to infect 10 mL of exponentially growing *E. coli* TG1 without shaking for 30 min at 37 °C. An aliquot of the same non-infected TG1 was plated on 2xYT-tetas a negative control. After infection, a 15 μ L aliquot of infected *E. coli*

was used to make a serial dilution (from 10^{-1} to 10^{-3}) in 150 μ L of 2xYT to determine the phage titer (output). One hundred and fifty microlitres of each dilution was spread on 2xYT-tet plates containing 12.5 mg/mL tetracycline. The remaining infected cultures (~10 mL) were centrifuged at 3,000 rpm for 15 min at 4 °C. The pellets were re-suspended in 300 μ L 2xYT, and spread on 2xYT/tetracycline plates for amplification and incubated overnight at 37 °C. The next morning, the cells were scraped from the plate using a plastic loop using 50 mL of 2xYT-Tet. Five millilitres of 2xYT-Tet were inoculated with 5 mL of collected cells and incubated at 30 °C for 5 hours with shaking at 220 rpm. The 2xYT culture was centrifuged at 5000 g for 30 min at 4°C to pellet the *E. coli*. The culture supernatant was passed through a 0.22 μ m sterile filter (Millipore, Nepean, ON), mixed with 1/5 the volume 20% (w/v) PEG 8K, 2.5 M NaCl solution and incubated on ice for 1 hour. The supernatant was centrifuged at 10,000 for 15 min at 4 °C to precipitate the phage. The resulting pellet was re-suspended in 200 μ L of 1X PBS AND stored at -20 °C.

3.2.3.3. Solid phase panning for VT-peptide fusion

Four rounds of panning were performed. Two wells of a NUNC Maxisorb ELISA plate (VWR) were coated with 100 μ L StartingBlock™ T20 (PBS) Blocking Buffer. Two extra wells were also coated with 100 μ L of 20 μ g/mL of streptavidin in sterile PBS, pH 7.4, the plate was incubated overnight at 4 °C. One hundred microlitres of amplified phage were pre-adsorbed with an irrelevant V_{HH} -VT-Bio followed by addition of 100 μ L of Blocking Buffer (1:1 volume ratio) and incubated overnight at 4 °C with continuous rotation. After overnight incubation, wells were emptied and washed twice with 1x PBS then 100 μ L of 20 μ g of peptide3-VT-Bio were added to the streptavidin wells and incubated at room temperature for two hours. Next, the wells were emptied and washed two times with 1x PBS then blocked

with StartingBlock buffer at room temperature for 2 hours. During this time, the pre-incubated phage was centrifuged at 13,000 g for 10 min at 4 °C and transferred to the blocking buffer coated well to adsorb non-specific binders and incubated for 2 hours. Subsequently, 100 µL of the pre-adsorbed phages were transferred to the VT-peptide captured well and incubated at room temperature for 1.5 hours. Unbound phages were removed by washing 5x, 7x, 10x and 12x with 300 µL of PBST 0.1% T-20, pH 5, for panning rounds 1, 2, 3 and 4 respectively, and then washed 5x, 7x, 10x and 12x with 300 µL PBS, pH 5, for panning rounds 1, 2, 3 and 4 respectively. In the fourth round, panning was performed using 10 µg/mL of C-IGFBP7 (C-terminal region of IGFBP7, BRI-Montreal, Canada) fragment protein and followed by the washing steps as mentioned above. In the first three rounds, phage were eluted with 100 µL of 100 mM triethylamine (TEA), added to the well and incubated at RT for 10 min. In the last round, phage were eluted first with ~10 µg/mL of C-IGFBP7 (recombinant C-fragments kindly provided by Protein Production Facility, NRC-BRI) then followed with 100 µL of TEA and the duplicate wells were eluted with ~10 µg/mL of rIGFBP7 then followed with 100 µL of TEA. Eluted phages were transferred to a microcentrifuge tube and vortexed with 50 µL of 1 M Tris-HCl, pH 7.4 to neutralize the TEA. One hundred and fifty microlitres of eluted phages were used to infect 10 mL of exponentially growing *E. coli* TG1 without shaking for 30 minutes at 37 °C. One hundred microlitres of the exponentially growing TG1 cells were kept for use as a negative control. After infection, a 15 µL aliquot of infected *E. coli* was used to make a serial dilution (from 10^{-1} to 10^{-3}) in 150 µL of 2xYT to determine the phage titer (output). One hundred and fifty microliters of each dilution was spread on 2xYT plates containing 12.5 mg/mL tetracycline. The remaining infected cultures (~10 ml) were centrifuged at 3,000 g

for 15 min at 4 °C. The pellets were re-suspended in 300 µL 2xYT and spread on 2xYT/tetracycline plates for amplification and incubated overnight at 37 °C. The next morning, the cells were collected from the plate using a plastic loop and 50 mL of 2xYT-Tet. Five millilitres of 2xYT-Tet were inoculated with 5 mL of collected cells and incubated at 30 °C for 5 hours with shaking at 220 rpm. The 2xYT culture was centrifuged at 5000 g for 30 min at 4°C to pellet the *E. coli*. The culture supernatant was passed through a 0.22 µm sterile filter (Millipore, Nepean, ON) and mixed with 1/5 the volume 20% (w/v) PEG 8K, 2.5 M NaCl solution and incubated on ice for 1 hour. The supernatant was centrifuged at 10,000 g for 15 min to precipitate the phage. The resulting pellet was resuspended in 200 µL of 1x PBS and stored at -20 °C.

3.2.4. ELISA

3.2.4.1. Peptide fusion and IGFBP7 protein monoclonal phage ELISA

After four rounds of panning, a total of 48 colonies (from the titer plate) from the fourth round of panning were screened for binding to rIGFBP7 using the standard phage ELISA protocol. For this, individual colonies were grown in a 96-well culture plate containing 250 µL/well of 2xYT supplemented with 12.5 µg/mL tetracycline at 30 °C overnight with shaking. A NUNC Maxisorb ELISA plate (VWR) was coated with 10 µg/mL of IGFBP7 diluted in sterile PBS, pH 7.4 and negative control wells were coated with StartingBlock™ buffer overnight at 4 °C. Next day, the wells were emptied and washed two times with 1x PBS, then blocked with blocking buffer at room temperature for two hours. At the same time, the culture plate was centrifuged at 1800 rpm for 20 min at 4 °C, and the phage supernatants were collected and used for monoclonal phage ELISA. One hundred microlitres of amplified phage supernatant were added to the wells, and incubated for 1.5

hours at room temperature. After the wells were washed five times with 300 μ L PBST, 100 μ L of anti-M13 mouse monoclonal IgG conjugated to HRP (GE Healthcare, Baie d'Urfé, QC) was added at 1:5000 dilution in blocking solution and incubated for one hour at room temperature. The wells were washed five times with 300 μ L 0.1% Tween 20-PBS. The reactions were developed with 100 μ L of TMB substrate (Pierce Biotechnology, Rockford, IL, USA) for about 10 min and then stopped with 100 μ L of 1 M phosphoric acid. The level of binding was determined spectrophotometrically at 450 nm on a standard ELISA plate reader (Thermo Multiskan FC 51119000). Clones with absorbance readings 3X greater than background readings were sequenced using the universal FDR-RP primer at the NRC-IBS sequence facility (National Research Council, Canada).

3.2.5. Subcloning, expression and purification of sdAbs in *E. coli*

3.2.5.1. V_HHs subcloning

All the clones showing strong binding to the IGFBP7 protein but with little or no binding to blocking buffer were subjected to DNA sequencing using PN2-FP (5'-CCCTCATAGTTA GCGTAA CGATCT-3') and FDR-RP (5'-CGGTATCAAGCTGTTTAAGAA ATTCAC-3') primers and ten V_HHs were subcloned into different expression vectors, namely, pVT2 for producing pentameric V_HH, and pSJF2 to produce monomeric V_HHs. Nine out of ten V_HHs were cloned into the pVT2 vector. The remaining V_HH was cloned in pMED2 (for monomeric V_HH) and pMED3 (for pentameric V_HH) vectors due to the presence of the internal *BbsI* and or *ApaI* restriction sites. V_HH genes were amplified from the phage vector in a total volume of 50 μ L by colony PCR using the following primers, VT-BbsI-F1 (5'-TAATAAGAAGACACCAGGCCAGGTAAAGCTGGAGGAGTCT-3'), *ApaI*-R (5'-AT TATTATGGGCCCTGAGGAGACGGTGACCTGGGTC-3') for cloning into the pVT2;

BamHI-R-(5'CGCGGGATCCTGAGGAGCGGTGACCTGGGTC-3') and VT-BbsI-F1 for cloning into the pSJF2; SfiI-L (5'CATGTGTAGATCGGCCAGCCGGCCGATGTGCA GCTGCAGGCGTCTG-3') and SfiI-R (5'CATGTGTAGATTCCTGGCCGGCCTGGC CTGAGGAGACGGTGACCTGGGTC-3') for cloning in pMED2 and pMED3 vector. An aliquot (~ 5 µL) from each PCR product was electrophoresed on a 1% agarose gel to confirm that the amplified products were of the correct size for the V_HHs (~ 450 bp). All the PCR products were purified using QIAGEN PCR Purification Kit (QIAGEN Inc., Mississauga, ON, Canada) and digested with the following restriction enzymes: *BbsI* and *Apa I* for pVT2, *BbsI* and *BamHI* for pSJF2 and *SfiI* for both pMED2 and pMED3. Then, all ten digested clone PCR products were ligated into the digested vectors as follows: 10 µL reaction volume was set up for each digested V_HH containing 1x DNA ligase reaction buffer, 1 unit T4 DNA ligase (Invitrogen, Carlsbad, California, USA), 90 ng pVT2, pSJF2, pMED2 and pMED3 vector, and 50 ng digested PCR product. The reactions were incubated for 1 hour at room temperature. After ligation, electrocompetent TG1 *E. coli* cells were transformed with the recombinant vectors. Two microlitres of each ligation product was added to 50 µL of electrocompetent TG1 *E. coli* cells (Stratagene, La Jolla, CA) and electroporated using Gene Pulser cuvette with a 0.1 cm gap (Bio-Rad Laboratories, Mississauga, ON) at 1800 V with 25 µF and 200 Ω. The transformed cells were immediately transferred into 1 mL SOC medium and incubated for 1 hour at 37 °C with slow shaking. Each 1 mL of transformed cells in SOC was plated on two preheated LB ampicillin plates (100 and 900 µl in each plate) containing 100 µg/mL ampicillin and the plates were incubated overnight at 32 °C. Colony PCR was performed to select the positive clones with correct V_HH size using the M13FP forward primer: 5'-GTAAAACGACGGCCAGT-3' and the M13RP reverse primer:

5'-CAGGAAACAGCTATGAC-3' (Operon, Eurofins USA). The expected size of the colony PCR product was ~950 base pairs for the pentamer vectors and ~700 base pairs for the monomer vectors. The PCR cycling conditions were as follows: 94 °C for 5 min; 30 cycles of: 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min; 72 °C for 7 min; 4 °C ∞. PCR products were resolved in a 1% (w/v) agarose gel to confirm the amplified product had the insert of correct size. Positive clones were sent for sequencing for further confirmation of their V_HH genes using M13RP and M13FP primers and Lasergene 6 software package was used for the analysis of sequencing data analysis (DNASTAR, Inc., Madison, Wisconsin, USA). Plasmid DNA and glycerol cell stocks were prepared and stored at -20 °C and -80 °C, respectively.

3.2.5.2. Soluble V_HH/pentamer expression and purification

3.2.5.2.1. Expression of pentaemric anti-peptide 3-VT, anti-peptide 4-VT and anti-IGFBP7 V_HHs

Single colonies of each clone were picked and transferred into 25 mL of LB/Amp (10 g/L Tryptone, 5 g/L yeast extract and 10 g/L NaCl) starter culture supplemented with 100 µg/mL Amp and 1% (w/v) glucose. Cultures were grown overnight at 37 °C while shaking at 220 rpm. For large-scale expression, 20 mL of starter culture was transferred into 1 L of M-9 medium/amp (10 µg/mL) medium supplemented with 5µg/mL vitamin B1, 0.4% (w/v) casamino acids, 0.2% (w/v) glucose, 0.1 mM CaCl₂ and 1 mM MgCl₂ and incubated at 220 rpm for 30 hours at 28 °C. The cultures were induced with 100 mL of 10x induction medium (12 g/L Tryptone, 24 g/L Yeast extract and 4 g/L Glycerol) , 150 µL of 1 M isopropyl β-d thiogalactopyranoside (IPTG) and 300 µL of 10 µg/mL ampicillin and left to incubate for another 60 hours at 220 rpm at 28 °C. After this period, cultures were centrifuged at 6,000

rpm (Beckman J2-21M/E centrifuge, Beckman Coulter Inc., Brea, CA; J-10 rotor) for 25 min at 4 °C. The harvested cells were re-suspended in 100 mL of ice-cold lysis buffer (50 mM tris-HCl at pH 8.0, 25 mM NaCl and complete EDTA-free protease inhibitor (Roche, Laval, Quebec) and stored at -20 °C overnight (Zhang, J. et al., 2004).

3.2.5.2.2. Expression of monomeric anti-peptide 3-VT, anti-peptide-4-VT and anti-IGFBP7 V_HHs

Single colonies of each clone were picked and transferred into 100 mL of LB/Amp (10 g/L Tryptone, 5 g/L yeast extract and 10 g/L NaCl) starter culture supplemented with 100 µg/mL Amp. Cultures were grown overnight at 37 °C while shaking at 220 rpm. For large-scale expression, 100 mL of starter culture was transferred into 900 mL of LB/Amp. Cultures were then grown at 32 °C with continuous shaking to an *A*₆₀₀/cm of 1, and induced by the addition of IPTG to 0.5 mM. Cultures were grown overnight at 32 °C with continuous shaking at 220 rpm.

3.2.5.2.3. Purification of pentamer V_HH antibodies

V_HH pentamer antibodies were extracted by the cell lysis method. The frozen cell pellets (see step 3.2.5.2.1 and 2) were thawed at room temperature with occasional shaking. To lyse the cells, 5 mL of freshly prepared lysozyme with 100 µg/mL (final concentration) (Sigma, Sigma-Aldrich, Oakville, ON) were added to the thawed cells. The suspension was incubated at room temperature for 30-50 minutes with occasional shaking. When the suspension became viscous, 300 µL of DNase I (Sigma, Sigma-Aldrich, Oakville, ON; 15 units/µL stock in 1 M MgCl₂) was added and the lysate was incubated for 20 to 30 minutes at room temperature until the suspension became watery. The lysate was centrifuged at 12,000 rpm (Beckman J2-21M/E centrifuge, J-17 rotor) for 30 minutes at 4 °C to separate

the soluble and insoluble fractions. Centrifugation was repeated with the soluble fraction until it became completely clear. The fraction which contained soluble V_HH pentabody was filtered through a 0.22 µm sterile filter (Millipore, Nepean, ON). Pentamerized V_HHs were purified by standard immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare). The recombinant proteins were eluted using a linear gradient with 500 mM imidazole buffer. Western blots and SDS-PAGE were done to confirm expression and purification quantity and quality, respectively. The tagged pentamer V_HH proteins were detected using anti-penta His monoclonal antibody (Qiagen, Mississauga, ON) diluted 10,000 fold followed by using Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (H+L) antibodies (GAM-AP) (1 : 5000) (Cedarlane, Burlington, Ontario) . Membranes were washed and developed with alkaline phosphatase substrate (AP Conjugate Substrate Kit; Bio-Rad, Mississauga, Ontario). Fractions containing pentamer V_HHs proteins were dialyzed overnight against SPR analysis buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA; HBS-E) with a 3,500 MW cut-off dialysis membrane. The absorbance of the proteins was measured at 280 nm (A₂₈₀) and the Expasy Protparam tool (<http://ca.expasy.org/tools/protparam.html>) was used to calculate the theoretical extinction coefficient (E) of each pentabody. The concentrations of the proteins were then determined using the following formula: $C = A_{260} / E$ where C is concentration in mg/ml, A is absorbance and E is extinction coefficient. The pentameric V_HHs were stored at 4 °C in PBS-3mM EDTA buffer.

3.2.5.2.4. Purification of monomer V_HH antibodies by Periplasmic protein extraction

Cultured cells were harvested by centrifuging at 6,000 rpm (Beckman J2-21M/E centrifuge, J-10 rotor) for 20 min at 4 °C. The supernatant was discarded and the cell pellet was placed on ice. The cell pellet was re-suspended in 30 mL ice-cold TES Buffer (0.2 M tris-HCl pH 8.0, 0.5 mM EDTA and 20% sucrose) and 3 µL of 100 mM PMSF and chilled on ice for 30 min. Forty millilitres of ice-cold 1/8 TES buffer was added to the cell suspension. The cell suspension was vortexed, chilled on ice for another 30 min, and centrifuged at 6000 rpm (Beckman J2-21M/E centrifuge, J-10 rotor) 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 filtered through a 0.22 µm filter and stored at 4 °C. SDS-PAGE and Western blotting were performed to confirm the expression of tagged protein. Monomeric V_HHs were purified by standard immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare). The recombinant proteins were eluted using a linear gradient with 500 mM imidazole buffer. Fractions containing monomer V_HH proteins were dialyzed overnight against SPR analysis buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA; HBS-E) with a 3,500 MW cut-off dialysis membrane. The absorbance of the proteins was measured at 280 nm (A₂₈₀) and the Expasy ProtParam tool (<http://ca.expasy.org/tools/protparam.html>) was used to calculate the theoretical extinction coefficient (E) of each pentabody. The concentrations of the proteins were then determined using the following formula: $C = A_{260} / E$ where C is concentration in mg/mL, A is absorbance and E is extinction coefficient. The purified monomer V_HHs proteins were stored at 4 °C.

3.2.6. Soluble ELISA

NUNC Maxisorb ELISA plate wells (VWR) were coated with 50 µg/mL of IGFBP7 diluted in sterile PBS, pH 7.4 and 20 µg/mL of peptide 3-VT and peptide 4-VT. Negative control wells were coated with StartingBlock™ T20 (PBS) Blocking Buffer (Thermo Fisher) overnight at 4 °C. The next day, the wells were emptied and washed two times with 1x PBS then blocked with StartingBlock™ T20 (PBS) Blocking Buffer at RT for two hours. One hundred microliters of 20 µg/mL of pentameric V_HH were added to the wells. As a negative control, 20 µg/mL of a non-related pentamer was used in the control wells and the plate was incubated for 1.5 hours at room temperature (RT). After the wells were washed five times with 300 µL PBST 0.1% T-20, 100 µL anti-c-Myc conjugated to horseradish peroxidase (HRP) (Bethyl laboratories, Tx., USA) was added at 1:10,000 dilution in StartingBlock™ T20 and incubated for 1 hour at RT. The wells were washed five times with 300 µL PBST 0.1% T-20. The reactions were developed with 100 µL of TMB substrate (Pierce Biotechnology, Rockford, IL) for about 10 min and then stopped with 100 µL of 1 M H₃PO₄. The level of binding was determined spectrophotometrically at 450 nm.

3.2.7. Kinetics analysis by surface Plasmon resonance (SPR)

Binding kinetic experiments were performed by SPR using a Biacore 3000 instrument (GE Healthcare, Baie d'Urfé, Quebec, Canada). Approximately 600 resonance units (1 RU = 1 pg/mm²) of human recombinant IGFBP7 was immobilized onto a research grade CM5 sensor chip (GE Healthcare). An ethanolamine blocked surface was used as a reference. Immobilization was carried out at a concentration of 50 µg/mL in 10 mM acetate pH 4.5 using the amine coupling kit supplied by the manufacturer. Size exclusion chromatography

(SEC) was used to eliminate any possible aggregates in the monomer (Superdex 75 column) and pentamer (Superdex 16/60 column) samples prior to Biacore analysis (figure 11). For the binding studies, analyses were carried out at 25 °C in 10 mM HEPES, pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005 % (v/v) surfactant P20. The flow rates used were 40 μ L/min and sample volumes of 20 μ L were used at RT unless otherwise stated. To regenerate the chip surface, it was washed thoroughly with the running buffer. Data were analyzed with BIAevaluation 4.1 software. All SPR studies were performed at the SPR facility at NRC-IBS by Henk van Faassen (NRC-IBS, Ottawa, Ontario, Canada).

3.2.8. Functional assay

3.2.8.1. Cell culture

The human pancreatic cell line, Panc-1, was obtained from the American Type Culture Collection. Panc-1 cells were grown at 37 °C in DMEM supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, USA) in a humidified atmosphere of 5% CO₂/95% air. Medium for Panc-1 cell line was changed every 3-4 days.

3.2.8.2. Anchorage-independent growth of Panc-1 on soft agar

Anchorage-independent growth of PANC-1 cells in the absence or presence of either P12 (3 μ M), IGFBP7 (300nM) or the combination of P12 (3 μ M) plus IGFBP7 (300nM) were examined in semi-solid agar. D-MEM containing 10% FBS was warmed to 48 °C and diluted with Bacto-Agar to make a 0.6% (w/v) agar solution. The bottom layer consisted of 0.3 mL of 0.6% (w/v) agar per well in a 24 well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The bottom layer was allowed to solidify for 30 min. before adding the top layer, which contained 0.6% (w/v) agar, 18,000 Panc-1 cells and 20x antibody treatments diluted in 0.15 mL D-MEM supplemented with 10% (v/v) FBS. After

the top layer solidified, 50 μ L of 1x antibody treatment was added and replaced every three days over the next 3 weeks. At the end of the experiment, phase contrast images (8 fields/well x 4 wells per treatment) were captured and visualized using an Olympus microscope (Olympus 1X81, Markham, ON, Canada). *In Vivo* and ImagePro 6.2 software packages were used to measure the total area (mm^2) and number of tumor colonies in each image.

3.2.8.3. Data analysis

To analyze the differences in total tumor colony area or number, one-way variance analysis followed by a student–Newman–Keul's post-hoc comparison of means was used (GraphPad Prism 5.0, USA). Differences with a P value ≤ 0.05 were considered to be statistically significant.

4. RESULTS

In this section, construction of a new biotinylated pentameric vector, expression and purification of VT-peptide fusion protein, isolation of V_HH sdAbs by panning, and characterization of anti-IGFBP7 llama V_HHs are presented.

4.1. Construction of pVT2-Human Hinge-Bio expression vectors

The original pentameric pVT2 plasmid vector was modified in order to introduce a biotinylation sequence containing a unique lysine residue site, GLNDIFETQKIEWHE (Scholle M., 2004). A total of 24 colonies were screened using M13RP and M13FP primers by colony PCR. Positive clones contained an insert of the expected size ~654 bp including the biotin signal sequence while the bands at ~530 bp were identified as negative (**Figure 5**). Positive clones were subjected to DNA sequencing and the sequencing data confirmed the presence of the biotinylation sequence. For the plasmid map, please refer to Appendix.

4.2. Peptide Antigen

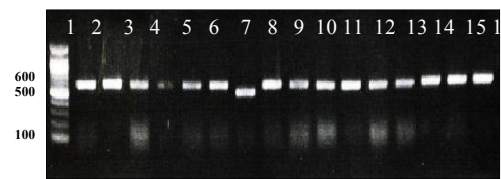
4.2.1. Cloning peptid coding sequence into pVT2-HuHg-Bio expression vector

The oligonucleotides encoding the target peptide were cloned into the pVT2-Human Hinge-Bio vector for pentameric expression in *E. coli*. A total of 16 colonies from each peptide were screened by colony PCR with M13RP and M13FP primers (see section 3.2.2.1.). For each peptide, four clones were identified as positive that contained an insert of the expected size of 700 bp and 714 bp, for peptide 3 and peptide 4, respectively (Figure 6). Sequencing data confirmed the presence of the respective peptide inserts.

