Generation of recombinant antibodies against membrane proteins by phage display

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Syed Hussain Mir

Frankfurt am Main, den 19, März 2007.

(Unterschrift)

to my naani

As Paul Ehrlich wrote more than a century ago

that..

'we have already caught a distinct glimpse of the land which we hope, nay, which we expect, will yield rich treasures for biology and therapeutics'. We see a jungle of technologies, old and new, stimulating each other: in the immediate future, most of them start with immunized animals.

(Proc. R. Soc.Lon.1900. 66, 424-448)

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Zusammenfassung

Integrale Membranproteine sind eine für die lebende Zelle essentielle Klasse von biologischen Molekülen. Sie erfüllen eine Vielzahl von lebenswichtigen Funktionen und sind für die Integrität und Lebensfähigkeit der Zelle notwendig. 20-30% der Strukturgene des menschlichen Genoms kodieren Membranproteine (Wallin und Heijne 1998). Seit der Veröffentlichung der ersten Röntgenkristallstruktur eines Membranproteins, des bakteriellen photosynthetischen Reaktionszentrums durch Michel, Huber und Deisenhofer vor mehr als 20 Jahren (Deisenhofer *et al.,* 1985), sind weniger als 130 Strukturen von integralen Membranproteinen publiziert worden. Im Gegensatz dazu stehen tausende Röntgenstrukturen von löslichen Proteinen, die in diesem Zeitraum veröffentlicht wurden.

Die geringe Zahl an Strukturen von Membranproteinen mit einer hohen Auflösung spricht für die Vielzahl von Schwierigkeiten und Herausforderungen, die damit verbunden sind (Hunte und Michel 2003). Um Kristallstrukturen bei hoher Auflösung zu bestimmen, werden hochgeordnete dreidimensionale Kristalle benötigt. Da aufgereinigte Membranproteine jedoch in solubilisierter Form vorliegen und daher mit einer großen Detergensmizelle umgeben sind, ist die freie Oberfläche für Kristallkontakte relativ gering. Die Ko-Kristallisation von Membranproteinen mit Hilfe von Antikörper-Fragmenten ist mittlerweile zu einer viel versprechenden Technik geworden, um qualitativ hochwertige Kristalle von Membranproteinen zu erhalten. Eine wesentliche Hürde dieser Technik ist jedoch die geringe Verfügbarkeit von geeigneten Antikörperfragmenten. Bisher wurde dazu die „traditionelle“ Hybridomatechnik zur Herstellung monoklonaler Antikörper verwendet. Die Methode ist schwierig in der Handhabung und sehr zeitaufwendig. Weiterhin ist aufgrund der hohen Sequenzhomologie von wichtigen Membranproteinen bei Säugetieren die Immunantwort bei vielen Zielproteinen sehr niedrig. Der Einsatz der *Phage Display* Technik bietet eine Möglichkeit diese Schwierigkeiten zu bewältigen.

Die *Phage Display* Technik basiert auf der Integration einer fremden DNA-Sequenz, z.B. einer Bibliothek von Antikörperfragmenten, in das Genom filamentöser Phagen. Diese Integration resultiert in der Darstellung (*Display*) dieser fremden Polypeptide auf der Oberfläche des Phagen (Smith 1985). Jeder dieser rekombinanten Phagen hat also theoretisch ein anderes Antikörperfragment auf seiner Oberfläche und gleichzeitig das zugehörige Gen. Der Phänotyp ist daher direkt an den Genotyp gekoppelt. In einem *Biopanning* können die

Phagen über den auf der Oberfläche dagestellten Antikörper durch Wechselwirkung mit dem immobilisierten Antigen aus dem milliardenfachen Hintergrund der irrelevanten Phagen isoliert werden.

Die *Phage Display* Technik ist mittlerweile eine Standardtechnik für die Selektion von Antikörperfragmenten gegen lösliche Zielproteine. Der Nutzen bei Membranproteinen ist bis jetzt noch sehr begrenzt, es gibt nur wenige erfolgreiche Beispiele für die Erzeugung von Antikörperfragmenten gegen Membranproteine. Die Erzeugung von spezifischen Antikörperfragmenten gegen Membranproteine stellt daher immer noch eine Herausforderung dar.

Das Ziel dieser Doktorarbeit ist die Entwicklung eines Antikörper *Phage Display* Systems, mit dem schnell und zuverlässig hoch affine Antikörperfragmente gegen Membranproteine erzeugt werden können. Diese sollen zur Ko-Kristallisation der Zielproteine geeignet sein. Es wurden zwei Lösungsansätze verfolgt: die Verwendung von naïven Bibliotheken sowie eine Antikörperbibliothek, die aus einem vorher immunisierten Tier gewonnen wurde.

Mit den naiven Antikörperbibliotheken wurden Antikörperfragmente gegen ein lösliches und ein Membranprotein erzeugt. Durch *Biopanning* der ETH-2 scFv-Phagemid Bibliothek gegen Cytochrom *c* aus *Saccharomyces cerevisiae* wurde das Antikörperfragment 3RE2-10scFv isoliert. Dieses Antikörperfragment zeigte eine spezifische Bindung im ELISA- und im Western-Blot, jedoch keinerlei Bindung im Gel-Filtrations-Experiment. 3RE-10 scFv bindet Spezies-spezifisch ein Epitop des partiell denaturierten Proteins mit vermutlich moderater Affinität.

Für die Selektion von Antikörperfragmenten gegen den G-Protein gekoppelten $ß_2$ adrenergen Rezeptor ($ß_2$AR) wurde die naive scFv-Phagemid Bibliothek Tomlinson I+J verwendet. Die Immobilisierung von $ß_2$AR in seiner nativen Konformation wurde durch die Kopplung der Biotinylierungsdomäne des Rezeptors an eine *core*-Streptavidin-Maxi-Sorp™ Oberfläche erreicht. Mittels *Panning*-Experimenten mit dieser Bibliothek wurde das Antikörperfragment B2-scFv isoliert. Dieses Antikörperfragment bindet $ß_2$AR im ELISA- und im *Blue-Native-PAGE*-Experiment, aber die Bindung ist sehr schwach. Die Sequenzanalyse von B2-scFv zeigt eine kurze CDR-H3-Domäne mit der Aminosäuresequenz

$G_{95}A_{96}G_{97}S_{98}$FDY. Durch die kurze Domäne lässt sich wahrscheinlich auch die niedrige Affinität des Antikörperfragments erklären. Die spezifische Bindung des B2-scFv könnte in einem nächsten Schritt durch Affinitätsreifung verbessert werden (Chowdhury und Pastan 1999, Boder *et al.,* 2000, Rajpal *et al.,* 2005). Zur Erhöhung der Affinität ist eine Vergrößerung der CDR-H3 Region durch zufällig eingefügte Aminosäuren ratsam.

In den beiden oben beschriebenen Fällen war es nicht möglich Antikörperfragmente zu isolieren, die eine ausreichend hohe Affinität für die Verwendung in Ko-Kristallisationsexperimenten aufweisen. Die Selektion aus naiven Bibliotheken führte zur Anreicherung von unspezifischen Phagenpopulationen. Die meisten dieser nicht-spezifischen Phagen enthielten keinerlei oder nur bruchstückhafte Gene für scFvs. Zudem wird durch die Anwesenheit von Detergens, welches für die Stabilisierung von Membranproteinen benötigt wird, die Adsorption von Phagen an Polystyrol Oberflächen verstärkt.

Ein alternativer Lösungsansatz war daher erforderlich. Es wurde eine Antikörperbibliothek aus dem Knochenmark und der Milz zuvor immunisierter Hühner erzeugt. Die Tiere wurden mit einer Mixtur der gereinigten Membrantransportproteine STNha und KefC aus *Salmonella typhimurium* immunisiert. Die Bibliothek im scFv Format wurde in den Vektor pComb3x Phagemid integriert. In der scFv DNA Bibliothek wurden V_L und V_H-Region mit zwei verschiedenen Verbindungstück-Formaten verbunden. Die kurze Verbindungstückvariante (SL) beinhaltet die Sequenz GQS_2RS_2. Die lange Variante (LL) besteht aus den Aminosäuren $GQS_2R(S_2G_4)_2S$. Die verschieden langen Verbindungsstücke wurden eingeführt, um dimere und monomere Formen der scFv zu erhalten, da die Verbindungsstücklänge einen starken Einfluss auf den oligomeren Zustand der scFv hat (Tang *et al.,* 1996, Arndt *et al.,* 1998).

Für die SL-scFv Bibliothek wurde eine Größe von $9{,}1 \times 10^7$ und für die LL-scFv-Bibliothek eine Größe von $1{,}2 \times 10^8$ erreicht. In der Literatur werden für eine *Immune Antibody Library* Größen ab 1×10^7 als ausreichend angegeben. Für eine naiven Bibliothek wird mindestens eine Größe von 1×10^{10} benötigt (Andris-Widhopf *et al.,* 2001). Ausgehend von den erhaltenen Größen konnte sowohl die SL-scFv- als auch LL-scFv Bibliothek als sehr gut geeignet für die weiteren Experiment bezeichnet werden.

Das *Panning* gegen STNhaA auf MaxiSorp™ Oberflächen führte zur Selektion einer Reihe von hoch affinen Antikörperfragmenten. Keines der 24 analysierten Antikörperfragmente zeigte unspezifische Bindungen. Eine sehr interessante Beobachtung war, dass mit einer Ausnahme (B2-scFv), sämtliche anti-STNhaA Antikörpfragmente auch an NhaA aus *Escherichia coli* binden. Diese Ergebnisse erstaunen jedoch nicht, wenn man den hohen Homologiegrad zwischen den beiden Membranproteinen (> 93%) in Betracht zieht. Die Spezies-spezifischen Eigenschaften des Antikörperfragmentes B2-scFv könnten für die Entwicklung von diagnostischen oder analytischen Werkzeugen gegen *S. typhimurium* sehr nützlich sein. Die Analyse der CDR-Sequenzen der 24 ausgewählten Antikörperfragmente ermöglichte eine Aufteilung in vier Gruppen. Mitglieder der ersten drei Gruppen binden STNhaA konformationsspezifisch. Je ein Mitglied dieser Gruppe wurde im Gelfiltrations-Experiment untersucht und es konnte eine stabile Ko-Komplexbildung mit STNhaA nachgewiesen werden. Die Antikörperfragmente der Gruppe IV binden an ein lineares Epitop, welches aus sieben Aminosäuren besteht: „EKHGRSP“. Diese Sequenz befindet sich in einer Verbindungschleife der Helix VIII und IX auf der zytoplasmatischen Seite des Transportproteins. Dies ist das erste Mal, dass ein Antikörper gegen dieses Epitop isoliert werden konnte.

Die ausgewählten Antikörperfragmente wurden in *E. coli* produziert und erfolgreich aus Periplasmaextrakten gereinigt. Die Ausbeute lag im Bereich zwischen 2-12 mg/ Liter Kultur. Die Analyse durch Oberflächenplasmonresonanz ausgewählter Antikörperfragmente ergab Affinitäten im sub-nanomolaren Bereich. Zum Beispiel beträgt die K_D für das Antikörperfragment F6scFv 78×10^{-12}. F6scFv wurde erfolgreich zur Ko-Kristallisation des Membrantransportproteins STNhaA verwendet.