Figure 5. PCR screening of pVT₂- HuHg-Bio.

(A) The screening of transformed colonies using M13RP and M13FP primers electrophoresed in a 1% agarose gel. Fragments of ~500 bp represent clones with no insert (lane 7), while fragments of ~654 bp represent clones harboring a biotin signal sequence (lanes 1-6 and 8-16). DNA ladder, 100 bp. (B) Schematic representation of pVT₂-HuHg-Bio primary structure.

A

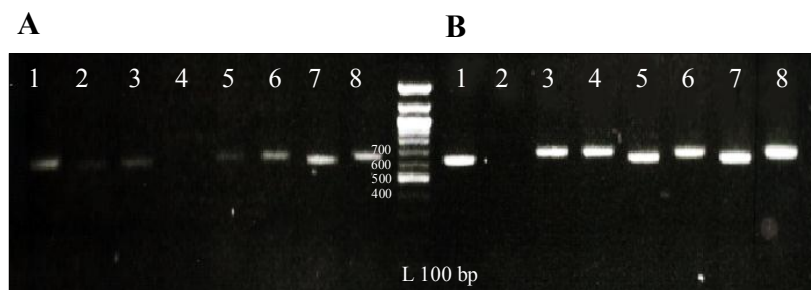


B



Figure 6. PCR screening of peptide-VT-Bio.

The transformed colonies were screened using M13RP and M13FP primers and electrophoresed in a 1% agarose gel. Fragments of ~654 bp represent clones with no insert, while fragments of ~700 bp represent clones harboring the peptide sequences. **(A)** Colony PCR of peptide 3 transformed clones (lanes 5, 6 and 8 are positive). **(B)** Colony PCR of peptide 4 transformed clones (lanes 3,4,6,8 are positive). DNA ladder, 100 bp. **(C)** Sequencing analysis of VT-peptide 3 and VT-peptide 4 clones. The amino acid sequence of VT-peptide 3 and VT-peptide 4 used in this study are aligned using the ClustalW method. Peptide sequence (shaded in grey), Biotin signal sequence (underlined).



C

VT-peptide3 -----**ALHEIPVKKGE****GAEL**-----GPAEPKSGDKTHTSPSPSTPDCVTGKVEYTKYNDEDTFTVKVGDKELFTNRRAN

VT-peptide4 **QASASAKITVVDALHEIPVK**-----GPAEPKSGDKTHTSPSPSTPDCVTGKVEYTKYNDEDTFTVKVGDKELFTNRRAN

VT-peptide3 LQSLLLSAQITGMTVTIKTNACHNNGGGFSEVIFRGGGSGLAGSEQKLISEEDLGSSGLNDIFETQKIEWHEGGGSENLY

VT-peptide4 LQSLLLSAQITGMTVTIKTNACHNNGGGFSEVIFRGGGSGLAGSEQKLISEEDLGSSGLNDIFETQKIEWHEGGGSENLY

VT-peptide3 FQGHHHHH

VT-peptide4 FQGHHHHH

4.2.2. Expression and purification of peptide fusion proteins

Expressions of VT-peptide 3 (clone 5) and VT-peptide 4 (clone 3) were performed and the presence of the fusion proteins were verified by Western blot. As shown in Figure 7, peptide-3 fusion and peptide-4 fusion proteins were successfully expressed and proteins of expected size were observed. Based on the amino acid composition, the theoretical molecular weights of the VT-peptide-3 and VT-peptide-4 plus tag sequence are predicted to be ~16.6 and ~17.12 kDa, respectively, using LaserGene DNA sequencing analysis (DNASTAR, Inc., Madison, Wisconsin). The bacterial lysate was loaded onto an IMAC column and purified. The elution of the ~17 kDa His₆-tagged fusion protein corresponded to a peak that eluted between the elution volumes of 24-35 mL. Eluted fractions were resolved by SDS-PAGE and stained with Coomassie blue R-250 which confirmed the expression of fusion protein (Figure 7 C.). The concentrations of peptide fusion proteins were measured spectrophotometrically and the yields were calculated using the extinction coefficient of the respective fusion proteins. The concentration of was calculated to be 0.35 mg/L for VT-peptide 4 and 2.3 mg/L for VT-peptide 3. The aggregation state of the purified VT-peptide3 and VT-peptide 4 was determined by size exclusion chromatography on Superdex 200 16/60 column. VT-peptide 3 showed a slight degree of aggregation however VT-peptide 4 showed no aggregation (Figure 8 A.). High concentrations of 2.0-3.0 mg/mL could contribute to aggregate formation in the VT-peptide 3 sample.

4.2.3. Mass spectrometry of peptide fusion proteins

The protein samples of VT-peptide-3 and VT-peptide-4 were run on LC-MS (Dr. John Kelly, NRC-IBS) and the LC-MS spectra are shown in Figure 8 (B). The biotinylated VT-peptide-3 is almost intact and has the correct molecular weight.

Figure 7. Western blot of peptide fusion protein expression.

(A) His-tagged VT-peptide-3 and VT-peptide-4 blots were probed with mouse anti-6-His IgG and goat anti-mouse mAb conjugated to alkaline phosphatase. Lane 1: 8 μ L pre-stained SDS-PAGE Broad Range Standard; Lane 2: VT-peptide-3; Lane 3: VT-peptide-4; Lane 4: positive control (biotinylated V_HH). (B) *In vivo* biotinylated VT-peptide-3 and VT-peptide-4 blot were probed with streptavidin conjugated to alkaline phosphatase. Lane 1: 8 μ L prestained SDS-PAGE Broad Range Standard; Lane 2: VT-peptide-3 (~2 μ g); Lane 3: VT-peptide-4 (~2 μ g). (C) SDS-PAGE of peptides fusion proteins. SDS-PAGE of eluted fractions collected during FPLC purification of VT-peptide-3 and VT-peptide-4. Molecular weight markers, M, in kDa. Lane 1-6 contains 10 μ L of VT-peptide-3 from fraction 21, 23, 25, 32 respectively. Lane 8-13 contains 10 μ L of VT-peptide-4 from fraction 21, 23, 25, 32 respectively.

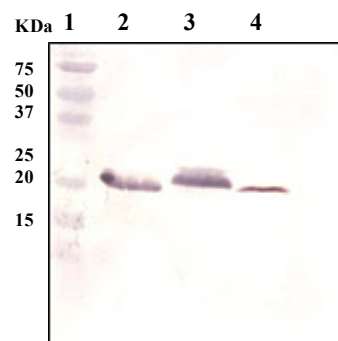
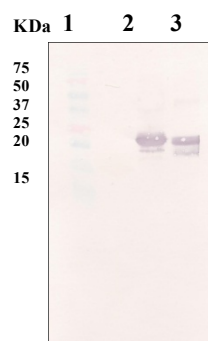
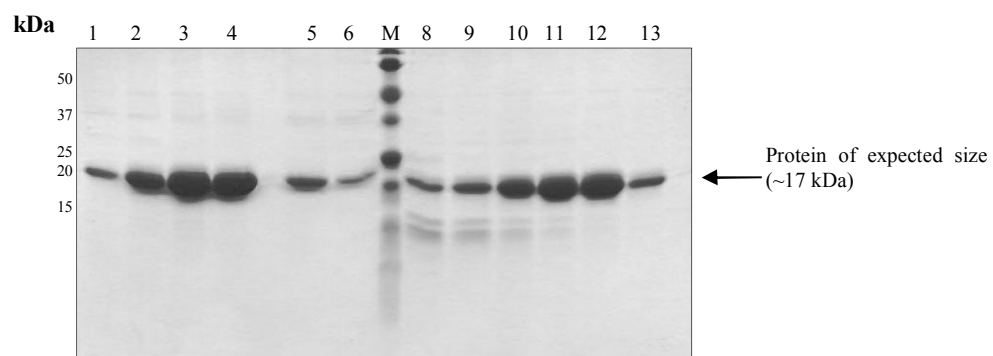
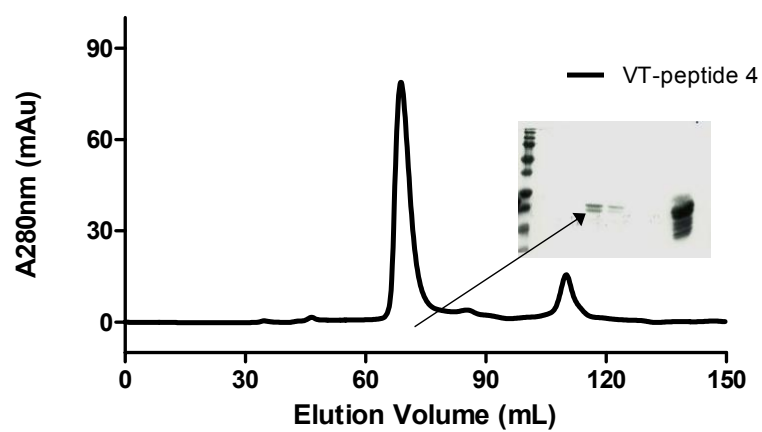
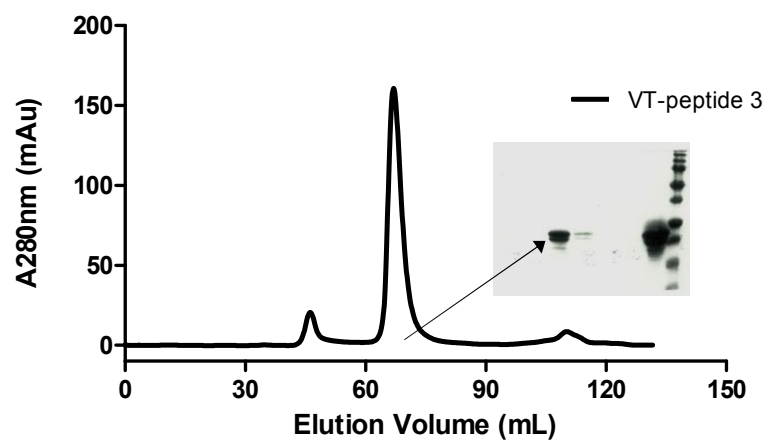
A**B****C**

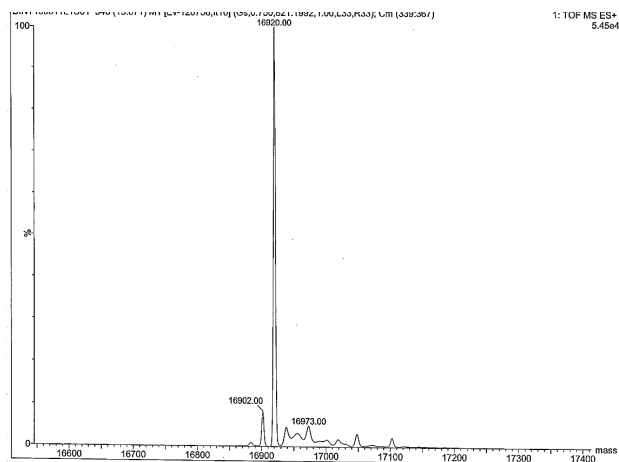
Figure 8. Size exclusion chromatography profiles of VT-peptide 3 and VT-peptide 4 proteins and Mass spectrometry profile.

(A) VT-fusion proteins were applied to a Superdex 200 16/60 column to analyze their aggregation state. Both peptides eluted at a volume of 66 mL. VT-peptide 3 shows some degree of aggregation based on the small peak eluted at approximately 45 mL whereas VT-peptide 4 shows no aggregation. (B) LC-MS spectra of the VT-peptide 3.

A



B



4.3. Panning I

4.3.1. Peptide panning using semi-automated KingFisher

The LAC-M naïve *Camelidae* library was used for panning and phage-rescued for panning. Four rounds of panning were performed to isolate naïve llama V_HH binders against two overlapping IGFBP7 peptides as described in section (3.2.3.1). In the first round of panning, 10 µg of VT-peptide 3 and 4 fusion proteins were anchored to streptavidin magnetic beads and incubated with 10¹² pre-adsorbed phage rescued from the naïve library. Three high speed washes were done to remove non-specific binders using a KingFisher Flex Magnetic Particle Processor. Antigen concentrations were reduced in the second, third and fourth rounds of panning while stringent washing conditions were increased. Table 3 shows the input and output phages from each round of panning.

4.3.2. IGFBP7 solid phase panning

For IGFBP7 panning, the naïve library was screened against IGFBP7 as described in section (3.2.3.2). Four consecutive rounds of panning were performed. After round 1, the recovered phage was further exposed to stringent condition by decreasing the concentration of IGFBP7 and by increasing the number of washes. Consequently, the output phage titer of the second round was decreased from 2.2 x 10⁶ to 7.1 x 10⁵ cfu. Despite the fact that the stringency condition was further increased in the third and fourth round of panning, the output titer increased to 2 x 10⁷ cfu. The results of this panning are shown in Table 3. As shown, the output phage titer dropped in the second round of panning for all three targets. However, the phage output increased between 40-900 times and remained unchanged.

Table 3. Phage titres of four rounds of panning against VT-peptides 3, VT-peptides 4 and IGFBP7.

<i>Round</i>	<i>VT-Peptide 3 (cfu)</i>	<i>VT-Peptide 4 (cfu)</i>	<i>IGFBP7 (cfu)</i>
Round 1			
Input	6×10^{12}	6×10^{12}	6×10^{12}
Output	1.3×10^7	2.8×10^6	2.2×10^6
Round 2			
Input	1.4×10^{12}	1.4×10^{12}	3.14×10^{11}
Output	1.1×10^5	3.9×10^5	7.1×10^5
Round 3			
Input	3×10^{12}	8×10^{11}	1.2×10^{12}
Output	9.1×10^7	2.2×10^7	2.8×10^7
Round 4			
Input	2.8×10^{12}	2.7×10^{12}	3.5×10^{12}
Output	6.13×10^7	4.72×10^7	2×10^7

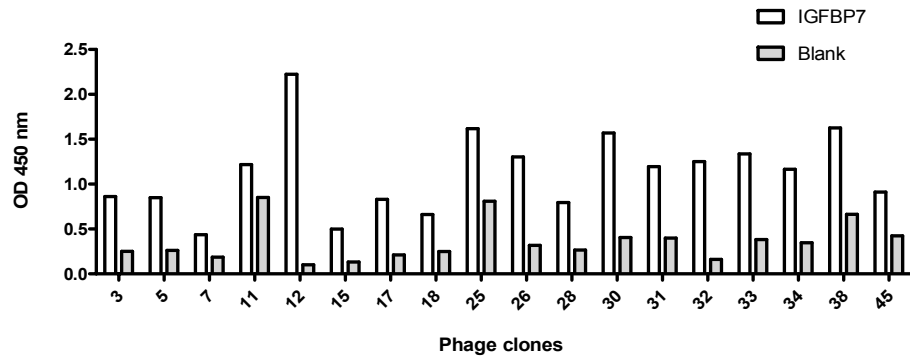
4.3.3. Monoclonal phage ELISA of anti-peptide and anti-IGFBP7

To identify specific V_HH binders against VT-peptide-3, VT-peptide-4 and IGFBP7, individual phage clones from the fourth round of panning were screened by monoclonal phage ELISA. A total of 48 clones for each antigen were picked at random and screened against immobilized IGFBP7. Bound phages were detected with anti-M13 IgG conjugated to HRP. Clones with an absorbance reading > 0.3 were considered positive for binding to IGFBP7 (Figure 9). For VT-peptide-3 about 35% (17/48) of the clones scored positive for binding to IGFBP7. For VT-peptide-4 about 17% (8/48) showed specific binding to IGFBP7. All 48 clones obtained from solid phase panning showed specific binding to IGFBP7 (Figure 9 not all data shown).

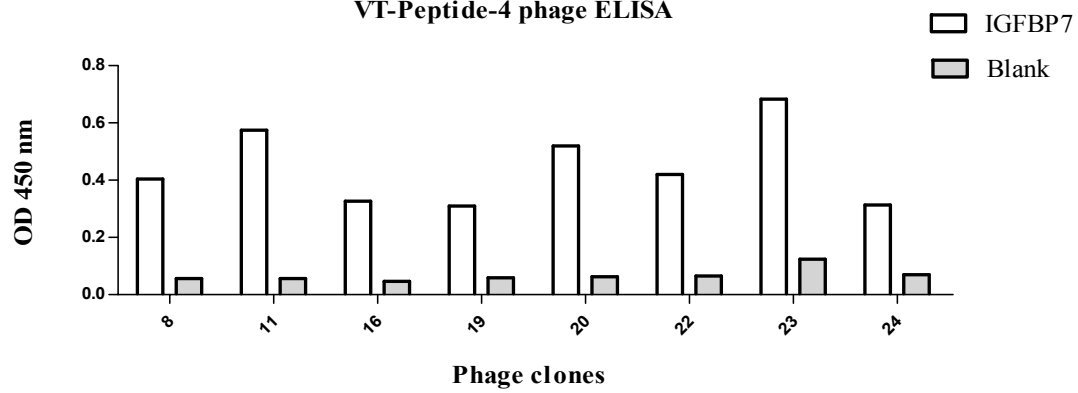
Figure 9. Monoclonal Phage ELISA of V_HHs obtained from the 4th round of panning.

Randomly selected individual colonies were grown and phage antibodies were prepared. Wells of a 96-well microtiter plate were coated with IGFBP7 (10 µg/mL) in PBS and incubated with phage supernatant. Bound phages were detected by anti-M13-HRP. Absorbance values at 450 nm were measured and subtracted from those of the background wells (no IGFBP7 coated). As shown, 17/48, 8/48 and 48/48 (not all data shown) phage clones specifically recognized IGFBP7. Clones with an absorbance reading > 0.3 were considered positive for α-IGFBP7 binding.

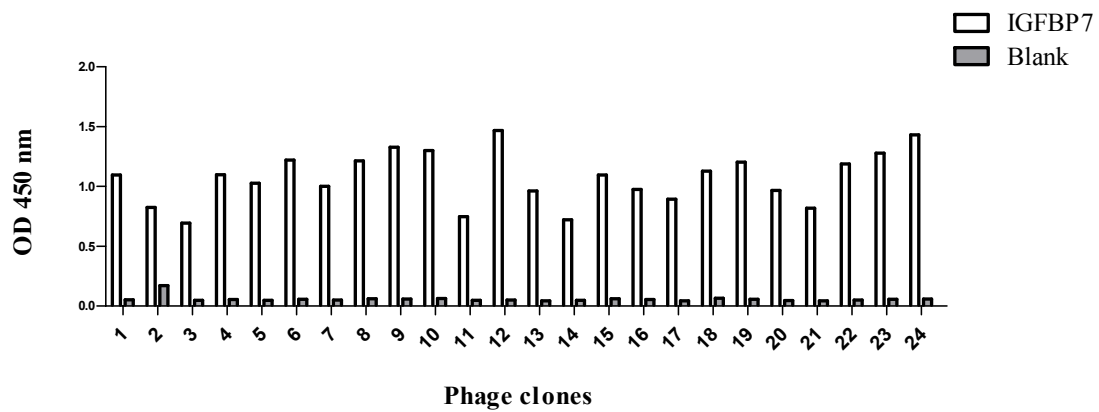
Peptide-3 phage ELISA



VT-Peptide-4 phage ELISA



IGFBP7 phage ELISA



4.3.4. Sequence analysis of round 4 anti-IGFBP7 V_HH clones

A total of nine clones considered positive for IGFBP7 binding by monoclonal phage ELISA were sequenced at NRC-IBS, Ottawa, Ontario, Canada using FdR-RP and PN2-FP primers. Analysis of the sequencing data revealed nine unique IGFBP7 binders having V_HH characteristics based on the presence of camelid V_HH hallmark residues at position 42, 49, 50, 52 and 92 (IMGT). Conventional VHs have conserved amino acid residues V, G, L and W at positions 42, 49, 50, and 52, respectively that interact with variable light domain. However, in camelid V_HHs those residues are replaced with more hydrophilic amino acids. All the anti-IGFBP7 clones have F, E and R at position 37, 44, and 45, respectively, confirming they are in fact V_HHs (data not shown). The isolated clones were clustered in nine groups and the pentameric V_HH clones were named P1, P2, P4, P5, P8, P9, P10, P11 and P12 and the monomeric clones were designated M2-2, M4-1, M5-1, M8-1, M9-2, and M12-1.

4.3.5. Large-scale expression and purification of V_HH clones

The positive V_HH sequences were cloned into the pVT2 vector for pentameric expression and into pSJF2 for monomeric expression in *E. coli*. Expressed proteins were purified from the periplasmic fractions (monomer) and the cell lysate (pentamer) by IMAC (Figure 15). The expression yields of purified V_HHs (both pentamer and monomer) were variable, ranging from 0.4 – 10 mg/L of bacterial culture (Table 4). The purified protein was further characterized by ELISA and surface plasmon resonance (SPR). Clone M12 and Clone P12 were further characterized in *in vitro* neutralization assays.

Purified V_HH fractions obtained after FPLC were combined. The average total volume of the combined fractions for each V_HH was around 10-15 mL. The combined fractions were dialyzed overnight and the concentration of the V_HHs was estimated using the theoretical molar absorbance coefficient. This value was calculated using ProtParam-ExPASy, Expert Protein Analysis System Proteomics Server of the Swiss Institute of Bioinformatics (<http://www.expasy.ch/tools/protparam.html>), while the molecular weights (MW) listed in the table were determined using DNASTAR, Inc. (Madison, Wisconsin, USA). Therefore, it was calculated that the concentration (C) of the dialyzed monomer and pentamer V_HH proteins was: Concentration (mg/mL): $\frac{A_{280}}{\Sigma} \times MW (Da)$

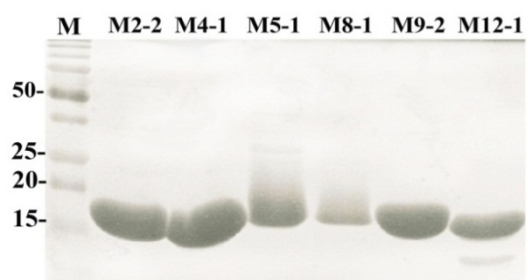
Table 4. Overview of isolated anti-IGFBP7 V_HHs from the naïve phage-display libraries

<i>anti-IGFBP7 Clone</i>	<i>MW (kDa)</i>	<i>Concentration (mg/mL)</i>
P1	117.76	6.0
P2	122.12	3.35
P4	121.54	2.23
P5	121.98	0.50
P8	122.02	0.40
P9	122.02	0.43
P10	121.81	1.70
P11	122.09	0.62
P12	121.63	1.13
M2-2	15.70	1.80
M4-1	15.59	1.80
M5-1	15.67	0.84
M8-1	15.67	0.50
M9-2	15.43	1.20
M12-1	15.62	1.50

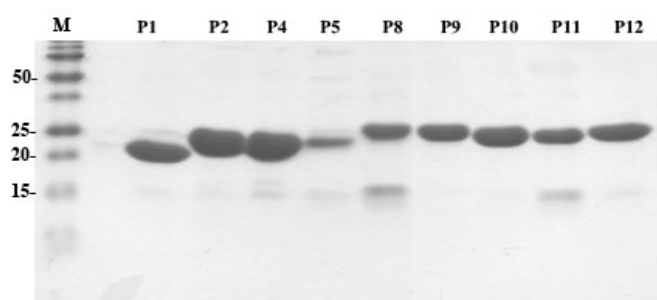
Figure 10. SDS-PAGE profile of purified V_HH.

Monomeric and pentameric V_HH isolated from the naïve llama phage display against VT-peptide3, VT-peptide4 and IGFBP7 were analyzed by SDS-PAGE. (A.) Purified monomeric V_HH clones with molecular weight ~15 kDa. (B.) Purified pentameric V_HHs with molecular weight ~25 kDa. Each lane contains 2-5 µg V_HH per lane. (M) Molecular weight markers in kDa. The 12.5% SDS-PAGE gel was stained with Coomassie blue R-25.

A.



B.



4.3.6. Size exclusion of V_HHs

Purified monomeric V_HH clones were analyzed to study their aggregation state by size exclusion chromatography using a Superdex 75 10/30 column. The major monomeric peaks eluted at 13 mL, the volume at which a protein of theoretically calculated molecular weight of ~15 kDa would elute using this column. There was slight aggregation found in M2-2, M4-1. M5-1 showed a small shoulder eluting at 16 mL, possibly due to the slight degradation of the His-tag. No aggregation was seen in the M8-1, M9-2 and M12-1 samples. The purified pentameric (P12) clone was also analyzed using a Superdex 200 16/60. The protein eluted at ~68 mL with no obvious aggregation (Figure 11).

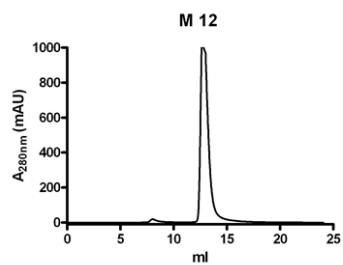
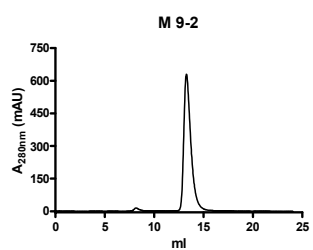
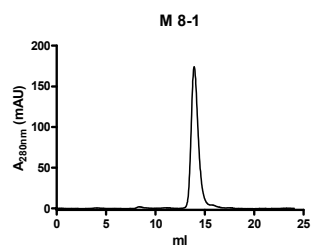
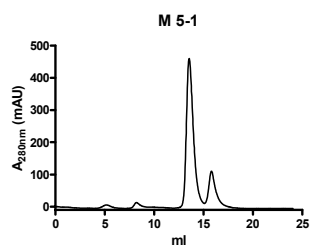
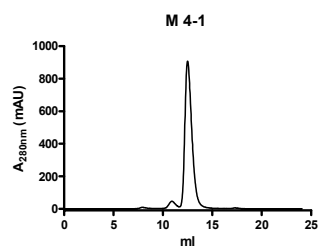
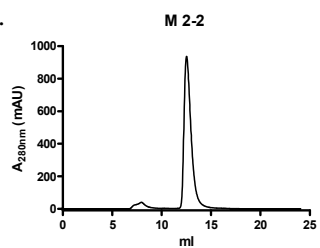
4.3.7. Soluble ELISA of anti-IGFBP7

The pentamerized V_HH clones were tested for their ability to bind both the recombinant VT-peptides and IGFBP7 in ELISA assays in which VT-peptide3, VT-peptide 4 and IGFBP7 were immobilized and purified pentameric V_HHs were specifically recognized and detected with anti-c-Myc-HRP conjugate. Soluble ELISA demonstrated that 7 of 9 V_HH clones were able to bind to the recombinant VT-peptide 3 and/or VT-peptide 4 except clone 1 (P1) and clone 4 (P4). The second soluble ELISA demonstrated that 8 of 9 V_HH clones were able to bind the parent protein IGFBP7 with the exception of clone 1 (P1) (Figure 12).

Figure 11. Size exclusion chromatography profiles of monomeric V_HH.

(A.) The purified V_HHs were applied to a Superdex 75 10/30 column to ensure that all behave as monomers and do not aggregate. Small aggregate peaks found in M2-1, M4-1 and M5-1 eluted at approximately 8 to 10 mL. (B.) Pentamer P12 was applied to a Superdex 200 16/60 and eluted at 68 mL with no aggregation.

A.



B.

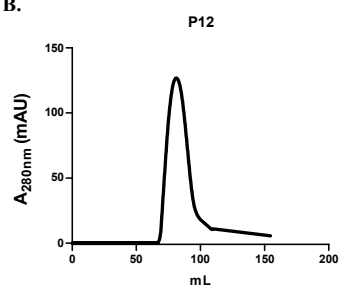
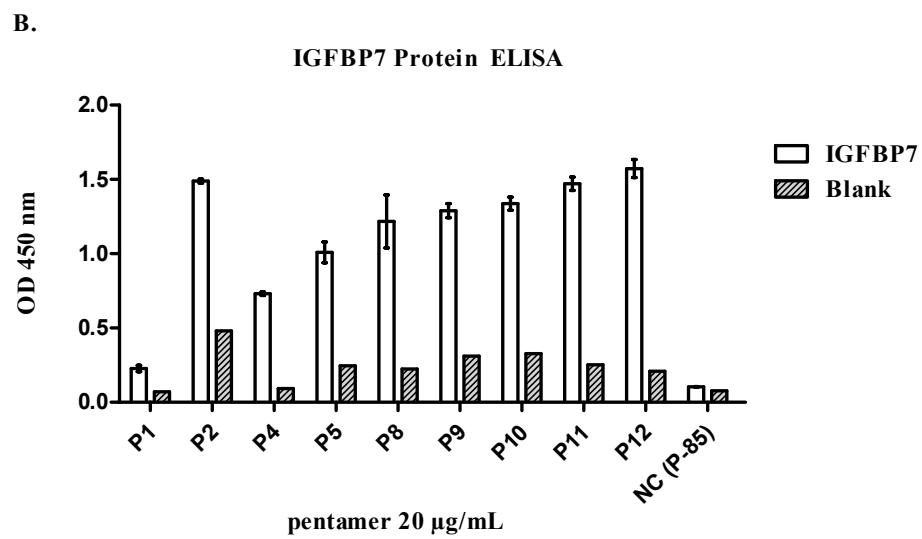
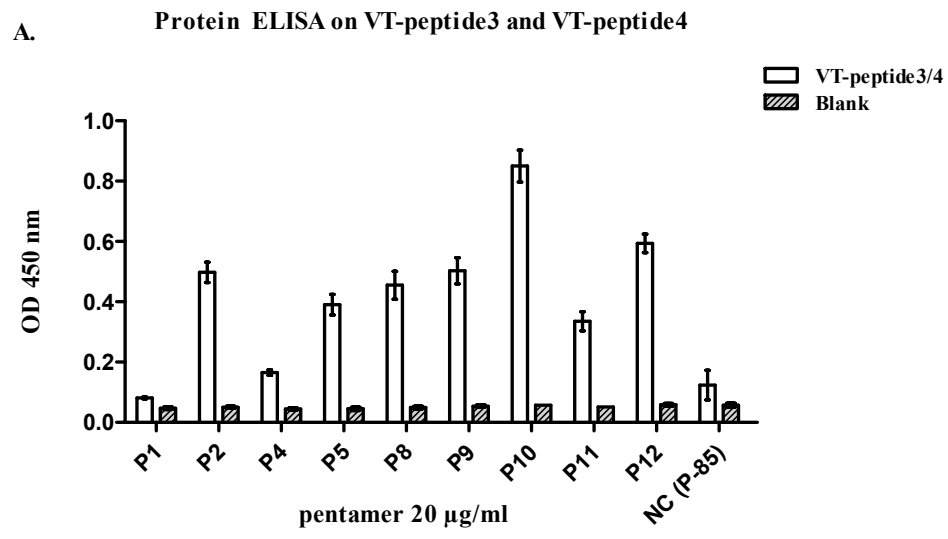


Figure 12. Soluble Protein ELISA of pentameric V_HH.

(A) Soluble Protein ELISA of pentameric V_HH against VT-peptide 3 or VT-peptide 4. **(B)** Soluble protein ELISA of V_HHs against IGFBP7. Purified pentamers (20 µg/mL) were incubated with immobilized antigen in microtiter wells and detected with anti-c-Myc-HRP. Negative control: pentabody 85-VT.



4.3.8. Surface Plasmon Resonance

Binding kinetics of anti-IGFBP7 V_HH binding to IGFBP7 were determined by surface plasmon resonance SPR (BiaCore™) analysis. Monomers were injected over Ovalbumin control and IGFBP7 bound to a CM5 sensor chip at various concentrations ranging from 1 to 5 μ M to generate the overlay sensogram of M2-2, M4-1, M5-1, M8-1 and M9-2 (Figure 13). The binding kinetics of V_HH clones are summarized in Table 5. All tested clones show binding affinity to immobilized IGFBP7 with fast dissociation rate and resulted in K_D values ranging from 100 nM to 1 μ M. From a previous study (Iqbal et al., 2010) and through all of these experiments, the recombinant IGFBP7 was observed that it is very unstable when it is stored at -20 °C. It was also observed that some non-related V_HH antibodies interact non-specifically with IGFBP7 as shown by SPR data. Therefore, another set SPR experiments were carried out with fresh aliquots of IGFBP7 stored at -80 °C with positive and single domain negative controls. Additionally, new buffer conditions were applied to test the binding with the clone that was isolated from IGFBP7 panning instead of the other peptide binders to avoid any possible chance of specific recognition to the pentameric domain, for example. In 150 mM NaCl, pH 7.4, the negative control showed some non-specific binding to IGFBP7 despite the fact that different pH ranges and different surfactant concentrations in wash buffer at pH 5 were used. However, when the salt concentration in the wash buffer was increased from 150 mM to 300 – 500 mM, the non-specific binding of the single domain negative control was almost abolished whereas the V_HH antibody M12 showed specific binding to IGFBP7 (Figure 14). This finding revealed that the salt concentration plays an important role in eliminating the non-specific binding of all V_HH tested to IGFBP7.

Therefore, the new washing conditions with low pH and 300 mM salt were applied in a new panning experiment in order to isolate specific V_HH antibodies.

Figure 13. Binding kinetics of monomeric V_HHs to IGFBP7 by SPR.

Purified recombinant IGFBP7 was immobilized on a CM5 sensor chip by amine-coupling and the monomeric V_HHs were injected over the surface at different concentration ranges (from 5 to 5000 nM), giving affinity constants ranging from 100 nM to 1 μ M.

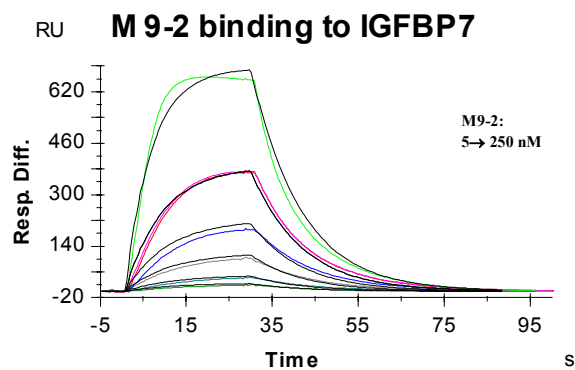
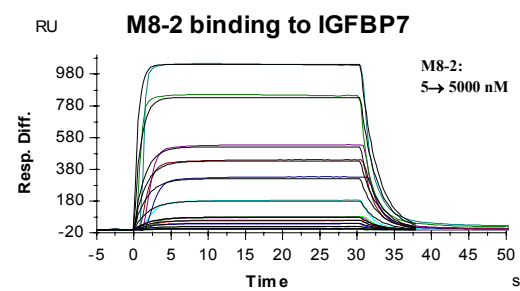
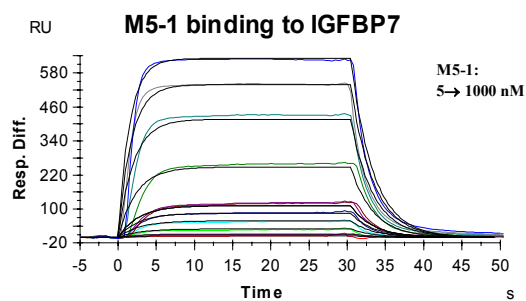
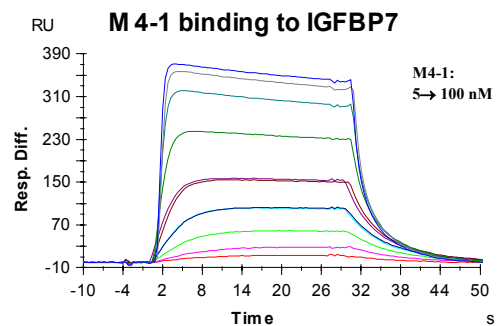
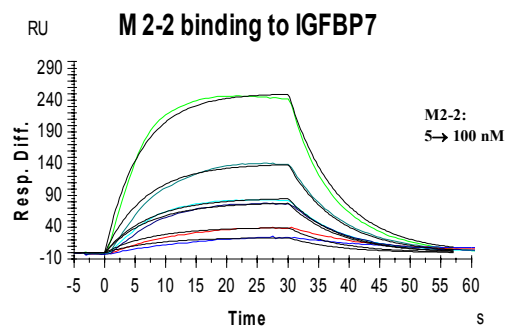


Table 5. Equilibrium dissociation constants (K_D s) of anti-IGFBP7 V_H H binders as determined by SPR.

IGFBP7 was immobilized on a CM5 sensor chip and binding kinetics were obtained from a 1:1 binding model of antibody:antigen interactions at different concentrations of each anti-IGFBP7 monomeric V_H Hs. The affinity constant (K_D), association (k_{on}) and dissociation (k_{off}) rate constants were determined by SPR analysis using BIACORE 3000.

Monomer	K_D (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	R_{max} (RU)	Theoretical R_{max}
M 2-2	210	4.8×10^5	1.23×10^{-1}	800	336
M 4-1	180	-	-	400	
M 5-1	930	3.19×10^5	3.4×10^{-1}	1200	
M 8-1	1570	2.71×10^5	4.84×10^{-1}	1400	
M 9-2	230	3.07×10^5	8.67×10^{-2}	1200	
M 12-1	100	-	-		

Figure 14. Binding kinetics of anti-IGFBP7 V_HH P12, negative control and positive control at different buffer conditions. Protein sequence of anti-IGFBP7 sdAb 4.43; CDR1, CDR2 and CDR3 are underlined.

Binding of monomers **M12** (anti IGFBP7) and **B4.3** (*Negative Control*) to IGFBP7

HEPES: 10mM

EDTA: 3mM

NaCl: 150mM

P-20: 0.005%

pH: 7.4

HEPES: 10mM

EDTA: 3mM

NaCl: 150mM

P-20: 0.05%

pH: 7.4

HEPES: 10mM

EDTA: 3mM

NaCl: 300mM

P-20: 0.005%

pH: 7.4

HEPES: 10mM

EDTA: 3mM

NaCl: 500mM

P-20: 0.005%

pH: 7.4

HEPES: 50mM

EDTA: 3mM

NaCl: 150mM

P-20: 0.005%

pH: 6.5

HEPES: 10mM

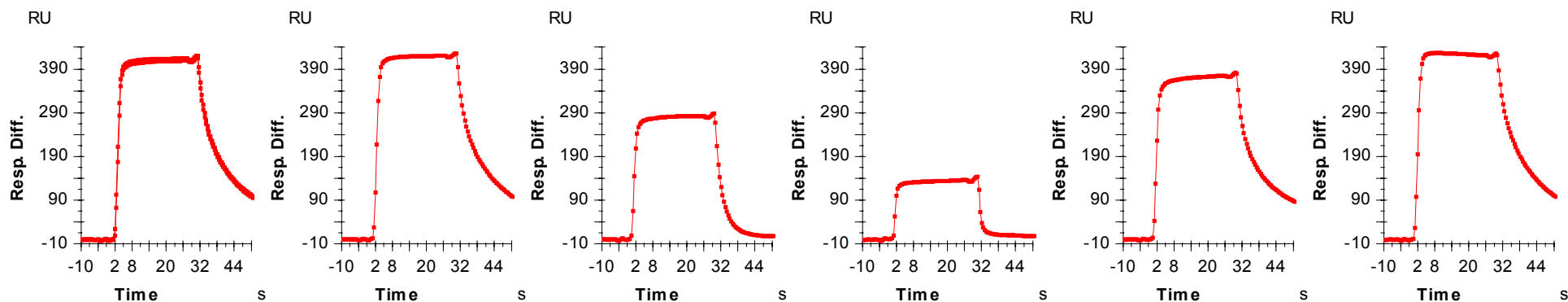
EDTA: 3mM

NaCl: 150mM

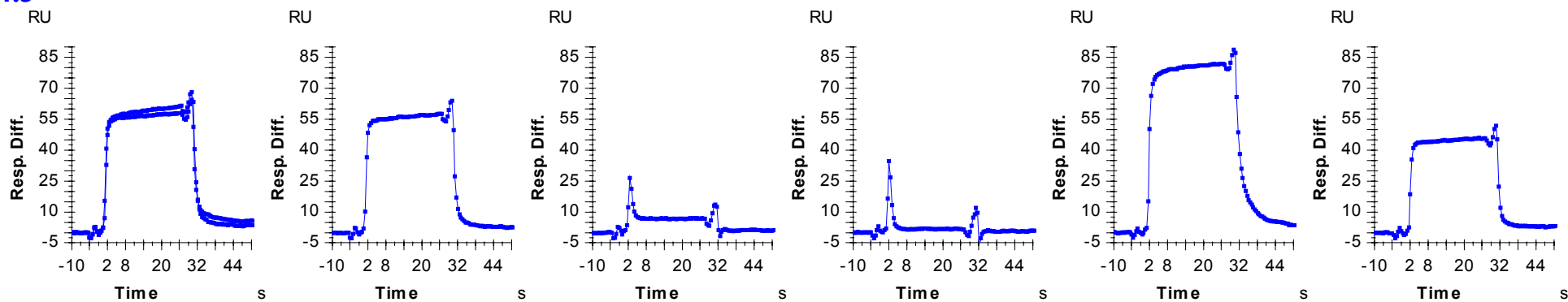
P-20: 0.005%

pH: 8.0

M-12

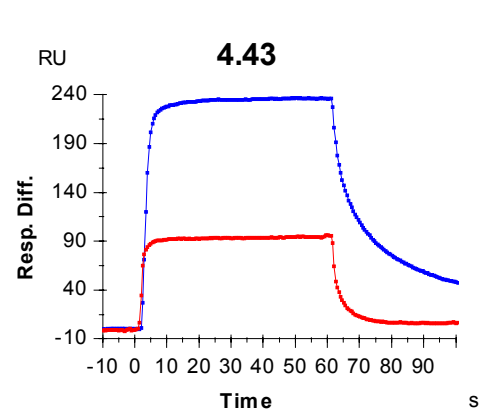


B4.3



Concentration: 500nM

Monomer 4.43 (500nM)



--- HBS-EP buffer
(NaCl: 150mM)

--- HBS-EP buffer
(NaCl: 300mM)

4.43 Sequence:

AIAIAVALAGFATVAQAQVKLEESGGGLVQAGGSLRLSCAAS
GRTSRRYAMGWFRQAPGKEREFVAGISRSGDGTHYAYSVKG
RFTISRDNAAANTVELQMNSLKPEDTAVYFCAAARTAFYYYG
NDYNYWGQGTQVTSS

4.4. Panning II: VT-peptide fusion solid phase panning

SPR analysis of isolated V_HHs against VT-peptide-3, VT-peptide-4 and IGFBP7 showed that there are some non-specific interactions between all V_HHs examined and IGFBP7. The origin of this non-specific binding remains to be elucidated. Based on the SPR data and optimized conditions where non-specific interaction of V_HH and IGFBP7 was abolished, a new panning strategy was established. Solid phase panning was performed essentially as described in section (3.2.3.3.) The microtiter plate was coated with Streptavidin and 20 µg/mL of VT-peptide-3. Four consecutive rounds of panning were performed. After round 1, the recovered phage was further exposed to stringent conditions by increasing the number of washes and by increasing the salt concentration of the pH 5 washing buffer to 300 mM NaCl. The stringency was further increased in the second and third round by decreasing the concentration of the VT-peptide-3 to 15 µg/mL and 10 µg/mL. A control pentabody (VT) was used in elution to remove any non-specific binders to VT domain followed by specific elution with C-IGFBP7 and IGFBP7. The low phage titer was most likely due to the stringent washing conditions. Despite the fact that the stringency condition was further increased in the third and fourth round of panning, the output titer was increased to 1.3×10^6 cfu. The results of this panning are shown in Table 6.

Table 6. Phage titres of four rounds of panning against VT-peptides 3

<i>Panning rounds</i>	<i>CFU/mL</i>
Round 1: Input	1.3×10^{13}
Output	1×10^3
Round 2: Input	9.36×10^{11}
Output	1.2×10^4
Round 3: Input	5.11×10^{12}
Output	9×10^4
Round 4: Input	2.87×10^{12}
Output	1.3×10^6

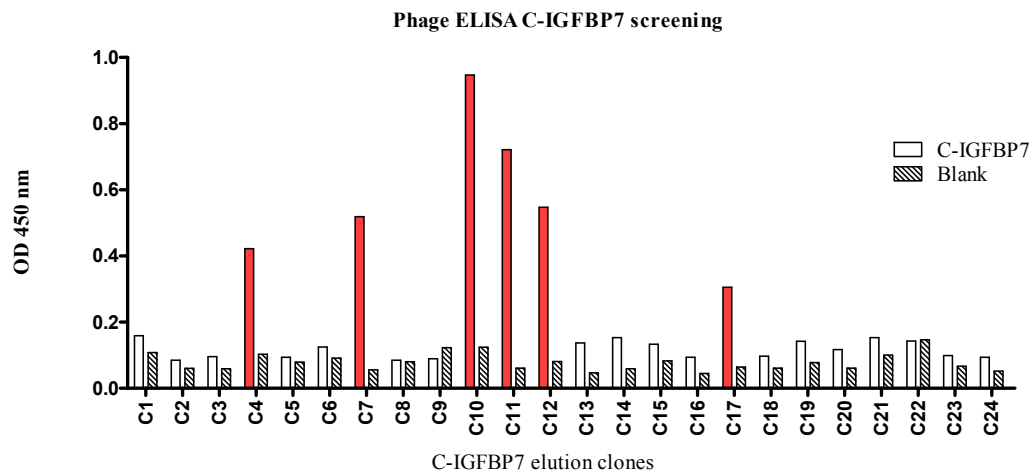
4.4.1. Monoclonal phage ELISA of VT-peptide-3 solid phase panning

Multiple phage ELISAs were performed under very stringent washing conditions using PBS buffer that contained 0.1% (v/v) Tween 20 and pH 5 in the case of C-IGFBP7 coated wells and 0.1% (v/v) Tween 20, pH 5 and 300 mM NaCl in the case of IGFBP7 coated wells. To identify specific V_HH binders against VT-peptide-3, the fourth round of panning was performed against C-IGFBP7. In the first set of panning, bound phages were eluted first with free C-IGFBP7 fragment (Figure 15, A) followed with TEA (Figure 15, B) and the second set were eluted with free IGFBP7 (Figure 16, A) followed by TEA (Figure 16, B). A total of 24 clones were picked randomly and screened against immobilized C-IGFBP7 and IGFBP7, respectively. Bound phages were detected with anti-M13 IgG conjugated to HRP. Clones with an absorbance reading > 0.3 were considered positive for α -IGFBP7 binding (Figure 15, Figure 16). For C-IGFBP7 panning, about 18.75 % (9/48) clones scored positive for binding to C-IGFBP7 and 58% (28/48) scored positive for binding to IGFBP7.