Die Selektion des $ß_2$AR spezifischen scFv in dieser Arbeit und die begrenzte Anzahl anderer Veröffentlichungen (Rubinstein *et al.,* 2003, Rothlisberg *et al.,* 2004) zeigen, dass die Möglichkeit besteht spezifische Antikörper gegen Membranproteine aus einer naiven Antikörper-Bibliothek zu erhalten. Die Erfolgsquote ist jedoch im Vergleich zur Anwendung der Bibliotheken bei löslichen Membranproteinen sehr viel geringer. Die Selektion der Antikörperbibliothek aus naiven Bibliotheken ist einfach. Im Gegensatz dazu erschweren schlechte Expressionsraten und niedrige Ausbeuten an gereinigten Protein die Anwendung im Bereich der Ko-Kristallisation. Zudem sind Antikörperfragmente mit niedrigen Affinitäten nicht geeignet, um stabile Ko-Komplexe für die Kristallisation zu bilden. Eine Möglichkeit

ist die Affinitätsreifung dieser Antikörperfragmente, um deren Affinität zum Zielprotein zu steigern.

Naive Bibliotheken von exzellenter Qualität und hoher Diversität wären für diese Strategie erforderlich. Für die akademische Forschung sind solche Bibliotheken schwer zu erhalten. Im Gegensatz dazu ist die Herstellung einer Bibliothek von immunisierten Tieren ein vielversprechender alternativer Lösungsansatz. In solchen Bibliotheken liegen spezifische Antikörperfragmente mit picomolaren bis sub-nanomolaren Affinitäten vor, so dass eine Selektion unter sehr stringenten Bedingungen erfolgen kann. Durch die Anreicherung von spezifischen und hoch affinen Antikörpern wird die erwähnte Detergensproblematik vernachlässigbar.

Diese Punkte konnten experimentell durch die Konstruktion der Anti-STNhaA/KefC scFv Bibliotheken aus immunisiertem Huhn unter Beweis gestellt werden. Die Selektion einer Reihe hoch affiner Antikörperfragmente zeigt, dass der gewählte Ansatz eine exzellenter Weg zur Gewinnung hoch spezifischer Antikörperfragmente ist. Die Eignung dieser Antikörperfragmente für die Ko-Kristallisation von Membranproteinen konnte mit dem Antikörperfragment F6scFv nachgewiesen werden.

Das Ziel die *Phage Display* Technik für die Verwendung von Membranproteinen zu optimieren, wurde erreicht. Es ist nun möglich mit einer Hochdurchsatztechnologie schnell und zuverlässig Zugang zu hoch affinen und spezifischen Antikörperfragmenten gegen Membranproteine zu erhalten. Nach dem heutigen Stand der Literatur ist es das erste Mal, dass hoch affine Antikörperfragmente gegen vollständige Membranproteine aus einem *Phage Display* Ansatz gewonnen wurden, bei dem die Antikörper-Bibliothek aus einem immunisierten Huhn gewonnen wurde. Diese Strategie kann ohne große Modifikationen für jedes beliebige Membranprotein übernommen werden.

Summary

Antibody fragment mediated co-crystallization technique has been proven a promising approach to obtain high quality crystals of membrane proteins. To date, a number of important high-resolution crystal structures of membrane proteins have been obtained by this method. The bottleneck of the technique is the low availability of antibody fragments suitable for co-crystallization. The traditional method of generating monoclonal antibodies by hybridoma technique is a lengthy and tedious process, also often limited by the lack of immune response against important human membrane protein targets in mice as they are fairly conserved across the mammalian kingdom. The technique of antibody phage display offers a promising alternative approach. The technique is well established for the selection of monoclonal antibody fragments against soluble protein targets, but its uses against membrane proteins are limited, indicating that technique against membrane proteins is still challenging.

The aim of this Ph.D thesis project was to establish an antibody phage display system suitable for fast access to antibody fragments suitable for co-crystallization and structural studies of membrane protein targets. In order to achieve this aim, two approaches were tested: i. the first involved the use of naïve antibody library, ii. the second involved the use of an immune antibody library.

In the first approach, use of the naïve antibody libraries was explored to select *binders* against a soluble and a membrane protein target. Biopanning of the ETH-2 scFv-phagemid library against yeast cytochrome *c* resulted in the isolation of a target-specific *binder* 3RE2-10 scFv. This scFv shows specific binding in ELISA as well as in Western-blot experiments of SDS-PAGE separated target protein, but not in gel-filtration binding assays. These observations indicate that 3RE2-10 is likely a moderate or low affinity *binder* that recognizes a semi-denatured epitope in a yeast-specific manner. For selection for a membrane protein target, a new scFv-phagemid library Tomlinson I+J was used against the G-protein coupled receptor β_2 adrenergic receptor (β_2AR). Purified β_2AR was presented in its native conformation during the library panning experiments to select antibodies for a native epitope. The immobilization of β_2AR in its active conformation was achieved by anchoring it to custom-made core streptavidin-MaxiSorp^{TM} surface via its biotinylation domain. The ligand binding assays performed on this immobilized β_2AR confirmed its active state. The panning of Tomlinson I + J scFv library against β_2AR resulted in isolation of the B2-scFv, a target-specific *binder*. B2-scFv was shown to bind β_2AR in ELISA as well as in Blue Native PAGE

gel-shift assays, but the binding was very low. These observations led us to the conclusion that B2-scFv is a specific but moderate or weak affinity *binder*. It binds to β_2AR in specific manner, and is a potential candidate for affinity maturation procedures.

In both of these cases, the antibody fragments isolated failed to form a stable co-complex with their targets in solution, a prerequisite for the suitability of these antibodies for co-crystallization experiments. One of the possible reasons hampering successful antibody selection from these naïve libraries against membrane-protein targets was found to be the high enrichment of nonspecific phage population during panning. Most often these non-specific binding phages carried a partial or no scFv insert. It was also observed that this unspecific adsorption of phages to the polystyrene surface is increased in the presence of detergents, which are required to maintain the active conformation of the membrane protein.

Due to the limitations of the naive libraries at hand, an alternative strategy was explored. An immune antibody library was generated from the spleen and bone marrow of chicken. Chickens were immunized with a mixture of two membrane proteins, STNhaA and KefC from *Salmonella typhimurium*. The library was constructed in scFv format in a pComb3x phagemid vector system. The panning of the library against STNhaA immobilized on MaxiSorp^{TM} surface resulted in the selection of a number of high quality *binders*. These monoclonal *binders* were analyzed for cross-reactivity i.e. binding to unrelated or closely related proteins. None of the 24 analyzed scFvs showed binding to any unrelated protein such as BSA or β_2AR. But interestingly all but one, are binding to NhaA from *E. coli*. These results were not unexpected as the STNhaA and NhaA from *E. coli* share more than 93% similarity. The only exception is B2-scFv that showed *Salmonella*-specific binding. The potential use of this scFv in *Salmonella* based pathogenesis and diagnosis will be explored.

The CDR-sequence analysis of the 24 randomly chosen anti-STNhaA scFvs, revealed the enrichment of four groups of *binders*, possibly binding on four different epitopes of STNhaA. The members of first three groups were all found to bind STNhaA in its native conformation. Representative members from the conformation-specific groups (group I-III) were tested by gel-filtration binding assays. Most of these STNhaA-specific scFvs are showing stable co-complex formation with the target in solution. On the other hand, all the members of group-IV turned out to be linear-epitope *binders*, as indicated by their ability to bind SDS-PAGE separated target protein in Western-blot experiments. The linear epitope of STNhaA to which these antibodies bind, was mapped and found to be the seven residues

"EKHGRSP" present on the cytoplasmic loop region that connects helix VIII and IX of NhaA. This is the first time an antibody against this epitope has been isolated.

The selected scFvs could be successfully produced and purified from *E. coli* periplasmic extracts. Purified yields of 2-12mg/L culture were obtained with individual scFvs. The surface plasmon resonance analysis of representative scFvs revealed the high-affinity binding nature of these antibodies. The average affinities determined for the representative scFvs are in sub-nanomolar range, e.g. the dissociation constant (K_D) values of 0.7 $x10^{-10}$ M^{-1} and 2.6 $x10^{-10}$ M^{-1} were obtained for F6-scFv and H4-scFv respectively. F6-scFv has already been shown to produce co-complex crystals with STNhaA suitable for X-ray diffraction studies.

Here, we have shown that the immunization of chicken combined with the phage-display antibody technique offers a very good perspective to obtain high-affinity *binders* against membrane proteins. Chicken as hosts for immunization might be advantageous to overcome the problem of low immunogenicity usually encountered in mice and rabbit against human membrane protein targets. The proof of principle, that these phage-display derived antibody fragments are suitable for co-crystallization of membrane proteins was also shown. To the best of my knowledge, it is the first time that the chicken immunization and phage display approach has been used to generate high-affinity antibodies against a full-length membrane protein suitable for its co-crystallization. In principle, the strategy can be applied to other membrane protein targets to obtain fast access to high affinity *binders*.

1. Introduction

1.1 Phage display

In 1915, Frederick W. Twort mentioned the possibility of bacterial viruses (Twort 1915), but it was Felix d Herelle in 1917, who demonstrated the role of viruses in bacterial lyses and gave the term "*bacteriophage*", the eaters of bacteria (Stent 2000). Bacteriophages have proven to be valuable tools in biological and biochemical research. The famous experimental evidence of DNA being the genetic material was also shown with the aid of bacteriophages (Hershey and Chase 1952). The treatment of bacterial infections with bacteriophages "phage therapy" commonly used in former USSR until 1940s is being re-considered presently due to the growing phenomenon of multi-drug resistance (MDR) in bacteria (Barrow and Soothill 1997, Stone 2002, Schoolinik *et al.,* 2004). Phages have led us to the development of various vectors for cloning and expression of foreign genes in heterologous systems. Commonly used by researchers is the group of filamentous temperate bacteriophages, like M13 and fd phages. This group of phages does not kill their host, because they are of non-lytic nature. They carry single stranded DNA as genetic material and are quite tolerant to foreign DNA insertions in their genome. Long before the advent of phage display technology, these filamentous phages were used in molecular biology laboratories for production of large quantities of single stranded DNA needed for sequencing (Messing 1983, Vieira and Messing 1987, Nicola *et al.,* 2006).

1.2 M13 filamentous phage *"biology and morphology"*

The most widely used phages for display of peptides and proteins belong to the group of filamentous bacteriophage collectively known as Ff phages, named so, as they all require bacterial F-pilus for infection. The group includes the closely related phages such as f1, fd and M13 filamentous phages, which share a more than 98% identical genome (Van Wezebbeek *et al.,* 1980). The Ff bacteriophages are members of the *Inoviridae* family. These long helical phages are about 7~10nm in diameter, while the length varies with the size of the ssDNA carried inside the phage particle. A wild type filamentous phage encapsulating a 6.4kb genome shapes up as a ~100nm long particle (Model and Russel 1988, Webster 1996).

The genome of the Ff phages is a 6.4 kb single-stranded circular DNA (ssDNA) that consists of a coding region and a non-coding *intergenic* (IG) region which contains the necessary sequences required for the replication and encapsulation of the genome (Beck and Zink 1981, Hill and Peterson 1982). The essential features of the IG region are the presence of the *origin of replication* (ori) and the *packaging signal* (PS). The PS is the 78 nucleotide long hairpin loop that is needed for packaging and orientation of the ssDNA inside the phage body. It is required for efficient assembly of phage particles (Russel and Model 1989, Marvin 1998).

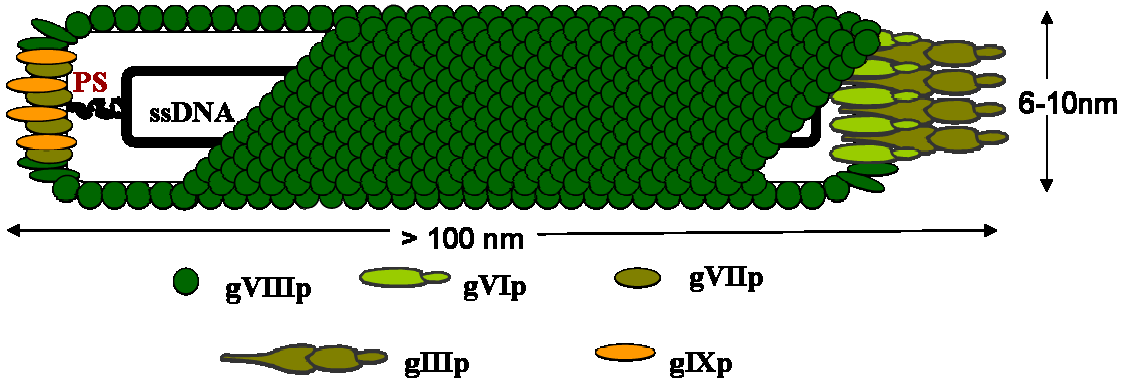


Figure 1: Schematic cartoon representation of structural features of a filamentous phage. M13 belongs to the group of filamentous bacteriophages that carry a copy of circular ssDNA as their genetic material. The phage body encapsulating its genetic material is a long barrel-like structure formed by major coat protein (gVIIIp). One end of the barrel is formed by few copies of gVIIp and gIXp proteins, while the other terminus is formed by gVIp and gIIIp, the minor coat proteins. The phage particle is about 6-10nm in diameter, and the length is around 100nm, but varies with length of the ssDNA. The figure is a modified adaptation from Winter 1994 and Marvin 1998.

The coding region of the genome comprises eleven genes (table 1). Three of them (gII, gV and gX) code for the proteins that are involved in the replication of the phage genome. The genes gII and gX code for proteins that are required for the generation of ssDNA. The product of gV is a ssDNA binding protein that maintains and protects the phage ssDNA in the host cytoplasm before being packaged into the phage envelop. Three other genes (gI, gIV and gXI) code for the proteins that are required for the export and assembly of the phage particle. They together form the phage assembly sites on the inner membrane of the host *Escherichia coli* (*E. coli*) (Feng *et al.,* 1997). Twelve to fourteen units of the gIVp protein form the trans-membrane cylindrical pore through which assembled phage particles are extruded out of the host cell (Linderoth *et al.,* 1997). Five of the genes (gIII, gVI, gVII, gVIII, and gIX) code for the capsid proteins that make up the filamentous phage body and are physically associated with the mature phage particle (Simons *et al.,* 1981, Marvin 1998).

Table 1: Ff type filamentous phage genome

Gene	Encoded protein-function	No. of (aa)	Position	Copies/phage
II	gIIp - DNA replication (nickase)	410	Host cytoplasm	
X	gXp - DNA replication	111	Host cytoplasm	
V	gVp - ssDNA binding protein	87	Host cytoplasm	
VIII	gVIIIp - major capsid protein	50	Phage surface	~ 2700
III	gIIIp - minor capsid protein	406	Phage terminus (head)	3 ~ 5
VI	gVIp - minor capsid protein	112	Phage terminus (head)	3 ~ 5
VII	gVIIp - minor capsid protein	33	Phage terminus (tail)	3 ~ 5
IX	gIXp - minor capsid protein	32	Phage terminus (tail)	3 ~ 5
I	gIp - assembly	348	Host inner membrane	
IV	gIVp - assembly (secretin)	405	Host outer membrane	
XI	gXIp - assembly	108	Host inner membrane	

The main tube-like body of the phage encasing the ssDNA genome is formed almost exclusively by the gVIIIp, *the major coat protein*, which is a 50 amino acid long polypeptide present in 2500~3000 copies per phage particle (Webster 2001). The rest of four capsid proteins each present at 4~5 copies per particle are identified as *the minor coat proteins*. These minor coat proteins are present exclusively at either of the two termini of the mature phage particle. Two of these minor coat proteins, i.e. gVIIp and gIXp form the one end towards which the PS motif of the ssDNA is oriented inside the phage particle (Fig. 1). This end is first to form during the phage synthesis and is first to protrude out of the host *E. coli* during phage secretion. The other end of the phage particle is formed by the gVIp and gIIIp minor coat proteins. This end of the particle is involved in the infection of host cells. The very ability of the filamentous phages to infect the F' positive bacteria is provided by the presence of the gIIIp minor coat protein. The gIIIp is a 406 residue polypeptide consisting of three discrete domains, the N-terminal '*N1*' and '*N2*', and the C-terminal '*CT*' domain (Bennett and Rakonjac 2006). The CT domain is embedded in the phage body and is essential for the integrity of the phage particle. The N1 and N2 are exposed on the surface. Both the N1 and N2 domains are essential for infection (Lubkowski *et al.,* 1998 and 1999, Karlsson *et al.,* 2003).

1.3 Life cycle of the filamentous phage *"from infection to assembly"*

The life cycle of the phage can be divided into three phases that start with its infection of the host bacterial cell, followed by replication of its ssDNA genome, assembly and release of new phage particles. The host cell starts producing the new phage particles already 10 minutes after infection (at 37^{o}C). A burst of about 1000 particles per infected host cell are produced in the first hour, and then 100 – 200 particles are secreted per generation. The filamentous phage does not kill i.e. lyse their host, but bacteria harboring phages inside them have a 50% reduced growth rate. Under optimal conditions, the infected bacteria can grow and divide while producing phage particles persistently.

1.3.1 Infection

Filamentous phages belonging to the Ff type e.g. M13, infect a wide variety of gram-negative bacteria that display F-pili on their surface. These bacteria carry the F-conjugative plasmid, which codes for the proteins that assemble the F-pili. The infection starts with the specific adsorption of a phage particle on the surface of the host bacteria. This adsorption is facilitated by binding of the bacterial F-pilus to the N2 domain of the phage capsid protein gIIIp (Deng *et al.,* 1999). The tightly packed domains of gIIIp are connected by glycine rich flexible linkers. Binding of the F-pilus to the N2-domain causes it to dislodge the tightly bound N1 domain that is now free to interact with the host TolA trans-membrane protein (Riechmann and Holliger 1997, Lubkowski *et al.,* 1999). The TolA acts as the co-receptor for the phage binding and, along with TolQ and TolR, is indispensable for phage infection (Click and Webster 1998). The binding of the N1 domain to TolA is followed by the regression of the F-pilus and disintegration of the coat proteins at the tip of the phage, which results in the disassembly of the phage particle and penetration of phage ssDNA genome into the host cytoplasm. The mechanism of this penetration is not fully understood.

1.3.2 Replication of the phage genome

Once the single-strand (+) circular DNA arrives in the cytoplasm, it is immediately acted upon by the host replication machinery and converted into a double-stranded (+/−) super-coiled molecule known as *replicative form* (RF) DNA. The (−) strand of the RF serves as the template for the expression of early genes. One of these genes (gIIp) code for a site specific nickase-ligase enzyme, which creates a nick in the (+) strand of RF. The resulting

free 3'-OH end is again acted upon by the bacterial enzymes to synthesize new (+) strand via a "*rolling-circle*" mode of replication. The displaced old (+) strand is re-circularized by the same phage enzyme gIIp, and is again converted into double stranded RF form. This process produces a new pool of progeny RF DNA molecules that can direct the synthesis of phage proteins, and can generate new (+) strand ssDNA as well (Webster 2001). One of these newly synthesized phage proteins is gVp, which is a single-strand DNA binding protein. The amount of gVp keeps on accumulating in the host cytoplasm. When its concentration reaches a critical level, it starts to bind the newly synthesized (+) ssDNA in a cooperative manner. The binding of gVp to ssDNA prevents its conversion to RF, thus maintaining the population of ssDNA in the host cytoplasm. This pool of ssDNA molecules each bound by hundreds of gVp dimers are the substrate for phage assembly.

1.3.3 Assembly of phage particles

The phage assembly is a process that involves the incorporation of newly synthesized ssDNA into the cylindrical tube of capsid proteins followed by their secretion out of the host cell. The assembly is a membrane associated affair that occurs in the cytoplasmic membrane at specific sites, where the inner and outer membranes are in close contact (Lopez and Webster 1985). These sites are thought to be composed of three phage coded proteins, where 12~14 units of gIVp form the cylindrical pore in the outer-membrane that interacts with multiple units of gXIp and gIp present in the cytoplasmic membrane (Linderoth *et al.,* 1997). The assembly of phage particles at these sites is initiated by gVIIp and gIXp together with gIp, all present in the inner cytoplasmic membrane. It is suggested by genetic evidence that the gIp interacts with 78 nucleotide hairpin *packaging signal* (PS) via its cytoplasmic N-terminal domain. This domain of gIp contains a conserved nucleotide binding motif (Feng *et al.,* 1999). It is hypothesized that gIp directs the orientation and positioning of capsid proteins (gVIIp, gIXp and gVIIIp) around the PS, thus forming the tip of the phage particle. Once the tip is formed, the particle is elongated by a successive replacement of gVp dimers around the ssDNA with gVIIIp, the major coat protein. The gVIIIp units are oriented in such a manner that the positively charged C-termini interact with the sugar phosphate backbone of the phage DNA. The process of elongation continues along the length of ssDNA. As the elongation continues, the phage particle protrudes out via the gIVp pore (Kazmierczak *et al.,* 1994). Once the complete ssDNA is encapsulated by gVIIIp, the elongation process is terminated by incorporation of gVIp and gIIIp. The insertion of these proteins results in the completion and release of the phage particle (Russel and Kazmierczak 1993, Rakonjac *et al.,* 1999).

1.4 Phage display "*the presentation of foreign polypeptides on phage surface*"

In the mid-1980s, Prof. George Smith at University of Missouri demonstrated that insertion of a foreign DNA segment into the f1 filamentous phage genome upstream of a coat-protein gene does result in the display of the foreign polypeptide on the surface of the phage in fusion with the coat protein. The phage displaying the foreign peptide on its surface can be easily isolated by simple means of affinity enrichment from millions of its counterparts not bearing the fusion peptide (Smith 1985). The technique currently known as "*phage display*" has been widely used since. The strength of the technique lies in the fact that it involves the direct physical linkage between the phenotype (*the polypeptide*) with its genotype (*the DNA*), as the phages bearing the foreign polypeptide on their surface carry the DNA encoding it in their genome.