Figure 15. Monoclonal Phage ELISA of anti-VT-peptide-3.

After the fourth round of panning, the bound phages were eluted with free C-IGFBP7 (**A**) and TEA (**B**) and selected against C-IGFBP7. Amplified phage were incubated with immobilized C-IGFBP7 (10 µg/mL) and washed 5 times with PBST 0.1%, pH 5 then detected with anti-M13-HRP. Absorbance values at 450 nm were measured and subtracted from those of the background wells (no C-IGFBP7 coated) absorbance reading > 0.3 were considered positive. Of 48 clones tested, 9 specifically recognized C-IGFBP7. Red bars represent the positive clones.

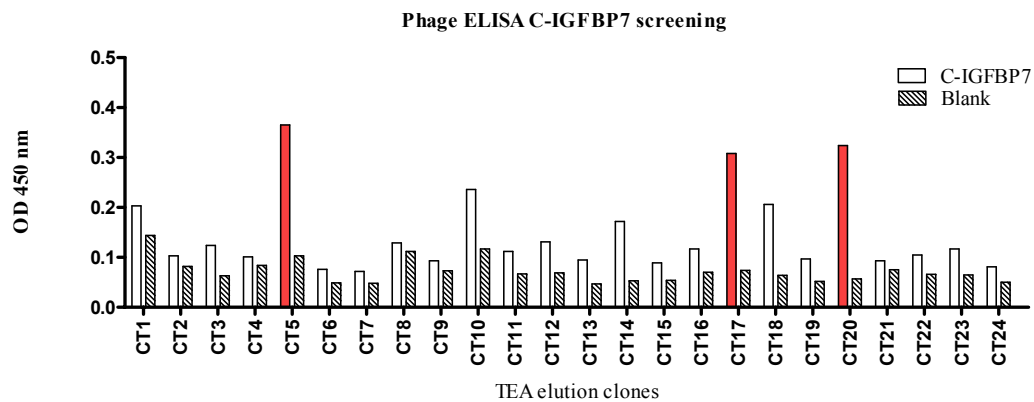
A



**washing with PBST 0.1% pH 5

Phage Clones

B



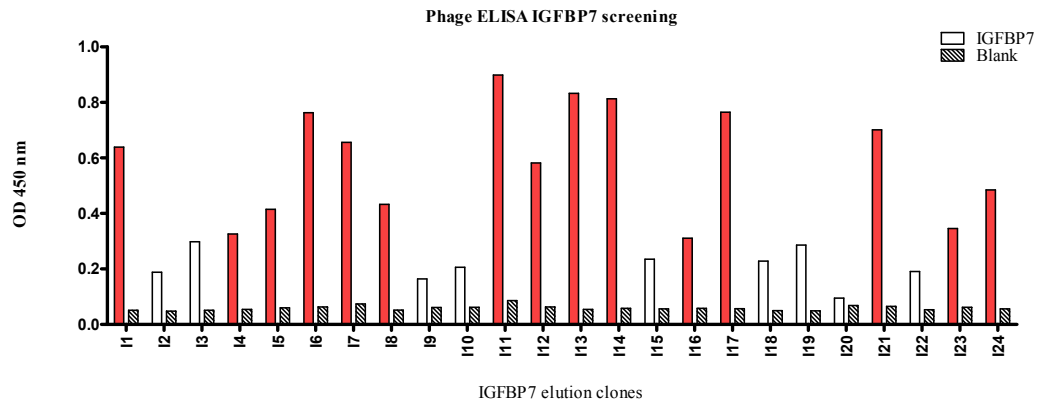
**washing with PBST 0.1% pH 5

Phage Clones

Figure 16. Monoclonal Phage ELISA of anti-IGFBP7.

Phage-displayed V_HH naïve library from the fourth round of panning eluted with free IGFBP7 (**A**) and TEA (**B**) and screened for binding against IGFBP7. Amplified phages from individual colonies were incubated with immobilized IGFBP7 (10 µg/mL). The wells were washed with PBST 0.1%, pH 5 containing 300 mM NaCl, then detected with anti-M13-HRP. Absorbance values at 450 nm were measured and subtracted from those of the background wells (no IGFBP7 coated) absorbance reading > 0.3 were considered positive. Of 48 clones tested, 28 specifically recognized IGFBP7. Red bars represent the positive clones.

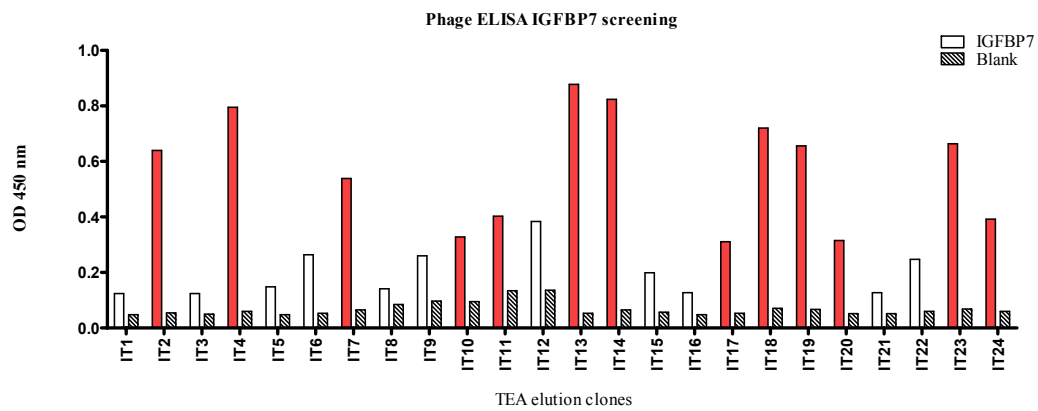
A



**washing with PBST 0.1% pH 5 + 300 mM NaCl

Phage Clones

B



**washing with PBST 0.1% pH 5 + 300 mM NaCl

Phage Clones

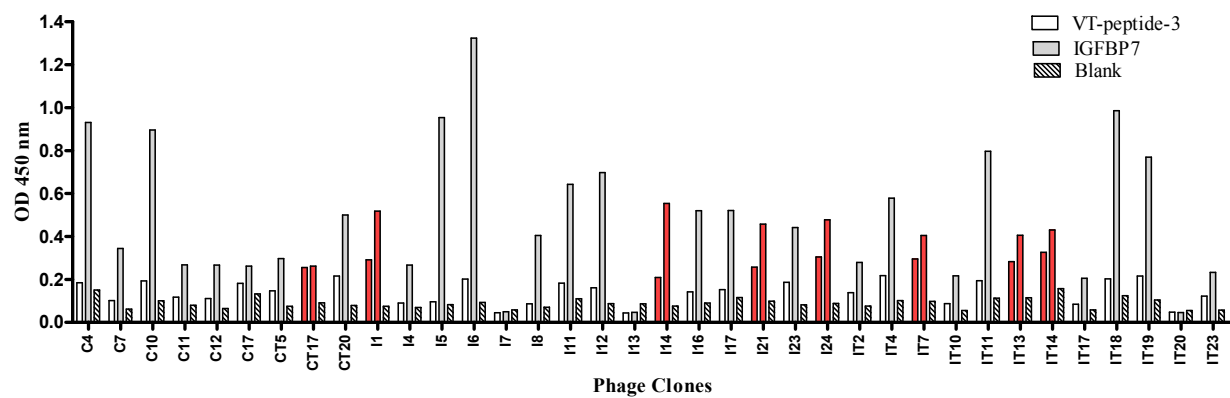
The selected positive clones from the first phage ELISA were further subjected to second phage ELISA and were screened against VT-peptide-3 and IGFBP7 respectively. In addition, stringent washing conditions were applied after each incubation by using wash buffer containing 0.1% (v/v) Tween 20, pH 5 and 300 mM NaCl and increasing the washing times in order to identify specific clones that only showed specific binding to VT-peptide-3 and IGFBP7 (Figure 17). Clones with an absorbance reading > 0.3 were considered positive for α -IGFBP7 and VT-peptide 3 binding. Eight clones were identified as specific V_HH binders and were subjected to DNA sequencing. The sequencing results showed that the panning resulted in the isolation of three unique V_HH binders. These three clones were designated V_HH7, V_HH21 and V_HH29.

Figure 17. Monoclonal Phage ELISA evaluating the binding of phage-displayed V_HHs selected against VT-peptide 3 and IGFBP7.

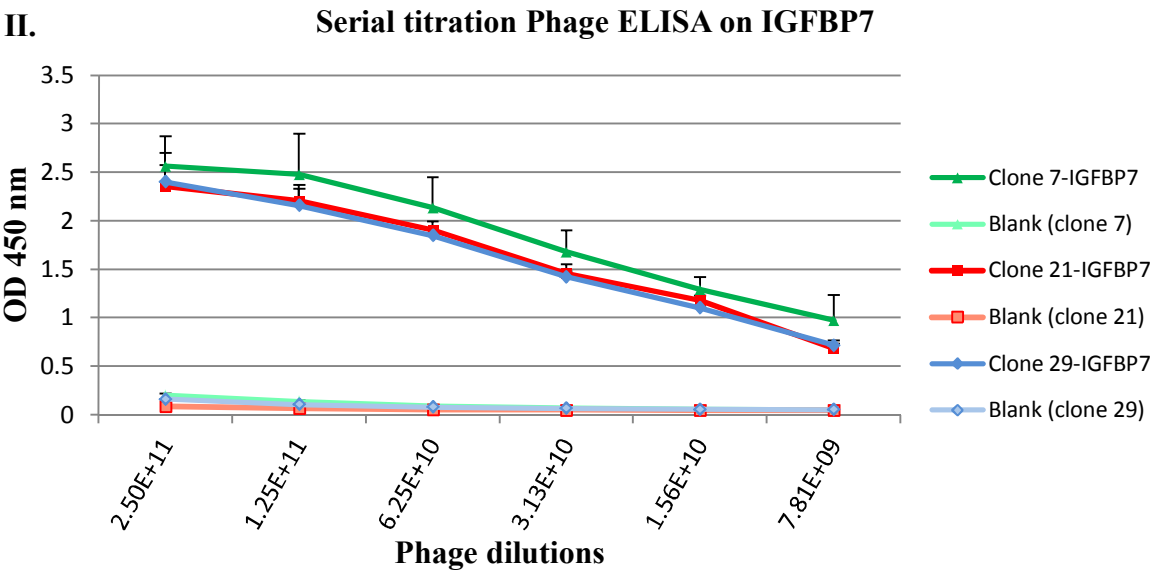
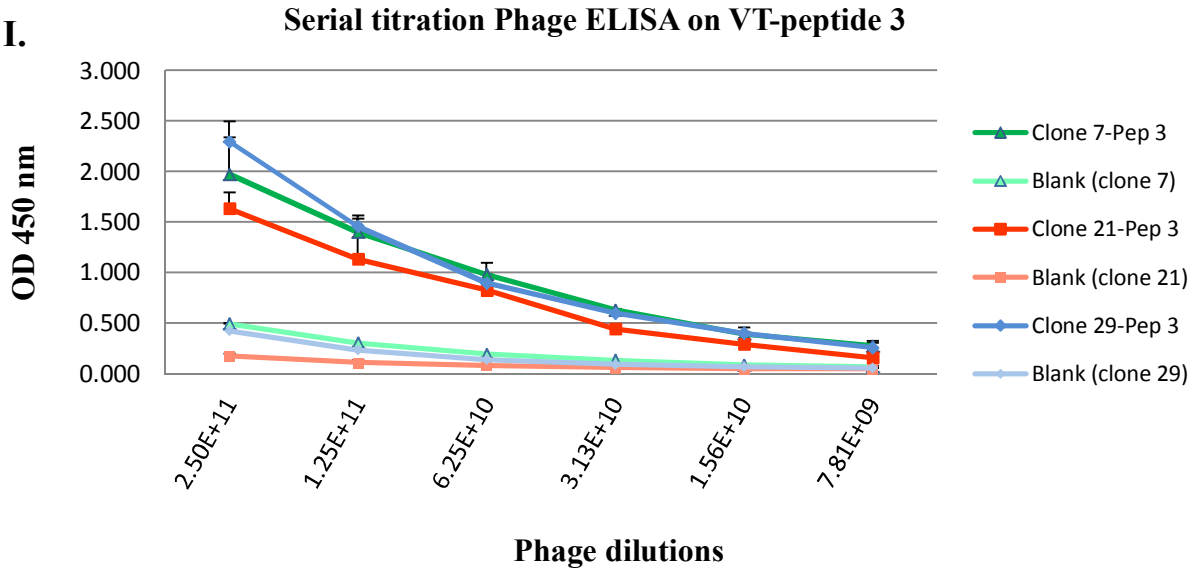
(A) Phage supernatants were prepared from growing overnight cultures of 36 clones and incubated with immobilized VT-peptide-3 and IGFBP7 followed by washing with buffer that contain 0.1% (v/v) Tween 20, pH 5 and 300 mM NaCl. Bound phages were detected with anti-M13-HRP. Absorbance values at 450 nm were measured and subtracted from those of the background wells (no IGFBP7 + no VT-peptide-3 coated) absorbance reading > 0.3 were considered positive. Of 36 clones tested, 8 specifically recognized both antigens. Red bars represent the positive clones. (B) Serial dilution of positive clones on VT-peptide 3 and IGFBP7. Phage antibodies were prepared from three selected positive clones namely 7, 21, and 29. 100 μ L of two-fold serial dilutions of phage from 2.5×10^{11} to 7×10^9 per well were prepared and added to wells coated with either VT-peptide-3 (I) or IGFBP7 (II). After washing with stringent conditions, bound phages were detected with anti-M13-HRP. The absorbance values were read at 450 nm. The absorbance value from the blank wells related to each clone is also shown.

A

Phage ELISA on VT-peptide 3 and IGFBP7



B



4.4.2. Sequence analysis of round four binders , protein expression and SEC

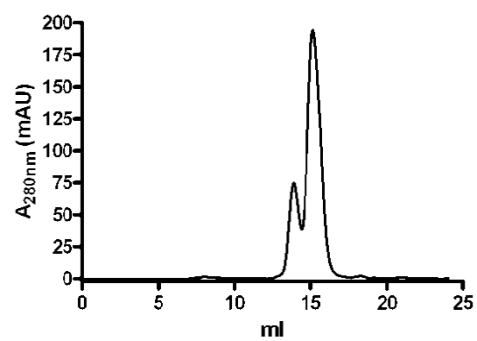
The second solid phase VT-peptide 3 panning resulted in three unique binders and sequencing data showed that these V_HHs also contain all of the llama V_HH hallmark residues. The three clones were named V_HH7, V_HH21, and V_HH29. All V_HH antibodies were cloned in a protein expression vector to be expressed as monomers and pentamers. The V_HH M7 was poorly expressed and not enough protein was derived for size exclusion analysis. Purified monomeric V_HHs M21 and M29 antibodies were analyzed to study their aggregation state by size exclusion chromatography (Superdex 75 10/30). The major monomeric peaks eluted at 13 to 15 mL which is within the range of expected molecular weight (~15 kDa). M21 had a small shoulder which can be due to the presence of unprocessed leader signal sequence in the V_HH population. There was some aggregation found in M29 (Figure 18). The purified pentameric (P7) clone was also analyzed by Superdex 200 column 10/30 and eluted at ~13 mL with no aggregation (Figure 18).

Figure 18. Size exclusion chromatography profile of V_HH clones.

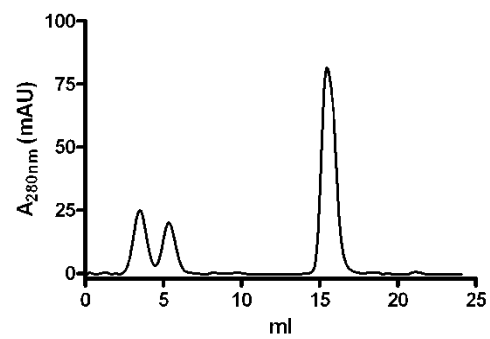
(A) The purified V_HHs were applied to a SuperdexTM 75 10/30 column to ensure that all behave as monomers and do not aggregate. Some aggregate peaks were observed in M2, which eluted at approximately 13 to 15 mL. (B) Pentamer P7 was applied to a Superdex 200 column 10/30 and eluted at 13 mL with no aggregation.

Superdex 75

M21

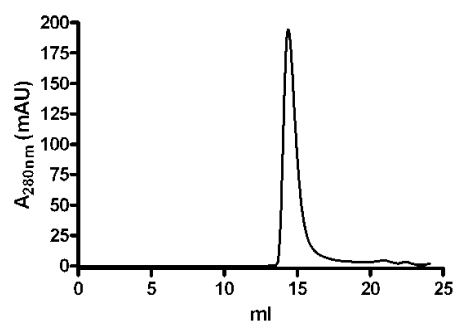


M29



Superdex 200

P7



4.4.3. SPR

SPR of M21 and M29 V_HH

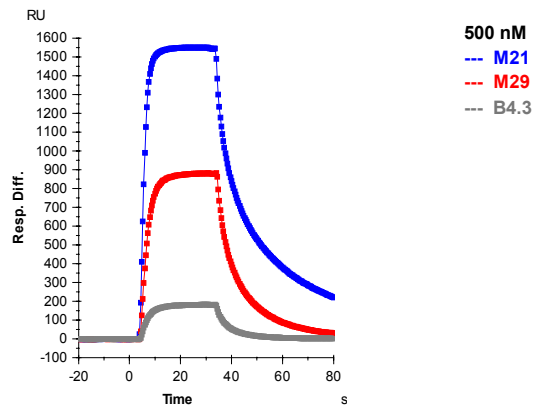
The new solid phase panning procedure resulted in three unique sequences: V_HH7, V_HH21 and V_HH29. The monomeric V_HH M21 and M29 were injected over Ovalbumin control and IGFBP7 bound to a CM5 sensor chip at various concentrations ranging from 1 to 500 nM to generate the overlay sensograms of M21 and M29 (Figure 20). Both V_HH bound to immobilized IGFBP7 with a K_D of 90 nM for M21 and 400 nM for M29. A negative control along with M21 and M29 were injected over IGFBP7 at the same concentration (500 nM) (Figure 20). Although, the single domain negative control, B4.3, showed binding to IGFBP7 at 150mM NaCl, this binding to IGFBP7 was prevented when the NaCl concentration was increased to 300mM (Figure 19). However, M21 and M29 remained bound to IGFBP7 at 300 mM NaCl. Increasing the salt concentration from 150 mM to 300 mM led to a lower binding constant, likely due to abolishment of specific ionic interactions (see Discussion). The third clone V_HH7 was expressed as a monomer and a pentamer but the monomer expression was insufficient for the SPR experiment and the pentamers were tested for binding under the new conditions with IGFBP7 (Figure 21).

Figure 19. The binding of negative control (B4.3), M21 and M29 to IGFBP7 at 150 mM and 300 mM NaCl.

M21, M29 and negative control were injected over a CM5 sensorchip with immobilized IGFBP7. The binding of negative control (B4.3) to IGFBP7 was almost abolished at 300 mM NaCl concentration; however the binding of M21 and M29 can withstand the high salt concentration. Note: Surface capacity had increased over time (500 nM M21 increased from 900RU to 1500RU, 500 nM M29 increased from 600RU to 800RU).

Binding of (Negative control: B4.3), M21 and M29 (500 nM) to IGFBP7

HBSEP (150 mM)



HBSEP (300 mM)

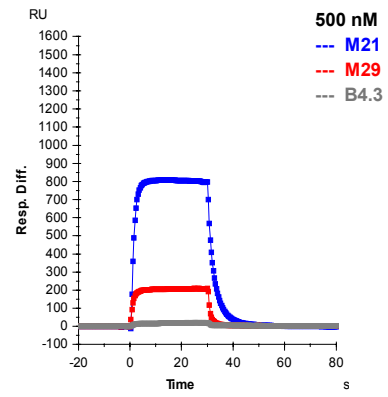
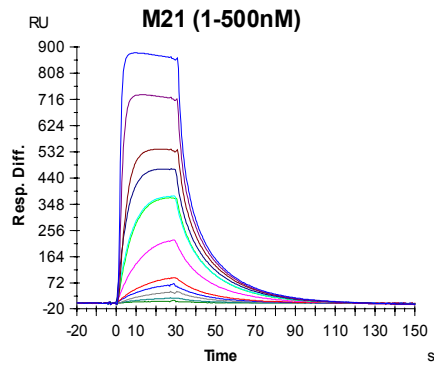


Figure 20. Binding kinetics of monomeric V_HHs (M21 and M29) to IGFBP7 by SPR.

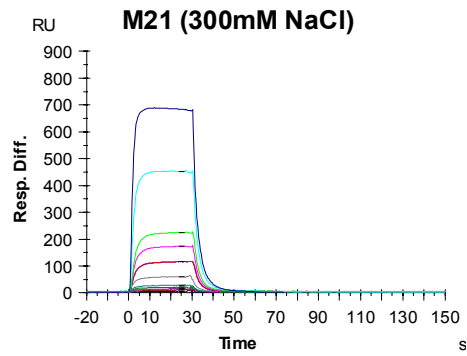
Purified recombinant IGFBP7 was immobilized on a CM5 sensor chip by amine-coupling and the monomeric V_HHs were injected over the surface at concentration ranges from 1-500 nM (shown on each sensorgram). Affinity constants 90 nM for M21 and 400 nM for M29 were observed.

M21

HBSEP (150 mM)



HBSEP (300 mM)



M29

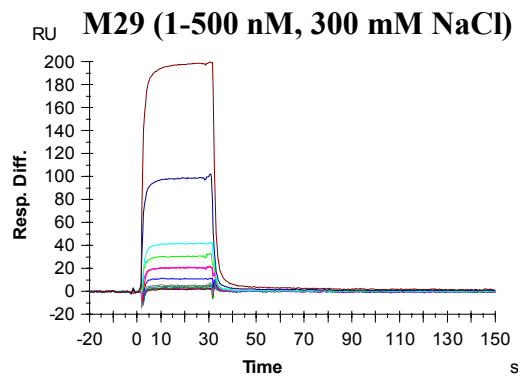
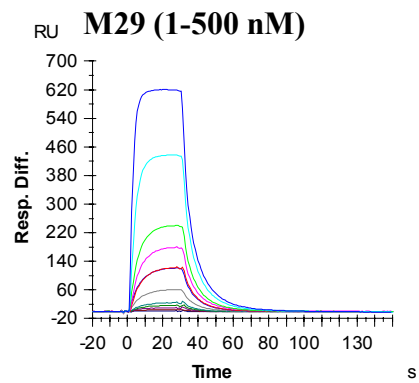


Figure 21. V_HH pentamer P7 binding to IGFBP7 surface.

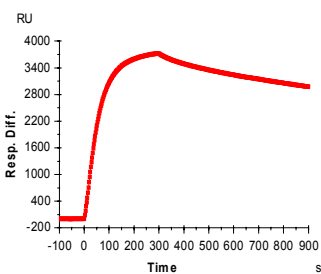
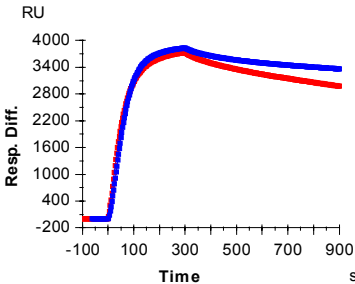
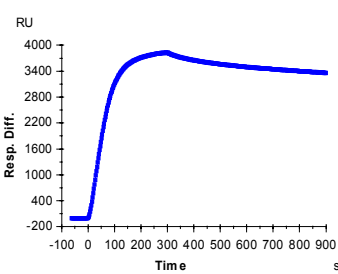
V_HH P7 was injected over the IGFBP7 at a concentration 20 nM and showed strong binding to IGFBP7 under both conditions (150 mM and 300 mM NaCl). The dissociation of the Pentamer P7 appeared to be a little faster when measured at the higher NaCl concentration of 300 mM.

Pentamer P7 (20 nM)

HBSEP (150 mM NaCl)

HBSEP (300 mM NaCl)

IGFBP7 surface

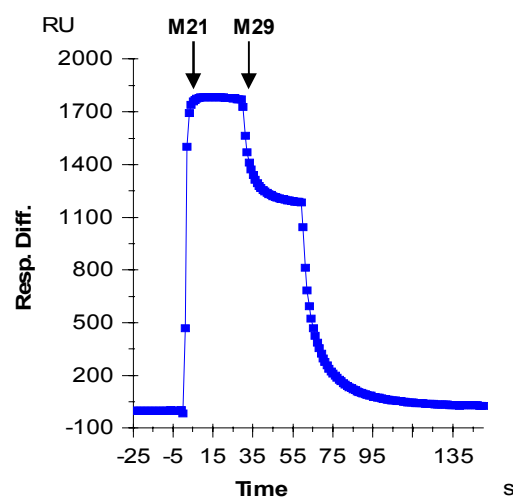


4.4.4. Epitope mapping of M21 and M29

SPR co-injection experiments were used to investigate whether M21 or M29 recognize the same or different epitopes on IGFBP7. M21 and M29 were added sequentially to IGFBP7 immobilized on a CM5 sensor chip. The first monomer (M21) was injected at a concentration of 2.5 μ M to insure that the immobilized IGFBP7 was saturated prior the injection of second monomer (M29). After saturation, M29 was injected at a concentration of 2.5 μ M over the M21-IGFBP7 complex (see Figure 22 arrow). The BIACORE sensorgram for M21 and M29 shows similar R_{max} ($\cong$ 1700-1800 RUs) values for individual V_H Hs with no increase in response upon injection of the M29, indicating that M21 and M29 antibodies bind an overlapping epitope on IGFBP7 (Figure 22). Binding to the same epitope was expected since both clones were isolated from a 15-mer peptide-3 panning.

Figure 22. Epitope mapping of M21 and M29.

Epitope mapping was performed by using SPR in order to confirm that M21 and M29 V_HHs bind to same epitope.



4.5. Functional study

4.5.1. Assessment of anti-IGFBP7 pentameric sdAb (P12) in a human pancreatic cancer (PANC-1) soft agar assay

In vitro anti-tumorigenic effect of anti-IGFBP7 pentameric sdAbs

Anchorage-independent growth of tumor cells is a hallmark of malignant transformation, correlating with tumorigenicity (Sato et al. 2007), and is considered one of the most precise and stringent *in vitro* assay for assessing malignant transformation of cells. Anchorage-independent growth assays are also the gold-standard for *in vitro* cancer therapeutic validation as they correlate with *in vivo* levels of tumorigenicity in *foxn1nu* mouse models (Pen A. et al., 2011).

This sensitive assay was employed for the therapeutic assessment of anti-IGFBP7 pentameric sdAb (P12) in inhibiting IGFBP7-induced PANC-1 colony growth in soft agar. The total area (mm^2) occupied by PANC-1 colonies per field was used as a measure of tumor growth. Control PANC-1 colonies showed slow growth in the three week time frame. As shown in Figures 23 and 24, incubation of PANC-1 with P12 alone ($3\mu\text{M}$) did not affect colony growth compared to control. The addition of IGFBP7 (300nM) induced a 1.8 fold increased in colony growth compared to control. However, this growth promoting effect of IGFBP7 (300nM) was completely blocked by the presence of P12 ($3\mu\text{M}$). No statistical difference was observed in the number of colonies between the different treatment groups.

Figure 23. The effect of anti-IGFBP7 P12 sdAbs on Panc-1 tumor colony formation grown in an anchorage-independent soft agar assay.

Representative photomicrographs of PANC-1 tumor colony growth in the presence of control (DMEM), P12 (3 μ M), IGFBP7 (300 nM) or the combination of P12 (3 μ M) plus IGFBP7 (300 nM).

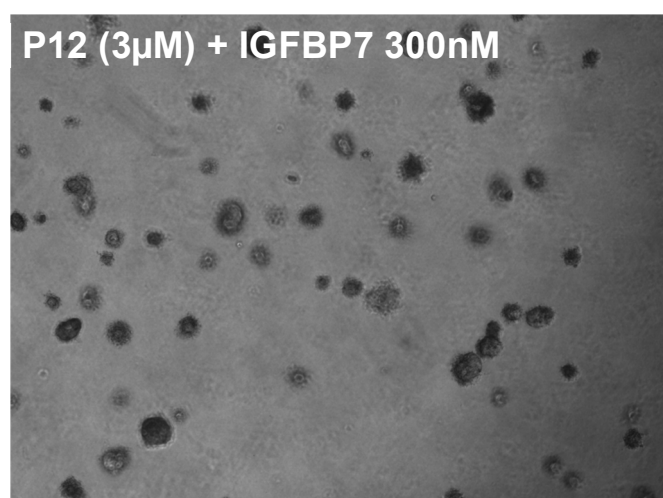
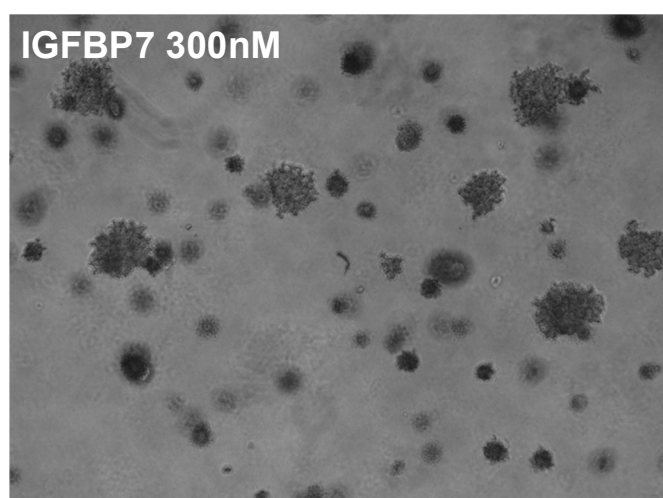
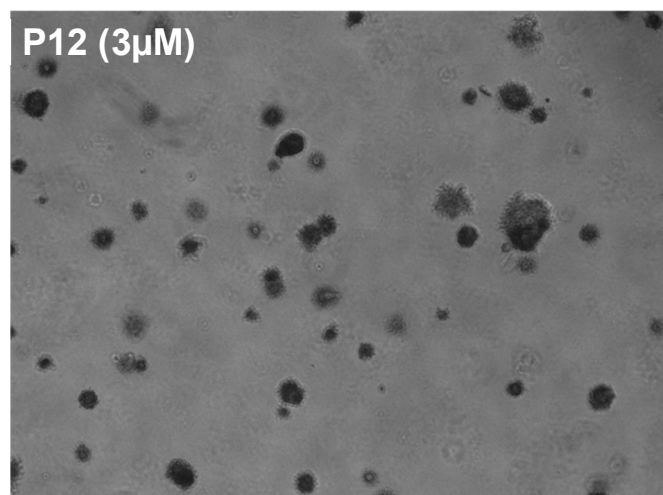
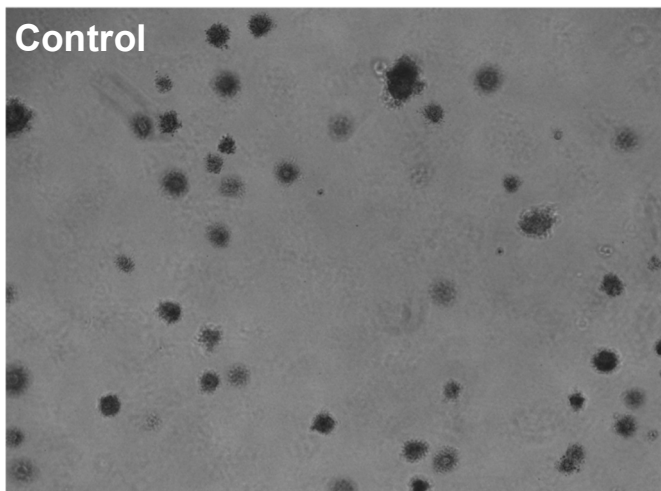
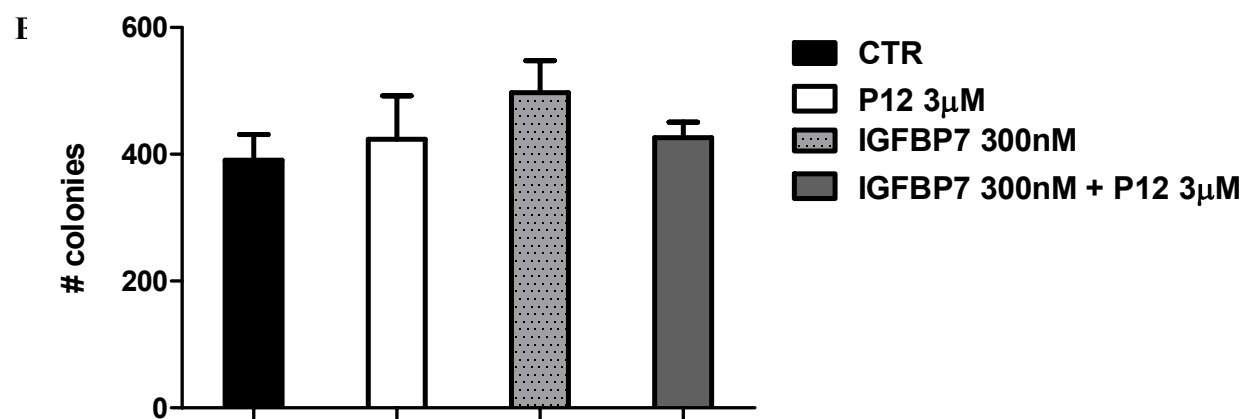
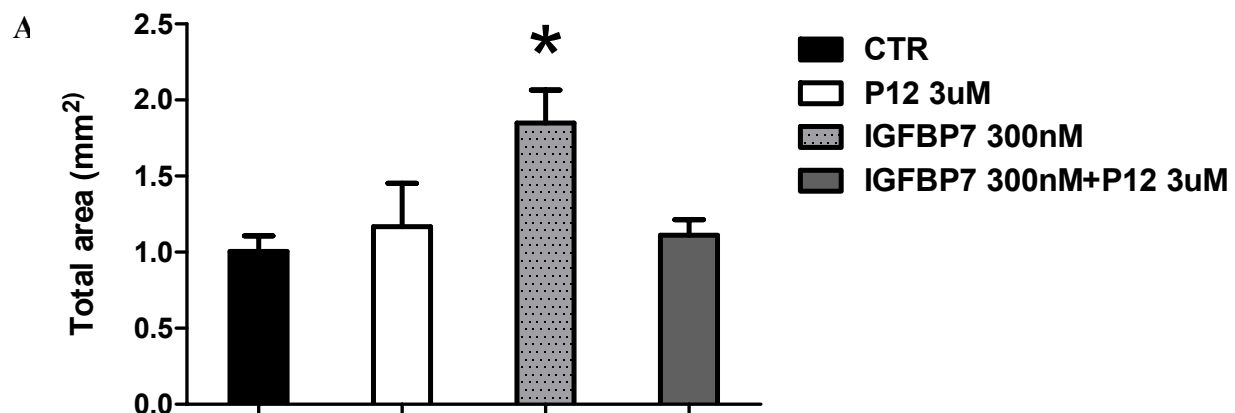


Figure 24. Quantitative analysis of anti-IGFBP7 P12 sdAbs on PANC-1 colony formation grown in an anchorage-independent soft agar assay.

Graphical illustration of (A) total area or (B) total number of colonies of PANC-1 colony growth in the presence of control (DMEM), P12 (3 μ M), IGFBP7 (300 nM), or the combination of P12 (3 μ M) plus IGFBP7 (300nM). Data is presented as mean $\pm$ SD, n=3-4 per group. Asterisks (*) indicate a statistical difference between treatment versus control (p<0.05).



5. DISCUSSION

Antibodies, known also as immunoglobulins, are the main elements of the humoral immune response and are involved in recognition of specific antigens on pathogens and, thereafter, launching an array of effector immune responses (by engaging other cells and elements of the immune system such as complement proteins, T lymphocytes, etc.) to effectively remove and destroy foreign invaders. These molecules have been recognized over a century ago as “magic bullets” for their specific disease targeting as well as their fundamental roles in the mammalian immune system (Jain et al., 2007). Among the main reasons for their attractiveness in biomedical and other areas of scientific research are their abilities to bind target molecules with high affinity and specificity, to perform as bi-functional/bi-specific molecules and to be engineered *in vitro* for the purpose of improving their biophysico-chemical properties or to be conjugated to toxin (Wu & Senter, 2005) or other effector molecules for diagnostic or therapeutic applications (Deyev & Lebedenko, 2009).

The discovery of hybridoma technology by Köhler and Milstein in 1975, by which antigen-specific-monoclonal antibodies are generated in tissue culture, rapidly enhanced our knowledge and understanding of antibody structure and function. It also expanded the applications of antibodies in medicine, as therapeutic and diagnostic reagents, as well as in other areas of research such as immunochemistry as detection reagents. In addition, advances in the fields of genetics and molecular biology led to the identification of antibody genes and their genetic recombination mechanism which provided opportunities to express and engineer antibodies and their fragments in simpler prokaryotic systems with desired

specificities for different applications. The introduction of phage display technology in 1985 by Smith (Smith G 1985) and successful display of antibody fragments on the phage surface in 1990 (MacCafferty et al. 1990) opened new era in the field of recombinant antibodies. This technology imitates the *in vivo* process of antibody expression by B lymphocytes and, therefore, provides a direct link between genotype (antibody gene cloned into phage genome) and phenotype (antibody binding domain on the phage surface). Antibody gene repertoires from immune/non-immune sources are cloned in phage or phagemid vectors and large libraries are constructed. Following the display of the antibody libraries on phage surface, antigen-specific phage antibodies are isolated by a selection process called “panning” in which a large pool of phage antibodies is exposed to immobilized (or in solution) target antigen and non-phage binders are removed by several washing steps. As a result, antibodies binding domains with high specificity and affinity for the desired targets can be isolated.

Panning of antibody phage display libraries is usually carried out on immobilized antigen coated onto a solid surface such as Nunc Maxisorp microtiter plate wells or immunotubes. This immobilizing method is easy, direct and can be applied widely for many antigens. However, some proteins can be denatured upon immobilization on plastic surfaces and their epitopes may not be correctly exposed for panning. Alternative methodologies have therefore been developed to increase the efficiency of antigen accessibility during panning. Biotinylation of the target antigens (peptide, protein) and direct capture on an immobilized streptavidin surface is one such method which greatly enhances the efficiency of coating and provides more uniform distribution of the antigen on the surface or on paramagnetic beads. Biotin is a water soluble vitamin referred to as vitamin H and has a molecular weight of

224.31 g/mol. Target protein and peptide are usually biotinylated by a chemical process and this involves a covalent linkage of a carboxyl group from biotin or biotin linker molecules and any free amine group from the protein or peptide. Alternatively, this process can be performed at a particular site *in vivo* by which the biotinylation reaction involves the formation of an amide linkage between the carboxyl group of biotin and the ϵ -amino group of lysine residues (Samols *et al.*, 1988) and is catalyzed by the enzyme biotin ligase also known as holocarboxylase synthetase, which is encoded by *birA* gene. Biotin ligase attaches biotin to the lysine residues in a specific signal sequence after the translation of protein (Smith *et al.*, 1999). In this study, the biotin signal sequence was incorporated into the pentameric pVT₂ and co-transformed with a plasmid encoding the *birA* gene. The newly constructed expression vector pVT₂-HuHg-Bio is very efficient for cloning, high yield expression and *in vivo* biotinylation of target peptide or protein domains. Two peptide fusion proteins (Table 1) were concurrently expressed with BirA ligase to direct *in vivo* biotinylation at their C-terminal regions. The biotinylated VT-peptides were successfully expressed in *E. coli* (AVB 100, 101), yielding 2.3 mg/L of purified pentameric peptide fusion proteins with little or no aggregation. Mass spectrometry data confirmed that VT-peptide 3 was intact and homogenous with a correct molecular weight with only one biotin molecule confirming the success of the biotinylation process (Figure 12). However, VT-peptide 4 showed more heterogenous protein populations. It is speculated that the smaller molecular weight protein peaks either resulted from bacterial protease degradation or alternative leader peptidase processing due to the similarity of the first three residues of peptide 4 with those of the OmpA leader sequence. By western blotting and SDS-PAGE, it

was shown that VT-peptide 3 fusion protein is highly stable at 4 °C even after four months of storage.

Isolation and functional characterization of anti-IGFBP7 V_HHs

In this work, biotinylated peptides were set to display in a pentameric format to isolate V_HH single domain antibodies from the LAC-M naïve library. The rationale behind this methodology of peptide display is two-fold. First, chemical peptide synthesis is a complicated and expensive process and amino acid composition determines the property of every individual peptide. Peptide solubility is a serious issue for peptides with a high content of hydrophobic amino acids and these peptides need to be solubilized in organic solvents such as DMSO (dimethyl sulfoxide). Consequently, synthesized peptides may not represent their original structure in the native protein and are not optimum targets for antibody panning. Secondly, the approach applied in this study allows easy cloning of peptide sequences through fusion to a bacterial protein and pentavalent display of peptide fusion proteins. Additionally, in contrast to random chemical biotinylation at any free amine group, one biotin molecule is added to the free amine group of the single lysine residue (Santala and Lamminmaki, 2004) within the biotinylation signal sequence located in the C-terminal region of the Verotoxin domain. The *in vivo* biotinylated VT-peptide fusions are therefore displayed unidirectionally upon capture on a Streptavidin surface, which is a critical factor in antigen accessibility during phage antibody panning. Advances in human genomic and proteomic research have led to the discovery of large numbers of gene-products for which no recombinant protein products are available. However, by applying bioinformatics tools and using available data from the protein data bank, it is possible to predict the protein structure and to design peptide sequences which are most likely located on the protein surface. We

believe that this approach of peptide display and its feasible unidirectional capturing can closely imitate the natural structure of peptides in parental proteins and enhance the possibility of isolating domain antibodies able to recognize the parental protein.

As a model antigen, IGFBP7 protein which is a therapeutically important cancer target was selected. IGFBP7 is a 31 kDa secreted protein that shares structural homology with a family of IGFBP-related proteins which includes IGFBP1-6. It has been demonstrated in several studies that IGFBP7 is over-expressed in tumor blood vessels with little or no expression in normal blood vessels, or in non-malignant angiogenic placental tissues (Akaogi et al., 1996, Pen et al., 2007). Previous work has suggested that IGFBP7 may have a pro-angiogenic role for HBEC, in that it increases the formation of capillary-like tubules in a matrigel assay (Iqbal et al., 2010). IGFBP7 has the potential to be an excellent biomarker for human GBM vessels that should be exploited using targeting moieties and non-invasive imaging. Two overlapping peptides (peptide-3 and peptide-4) located in the C-terminal region of IGFBP7 were designed and cloned upstream of the gene encoding the Verotoxin B-subunit of *E. coli* (Zhang J. et al, 2004). The purified VT-peptide 3 and 4 along with recombinant IGFBP7 were used for panning. Phage antibodies rescued from the LAC-M naïve llama library were used for panning against the biotinylated VT-peptides that were captured on streptavidin beads as well as recombinant IGFBP7 coated on a single ELISA well. Anti-peptide and IGFBP7 V_HH binders were enriched after several rounds of panning where highly selective pressure was applied through decreasing the concentration of VT-peptides and IGFBP7 and increasing the number of washes. Moreover, phage libraries were depleted of VT domain and blocking buffer binders by overnight pre-adsorption followed by centrifugation. After four rounds of panning, monoclonal phage ELISA showed positive

binding signals and approximately 51% of individual clones showed specific binding to IGFBP7 (Figure 14). VT-peptides and IGFBP7 protein panning revealed nine unique sequences all determined to be V_HH antibodies based on the presence of the tetrad hallmark residues at positions 37, 44, 45, and 47 (Muyldermans et al., 1994). All of the anti-IGFBP7 antibodies were cloned into monomeric and pentameric protein expression vectors and all V_HHs were successfully expressed in *E. coli* and purified using IMAC. Expression yields of 0.4-10 mg/L were observed, which was enough for kinetic measurements and *in vitro* neutralization assays.

The binding kinetics of anti-IGFBP7 antibodies were determined by SPR and revealed that the isolated clones (M2-2, M4-1, M5-1, M8-1, M9-2 and M12-1) showed binding to immobilized IGFBP7 with K_D values ranging from 100 nM to 1 μ M (Table 5), which is expected from naive single domain antibody libraries (Tanha et al., 2002; Monegal et al., 2009; Dong et al., 2010). However, when the binding to IGFBP7 were examined using negative and positive control V_HH antibodies, I observed that several non-related V_HHs which were isolated from a different immune library showed non-specific binding to IGFBP7 (Figure 19) (data not shown). Therefore, I decided to repeat the binding kinetic experiment using anti-IGFBP7 V_HH (P12) that was isolated against IGFBP7, B4.3 as a single domain negative control (Hussack G. et al., 2012) and V_HH 4.43, isolated previously from an immune llama library (Iqbal U. et al., 2010), as a positive control for IGFBP7 using a number of different buffer systems to study their influence on the antigen-antibody as well as non-specific interactions. Changing the pH value and detergent concentration of the HEPES buffers did not abolish the non-specific binding of the negative control. However, when the salt concentration was increased from 150 mM to 300-500 mM, the non-specific

interaction of sdAb negative control to IGFBP7 was eliminated. This suggests non-specific ionic interactions between non-related V_HH antibodies and IGFBP7. As shown in Figure 19, under higher salt concentrations, specific interactions of both positive control 4.43 V_HH and M12 V_HH with IGFBP7 are also compromised. This suggests that the presence of the charged residues in CDR regions (five residues in CDR1 and CDR3 of V_HH4.43 and four residues in CDR3 of M12 V_HH) are important for binding to IGFBP7 and increasing salt concentration disrupts this type of binding (Figure 19). Surprisingly, the affinity of V_HH (M12-1) from the LAC-M naive library is similar to the affinity of V_HH 4.43 antibody which was isolated from a llama V_HH immune library (Iqbal et al., 2010).