Initially, the technique was limited to display large libraries of small peptides (Smith 1985, Parmley and Smith 1988, Scott and Smith 1990). By the early 1990s, the developments in this field led to the successful display of larger proteins such as single chain antibody fragments (scFv) (McCafferty *et al.,* 1990), Fab antibody fragments (Barbas *et al.,* 1991a, Hoogenboom *et al.,* 1991), and human growth hormone (Lowman *et al.,* 1991). The scope of the technique is no more limited to the selection of peptides and proteins. Instead, it has acquired its value in a wide variety of research activities that require the study of protein-protein, protein-peptide, and even DNA-protein interactions. Phage display has been used for the evolution and selection of tight-binding enzyme variants (Pederson *et al.,* 1998), and for the generation and selection of DNA binding proteins, such as isolation of zinc-finger protein domains that can down or up-regulate certain genes by binding to their regulatory DNA sequences (Segal *et al.,* 1999). Phage-displayed random peptide libraries have been successfully used to map the antibody epitopes, to create novel peptides with diagnostic and therapeutic-drug value (Watt 2006), to map cell surface receptor proteomes (Pasqualini and Ruoslahti 1996, Valadon *et al.,* 2006), and to develop biologically active peptides to regulate target receptors (Koivunen *et al.,* 1999, Eichler 2005). "Gene-fragment" cDNA libraries have been successfully displayed on the phage surface to isolate binding domains for various cell receptors and antibodies (Jacobsson and Frykberg 1996, Sergeeva *et al.,* 2006). The utility of phage display in the study of infectious diseases was earlier shown with the generation of a *Plasmodium falciparum* whole genome fragment library and its panning against erythrocyte membrane proteins (Lauterbach *et al.,* 2003), and has been recently reviewed (Mullen *et al.,* 2006). Among the novel uses of phage display is the incorporation of specific, nucleating

peptides on the filamentous phage surface that provide a viable template for the directed synthesis of novel semiconducting magnetic materials (Whaley *et al.,* 2000, Mao *et al.,* 2004).

Withstanding all the above versatile uses and benefits, the most fruitful applications of the phage display system has been and still is the generation of antibody libraries and the selection of monoclonal antibodies from them (Brekke and Sandlie 2003). It permits to bypass the tedious hybridoma technology (Burton and Barbas 1994, Griffiths *et al.,* 1994) and in other cases the immunization (Marks *et al.,* 1991, Lerner *et al.,* 1992). Phage display has also been often also used to improve the affinity of existing antibodies (Chowdhury and Pastan 1999). For more detailed reports of diverse applications of phage display technology see the following reviews (Bradbury and Marks 2004, Sergeeva *et al.,* 2006).

1.4.1 Phage display in comparison with alternative display systems

A number of display systems have evolved over the years with a potential to complement the phage display system. All these display techniques maintain the fundamental principle of having the direct physical linkage between the phenotype and the genotype encoding it. They also share characteristic features such as the scope of generating molecular display libraries of vast diversity and enormous size, and the ease of selection of a candidate phenotype from them with desired properties. These alternative display techniques include *cell-based* yeast display (Border and Wittrup 1997, Feldhaus and Siegel 2004) or bacterial display (Chen *et al.,* 2001, Harvey *et al.,* 2004) and *in-vitro* based ribosome (Mattheakis *et al.,* 1994) or RNA display systems (Roberts and Szostak 1997).

Phage display in comparison with these alternative display techniques has many advantages and some limitations also. The main advantages accounting for its widespread use are its robustness and simplicity, and the ease with which the libraries can be generated and selected for target-specific *binders*. The phage particles are stable over a wide range of environmental conditions. One of the limitations of the technique is the requirement for bacterial transformation, the bottleneck step that restricts the size of the library. For the generation of combinatorial display libraries of large diversity, naked DNA needs to be transformed into host *E. coli* cells. In spite of significant developments in prokaryotic transformation protocols such as improved electroproration method, the size of a phage display library remains restricted often to 10^{10} to 10^{11} members. *In-vitro* techniques such as *Ribosome* or *mRNA display* do not require any transformation step, hence, they are advantageous when a library of larger diversity is required (Hanes *et al.,* 2000, Keefe and Szostak 2001, Lipovsek and Pluckthun 2004).

1.5 Phage display vectors "*phage or phagemid*"

Two most commonly used systems for phage display are based on the filamentous M13 phage genome. The first category employs "*phage based vectors*" and the second uses its counterpart "*phagemid based vectors*" (O'Connell *et al.,* 2002). Each of these systems utilizes one of the surface coat proteins (gIIIp of gVIIIp) to display the foreign polypeptide on the surface of the phage particle. Both of these systems have been successfully used to generate molecular protein or peptide display libraries of considerable size and diversity. A detailed classification of all available phage display vectors is provided by Smith and his co-workers (Simith and Petrenko 1997).

The phage based vectors (Smith 1985) contain most of the genetic information of a wild-type phage genome, such as all genes coding for the regulatory and structural coat proteins. Such systems are self sufficient for propagation and assembly of phage particles, and do not require any assistance from the helper phage. Because of the limitations on the size of the foreign DNA insertion and levels of expression, these vector systems are more suitable for the display of small polypeptides. Thus phage vectors have been successfully used for generation of peptide display libraries (Cwirla *et al.,* 1990, Scott and Smith 1990, Kay *et al.,* 1993).

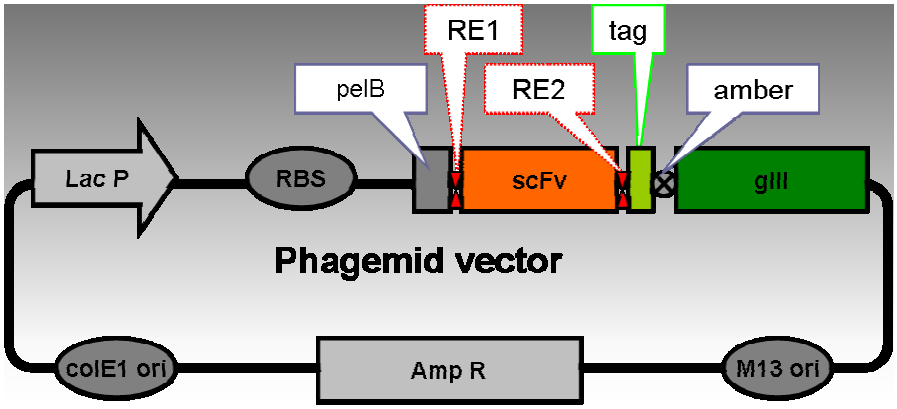


Figure 2: Schematic representation of a phagemid vector. A typical phagemid vector carries the following features: 1. the phage origin of replication (*M13 ori*) 2. the DNA sequence PS (*packaging signal*) 3. the gene coding for the coat protein for the fusion and surface display of the foreign polypeptide, such as, the minor coat protein (gIIIp). Phagemid vectors also carry bacterial origin of replication (*colE1 ori*) that allows it to be carried and maintained like a plasmid in the host cell, a cloning and expression cassette consisting of a promoter region (usually *LacP*), a ribosome binding sequence (*RBS*), a leader sequence (*pelB*) that directs the translated polypeptide to the periplasmic space.

The phagemid-based vector system combines the features of a plasmid and that of a phage together (Bass *et al.,* 1990, Breitling *et al.,* 1991, Winter *et al.,* 1994). In the host bacterium, it is maintained as double-stranded DNA plasmid that can be isolated and produced in large quantities. On the other hand, it can be produced as single-stranded DNA and packaged into phage particles. In a phagemid, most of the genome that encodes for regulatory and structural proteins is deleted to facilitate the insertion of foreign DNA segments of considerably larger size, whereas those sequences that are required for propagation and formation of phage particle are retained. The phagemid genome consists of a phage origin of replication (*M13 ori*) for propagation of phage particles, and the DNA sequence PS, *the packaging signal*. It also carries one of the genes (usually gIIIp or gVIIIp) coding for the phage coat protein for fusion display of the foreign polypeptide.

Since a phagemid vector is deficient in all other structural genes of the wild-type phage, it requires the assistance of a helper phage to form a phage particle. The helper phage, usually a wild-type M13 phage with defective origin of replication and defective PS (Russel and Model 1989) such as M13KO7 or VCSM13, supplies all the required structural and coat proteins for the assembly and formation of a mature and infective phage particle (Russel and Model 1986). These mature phage particles enclose the phagemid genome along with the inserted foreign DNA segment inside them, and display the foreign polypeptide on their surface. One of the important features of current generation phagemid vectors is the presence of an *amber* (stop) codon in-between the insert and geneIII sequence. This allows the expression of soluble (not fused to gIIIp) form of the foreign gene (i.e. scFv), when grown in a non-suppressor strain of *E. coli* such as HB2151, and allows the expression of fusion protein when grown in suppressor strain of *E. coli* such as XL1-blue. The common features of a general phagemid vector are shown (Fig. 2).

The smaller size and high transformation efficiency of phagemids have made them the vectors of choice for the generation of large sized phage display libraries. As compared to phage vectors they have less tendency to delete the non-essential DNA, hence are more tolerant to large size insertions. DNA fragments up to 1.4 kbp (i.e. Fab) are quite soundly maintained and expressed in phagemid vectors (Barbas and Lerner 1991b).

1.6 Types of display on the surface of filamentous phages

For the display on the phage surface, the gene segments encoding the foreign polypeptide can be fused to any gene that codes for a coat protein. Among the capsid proteins that are used for the surface display of foreign proteins, gIIIp has been the most preferable target, followed by gVIIIp (Bass *et al.,* 1990, Iannolo *et al.,* 1995). Although not widely used, other minor coat proteins gVIp, gVIIp, and gIXp have also been exploited for the development of phage display systems (Gao *et al.,* 1999, Gao *et al.,* 2002). The fusion display with either the gVIIp or gIXp minor coat proteins does have an attractive advantage, since both these proteins are located at the end of the phage particle that is not involved in bacterial infection (Webster 1996, Kwasnikowski *et al.,* 2005). Such fusions will not hinder the infectivity of the phage particles.

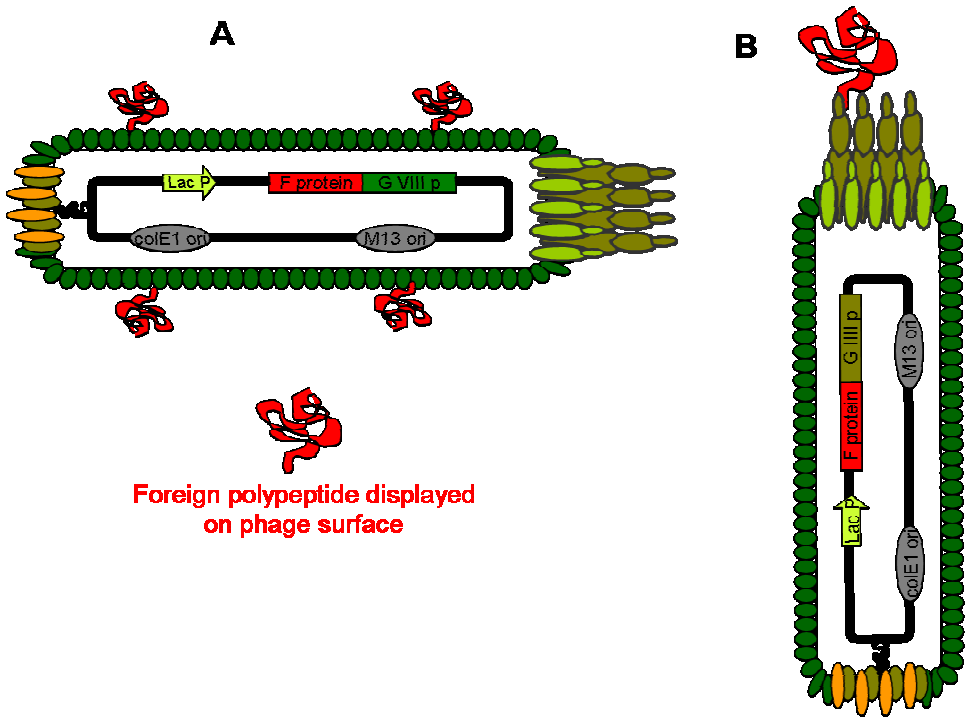


Figure 3: Display of foreign polypeptide on the surface of filamentous phages. A. Fusion with gVIIIp, *the major coat protein* **B.** Fusion with gIIIp, *the minor coat protein*. The figures are adapted from Winter 1994 and Marvin 1998.