The SPR data and optimized buffering conditions (salt concentration effect) led me to design and develop an alternative panning strategy to bypass non-specific interaction of V_HH with IGFBP7 protein and isolate V_HHs which specifically interact with this antigen only through CDR regions. To start, the phage library was pre-adsorbed overnight with a non-related pentabody to deplete VT domain binders. The panning conditions were essentially similar to those used initially except that VT-peptide 3 was captured on Streptavidin coated microtiter plates and the fourth round of panning was performed against the recombinant C-fragment of IGFBP7. Additionally, highly stringent conditions were applied during panning by increasing the number of washes after each round and using washing buffer that contain 300 mM NaCl, pH 5.0. The overall phage titer after each round was lower when compared to the previous panning, most likely due to the stringent washing conditions (Table 6). The new panning was also performed in duplicate: in the first well, bound phages were eluted with C-IGFBP7 followed by TEA elution whereas in the second well phages were eluted with IGFBP7 protein followed by TEA elution. The monoclonal phage ELISA on phages

eluted from the first and second ELISA wells showed that approximately 19% and 58% of individual clones had specific binding to C-IGFBP7 and IGFBP7, respectively (Figure 20 and 21). Selected positive clones were also tested for their specific binding to VT-peptide 3 and IGFBP7 simultaneously by the phage ELISA which resulted in eight clones recognizing both peptide and protein antigens (Figure 22). DNA sequence analysis showed that two clones (CT17 and I21) share the same DNA sequence though they were eluted differently. DNA sequence analysis also revealed that in total there were three unique sequences ($V_{\text{H}}\text{H7}$, $V_{\text{H}}\text{H21}$ and $V_{\text{H}}\text{H29}$) and were all determined to be $V_{\text{H}}\text{H}$ antibodies based on the presence of the tetrad hallmark residues at positions 37, 44, 45, and 47 (Muyldermans et al., 1994).

The binding kinetics of anti-IGFBP7 $V_{\text{H}}\text{H}$ antibodies were determined by SPR using the new washing buffer that contains 300 mM NaCl. Two monomeric VHHs, M21 and M29, and a negative control, B4.3, were tested against IGFBP7 (Figure 25). The single domain negative control showed almost no binding whereas M21 and M29 showed strong binding to the immobilized IGFBP7 with a K_{D} of 90 nM for M21 and 400 nM for M29. These affinity data are in the range of some other $V_{\text{H}}\text{H}$ domain antibodies isolated from naive llama single domain libraries against protein targets (Monegal et al., 2009; Dong et al., 2010). In order to examine the avidity effect, $V_{\text{H}}\text{H7}$ was expressed as pentamer and its binding was analyzed by SPR. The pentamerization of $V_{\text{H}}\text{H}$ (P7) resulted in strong binding to IGFBP7 under both salt conditions (150 mM and 300 mM NaCl), indicating the existence of multiple antibody-antigen interactions. The dissociation of the pentamer P7 appeared to be a little faster when measured at the higher (300 mM) NaCl concentration (Figure 27).

Epitope mapping by SPR showed that V_HH 21 and V_HH 29 most likely target the same epitopes on IGFBP7. This result was expected since both V_HHs were isolated from panning against VT-peptide 3 which is 15 amino acids in length and it seems that both V_HHs are competing for binding to the same region.

Finally, V_HH12, in pentameric format, was used in a functional anchorage-independent growth cell assay to examine its anti-IGFBP7 effect. This assay showed that colony growth of PANC-1 was induced by 300 nM of IGFBP7 in soft agar. The mechanism of anchorage-independent cell growth remains unknown and needs to be investigated. However, the growth-promoting effect of IGFBP7 on PANC-1 cells was completely blocked by the presence of 3 μ M anti-IGFBP7 P12 pentamer (Figure 29, 30). These findings strongly indicate that P12 binds and inhibits the growth promoting effect of IGFBP7. Therefore, V_HH12, and its higher affinity matured version, and its fluorescently labeled format may be very promising imaging/therapeutic reagents for brain tumor angiogenesis because of their small size, which may result in better tumor penetration, which are highly advantageous in *in vivo* molecular imaging applications (Lin et al., 2011).

6. CONCLUSION

The aim of this project was to establish a bacterial-based multivalent display platform technology for antigens (peptide and small protein domains) originating from humans and other mammals. The biotinylated peptide/protein-fusions are captured unidirectionally on a Streptavidin-coated surface and used for the panning of antibody phage display libraries. Single domain antibodies are an ideal choice for targeting of brain tumors, as they can easily cross the blood-brain barrier (Muruganandam et al., 2002), are able to deeply penetrate into

dense tissue and are rapidly cleared from the body. To examine the effectiveness of displayed peptide antigens for panning, two C-terminal-peptides of IGFBP7 were selected as a model antigens. The peptides were successfully expressed as fusion proteins to bacterial pentamerization domains and biotinylation *in vivo*. Protein expression was confirmed by Western blotting and proteins were characterized using mass spectrometry. An existing llama naïve phage display library was panned against the peptide fusion proteins as well as with the recombinant IGFBP7. Peptide fusion proteins and IGFBP7 specific V_HHs were isolated, expressed and characterized. Surface plasmon resonance (SPR) analyses showed that the isolated V_HHs antibodies had dissociation constants (K_D) in a micromolar to nanomolar range. However, some non-related sdAbs isolated from other libraries showed binding to the target antigen IGFBP7. This non-specific interaction has not been reported before and requires further investigation. Therefore, several washing buffers were examined in order to identify the best conditions for isolating IGFBP7-specific-V_HHs and, at the same time, avoiding non-CDR based binding of irrelevant V_HH antibodies to IGFBP7. SPR analyses revealed that the addition of 300-500 mM NaCl concentration in low pH was able to eliminate the non-specific binding of all V_HHs to the IGFBP7 protein. Our lead binder, V_HH12, tolerates the high salt concentration effect and showed specific binding to IGFBP7, although the binding was compromised to a certain extent due, most likely, to the presence of charged amino acids in CDR regions. Based on the SPR data and optimized buffer conditions, a new panning strategy was established. After four rounds of panning under stringent conditions, three unique binders were isolated which showed specific binding in SPR analysis under 300 mM NaCl and had dissociation constants (K_D) in the nanomolar range (90-400 nM). Furthermore, V_HH12 was pentamerized (P12) and used in cell-based

functional studies. Using an anchorage-independent growth cell assay, it was shown that PANC-1 cell growth could be blocked by anti-IGFBP7 P12 pentamer. Thus, V_HH12 (M12 or P12) provides the basis for developing a novel imaging/therapeutic reagent for targeting and treating brain tumor angiogenesis in early stages of tumorigenesis and can also be used as a molecular tool to monitor the degree of angiogenesis in gliomas which may help to improve the clinical management of brain tumors.

7. FUTURE DIRECTIONS AND RECOMMENDATIONS

The following are some recommendations and possible directions for future work on this project, based on the successful work carried out here and from recent published literature.

1- On the isolation of anti-IGFBP7

To isolate high affinity anti-IGFBP7 V_HHs, it is suggested to immunize a camel or llama with the recombinant IGFBP7 and/or with recombinant N- and C-fragments of IGFBP7 protein and construct an immune heavy chain library. Immunization with all of the three domains, amino-terminal domain, Kazal domain and C-terminal domain (Hwa V. et al., 1999), will maximize the chance of isolating domain-specific single domain antibodies. IGFBP7 is naturally cleaved by the membrane-bound matriptase 1 into two chains forming N-terminal domain and C-terminal domain (Ahmed et al., 2006). Both cleaved and uncleaved forms modulate the biological function of IGF (Ahmed et al., 2003). Once the immune library is constructed, selective pressure and washing conditions under high salt concentration and low pH must be applied during the panning process. This type of approach can lead to successful isolation of specific-binders against IGFBP7 target, as evidenced by the SPR experiment.

2- On enhancing the affinity and the efficacy of V_HHs

As shown previously by others (Hawkins R. et al., 1992; Maynard J. et al., 2000) the affinity of V_HHs could be improved by CDR randomization and site-directed mutagenesis. Following construction of mutant libraries and panning experiments, affinity matured V_HHs will be analyzed by SPR. The V_HH antibodies with improved affinities can then be used in cell-based functional studies. The anti-IGFBP7 V_HH antibodies can also be radiolabeled for optical imaging applications.

3. On determining the blocking mechanism of anti-IGFBP7 V_HHs

From the *in vitro* anti-tumorigenic effect of anti-IGFBP7 (M12, P12), the cell stimulatory effect of IGFBP7 was completely blocked by the presence of P12. The neutralizing mechanism will be further investigated through collaborations with Dr. Maria Moreno and Dr. Umar Iqbal at NRC-HHT. Additionally, the anti-IGFBP7 effects of new V_HH clones (M7, M21 and M29) need to be investigated using the same cell-based functional assay.

4. On *in vivo* animal optical imaging studies using anti-IGFBP7 V_HHs

To assess the imaging efficacy, *in vivo* animal studies need to be performed using the *foxn1nu* CD1 mouse model transplanted with orthotopic glioblastoma tumor (Iqbal et al., 2010). This involves administration of a labelled single domain antibody via the tail vein and subjects the animals to *in vivo* imaging tools. The primary result would show the detection of high optical signals of the specific V_HH in brain tumor.

8. CONTRIBUTIONS OF COLLABORATORS

The following section details individual collaborators and their role in this thesis.

1. Dr. John Kelly (NRC-IBS) assisted with mass spectrometry experiments.
2. Sonia Leclerc (NRC) performed all DNA sequencing.
3. Dr. Denis L'Abbé (Genomics and Health Initiative, NRC) provided recombinant IGFBP7.
4. Dr. Roger MacKenzie, Henk van Fassen (NRC) assisted with SEC and SPR analyses.
5. King Saud University for sponsoring me and funding my research.

REFERENCES

- Ahmed S, Jin X, Yagi M, Yasuda C, Sato Y, Higashi S *et al.* (2006). Identification of membrane-bound serine proteinase matriptase as processing enzyme of insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1/angiomodulin/mac25). *FEBS J* 273: 615–627.
- Ahmed, S., Yamamoto, K., Sato, Y., Ogawa, T., Herrmann, A., Higashi, S., & Miyazaki, K. (2003). Proteolytic processing of IGFBP-related protein-1 (TAF/angiomodulin/mac25) modulates its biological activity. *Biochem Biophys Res Commun*, 310(2), 612-618.
- Akaogi K, Okabe Y, Funahashi K, Yoshitake Y, Nishikawa K, Yasumitsu H *et al.* (1994). Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. *Biochem Biophys Res Commun* 198: 1046–1053.
- Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K *et al.* (1996). Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. *Proc Natl Acad Sci USA* 93: 8384–8389
- Algate, Paul, Baldridge, Jory, & Mossman, Sally. (2006). *Antigens Making and Using Antibodies* (pp. 7-25): CRC Press.
- Amersdorfer, P., Wong, C., Smith, T., Chen, S., Deshpande, S., Sheridan, R., & Marks, J. D. (2002). Genetic and immunological comparison of anti-botulinum type A antibodies from immune and non-immune human phage libraries. *Vaccine*, 20(11-12), 1640-1648.
- Angeletti, R. H. (1999). Design of useful peptide antigens. *J Biomol Tech*, 10(1), 2-10.
- Antibody Phage Display. In Philippa M. O'Brien & Robert Aitken (Eds.), (Vol. 178, pp. 133-145): Humana Press.
- Arbabi-Ghahroudi (a), M., Tanha, J., & MacKenzie, R. (2009). Isolation of monoclonal antibody fragments from phage display libraries. *Methods Mol Biol*, 502, 341-364. doi: 10.1007/978-1-60327-565-1_20
- Arbabi-Ghahroudi, M., MacKenzie, R., & Tanha, J. (2009). Selection of non-aggregating VH binders from synthetic VH phage-display libraries. *Methods Mol Biol*, 525, 187-216, xiii. doi: 10.1007/978-1-59745-554-1_10
- Arbabi-Ghahroudi, M., Tanha, J., & MacKenzie, R. (2005). Prokaryotic expression of antibodies. *Cancer Metastasis Rev*, 24(4), 501-519. doi: 10.1007/s10555-005-6193-1

- Barat, B., & Wu, A. M. (2007). Metabolic biotinylation of recombinant antibody by biotin ligase retained in the endoplasmic reticulum. *Biomol Eng*, 24(3), 283-291. doi: 10.1016/j.bioeng.2007.02.003
- Barbas, C. F., 3rd, Bain, J. D., Hoekstra, D. M., & Lerner, R. A. (1992). Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc Natl Acad Sci U S A*, 89(10), 4457-4461.
- Barbas, C. F., 3rd, Kang, A. S., Lerner, R. A., & Benkovic, S. J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci U S A*, 88(18), 7978-7982.
- Benhar, I., Azriel, R., Nahary, L., Shaky, S., Berdichevsky, Y., Tamarkin, A., & Wels, W. (2000). Highly efficient selection of phage antibodies mediated by display of antigen as Lpp-OmpA' fusions on live bacteria. *J Mol Biol*, 301(4), 893-904. doi: 10.1006/jmbi.2000.4021
- Boel, E., Bootsma, H., de Kruif, J., Jansze, M., Klingman, K. L., van Dijk, H., & Logtenberg, T. (1998). Phage antibodies obtained by competitive selection on complement-resistant *Moraxella* (*Branhamella*) *catarrhalis* recognize the high-molecular-weight outer membrane protein. *Infect Immun*, 66(1), 83-88.
- Bradbury, A. R. (2010). The use of phage display in neurobiology. *Curr Protoc Neurosci*, Chapter 5, Unit 5 12. doi: 10.1002/0471142301.ns0512s51
- Bradbury, A. R., & Marks, J. D. (2004). Antibodies from phage antibody libraries. *J Immunol Methods*, 290(1-2), 29-49. doi: 10.1016/j.jim.2004.04.007
- Braitbard, O., Glickstein, H., Bishara-Shieban, J., Pace, U., & Stein, W. D. (2006). Competition between bound and free peptides in an ELISA-based procedure that assays peptides derived from protein digests. *Proteome Sci*, 4, 12. doi: 10.1186/1477-5956-4-12
- Burger, A. M., Leyland-Jones, B., Banerjee, K., Spyropoulos, D. D., & Seth, A. K. (2005). Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer. *Eur J Cancer*, 41(11), 1515-1527. doi: 10.1016/j.ejca.2005.04.023
- Butler, J. E., Ni, L., Brown, W. R., Joshi, K. S., Chang, J., Rosenberg, B., & Voss, E. W., Jr. (1993). The immunochemistry of sandwich ELISAs--VI. Greater than 90% of monoclonal and 75% of polyclonal anti-fluoresceyl capture antibodies (CAbs) are denatured by passive adsorption. *Mol Immunol*, 30(13), 1165-1175.
- Butler, J. E., Ni, L., Nessler, R., Joshi, K. S., Suter, M., Rosenberg, B., Cantarero, L. A. (1992). The physical and functional behavior of capture antibodies adsorbed on polystyrene. *J Immunol Methods*, 150(1-2), 77-90. doi: 10.1016/0022-1759(92)90066-3

- Cai, X., & Garen, A. (1995). Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. *Proc Natl Acad Sci U S A*, 92(14), 6537-6541.
- Carmen, S., & Jermutus, L. (2002). Concepts in antibody phage display. *Brief Funct Genomic Proteomic*, 1(2), 189-203.
- Chames, P., Hoogenboom, H. R., & Henderikx, P. (2002). Selection of antibodies against biotinylated antigens. *Methods Mol Biol*, 178, 147-157.
- Chames, Patrick, & Baty, Daniel. (2010). Phage Display and Selections on Biotinylated Antigens (pp. 151-164).
- Chopin, M. C., Rouault, A., Ehrlich, S. D., & Gautier, M. (2002). Filamentous phage active on the gram-positive bacterium *Propionibacterium freudenreichii*. *J Bacteriol*, 184(7), 2030-2033.
- Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., . . . et al. (1989). Conformations of immunoglobulin hypervariable regions. *Nature*, 342(6252), 877-883. doi: 10.1038/342877a0
- Clackson, T., Hoogenboom, H. R., Griffiths, A. D., & Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature*, 352(6336), 624-628. doi: 10.1038/352624a0
- Coligan, J. E., Tam, J. P., & Shao, J. (2001). Production of antipeptide antisera. *Curr Protoc Neurosci*, Chapter 5, Unit 5 6. doi: 10.1002/0471142301.ns0506s00
- Conrath, K., Vincke, C., Stijlemans, B., Schymkowitz, J., Decanniere, K., Wyns, L., Loris, R. (2005). Antigen binding and solubility effects upon the veneering of a camel VHH in framework-2 to mimic a VH. *J Mol Biol*, 350(1), 112-125. doi: 10.1016/j.jmb.2005.04.050
- Coomber, David W. J (2002). Panning of antibody phage-display libraries. Standard protocols. *Methods Mol Biol*, 178, 133-145.
- Coppieters, K., Dreier, T., Silence, K., de Haard, H., Lauwereys, M., Casteels, P., . . . Rottiers, P. (2006). Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. *Arthritis Rheum*, 54(6), 1856-1866. doi: 10.1002/art.21827
- Cortez-Retamozo, V., Backmann, N., Senter, P. D., Wernery, U., De Baetselier, P., Muyldermans, S., & Revets, H. (2004). Efficient cancer therapy with a nanobody-based conjugate. *Cancer Res*, 64(8), 2853-2857.
- Cortez-Retamozo, V., Lauwereys, M., Hassanzadeh Gh, G., Gobert, M., Conrath, K., Muyldermans, S., Revets, H. (2002). Efficient tumor targeting by single-domain antibody fragments of camels. *Int J Cancer*, 98(3), 456-462.

Cossins, A. J., Harrison, S., Popplewell, A. G., & Gore, M. G. (2007). Recombinant production of a VL single domain antibody in *Escherichia coli* and analysis of its interaction with peptostreptococcal protein L. *Protein Expr Purif*, 51(2), 253-259. doi: 10.1016/j.pep.2006.07.013

Cwirla, S. E., Peters, E. A., Barrett, R. W., & Dower, W. J. (1990). Peptides on phage: a vast library of peptides for identifying ligands. *Proc Natl Acad Sci U S A*, 87(16), 6378-6382.

Daugherty, P. S., Chen, G., Olsen, M. J., Iverson, B. L., & Georgiou, G. (1998). Antibody affinity maturation using bacterial surface display. *Protein Eng*, 11(9), 825-832.

Daugherty, P. S., Olsen, M. J., Iverson, B. L., & Georgiou, G. (1999). Development of an optimized expression system for the screening of antibody libraries displayed on the *Escherichia coli* surface. *Protein Eng*, 12(7), 613-621.

Davies, J., & Riechmann, L. (1994). 'Camelising' human antibody fragments: NMR studies on VH domains. *FEBS Lett*, 339(3), 285-290.

Davies, J., & Riechmann, L. (1996). Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability. *Protein Eng*, 9(6), 531-537.

Davies, J., Dawkes, A. C., Haymes, A. G., Roberts, C. J., Sunderland, R. F., Wilkins, M. J., Edwards, J. C. (1994). A scanning tunnelling microscopy comparison of passive antibody adsorption and biotinylated antibody linkage to streptavidin on microtiter wells. *J Immunol Methods*, 167(1-2), 263-269.

Davies, Julian, & Riechmann, Lutz. (1996). Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability. *Protein Eng*, 9(6), 531-537. doi: 10.1093/protein/9.6.531

De Genst, E., Saerens, D., Muyldermans, S., & Conrath, K. (2006). Antibody repertoire development in camels. *Dev Comp Immunol*, 30(1-2), 187-198. doi: 10.1016/j.dci.2005.06.010

De Genst, E., Silence, K., Decanniere, K., Conrath, K., Loris, R., Kinne, J., Wyns, L. (2006). Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci U S A*, 103(12), 4586-4591. doi: 10.1073/pnas.0505379103

de Marco, A. (2011). Biotechnological applications of recombinant single-domain antibody fragments. *Microb Cell Fact*, 10, 44. doi: 10.1186/1475-2859-10-44

Degeorges, A., Wang, F., Frierson, H. F., Jr., Seth, A., & Sikes, R. A. (2000). Distribution of IGFBP-rP1 in normal human tissues. *J Histochem Cytochem*, 48(6), 747-754.

Demain, A. L., & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnol Adv*, 27(3), 297-306. doi: 10.1016/j.biotechadv.2009.01.008

- Desmyter, A., Transue, T. R., Ghahroudi, M. A., Thi, M. H., Poortmans, F., Hamers, R., Wyns, L. (1996). Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. *Nat Struct Biol*, 3(9), 803-811.
- Deyev, S. M., & Lebedenko, E. N. (2009). Modern Technologies for Creating Synthetic Antibodies for Clinical application. *Acta Naturae*, 1(1), 32-50.
- Dhillon, J. K., Drew, P. D., & Porter, A. J. (1999). Bacterial surface display of an anti-pollutant antibody fragment. *Lett Appl Microbiol*, 28(5), 350-354.
- Diamandis, E. P., & Christopoulos, T. K. (1991). The biotin-(strept)avidin system: principles and applications in biotechnology. *Clin Chem*, 37(5), 625-636.
- Diskin, R., Scheid, J. F., Marcovecchio, P. M., West, A. P., Jr., Klein, F., Gao, H., Bjorkman, P. J. (2011). Increasing the Potency and Breadth of an HIV Antibody by Using Structure-Based Rational Design. *Science*. doi: 10.1126/science.1213782
- Dong, Jianbo, Thompson, Aaron A., Fan, Yongfeng, Lou, Jianlong, Conrad, Fraser, Ho, Mengfei, Marks, James D. (2010). A Single-Domain Llama Antibody Potently Inhibits the Enzymatic Activity of Botulinum Neurotoxin by Binding to the Non-Catalytic α -Exosite Binding Region. *Journal of Molecular Biology*, 397(4), 1106-1118. doi: 10.1016/j.jmb.2010.01.070
- Duan, C. (2002). Specifying the cellular responses to IGF signals: roles of IGF-binding proteins. *J Endocrinol*, 175(1), 41-54.
- Duan, Z., & Siegmundfeldt, H. (2010). An efficient method for isolating antibody fragments against small peptides by antibody phage display. *Comb Chem High Throughput Screen*, 13(9), 818-828.
- Dufner, P., Jermutus, L., & Minter, R. R. (2006). Harnessing phage and ribosome display for antibody optimisation. *Trends Biotechnol*, 24(11), 523-529. doi: 10.1016/j.tibtech.2006.09.004
- Early, P., Huang, H., Davis, M., Calame, K., & Hood, L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell*, 19(4), 981-992. doi: 10.1016/0092-8674(80)90089-6
- El-Aneed, A., & Banoub, J. (2006). Proteomics in the diagnosis of hepatocellular carcinoma: focus on high risk hepatitis B and C patients. *Anticancer Res*, 26(5A), 3293-3300.
- Finishing the euchromatic sequence of the human genome. (2004). *Nature*, 431(7011), 931-945. doi: 10.1038/nature03001
- Fox, J. L., & Klass, M. (1989). Antigens produced by recombinant DNA technology. *Clin Chem*, 35(9), 1838-1842.

Fuchs, P., Breitling, F., Dubel, S., Seehaus, T., & Little, M. (1991). Targeting recombinant antibodies to the surface of *Escherichia coli*: fusion to a peptidoglycan associated lipoprotein. *Biotechnology (N Y)*, 9(12), 1369-1372.

Goldman, E. R., Anderson, G. P., Liu, J. L., Delehanty, J. B., Sherwood, L. J., Osborn, L. E., Hayhurst, A. (2006). Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library. *Anal Chem*, 78(24), 8245-8255. doi: 10.1021/ac0610053

Grant, G. A. (2003). Synthetic peptides for production of antibodies that recognize intact proteins. *Curr Protoc Immunol*, Chapter 9, Unit 9 2. doi: 10.1002/0471142735.im0902s55

Greenberg, Andrew S., Avila, David, Hughes, Marianne, Hughes, Austin, McKinney, E. Churchill, & Flajnik, Martin F. (1995). A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. [10.1038/374168a0]. *Nature*, 374(6518), 168-173.

Griffiths, A. D., Malmqvist, M., Marks, J. D., Bye, J. M., Embleton, M. J., McCafferty, J. (1993). Human anti-self antibodies with high specificity from phage display libraries. *EMBO J*, 12(2), 725-734.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J*, 13(14), 3245-3260.

Harmsen, M. M., Ruuls, R. C., Nijman, I. J., Niewold, T. A., Frenken, L. G., & de Geus, B. (2000). Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. *Mol Immunol*, 37(10), 579-590.

Harmsen, M. M., van Solt, C. B., van Zijderveld-van Bommel, A. M., Niewold, T. A., & van Zijderveld, F. G. (2006). Selection and optimization of proteolytically stable llama single-domain antibody fragments for oral immunotherapy. *Appl Microbiol Biotechnol*, 72(3), 544-551. doi: 10.1007/s00253-005-0300-7

Harmsen, Michiel M., Ruuls, Robin C., Nijman, Isa c J., Niewold, Theodoor A., Frenken, Leon G. J., & de Geus, Bernard. (2000). Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. *Mol Immunol*, 37(10), 579-590. doi: 10.1016/s0161-5890(00)00081-x

Harrison, R. A., Hasson, S. S., Harmsen, M., Laing, G. D., Conrath, K., & Theakston, R. D. (2006). Neutralisation of venom-induced haemorrhage by IgG from camels and llamas immunised with viper venom and also by endogenous, non-IgG components in camelid sera. *Toxicon*, 47(3), 364-368. doi: 10.1016/j.toxicon.2005.10.017

Harvey, B. R., Georgiou, G., Hayhurst, A., Jeong, K. J., Iverson, B. L., & Rogers, G. K. (2004). Anchored periplasmic expression, a versatile technology for the isolation of high-

affinity antibodies from Escherichia coli-expressed libraries. *Proc Natl Acad Sci U S A*, 101(25), 9193-9198. doi: 10.1073/pnas.0400187101

Hassanzadeh-Ghassabeh, G., Saerens, D., & Muyldermans, S. (2011). Generation of anti-infectome/anti-proteome nanobodies. *Methods Mol Biol*, 790, 239-259. doi: 10.1007/978-1-61779-319-6_19

Hawkins, R. E., Russell, S. J., & Winter, G. (1992). Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J Mol Biol*, 226(3), 889-896.

He, M., & Khan, F. (2005). Ribosome display: next-generation display technologies for production of antibodies in vitro. *Expert Rev Proteomics*, 2(3), 421-430. doi: 10.1586/14789450.2.3.421

He, M., & Taussig, M. J. (1997). Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. *Nucleic Acids Res*, 25(24), 5132-5134.

Holliger, P., & Hudson, P. J. (2005). Engineered antibody fragments and the rise of single domains. *Nat Biotechnol*, 23(9), 1126-1136. doi: 10.1038/nbt1142

Hoogenboom, H. R. (2005). Selecting and screening recombinant antibody libraries. *Nat Biotechnol*, 23(9), 1105-1116. doi: 10.1038/nbt1126

Hoogenboom, H. R., & Winter, G. (1992). By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J Mol Biol*, 227(2), 381-388.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., & Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res*, 19(15), 4133-4137.

Hoogenboom, Hennie R., de Bruijne, Adriaan P., Hufton, Simon E., Hoet, René M., Arends, Jan-Willem, & Roovers, Rob C. (1998). Antibody phage display technology and its applications. *Immunotechnology*, 4(1), 1-20. doi: 10.1016/s1380-2933(98)00007-4

Hudson, P. J. (1998). Recombinant antibody fragments. *Curr Opin Biotechnol*, 9(4), 395-402.

Hudson, P. J., & Souriau, C. (2003). Engineered antibodies. *Nat Med*, 9(1), 129-134. doi: 10.1038/nm0103-129

Hussack, G., Keklikian, A., Alsughayyir, J., Hanifi-Moghaddam, P., Arbabi-Ghahroudi, M., van Faassen, H., Tanha, J. (2012). A V(L) single-domain antibody library shows a high-

propensity to yield non-aggregating binders. *Protein Eng Des Sel*, 25(6), 313-318. doi: 10.1093/protein/gzs014

Hwa, V., Oh, Y., & Rosenfeld, R. G. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev*, 20(6), 761-787.

Ionescu, R. M., Vlasak, J., Price, C., & Kirchmeier, M. (2008). Contribution of variable domains to the stability of humanized IgG1 monoclonal antibodies. *J Pharm Sci*, 97(4), 1414-1426. doi: 10.1002/jps.21104

Iqbal, U., Albaghdadi, H., Luo, Y., Arbabi, M., Desvaux, C., Veres, T., Abulrob, A. (2010). Molecular imaging of glioblastoma multiforme using anti-insulin-like growth factor-binding protein-7 single-domain antibodies. *Br J Cancer*, 103(10), 1606-1616. doi: 10.1038/sj.bjc.6605937

Iqbal, U., Trojahn, U., Albaghdadi, H., Zhang, J., O'Connor-McCourt, M., Stanimirovic, D., Abulrob, A. (2010). Kinetic analysis of novel mono- and multivalent VHH-fragments and their application for molecular imaging of brain tumours. *Br J Pharmacol*, 160(4), 1016-1028. doi: 10.1111/j.1476-5381.2010.00742.x

J Brichta, M Hnilova, T Viskovic (2005). Generation of hapten-specific recombinant antibodies : antibody phage display technology : a review. *Veterinarni Medicina* 50(6), 231-252.

Jain, M., Kamal, N., & Batra, S. K. (2007). Engineering antibodies for clinical applications. *Trends Biotechnol*, 25(7), 307-316. doi: 10.1016/j.tibtech.2007.05.001

Janeway, C. (2005). *Immunobiology: The Immune System in Health and Disease*: Garland Science.

Jenkins, N., & Curling, E. M. (1994). Glycosylation of recombinant proteins: problems and prospects. *Enzyme Microb Technol*, 16(5), 354-364.

John, McCafferty. (1996). Chapter 15 - Phage Display: Factors Affecting Panning Efficiency. In K. Kay Brian, Winter Jill, Jill Winter John McCaffertyA2 - Brian K. Kay & McCafferty John (Eds.), *Phage Display of Peptides and Proteins* (pp. 261-276). Burlington: Academic Press.

Johns, M., George, A. J., & Ritter, M. A. (2000). In vivo selection of sFv from phage display libraries. *J Immunol Methods*, 239(1-2), 137-151.

Johnson, I. S. (1983). Human insulin from recombinant DNA technology. *Science*, 219(4585), 632-637.

Jones, J. I., & Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev*, 16(1), 3-34.

Jones, Peter T., Dear, Paul H., Foote, Jefferson, Neuberger, Michael S., & Winter, Greg. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. [10.1038/321522a0]. *Nature*, 321(6069), 522-525.

Joosten, V., Lokman, C., Van Den Hondel, C. A., & Punt, P. J. (2003). The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. *Microb Cell Fact*, 2(1), 1.

Jung, S., & Pluckthun, A. (1997). Improving in vivo folding and stability of a single-chain Fv antibody fragment by loop grafting. *Protein Eng*, 10(8), 959-966.

Jung, Sabine, Honegger, Annemarie, & Plückthun, Andreas. (1999). Selection for improved protein stability by phage display. *J Mol Biol*, 294(1), 163-180. doi: 10.1006/jmbi.1999.3196

Kabat, E.A., Te Wu, T., Gottesman, K.S., & Foeller, C. (1992). *Sequences of Proteins of Immunological Interest*: Diane Publishing Company.

Kang, A. S., Barbas, C. F., Janda, K. D., Benkovic, S. J., & Lerner, R. A. (1991). Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc Natl Acad Sci U S A*, 88(10), 4363-4366.

Kehoe, J. W., & Kay, B. K. (2005). Filamentous phage display in the new millennium. *Chem Rev*, 105(11), 4056-4072. doi: 10.1021/cr000261r

Khandwala, H. M., McCutcheon, I. E., Flyvbjerg, A., & Friend, K. E. (2000). The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocr Rev*, 21(3), 215-244.

Kieny, M. P., Lathe, R., Riviere, Y., Dott, K., Schmitt, D., Girard, M., Lecocq, J. (1988). Improved antigenicity of the HIV env protein by cleavage site removal. *Protein Eng*, 2(3), 219-225.

Kim, A. Y., & Blaschek, H. P. (1991). Isolation and characterization of a filamentous viruslike particle from *Clostridium acetobutylicum* NCIB 6444. *J Bacteriol*, 173(2), 530-535.

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Virnekas, B. (2000). Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol*, 296(1), 57-86. doi: 10.1006/jmbi.1999.3444

Kohler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256(5517), 495-497.

- Koide, A., Wojcik, J., Gilbreth, R. N., Reichel, A., Piehler, J., & Koide, S. (2009). Accelerating phage-display library selection by reversible and site-specific biotinylation. *Protein Eng Des Sel*, 22(11), 685-690. doi: 10.1093/protein/gzp053
- Kong, R., Li, H., Bibollet-Ruche, F., Decker, J. M., Zheng, N. N., Gottlieb, G. S., Shaw, G. M. (2011). Broad and Potent Neutralizing Antibody Responses Elicited in Natural HIV-2 Infection. *J Virol*. doi: 10.1128/jvi.06155-11
- Kontermann, R., & Dübel, S. (2010). *Antibody Engineering*: Springer.
- Konthur, Z., Hust, M., & Dubel, S. (2005). Perspectives for systematic in vitro antibody generation. *Gene*, 364, 19-29. doi: 10.1016/j.gene.2005.05.042
- Konthur, Zoltán, Wilde, Jeannine, & Lim, Theam Soon. (2010). Semi-automated Magnetic Bead-Based Antibody Selection from Phage Display Libraries (pp. 267-287).
- Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Morgan, M. J. (2001). Initial sequencing and analysis of the human genome. *Nature*, 409(6822), 860-921.
- Larrick, J. W., Danielsson, L., Brenner, C. A., Abrahamson, M., Fry, K. E., & Borrebaeck, C. A. (1989). Rapid cloning of rearranged immunoglobulin genes from human hybridoma cells using mixed primers and the polymerase chain reaction. *Biochem Biophys Res Commun*, 160(3), 1250-1256.
- Lee, B. S., Huang, J. S., Jayathilaka, G. D., Lateef, S. S., & Gupta, S. (2010). Production of anti-peptide antibodies. *Methods Mol Biol*, 657, 93-108. doi: 10.1007/978-1-60761-783-9_7
- Lee, Sang Yup, Choi, Jong Hyun, & Xu, Zhaohui. (2003). Microbial cell-surface display. *Trends Biotechnol*, 21(1), 45-52. doi: 10.1016/s0167-7799(02)00006-9
- Leon, S. P., Folkerth, R. D., & Black, P. M. (1996). Microvessel density is a prognostic indicator for patients with astroglial brain tumors. *Cancer*, 77(2), 362-372. doi: 10.1002/(sici)1097-0142(19960115)77:2<362::aid-cnrc20>3.0.co;2-z
- Lin, X., Xie, J., & Chen, X. (2011). Protein-based tumor molecular imaging probes. *Amino Acids*, 41(5), 1013-1036. doi: 10.1007/s00726-010-0545-z
- Lo, B.K.C. (2004). *Antibody Engineering: Methods and Protocols*: Humana Press.
- Lo, Benny K. (2003). Antibody Humanization by CDR Grafting (Vol. 248, pp. 135-159).
- Löfblom, John. (2011). Bacterial display in combinatorial protein engineering. *Biotechnology Journal*, 6(9), 1115-1129. doi: 10.1002/biot.201100129

Loomans, E. E., Gribnau, T. C., Bloemers, H. P., & Schielen, W. J. (1998). Adsorption studies of tritium-labeled peptides on polystyrene surfaces. *J Immunol Methods*, 221(1-2), 131-139.

Male, D.K., Brostoff, J., & Roth, D.B. (2006). *Immunology*: Mosby Elsevier.

Marks J.D., Hoogenboom H.R., Bonnert T.P., McCafferty, J., Griffiths A.D., Winter G. . (1991). By-passing immunization human antibodies from V-gene libraries displayed on phage. *Journal of Molecular Biology*, 222, 581–597

Massoner, P., Ladurner-Rennau, M., Eder, I. E., & Klocker, H. (2010). Insulin-like growth factors and insulin control a multifunctional signalling network of significant importance in cancer. *Br J Cancer*, 103(10), 1479-1484. doi: 10.1038/sj.bjc.6605932

Maynard, J., & Georgiou, G. (2000). Antibody engineering. *Annu Rev Biomed Eng*, 2, 339-376. doi: 10.1146/annurev.bioeng.2.1.339

McCafferty, J., Griffiths, A. D., Winter, G., & Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, 348(6301), 552-554. doi: 10.1038/348552a0

McCafferty, John, & Johnson, Kevin S. (1996). Chapter 6 - Construction and Screening of Antibody Display Libraries. In K. Kay Brian, Winter Jill, Jill Winter John McCaffertyA2 - Brian K. Kay & McCafferty John (Eds.), *Phage Display of Peptides and Proteins* (pp. 79-111). Burlington: Academic Press.

Meulemans, E. V., Slobbe, R., Wasterval, P., Ramaekers, F. C., & van Eys, G. J. (1994). Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. *J Mol Biol*, 244(4), 353-360. doi: 10.1006/jmbi.1994.1735

Monegal, Ana, Ami, Diletta, Martinelli, Chiara, Huang, He, Aliprandi, Marisa, Capasso, Paola, de Marco, Ario. (2009). Immunological applications of single-domain llama recombinant antibodies isolated from a naïve library. *Protein Engineering Design and Selection*, 22(4), 273-280. doi: 10.1093/protein/gzp002

Moreno MJ, Ball M, Andrade MF, McDermid A, Stanimirovic D (2006) Insulin-like growth factor binding protein-4 (IGFBP-4) is a novel anti-angiogenic and anti-tumorigenic factor secreted by dibutyryl cyclicAMP (dB-cAMP)-differentiated glioblastoma cells. *Glia* 53(8):845-57.

Mullen, L. M., Nair, S. P., Ward, J. M., Rycroft, A. N., & Henderson, B. (2006). Phage display in the study of infectious diseases. *Trends Microbiol*, 14(3), 141-147. doi: 10.1016/j.tim.2006.01.006

- Muller, D., Karle, A., Meissburger, B., Hofig, I., Stork, R., & Kontermann, R. E. (2007). Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. *J Biol Chem*, 282(17), 12650-12660. doi: 10.1074/jbc.M700820200
- Murphy, M., Pykett, M. J., Harnish, P., Zang, K. D., & George, D. L. (1993). Identification and characterization of genes differentially expressed in meningiomas. *Cell Growth Differ*, 4(9), 715-722.
- Muyldermans, S. (2001). Single domain camel antibodies: current status. *J Biotechnol*, 74(4), 277-302.
- Muyldermans, S., & Lauwereys, M. (1999). Unique single-domain antigen binding fragments derived from naturally occurring camel heavy-chain antibodies. *J Mol Recognit*, 12(2), 131-140. doi: 10.1002/(sici)1099-1352(199903/04)12:2<131:aid-jmr454:3
- Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J.A.R.G., & Hamers, R. (1994). Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Eng*, 7(9), 1129-1135. doi: 10.1093/protein/7.9.1129
- Muyldermans, S., Baral, T. N., Retamozzo, V. C., De Baetselier, P., De Genst, E., Kinne, J., Saerens, D. (2009). Camelid immunoglobulins and nanobody technology. *Vet Immunol Immunopathol*, 128(1-3), 178-183. doi: 10.1016/j.vetimm.2008.10.299
- Muyldermans, Serge. (2001). Single domain camel antibodies: current status. *Reviews in Molecular Biotechnology*, 74(4), 277-302. doi: 10.1016/s1389-0352(01)00021-6
- Nguyen, Viet Khong, Hamers, Raymond, Wyns, Lode, & Muyldermans, Serge. (2000). Camel heavy-chain antibodies: diverse germline VHH and specific mechanisms enlarge the antigen-binding repertoire. [10.1093/emboj/19.5.921]. *EMBO J*, 19(5), 921-930.
- Nicosia, R. F., Nicosia, S. V., & Smith, M. (1994). Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. *Am J Pathol*, 145(5), 1023-1029.
- Noppe, W., Plieva, F., Galaev, I. Y., Pottel, H., Deckmyn, H., & Mattiasson, B. (2009). Chromato-panning: an efficient new mode of identifying suitable ligands from phage display libraries. *BMC Biotechnol*, 9, 21. doi: 10.1186/1472-6750-9-21
- Orlandi, R., Gussow, D. H., Jones, P. T., & Winter, G. (1989). Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc Natl Acad Sci U S A*, 86(10), 3833-3837.

Oshima, M., & Atassi, M. Z. (1989). Comparison of peptide-coating conditions in solid phase plate assays for detection of anti-peptide antibodies. *Immunol Invest*, 18(7), 841-851.

Padlan, E. A. (1994). Anatomy of the antibody molecule. *Mol Immunol*, 31(3), 169-217.

Paschke, M. (2006). Phage display systems and their applications. *Appl Microbiol Biotechnol*, 70(1), 2-11. doi: 10.1007/s00253-005-0270-9

Payne, Gillian. (2003). Progress in immunoconjugate cancer therapeutics. *Cancer Cell*, 3(3), 207-212. doi: 10.1016/s1535-6108(03)00057-6

Pen, A., Moreno, M. J., Martin, J., & Stanimirovic, D. B. (2007). Molecular markers of extracellular matrix remodeling in glioblastoma vessels: microarray study of laser-captured glioblastoma vessels. *Glia*, 55(6), 559-572. doi: 10.1002/glia.20481

Pen, A., Durocher, Y., Slinn, J., Rukhlova, M., Charlebois, C., Stanimirovic, D. B., & Moreno, M. J. (2011). Insulin-like growth factor binding protein 7 exhibits tumor suppressive and vessel stabilization properties in U87MG and T98G glioblastoma cell lines. *Cancer Biol Ther*, 12(7), 634-646.

Pyun, J. C., Cheong, M. Y., Park, S. H., Kim, H. Y., & Park, J. S. (1997). Modification of short peptides using epsilon-aminocaproic acid for improved coating efficiency in indirect enzyme-linked immunosorbent assays (ELISA). *J Immunol Methods*, 208(2), 141-149.

Radosevic, Katarina, & Ewijk, Willem. (2002). Subtractive Isolation of Single-Chain Antibodies Using Tissue Fragments

Rakonjac, J., Bennett, N. J., Spagnuolo, J., Gagic, D., & Russel, M. (2011). Filamentous bacteriophage: biology, phage display and nanotechnology applications. *Curr Issues Mol Biol*, 13(2), 51-76.

Revets, H., De Baetselier, P., & Muyldermans, S. (2005). Nanobodies as novel agents for cancer therapy. *Expert Opin Biol Ther*, 5(1), 111-124. doi: 10.1517/14712598.5.1.111

Riechmann, L., & Muyldermans, S. (1999). Single domain antibodies: comparison of camel VH and camelised human VH domains. *J Immunol Methods*, 231(1-2), 25-38.

Roitt, I.M., Brostoff, J., & Male, D.K. (2001). *Immunology*: Mosby.

Rondot, S., Koch, J., Breitling, F., & Dubel, S. (2001). A helper phage to improve single-chain antibody presentation in phage display. *Nat Biotechnol*, 19(1), 75-78. doi: 10.1038/83567

Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schermelleh, L., Gahl, A., . . . Leonhardt, H. (2006). Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat Methods*, 3(11), 887-889. doi: 10.1038/nmeth953

- Rothe, A., Hosse, R. J., & Power, B. E. (2006). Ribosome display for improved biotherapeutic molecules. *Expert Opin Biol Ther*, 6(2), 177-187. doi: 10.1517/14712598.6.2.177
- Rouet, Romain, Lowe, David, Dudgeon, Kip, Roome, Brendan, Schofield, Peter, Langley, David, Christ, Daniel. (2012). Expression of high-affinity human antibody fragments in bacteria. [10.1038/nprot.2011.448]. *Nat. Protocols*, 7(2), 364-373.
- Roux, K. H., Greenberg, A. S., Greene, L., Strelets, L., Avila, D., McKinney, E. C., & Flajnik, M. F. (1998). Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. *Proc Natl Acad Sci U S A*, 95(20), 11804-11809.
- Russel, M., Lowman, H.B., and Clackson, T. (2004) Introduction to phage biology and phage display. In *Phage Display – A Practical Approach*. Clackson, T. and Lowman, H.B. (eds). Oxford: Oxford University Press, pp. 1-26.
- Saerens (C), Dirk, Ghassabeh, Gholamreza Hassanzadeh, & Muyldermans, Serge. (2008). Single-domain antibodies as building blocks for novel therapeutics. *Current Opinion in Pharmacology*, 8(5), 600-608. doi: 10.1016/j.coph.2008.07.006
- Saerens, D., Conrath, K., Govaert, J., & Muyldermans, S. (2008). Disulfide bond introduction for general stabilization of immunoglobulin heavy-chain variable domains. *J Mol Biol*, 377(2), 478-488. doi: 10.1016/j.jmb.2008.01.022
- Saerens, D., Ghassabeh, G. H., & Muyldermans, S. (2008). Antibody technology in proteomics. *Brief Funct Genomic Proteomic*, 7(4), 275-282. doi: 10.1093/bfgp/eln028
- Saerens, D., Kinne, J., Bosmans, E., Wernery, U., Muyldermans, S., & Conrath, K. (2004). Single domain antibodies derived from dromedary lymph node and peripheral blood lymphocytes sensing conformational variants of prostate-specific antigen. *J Biol Chem*, 279(50), 51965-51972. doi: 10.1074/jbc.M409292200
- Saerens, D., Stijlemans, B., Baral, T. N., Nguyen Thi, G. T., Wernery, U., Magez, S., Conrath, K. (2008). Parallel selection of multiple anti-infectome Nanobodies without access to purified antigens. *J Immunol Methods*, 329(1-2), 138-150. doi: 10.1016/j.jim.2007.10.005
- Saerens, Dirk, Huang, Lieven, Bonroy, Kristien, & Muyldermans, Serge. (2008). Antibody Fragments as Probe in Biosensor Development. *Sensors*, 8(8), 4669-4686.
- Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Haase, F. C., & Wood, H. G. (1988). Evolutionary conservation among biotin enzymes. *J Biol Chem*, 263(14), 6461-6464.
- Santala, V., & Lamminmaki, U. (2004). Production of a biotinylated single-chain antibody fragment in the cytoplasm of *Escherichia coli*. *J Immunol Methods*, 284(1-2), 165-175.