1.6.1 Display in fusion with gIIIp

The fusion with the minor coat protein gIIIp has been the most frequently used format for phage display. Such a fusion allows the stable insertion and display of small peptides as well as large proteins in both phages (Parmley and Smith 1988, McCafferty *et al.,* 1990, Scott and Smith 1990) as well as in phagemid based vector systems (Bass *et al.,* 1990, Hoogenboom *et al.,* 1991). Large diversity molecular libraries of polypeptides and antibodies

have been made using gIIIp fusion display (Smith 1993, Winter *et al.,* 1994, Hoogenboom 1997). In a phagemid system, wild-type gIIIp supplied by the helper phage competes with the fusion-gIIIp coded by the phage genome during the assembly of the phage particles. The majority of the progeny phage population carries no or single copy of fusion protein. Therefore, in a gIIIp-fusion display the prevalent numbers of phage particles exhibit a monovalent display of fusion protein (Fig. 3), that in-effect promotes the selection for high affinity *binders*. Most of the current generation phage display vector systems (i.e. phagemid based pComb3x, pHEN2, pDN322, pCANTAB, and phage based M13KE are all based upon gIIIp fusion display.

1.6.2 Display in fusion with gVIIIp

The second most frequently used target for the surface display has been the fusion with major coat protein gVIIIp. Since the phage coat consists of thousands of copies of gVIIIp, the fusion of foreign gene segment with it results in a multivalent display. The gVIIIp fusion has been most successful for the display of small polypeptides (< 10 mer) (Iannolo *et al.,* 1995, Malik *et al.,* 1996). Fusions of small peptides with gVIIIp have been used to create landscape phage libraries that display the fusion peptide all over the phage body (Petrenko *et al.,* 1996, Petrenko and Smith 2000). In such libraries, where display occurs nearly on every copy of gVIIIp, the size of the displayed peptide is usually limited to 6-8 mer residues (Malik *et al.,* 1996). Fusion with larger peptides (>12mer) or bigger proteins is less tolerated and also less efficiently packaged into the phage. However, larger proteins such as Fab fragments (> 50 kDa) have been displayed in fusion with gVIIIp by some workers (Barbas and Lerner 1991b, Kang *et al.,* 1991). In all such cases phages are thought to be bearing only few (<20) copies of fusion protein on their surface. And such big inserts are more prone to deletion as compared to gIIIp-fusion system. These phages have also reduced production levels partially because of the hindrance occurring due to limited pore size of gIVp channel during the phage secretion (Marciano *et al.,* 1999).

1.7 Phage display of antibodies

Antibodies are central molecules of the vertebrate immune system with two unique features: i. *high precision target location*, i.e. the ability to bind their target antigen with a high degree of specificity and required affinity, ii. *target annihilation*, i.e. the ability to mediate elimination of the antigen bearing target, either by complement lysis or by antibody dependent cell-mediated cytolysis (ADCC). These two extraordinary features lie in two

separate domains of an antibody molecule. The former property is by virtue of the variable Fab domain, and the later is due to the constant Fc (*fragment crystalline*) domain. For membrane protein structural biology, we are hereby interested only in their ability to bind the target antigen molecule with high specificity and affinity.

1.7.1 Antibody structure

Antibodies belong to a family of glycosylated proteins known as immunoglobulins. Each monomeric immunoglobulin molecule consists of four polypeptides (*two identical heavy and two identical light chains*) arranged in "Y" shape, tied together by intra and inter-chain disulphide bonds. Based on the amino acid sequence, both the light and heavy chain polypeptides comprise of discrete constant and variable domains. Diversity of the antibody population is by virtue of their variable domains (Braden *et al.,* 1998, Richard *et al.,* 2000).

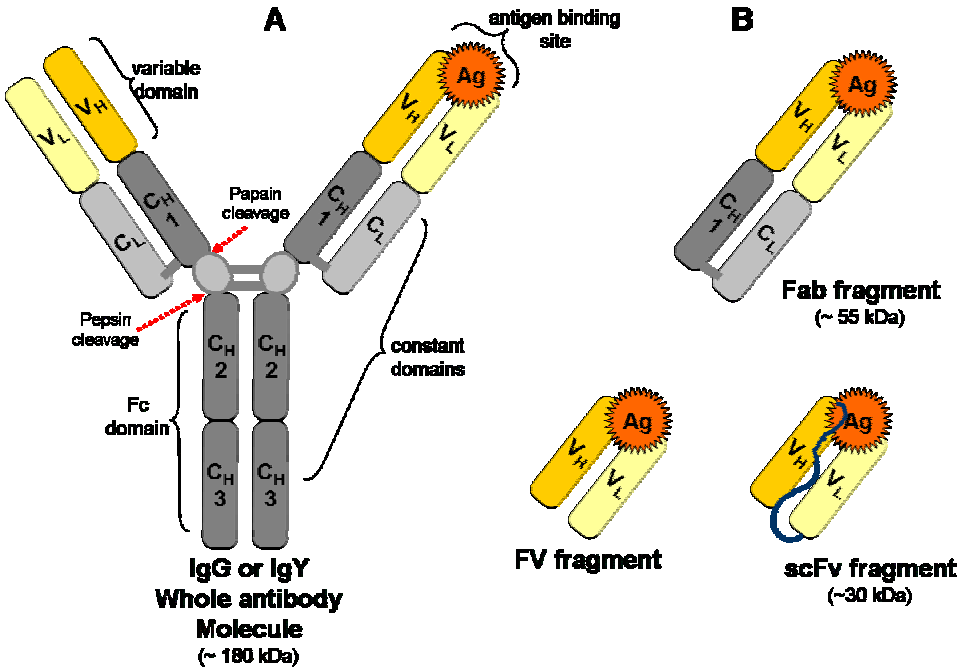


Figure 4: Schematic representation of structural features of IgG/IgY antibodies. A. Whole antibody molecule. **B.** Recombinant antibody fragments: Fv (*fragment variable*), Fab (*fragment antigen binding*) and scFv (*single chain fragment variable*).

The most common immunoglobulin class in human and mouse is IgG, and its counterpart in chicken is known as IgY. The light chain of IgG consists of a single constant (C_L) and a variable (V_L) domain, while the heavy chain contains three constant domains (C_H1, C_H2, *and* C_H3) and a variable domain (V_H). The light chain C_L domain is connected to C_H1 domain of heavy chains by an inter-chain disulphide bond. The two heavy chains are held together by the *hinge* region inter-chain disulphide bonds (Fig. 4A). The IgG molecule is

bivalent, i.e. contains two antigen binding sites, each formed by a V_H and a V_L domain (Burton 1987).

Each variable domain is further characterized into four less variable "framework" (FR1, FR2, FR3, FR4) regions separated by three hypervariable segments known as "complementarity determining regions" (CDR1, CDR2, CDR3). The antigen binding site is formed by the six CDR loops (*three from V_L and three from V_H*) mounted upon the compact conserved scaffold of β-sheets formed by the framework segments. The CDRs differ in length and sequence among different antibodies. The specificity and affinity of the antigen binding sites is principally contributed by the structures of these six juxtaposed hypervariable CDR region loops (Chothia *et al.,* 1989, Padlan 1993, Richard *et al.,* 2000).

1.7.2 Recombinant antibody fragments

The whole antibody molecule i.e. IgG is not suitable for phage display due its larger size. The most favored forms of antibody used in 'phage display' are the smaller fragments such as scFv or Fab (Bird *et al.,* 1988, Skerra and Plückthun 1988, Hudson 1998). Both of these fragments retain the antigen-binding properties of the parent antibody molecule. The scFv molecule consists of a V_H and a V_L domain covalently linked by a flexible linker region, while the Fab fragment has constant regions one each from heavy and light chain (Fig. 4B). Due to their antigen binding property and smaller size, these fragments have become the molecules of choice for the development of novel and unique therapeutics and diagnostic laboratory reagents, whereas their shape and size makes them suitable for co-crystallization experiments (Hunte and Michel 2002).

1.7.3 Antibody libraries *"on the phage surface"*

Phage antibody is a filamentous phage displaying an antibody molecule (usually a scFv or Fab) on its surface. And the phage antibody library is a pool of such phages, each of which display a different antibody fragment on their surface while encapsulating inside the genetic information of these antibodies. The phage libraries are constructed by cloning a library of antibody genes into a displaying vector such as a phage or a phagemid. Based on the source of antibody genes two main types of libraries that have been developed and widely used are immune and naïve libraries (Hoogenboom and Winter 1992, Nissim *et al.,* 1994).

Naïve libraries generated from non-immunized sources are not biased to any particular antigen, thus providing the feasibility of obtaining antibodies against a vast array of antigens from a single library. Therefore, they are also referred as *'single-pot'* antibody libraries. Naïve libraries can be further classified based on the source of the variable gene fragments utilized to generate it:

1. "*Natural naïve library*", where the source of the library diversity is the pool of rearranged variable (V) gene segments (Marks *et al.,* 1991, Vaughan *et al.,* 1996).
2. *"Semi-synthetic naïve library"*, where the library diversity is generated by germline V-gene segments in which one or more CDRs are added or diversified *in-vitro* by random PCR (Griffiths *et al.,* 1994, Knappik *et al.,* 2000). The scFv-phage antibody libraries (*ETH-2* and *Tomlinson I+J*) used during the study of this thesis are semi-synthetic naïve type of libraries.

Immune libraries are generated with variable (V) gene segments obtained from an immunized source (Barbas *et al.,* 1991a, Ames *et al.,* 1994, Amersdorfer and Marks 2000). Such libraries have a strong bias towards antigen-specific antibodies. During the process of immunization, the stimulation by antigen drives the specific subtle lymphocytes (memory B-cells) to turn into highly-proliferative plasma B-cells. This process results in the upsurge of the antigen-specific B-cell population, and also the dramatic increase in the affinities of the progeny via somatic hypermuation. Such populations of activated B-cells not only contain elevated levels of mRNA as compared to resting B-cells (Hoogenboom and Winter 1992), but such a mRNA is rich in V_H and V_L genes that are predisposed to create high affinity antigen binding combinations (Clackson *et al.,* 1991, Hawkins and Winter 1992a).