- Sato Y, Chen Z, Miyazaki K (2007) Strong suppression of tumor growth by insulin-like growth factor-binding protein-related protein 1/tumor-derived cell adhesion factor/mac25. *Cancer Sci* 98(7):1055-1063.
- Scholle, M. D., Collart, F. R., & Kay, B. K. (2004). In vivo biotinylated proteins as targets for phage-display selection experiments. *Protein Expr Purif*, 37(1), 243-252. doi: 10.1016/j.pep.2004.05.012
- Scott, J. K., & Smith, G. P. (1990). Searching for peptide ligands with an epitope library. *Science*, 249(4967), 386-390.
- Sidhu, S. S. (2001). Engineering M13 for phage display. *Biomol Eng*, 18(2), 57-63.
- Sidhu, S.S. (2005). Phage display in biotechnology and drug discovery: CRC Press/Taylor & Francis.
- Skerra, A., & Pluckthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science*, 240(4855), 1038-1041.
- Smith, B. J., Popplewell, A., Athwal, D., Chapman, A. P., Heywood, S., West, S. M., Sutters, A. (2001). Prolonged in vivo residence times of antibody fragments associated with albumin. *Bioconjug Chem*, 12(5), 750-756.
- Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, 228(4705), 1315-1317.
- Smith, G. P., & Petrenko, V. A. (1997). Phage Display. *Chem Rev*, 97(2), 391-410.
- Soderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A. M., Borrebaeck, C. A. (2000). Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat Biotechnol*, 18(8), 852-856. doi: 10.1038/78458
- Sonntag, William E., Lynch, Colleen, Thornton, Phillip, Khan, Amir, Bennett, Sean, & Ingram, Rhonda. (2000). The effects of growth hormone and IGF-1 deficiency on cerebrovascular and brain ageing. *Journal of Anatomy*, 197(4), 575-585. doi: 10.1046/j.1469-7580.2000.19740575.x
- Spada, S., Krebber, C., & Pluckthun, A. (1997). Selectively infective phages (SIP). *Biol Chem*, 378(6), 445-456.
- Spinelli, S., Frenken, L. G., Hermans, P., Verrips, T., Brown, K., Tegoni, M., & Cambillau, C. (2000). Camelid heavy-chain variable domains provide efficient combining sites to haptens. *Biochemistry*, 39(6), 1217-1222.

- Tabares-da Rosa, S., Rossotti, M., Carleiza, C., Carrion, F., Pritsch, O., Ahn, K. C., Gonzalez-Sapienta, G. (2011). Competitive selection from single domain antibody libraries allows isolation of high-affinity antihapten antibodies that are not favored in the llama immune response. *Anal Chem*, 83(18), 7213-7220. doi: 10.1021/ac201824z
- Tamura, K., Hashimoto, K., Suzuki, K., Yoshie, M., Kutsukake, M., & Sakurai, T. (2009). Insulin-like growth factor binding protein-7 (IGFBP7) blocks vascular endothelial cell growth factor (VEGF)-induced angiogenesis in human vascular endothelial cells. *Eur J Pharmacol*, 610(1-3), 61-67. doi: 10.1016/j.ejphar.2009.01.045
- Tanha, J., Dubuc, G., Hirma, T., Narang, S. A., & MacKenzie, C. R. (2002). Selection by phage display of llama conventional V(H) fragments with heavy chain antibody V(H)H properties. *J Immunol Methods*, 263(1-2), 97-109.
- Tanha, J., Xu, P., Chen, Z., Ni, F., Kaplan, H., Narang, S. A., & MacKenzie, C. R. (2001). Optimal design features of camelized human single-domain antibody libraries. *J Biol Chem*, 276(27), 24774-24780. doi: 10.1074/jbc.M100770200
- To, R., Hirma, T., Arbabi-Ghahroudi, M., MacKenzie, R., Wang, P., Xu, P., . . . Tanha, J. (2005). Isolation of monomeric human V(H)s by a phage selection. *J Biol Chem*, 280(50), 41395-41403. doi: 10.1074/jbc.M509900200
- Trill, John J., Shatzman, Allan R., & Subinay, Ganguly. (1995). Production of monoclonal antibodies in COS and CHO cells. *Current Opinion in Biotechnology*, 6(5), 553-560. doi: 10.1016/0958-1669(95)80092-1
- Utsumi, Sayaka, & Karush, Fred. (1964). The Subunits of Purified Rabbit Antibody*. *Biochemistry*, 3(9), 1329-1338. doi: 10.1021/bi00897a024
- Vajkoczy P, Menger MD. (2000). Vascular microenvironment in gliomas. *J Neurooncol* 50: 99–108.
- van der Linden, R. H., Frenken, L. G., de Geus, B., Harmsen, M. M., Ruuls, R. C., Stok, W., Verrips, C. T. (1999). Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta*, 1431(1), 37-46.
- van der Linden, R., de Geus, B., Stok, W., Bos, W., van Wassenaar, D., Verrips, T., & Frenken, L. (2000). Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama. *J Immunol Methods*, 240(1-2), 185-195.
- van Duijn, G. J., van Biert, R., Bleeker-Marcelis, H., Van Boeijen, I., Adan, A. J., Jhakrie, S., & Hessing, M. (2002). Detection of genetically modified organisms in foods by protein- and DNA-based techniques: bridging the methods. *J AOAC Int*, 85(3), 787-791.
- Van Regenmortel, M. H. (2001). Antigenicity and immunogenicity of synthetic peptides. *Biologicals*, 29(3-4), 209-213. doi: 10.1006/biol.2001.0308

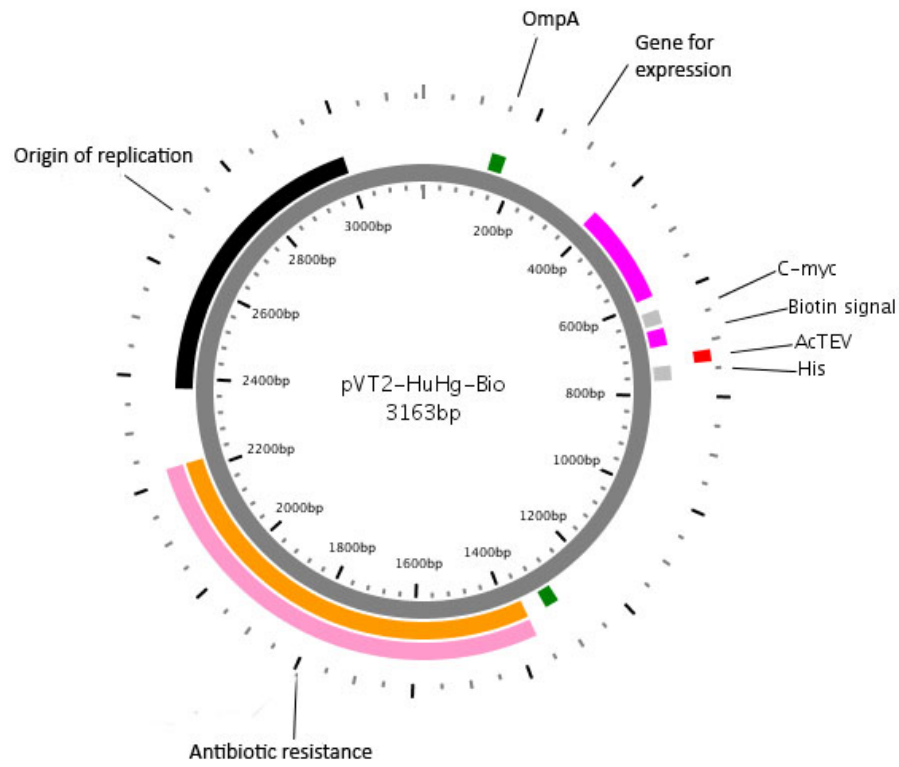
- Vanlandschoot, P., Stortelers, C., Beirnaert, E., Ibanez, L. I., Schepens, B., Depla, E., & Saelens, X. (2011). Nanobodies(R): New ammunition to battle viruses. *Antiviral Res.* doi: 10.1016/j.antiviral.2011.09.002
- Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., Johnson, K. S. (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol*, 14(3), 309-314. doi: 10.1038/nbt0396-309
- Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Zhu, X. (2001). The sequence of the human genome. *Science*, 291(5507), 1304-1351. doi: 10.1126/science.1058040
- Vu, K. B., Ghahroudi, M. A., Wyns, L., & Muyldermans, S. (1997). Comparison of llama VH sequences from conventional and heavy chain antibodies. *Mol Immunol*, 34(16-17), 1121-1131.
- Walter, G., Konthur, Z., & Lehrach, H. (2001). High-throughput screening of surface displayed gene products. *Comb Chem High Throughput Screen*, 4(2), 193-205.
- Ward, E. S., Gussow, D., Griffiths, A. D., Jones, P. T., & Winter, G. (1989). Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature*, 341(6242), 544-546. doi: 10.1038/341544a0
- Ward, R. L., Clark, M. A., Lees, J., & Hawkins, N. J. (1996). Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. *J Immunol Methods*, 189(1), 73-82.
- Watters, J. M., Telleman, P., & Junghans, R. P. (1997). An optimized method for cell-based phage display panning. *Immunotechnology*, 3(1), 21-29.
- Wesolowski, J., Alzogaray, V., Reyelt, J., Unger, M., Juarez, K., Urrutia, M., Koch-Nolte, F. (2009). Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol*, 198(3), 157-174. doi: 10.1007/s00430-009-0116-7
- Wetterau, L. A., Moore, M. G., Lee, K. W., Shim, M. L., & Cohen, P. (1999). Novel aspects of the insulin-like growth factor binding proteins. *Mol Genet Metab*, 68(2), 161-181. doi: 10.1006/mgme.1999.2920
- Whaley, S. R., English, D. S., Hu, E. L., Barbara, P. F., & Belcher, A. M. (2000). Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly. *Nature*, 405(6787), 665-668. doi: 10.1038/35015043
- Willats, W. G. (2002). Phage display: practicalities and prospects. *Plant Mol Biol*, 50(6), 837-854.

- Willuda, J., Honegger, A., Waibel, R., Schubiger, P. A., Stahel, R., Zangemeister-Wittke, U., & Pluckthun, A. (1999). High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. *Cancer Res*, 59(22), 5758-5767.
- Wingren, C., & Borrebaeck, C. A. (2004). High-throughput proteomics using antibody microarrays. *Expert Rev Proteomics*, 1(3), 355-364. doi: 10.1586/14789450.1.3.355
- Winter, G., Griffiths, A. D., Hawkins, R. E., & Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annu Rev Immunol*, 12, 433-455. doi: 10.1146/annurev.iy.12.040194.002245
- Wu, A. M., & Senter, P. D. (2005). Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol*, 23(9), 1137-1146. doi: 10.1038/nbt1141
- Wu, A. M., & Senter, P. D. (2005). Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol*, 23(9), 1137-1146. doi: 10.1038/nbt1141
- Yamanaka, Y., Wilson, E. M., Rosenfeld, R. G., & Oh, Y. (1997). Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. *J Biol Chem*, 272(49), 30729-30734.
- Yamauchi, T., Umeda, F., Masakado, M., Isaji, M., Mizushima, S., & Nawata, H. (1994). Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. *Biochem J*, 303 (Pt 2), 591-598.
- Yang, Feixue, Ventura-Holman, Tereza, Waldbieser, Geoffrey C., & Lobb, Craig J. (2003). Structure, genomic organization, and phylogenetic implications of six new VH families in the channel catfish. *Mol Immunol*, 40(5), 247-260. doi: 10.1016/s0161-5890(03)00143-3
- Yau, K. Y., Lee, H., & Hall, J. C. (2003). Emerging trends in the synthesis and improvement of hapten-specific recombinant antibodies. *Biotechnol Adv*, 21(7), 599-637.
- Zhang, J., Li, Q., Nguyen, T. D., Tremblay, T. L., Stone, E., To, R., Roger MacKenzie, C. (2004). A pentavalent single-domain antibody approach to tumor antigen discovery and the development of novel proteomics reagents. *J Mol Biol*, 341(1), 161-169. doi: 10.1016/j.jmb.2004.05.069
- Zhang, J., Tanha, J., Hiram, T., Khieu, N. H., To, R., Tong-Sevinc, H., MacKenzie, C. R. (2004). Pentamerization of single-domain antibodies from phage libraries: a novel strategy for the rapid generation of high-avidity antibody reagents. *J Mol Biol*, 335(1), 49-56.

Zhu, X., Wang, L., Liu, R., Flutter, B., Li, S., Ding, J., Gao, B. (2010). COMBODY: one-domain antibody multimer with improved avidity. *Immunol Cell Biol*, 88(6), 667-675. doi: 10.1038/icb.2010.21

Appendix

pVT2-HuHg-Bio map



Created using PlasMapper

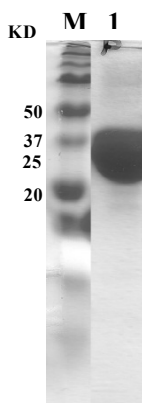
Genetic organization of pVT2-HuHg-Bio vector. The circular map shows the positions of lac operon promoter, the antibiotic-resistance gene (ampicillin amp R) and the origin of replication.

Recombinant IGFBP7 protein

Purified full-length IGFBP7 protein was provided by Dr. Denis L'Abbé (Genomics and Health Initiative, NRC). The full cDNA encoding IGFBP7 (GeneBank BC017201) in the vector pOTB7 was purchased from ATCC. Using this as template, *igfbp7* cDNA fragment was PCR amplified using forward primer 5'-TCG AAT TCC CGC CAT GGA GCG GCC GTC G-3' and reverse primer 5'- TAG GGA TCC TAG CTC GGC ACC TTC ACC T-3'. The PCR product was then digested with *EcoRI* and *BamHI* restriction enzymes and inserted into the vector pTT5SH8Q2 (in the frame with Streptag-II and polyhistidine epitopes) to yield IGFBP7/ pTT5SH8Q2. A stably transfected human embryonic kidney 293 cell line expressing IGFBP7 was established. Expressed protein was purified by immobilized metal affinity chromatography. The purified recombinant protein had the expected size of ~ 33 KDa (Pen A. et. al., 2011).

Recombinant IGFBP7 SDS-PAGE

The gene encoding the IGFBP7 protein was cloned in a mammalian pOTB7 expression vector and the recombinant protein was expressed in human embryonic kidney 293 cell line and purified from the supernatant by immobilized metal affinity chromatography. IGFBP7 was kindly provided by Dr. Denis L'Abbé (GHI, NRC).



SDS-PAGE profile of purified rIGFBP7. IGFBP7 was expressed in human embryonic kidney 293 cell line and purified from supernatant by IMAC. The purified recombinant protein had the expected size (~ 33 kDa) (Pen A. et. al., 2011). Lane 1 contains 10 µg of IGFBP7 . Molecular weight markers, M, in kDa.

Functional assay II:

***In vitro* anti-tumorigenic effect of anti-IGFBP7 pentameric and monomeric V_HH 12**

Cell culture

The human pancreatic cell line, PANC-1, was obtained from the American Type Culture Collection (ATCC, CRL-1469). PANC-1 cells were grown in D-MEM supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, USA) in humidified atmosphere of 5% CO₂/95% air at 37 °C. Culture media was replaced every 3-4 days.

Anchorage-independent growth of Panc-1 cells in soft agar

Anchorage-independent growth of PANC-1 cells was evaluated in semi-solid agar in the absence or presence of either M12 (3 µM) or P12 (3 µM) antibodies. Approximately 15x10⁵ cells ± treatment were resuspended in 150 mL growth media (D-MEM + 10% (v/v) FBS) containing 0.6% (w/v) agar and seeded into wells of a 24-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) previously layered with 250 mL of 0.6% (w/v) agar. The solidified cell layer was covered with 50 mL D-MEM ± treatment, which was replaced every three days over a 3 week period. At the end of the experiment, phase contrast images (8 fields/well x 4 wells per treatment) were captured and visualized using an Olympus microscope (Olympus 1X81, Markham, ON, Canada). To measure cell viability at the end of the experiment, 50 mL of Alamar Blue (Cedarlane, Burlington, Canada) was added to each well and fluorescence readings (530 nm excitation and 590 nm emission) were measured after a 180 min period of incubation.

Data analysis

Statistical significance among groups was performed using one-way variance analysis followed by a student–Newman–Keul's post-hoc comparison of means (GraphPad Prism 5.0, USA). Differences with a P value ≤ 0.05 were considered to be statistically significant.

Results:

***In vitro* anti-tumorigenic effect of anti-IGFBP7 pentameric and monomeric V_HH**

This sensitive assay was employed to assess the therapeutic capacity of anti-IGFBP7 monomeric (M12) and pentameric (P12) V_HH to inhibit tumor colony growth in soft agar. The PANC-1 cell colonies in the control group varied in size from large to small and exhibited a diffuse morphology, with a large number of cells disseminated around the tumor, indicative of a migratory phenotype (Figure A). PANC-1 cell line expresses IGFBP7 and this was confirmed by Western blot. PANC-1 cells that were treated with M12 formed colonies of medium and small size. These colonies were tighter and almost encapsulated compared to the control colonies. This may be indicative of a reduction in the migratory capacity of these cells. In addition, PANC-1 cells that were treated with P12 were mostly of small size and of tight/encapsulated morphology (Figure A) indicating that P12 and M12 V_HH were able to bind the endogenous IGFBP7 that expressed in PANC-1 cell, minimizing their migratory growth.

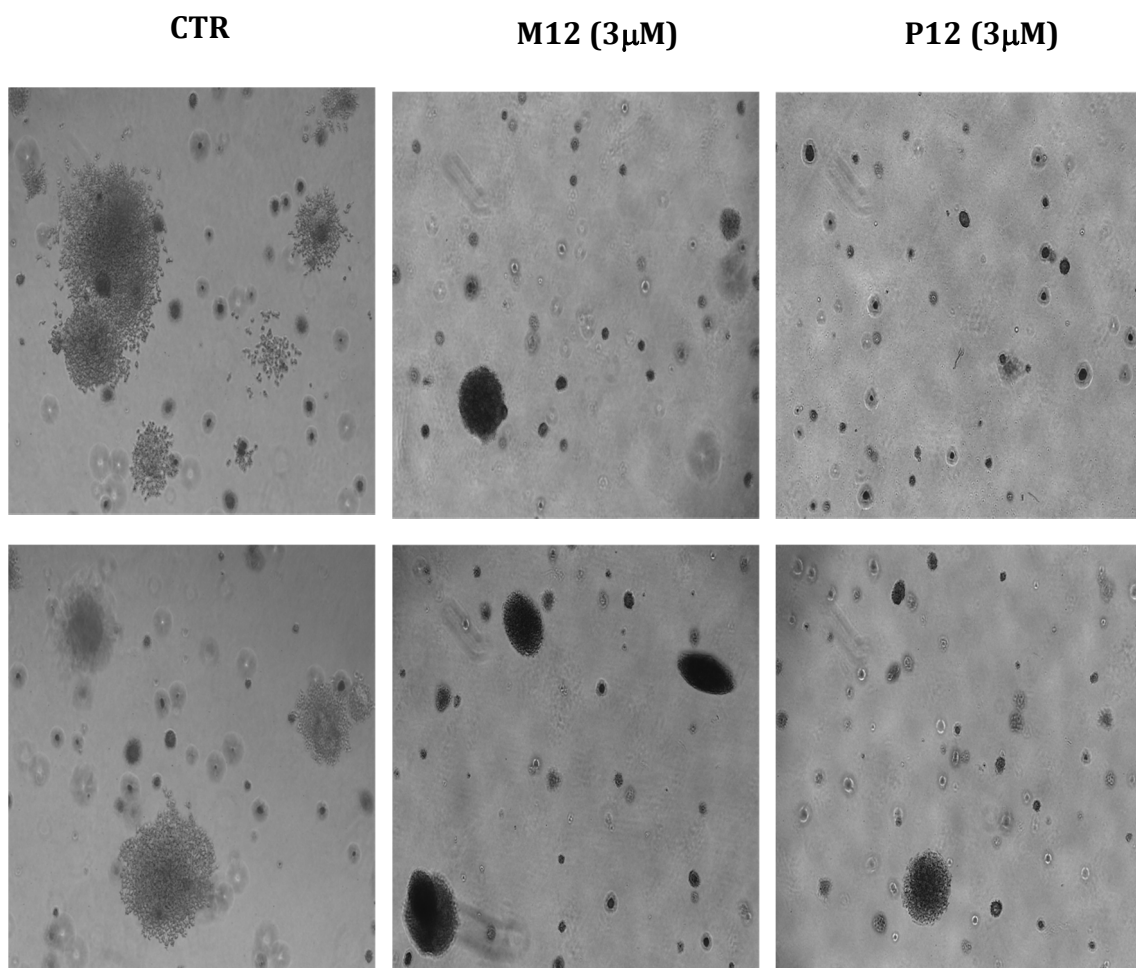


Figure A. The effect of anti-IGFBP7 monomeric (M12) and pentameric (P12) sdAbs on PANC-1 tumor colony formation in an anchorage-independent soft agar assay. Representative photomicrographs of PANC-1 tumor colony growth in the presence of control (DMEM), M12 (3 μ M) and P12 (3 μ M).

Quantification of PANC-1 cell growth in soft agar using the metabolic dye Alamar Blue

At the end of the soft agar experiment, cell viability was measured by adding 50 μ L of Alamar Blue to each well and measuring the fluorescence after a 180 min period of incubation. Alamar Blue is a redox indicator that yields a fluorescent signal in response to metabolic activity. As shown in the Figure B, M12 and P12 significantly reduced by ~58% and 72%, the viability of Panc-1 cells grown in soft-agar, respectively (Figure B).

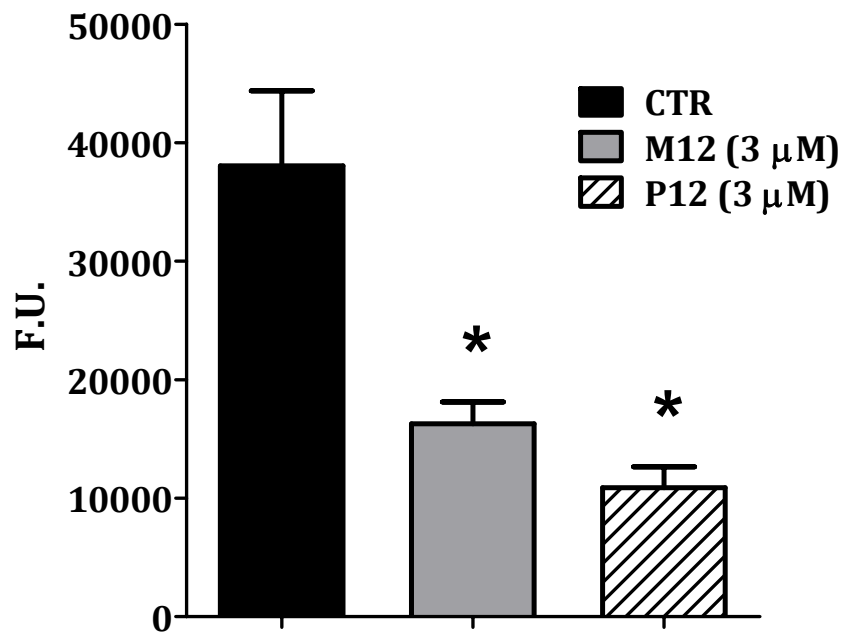


Figure B. Quantification analysis of PANC-1 cell growth in soft agar assay using the metabolic dye Alamar Blue. Graphical illustration of the metabolic activity of Panc-1 cells grown in presence of control (DMEM), M12 (3 μ M) and P12 (3 μ M). Data are presented as mean $\pm$ SD, n=3-4 per group. Asterisks (*) indicate a statistical difference between treatment versus control ($p < 0.05$).