1.7.4 Biopanning

The simplified process of selection of a rare phage with the desired properties from a library of phages consisting of hundreds of millions of candidates is known as *"biopanning"*, the term given by G.P. Smith in the very first paper of phage display (Smith 1985). Biopanning is a methodology that mimics the natural immune system for the selection and enrichment of specific *binders*. The process is based upon affinity enrichment that involves repeated rounds of selections and amplification of specific *binders*.

A pool of phages bearing a variety of antibody fragments on their tips is incubated with the target molecule (protein, peptide, DNA) that is usually immobilized on a solid

surface. The loosely and non-specifically bound phages are eliminated by washing. The specific *binders* are eluted, and infected into host *E. coli* for propagation and amplification. These selected and amplified phages are rescued from the host *E. coli* by helper phage superinfection and applied for the next round of selection (Fig. 5). After few (usually 2-4) rounds of selection, a population of phages is obtained that is enriched for specific *binders*. Depending upon the quality of the initial library, the degree of enrichment achieved could vary from 10^3 to 10^5 fold per round of selection.

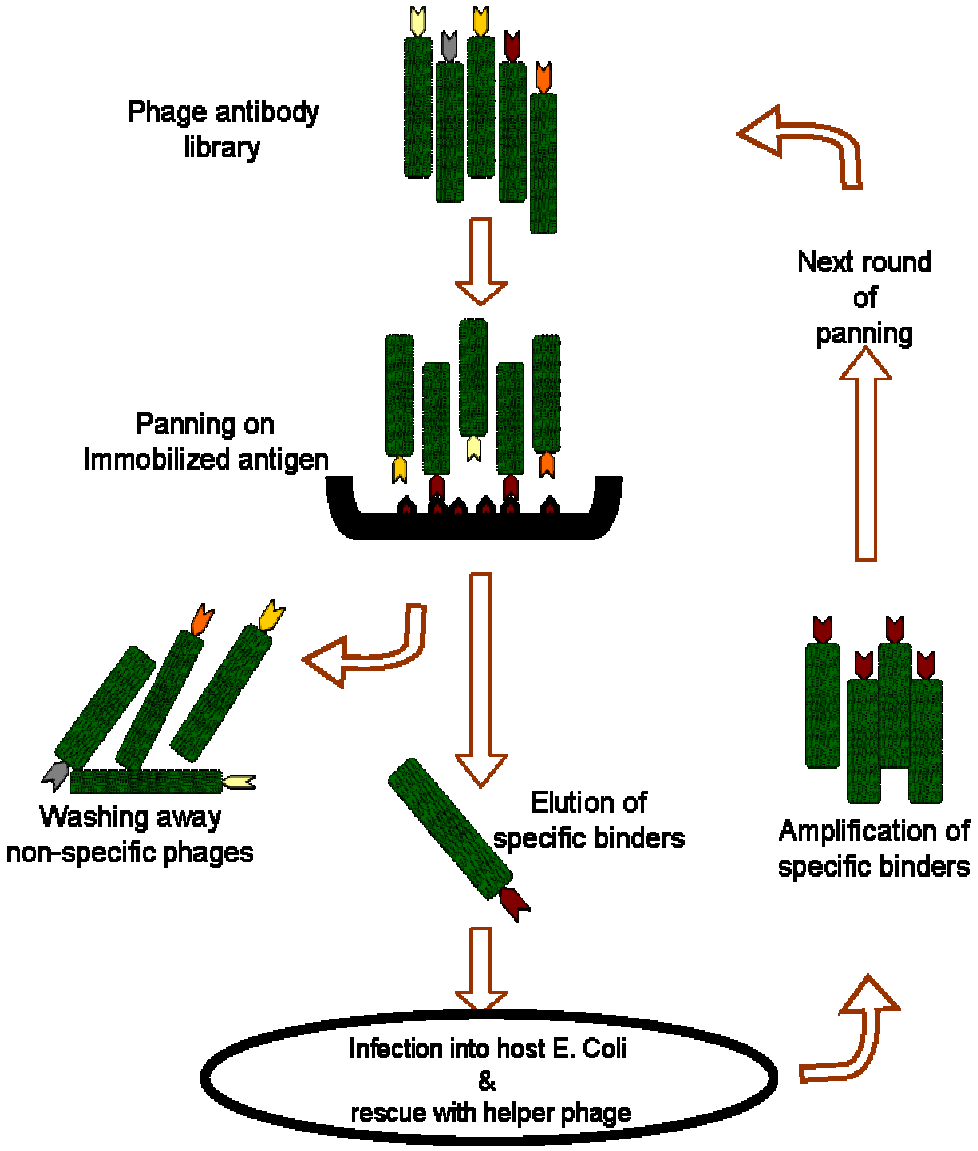


Figure 5: Schematic outline of biopanning. The process involves multiple rounds of selection, elution, amplification and rescue (see text for details).

1.8 Membrane protein crystallization

No other biological molecule of nature performs sophisticated functions as diverse as that of the proteins. Proteins are involved in every possible activity that in essence forms the basis of the cellular life. It is amazing to consider that just twenty amino acids in various permutations and combinations can give rise to the thousands of proteins each with unique functional characteristics. Such a functional uniqueness of a protein lies not only in the primary structure the amino acid sequence, but mostly on the arrangement of these amino acid residues in the three-dimensional space. Among the various structural biology techniques, X-ray crystallography has been at the forefront to elucidate the structural details of the proteins at near atomic resolution. These structures have helped us to understand the intricacies and molecular mechanisms behind the diverse functional characteristics of these proteins.

Among the very large population of the protein world, is a category of proteins that are integrated in biological membranes known as integral membrane proteins. These membrane proteins perform a wide range of vital cellular functions such as signal transduction and transport of molecules across the biological membranes. They are essential in generation and dissipation of cellular energy as well as to maintain cellular integrity. Membrane proteins are abundant and present in all cellular organisms, but their high resolution crystallographic structures are rare. Less than 130 integral membrane protein structures have been published since the first breakthrough, when Michel and co-workers determined the structure of bacterial photosynthetic reaction center more than 20 years ago (Deisenhofer *et al.,* 1985). Considering the fact that about one third of the human genome is predicted to code for membrane proteins (Wallin and Heijne 1998), yet a tiny fraction of structures published till now belong to them. The availability of not so many high resolution structures of membrane proteins is an indication of the difficulties and challenges involved in this process (Hunte and Michel 2003). The problems encountered during the process of membrane protein structure determination are the following:

a. The difficulty in obtaining a membrane protein in adequate quantity and of sufficient quality from their natural sources. Although with the advent of recombinant DNA technology, the production of recombinant membrane proteins in expression systems has been of immense help.
b. The intricacies of handling membrane proteins due to their amphiphatic nature, they are delicate and highly unstable once taken out of their native membrane environment.

c. The difficulty in obtaining good quality membrane protein crystals for X-ray diffraction measurements. The complexities are mainly due to scant hydrophilic surface area required for the crystal contacts and the lack of rigidness in the protein molecule. The flexible domains common in membrane often hamper the crystal formation.

1.8.1 Antibody fragment mediated membrane protein crystallization

The strategy to increase the probability of getting well-ordered crystals by enlarging the polar surface of a membrane protein with the aid of antibody fragments was first devised by Michel and colleagues (Ostermeier *et al.,* 1995a). The potential and importance of the strategy can be assessed by the fact that since then X-ray structures of six different proteins in complex with antibody fragments have been published (Hunte *et al.,* 2000, Zhou *et al.,* 2001, Dutzler *et al.,* 2003).

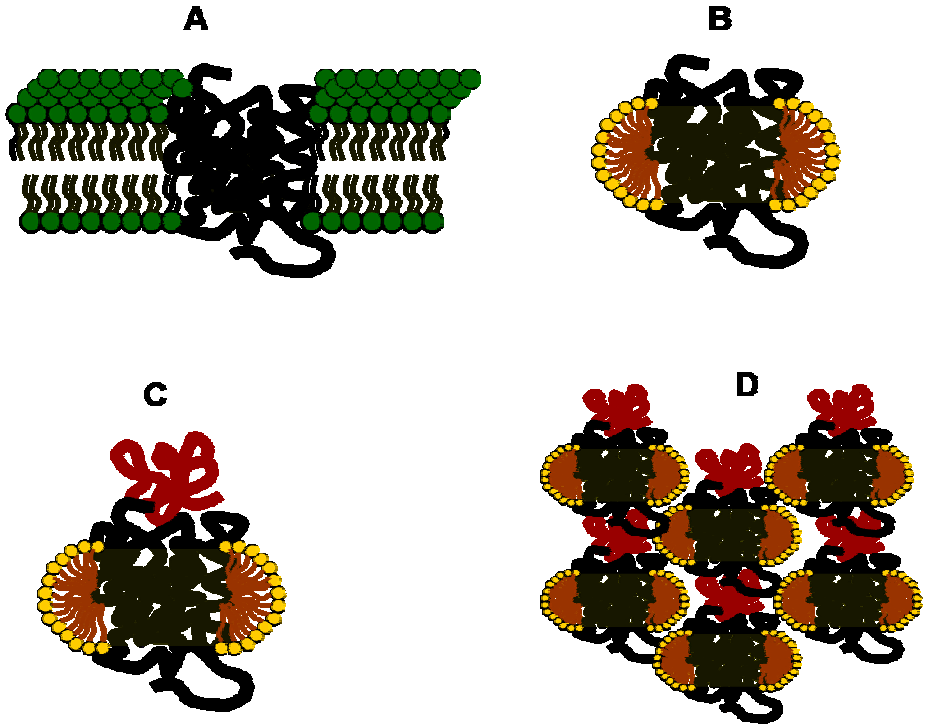


Figure 6: Antibody fragment mediated crystallization of membrane proteins. Cartoon representation of a membrane protein in **A.** a biological membrane, **B.** in detergent micelle solubilised form, **C.** in complex with antibody fragment and **D.** in a type II crystal packing. The figure is a modified adaptation from Hunte 2003.

In order to obtain well ordered three-dimensional crystals, membrane proteins solubilised with detergents are incubated with antibody fragments (Fab or Fv) and purified in complex with them. The purified antibody-membrane-protein-detergent micelle ternary complexes usually form *type II* three-dimensional crystals (Hunte and Michel 2002). The antibody fragments facilitate the well ordered crystal growth by enlarging the hydrophilic

surface area of the membrane protein required for crystal contacts, while expanding the space that accommodates the detergent micelle in the crystal lattice (Fig. 6). They also confer the much needed rigidity to the membrane protein by binding to it, and interlocking its flexible loops in a fixed conformation. Generally a whole IgG antibody molecule (~150kDa) is not appropriate for co-crystallization attempts, primarily because of its flexible hinge region that is detrimental for crystal growth (Lesk and Chothia 1988). Secondly, their bi-valency is in most cases unfavorable.

Up to now, all successful examples of membrane protein structure determination by antibody-mediated crystallization used antibody fragments generated from monoclonal antibodies either by papain digestion or by recombinant antibody technology. These monoclonal antibodies were obtained from hybridomas generated by the fusion of pre-defined plasma B-lymphocytes obtained from immunized mice with the plasmacytoma cells (Kohler and Milstein 1975). Whereas papain cleavage generates Fab fragments, smaller antibody fragments such as Fv or scFv can be produced with recombinant antibody technology (Plückthun and Skerra 1989). The light and heavy chain variable gene segments obtained from hybridoma cells can be expressed in *E. coli* (Plückthun 1997, Venturi *et al.,* 2002). Both strategies require the arduous task of raising and screening of hybridomas, very often the bottle-neck step. The proteolytic Fab production often faces the problems like inadequate quality and homogeneity of Fab species due to inefficient or partial papain digestion.

A solution to overcome these obstacles is offered by the development of phage display antibody systems. Recombinant antibody fragments such as scFv or Fab can be generated against membrane protein targets using phage display technique. Recombinant antibody fragments have been isolated from a Fab based synthetic naïve library against $p185^{erbB-2}$, a transmembrane glycoprotein (Griffiths *et al.,* 1994, Sawyer *et al.,* 1997). An immune Fab based library generated from HIV-type1 seropositive individuals was used to isolate *binders* against detergent solubilised HIV-1 Env glycoprotein (Burton *et al.,* 1991, Labrijn *et al.,* 2002). A scFv based synthetic-phagemid library was panned against the detergent solubilised mitochondrial complex I, and a *binder* to its 51-kDa subunit was isolated (Rubinstein *et al.,* 2003). In another example, an antibody library was constructed with an aim to select *binders* suitable for the stabilization and crystallization of membrane proteins (Rothlisberger *et al.,* 2004). These examples demonstrate the feasibility of using phage display system to select antibody fragments against membrane protein targets. But the examples are very few when compared to thousands available for soluble proteins, against which the technique is well established (Sergeeva *et al.,* 2006). The not so frequent use of phage display against

membrane proteins is an indication that the technique against them is still challenging and difficult. The difficulties are mainly due to the problematic nature of the membrane protein itself. The technique needs to be optimized against membrane proteins in order to have fast excess to antibody fragments suitable for their co-crystallization.

1.9 Aim of this work

To establish the antibody-mediated strategy as a general tool for the crystallization of membrane proteins, fast access to suitable antibody fragments is needed. The usual method of raising monoclonal antibodies by classical hybridoma technique is tedious and time consuming. In addition, most of the biologically important human membrane proteins are fairly conserved in mice, and hence do not elicit the desired immunogenic response in them. Phage display has the potential to overcome these problems. The technique is well established for selecting antibody fragments against soluble proteins, but is still in its infancy concerning membrane protein targets. Part of the problem is the very nature of membrane proteins. Their sticky hydrophobic surfaces and scanty polar regions do often result in poor selection from antibody phage libraries.

The goal of this work was to device a phage display of antibodies against membrane protein targets as a reliable and fast approach to obtain high affinity antibody fragments suitable for their co-crystallization.

2. Results

There are two options to select antibody fragments with the phage display technique, based on naïve or immunized libraries. The naïve libraries are more versatile as they can be used for all antigens and generation of antibodies would be faster. Therefore, the use of the naïve library ETH-2 was explored.

The ETH-2 antibody library is a phagemid based human antibody library in scFv format. The library uses a single functional heavy chain gene (V_H –DP47) and two functional light chain genes, a *kappa* (V_L –DPK22), and a *lambda* (V_L –DPL16) gene. These heavy and light chains form one of the most stable and highly expressing framework scaffolds. The diversity of the ETH-2 library has been generated *in-vitro* by randomizing the respective CDR3 sites of the heavy (V_H) and light chain (V_L) genes (Pinni *et al.,* A 1998). In these libraries pDN322 phagemid vector has been used (appendix A-2).

2.1 Selection from ETH-2 library

The soluble protein yeast cytochrome *c* (cyt *c*) was chosen as first target to select antibodies from the ETH-2 library to test the performance of the library and of the selection protocols.

2.1.1 Panning and selection against yeast cyt *c*

Phage-scFvs were packaged with hyperphages and used for selection against immobilized yeast cyt *c*. The packaging with g-III deleted hyperphages result in more efficient display of antibody fragments on the phage surface (Rondot *et al.,* 2001). The details of the selection process are provided in the methods (section 4.4). Briefly summarizing here, cyt *c* was immobilized on MaxiSorp^{TM} polystyrene surface in 96 well microtiter plates. Phages from all four sub-libraries (A-D) were pooled and applied for multiple rounds of panning and selection. After every round of panning the bound phage population was eluted in two steps, first with trypsin treatment (E1) followed by pH elution (E2). The phages obtained from the two elution steps were pooled and used for the next round of selection. The Fourth round was performed only with the phages eluted by E1. The first information about the proper enrichment of *binders* across the panning rounds was observed by an increased ratio of the number of eluted phages versus the number of applied phages (table 2).

Table 2: Summary of anti-cyt *c* panning of ETH-2 scFv library

Panning round	Total input Phage (TU)	Total output Phage (TU)*	No. of washing steps**	Total duration of washing (minutes)	Enrichment ratio (output / input) $x10^{-8}$	Enrichment factor over previous round
1.	~ $2x10^{10}$	~ 250	5+5	10	1	
2.	~ $6x10^{9}$	~ $8x10^{3}$	8+8	16	133	~ 100
3.	~ $8x10^{9}$	~ $2x10^{4}$	15+15	30	250	~ 2
4.	~ $7x10^{9}$	~ $5x10^{5}$	20+15	30	7140	~ 28

(The TU *transforming units* reflect the number of phage particles), * Total output of phages eluted by trypsin (E1) + by pH (E2). ** Washing steps: first digit indicates washing with PBST (0.1% tween-20), second digit indicates washing by PBS only.

2.1.2 Analysis of cyt *c*-specific enrichment

Phage-scFvs obtained after different rounds of panning were tested in polyclonal phage ELISA (section 4.4.8) to check for the specific enrichment of *binders*. A relevant increase in target specific ELISA signal was observed across the panning rounds (Fig. 7).

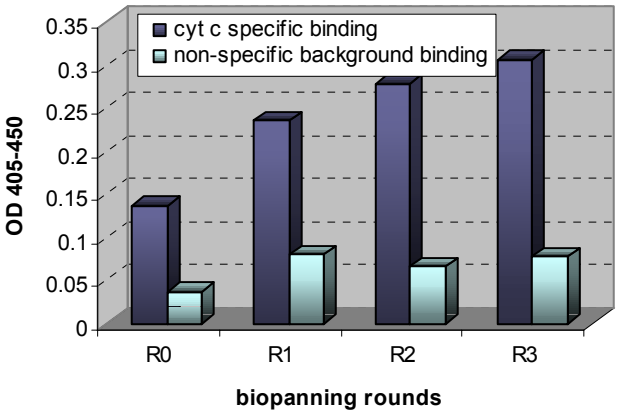


Figure 7: Antigen-specific enrichment of *binders*. The polyclonal-phage ELISA analysis shows anti-cyt *c* enrichment across the panning rounds. Microtiter plates (MaxiSorp™) were coated with yeast cyt *c* solution (50µg/ml). No antigen was coated for non-specific background binding measurement. The microtiter plates were blocked and incubated with cleared culture supernatants from overnight amplifications of phage-scFvs. Bound phages were detected with anti-M13 HRPO conjugate antibody.

In order to analyze the cyt *c* specific enrichment, the eluted phages from third and fourth rounds of selection were infected into XL1-Blue *E. coli* cells and plated to obtain single colonies. Ten to twenty single colonies were randomly picked and grown for phage production. The monoclonal phage populations thus obtained were checked by ELISA for binding against immobilized cyt *c*. Most of the fourth round clones did not show any relevant antigen binding (data not shown). In comparison, half of the third-round clones, which were obtained by pH elution i.e. 3RE2-5, 3RE2-8 and 3RE2-10, showed relevant positive antigen-specific binding (Fig. 8).

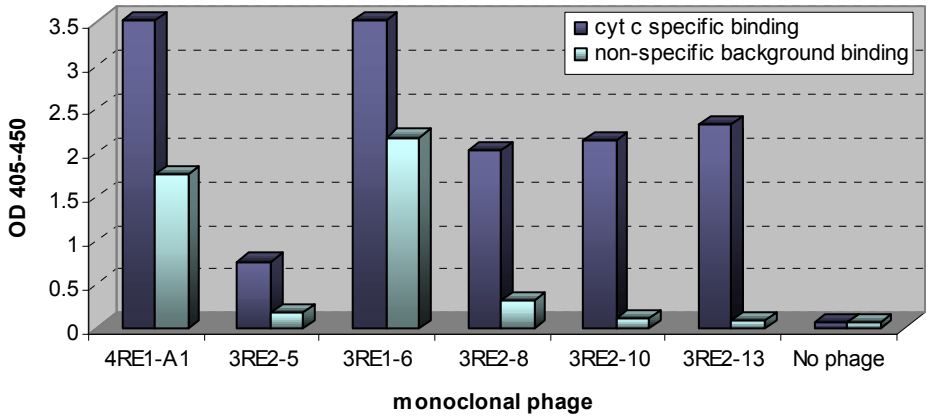


Figure 8: Characterization of representative clones. Representative monoclonals obtained after anti-cyt *c* selection were analyzed by phage-ELISA. The ELISA was performed as in section (4.4.12), except that for coating lower antigen concentrations (20µg/ml) were used. Cleared supernatants from overnight amplifications of monoclonal phage-scFvs were checked for binding. Antigen bound phage were probed with mouse anti-M13 antibody, that in turn was detected by anti-mouse alkaline phosphatase conjugate antibody. The OD values here represent the average of 3 ELISA wells for each sample.

2.1.3 Effect of K07 versus hyperphage packaging

The use of gIIIp mutant helper phages such as hyperphage (Δ gIIIp) is aimed to drive the system to use the only available source of pIII that is supplied by the phagemid, i.e. the pIII-scFv fusion protein. This should result in the increase of the phage population that displays the scFv protein. Such phages should carry the multiple copies of scFv on their surface, i.e. an increased avidity effect (Rondot *et al.,* 2001).

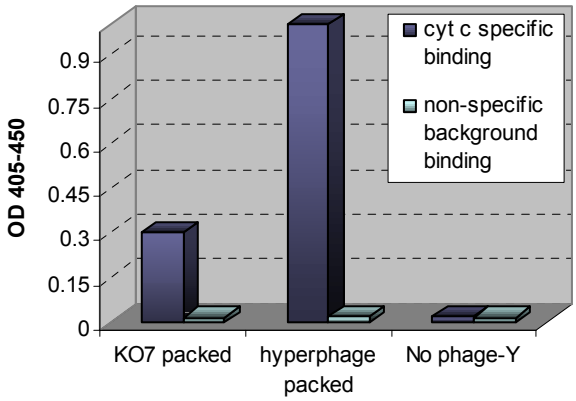


Figure 9: Effect of K07 versus hyperphage packaging. Comparative antigen-binding characteristics in ELISA of anti-cyt *c* 3RE2-10 scFv phage when packaged with two different helperphage systems. ELISA microtiter plates (MaxiSorp™) were coated with antigen (yeast cyt *c*), and probed with the respective phage preparation as done in previous monoclonal phage ELISA. The OD values here represent the average of 2 ELISA wells for each sample.

Anti-yeast cyt *c* 3RE2-10 phage was rescued and packaged separately with wild-type helper phage (M13K07) and hyperphage. Both phage-scFvs were purified by PEG/NaCl and checked for binding to the target antigen yeast cyt *c* by phage ELISA. The hyperphage packaged 3RE2-10 phage shows three-fold increased antigen-binding as compared to the M13KO7 packaged ones (Fig. 9). These results signify the importance of the hyperphage system for the selection of naïve phage antibody library containing low or moderate affinity *binders*.

The hyperphage system can not be used with the pComb3x phagemid display system that was used for the generation of chicken antibody library (reported in later sections). This is because the gIIIp present in pComb3x system lacks the N1 and N2 domains that are required for infection of host cell. Hence, the packaging of pComb3x phagemid with hyperphage would produce non-infectious phage particles.

2.1.4 Colony-PCR analysis

The presence of scFv-DNA insert in the single clones obtained from anti-yeast cyt *c* selection from the ETH-2 library was checked by colony-PCR using flanking forward and reverse primers. For each clone, a well-separated XL1-Blue or HB2151 *E. coli* colony carrying the respective phagemid-scFv was taken and directly resuspended in a PCR reaction mixture (section 4.2.9). Samples were analyzed by agarose gel electrophoresis (Fig. 10).

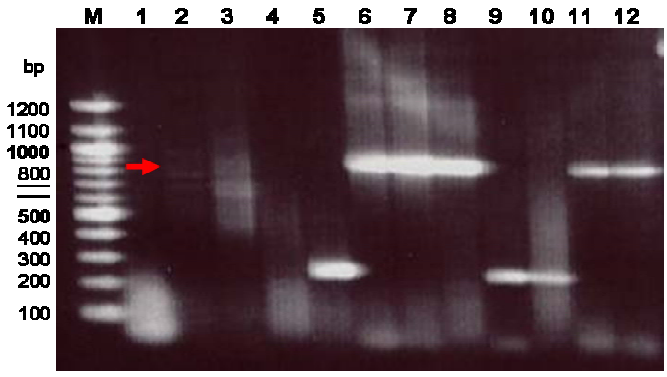


Figure 10: Characterization of the ETH-2 library clones by colony PCR. Single bacterial colonies harboring phagemid clones, selected after anti-cyt *c* panning was directly checked for the presence of scFv DNA insert. For PCR, specific primers (*LMB3 and fdSeq2*) were used. *Lane profile,* lane M: 100bp marker (NEB), Lane 1- 4: single-primer or no-template control reactions. Lane 5, 6, 7 and 8: are 3RE1-6, 3RE2-8, **3RE2-10** and 3RE2-13 clones in XL1-blue cells. Lane 9, 10, 11 and 12: are 4RE3-L1, 3RE1-6, 3RE2-8 and 3RE2-13 clones in HB2151 *E. coli* cells.

The presence of full length scFv (~ 750bp) insert in clones 3RE2-10, 3RE2-8 and 3RE2-13 was indicated by a ~ 950bp PCR product. The clones 4RE1-A1 and 3RE1-6 show a ~ 250bp PCR product indicating the absence of a scFv insert. Incidentally, these clones show the highest non-specific background signals in the ELISA experiment (Fig. 8). The presence of an intermediate size PCR product (data not shown) in some clones indicate the presence of partial scFv inserts corresponding to either heavy (V_H) or light (V_H) chain DNA segments.

Overall, the presence of full length scFv insert was more frequent in clones that were obtained after pH elution (i.e. E2) as compared to trypsin treatment (i.e. E1). Out of 20 clones checked for the pH-eluted third round phages, all but one carried full-length scFv insert, whereas only half of the clones from third round and none from the fourth round carried the full-length PCR product when eluted by trypsin. It is likely that the internal trypsin-cleavage sites of miss-folded or half scFv inserts are accessible to trypsin, and hence such phages are predominantly eluted.

These results were verified by DNA-sequence analysis of representative clones. The ELISA positive clones 3RE2-8, 3RE2-10 and 3RE2-13 were found to be carrying the same scFv-insert sequence (appendix A-1). Therefore, only 3RE2-10 was used for further analysis and characterization.

2.1.5 Expression and IMAC purification of 3RE2-10 scFv

The scFv from the clone 3RE2-10 scFv were produced in soluble format, i.e. not fused to phage pIII, by periplasmic expression in *E. coli* strain HB2151 (section 4.5.1). This non-suppressor strain produces soluble scFvs, as they do not suppress the amber codon present between the scFv and gIIIp. After periplasmic extraction, the scFv were purified using Ni-NTA agarose resin, since the scFv protein carries a 6xHis tag on its C-terminus. The single step immobilized metal affinity chromatography (IMAC) purification resulted in homogenous species of scFv-protein observed as a single band of ~29 kDa size on SDS-PAGE analyzed with silver staining (Fig. 11A). The anti-His tag Western-blot analysis showed small amounts of degradation products (Fig. 11B) which were not visible on silver-stained SDS-PAGE.

The yield of purified scFv protein determined by bicinchoninic acid assay (BCA) was ~ 700µg/ L culture.

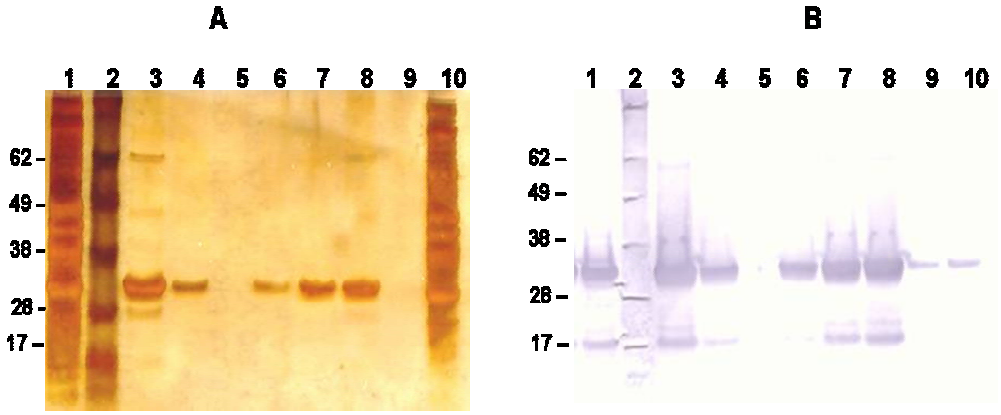


Figure 11: Purification of 3RE2-10 scFv. **A.** Silver stained SDS-PAGE (Nu-PAGE, 4-12% gradient Bis-Tris gel, 1xMES running buffer) **B**. Anti-His tag western blot of the same. The 3RE2-10 $scFv_{HIS}$ protein was purified from periplasmic extracts by IMAC as described (section 4.5.4). Lane 1: periplasmic extract, Lane 2: molecular weight marker, Lane 3 and 4: pooled elution fraction samples after and before 10kD cutoff concentration and Lanes 6-7: individual elution fractions, Lane 9: wash fraction and Lane 10: flow-through fraction. 10µl of sample were used for per lane.

2.1.6 Species specific binding of anti-cyt *c* 3RE2-10 scFv

Binding of the IMAC-purified scFv 3RE2-10 to native cyt *c* was analyzed by mixing scFv and antigen in stoichiometric ratio in solution and probing complex formation by gel filtration on Superdex-75 column (SMART system). No binding was observed as cyt *c* and scFv eluted in two separate peaks (data not shown), an indication that a stable co-complex was not formed in solution. The likely reasons are that 3RE2-10 scFv is either a low affinity *binder*, or it recognizes a non-native form of the antigen.

Therefore, the binding of the scFv was tested by Western-blot analysis of SDS-PAGE separated cyt *c*. To analyze if the scFv recognizes a partially denatured form of the antigen, Western blot analysis was performed using a renaturation protocol (Hunte 1993). This protocol involves the incubation of the gel in a renaturation buffer containing 20% glycerol before the protein transfer onto the PVDF membrane at basic pH (pH-10). The protocol allows the protein to partially refold before the transfer (section 4.5.6).

3RE2-10 scFv binds to yeast and horse heart cytochrome *c* when analyzed with the renaturation Western-blot of SDS-PAGE separated samples (Fig. 12b). It was observed that the scFv binds to yeast cyt *c* with much higher intensity than to the horse heart homologue. In a normal Western-blot analysis, the binding signals were very low for yeast cyt *c* and absent for horse heart counterpart (data not shown). These results suggest a species-specific binding nature of 3RE2-10 scFv, possibly recognizing a semi-denatured target epitope. The preference of the scFv for yeast cyt *c* was also confirmed by ELISA (Fig. 12c). The partial denaturation

of the cyt *c* could be explained by the immobilization on polystyrene surface under alkaline conditions.

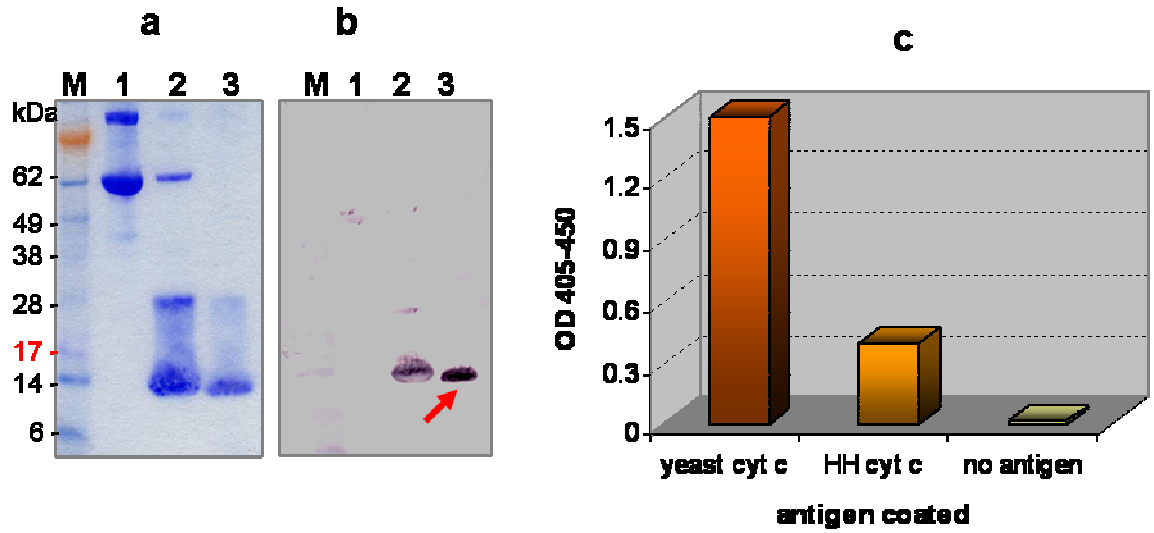


Figure 12: Binding analysis of 3RE2-10 scFv to yeast cyt *c*. **a**. Coomassie stained SDS-PAGE gel and **b.** subsequent western blot obtained after transfer at basic pH (renaturation protocol). Lane-1: BSA (10µg/lane). Lane-2: Horse heart cyt *c* (10µg/lane). Lane 3: Yeast cyt *c* (5µg/lane). The blotted membrane was incubated with the purified 3RE2-10 scFv, and probed with anti-FLAG tag M2 antibody. The scFv carries a C-terminal FLAG tag. The secondary antibody binding was detected by using anti-mouse alkaline phosphate conjugate antibody. **c.** comparative ELISA analysis of 3RE2-scFv binding to yeast and horse heart cyt *c*. In this experiment, microtiter plates (MaxiSorp™) were coated with equal amounts (2mg/ml) of cyt *c* from yeast and horse heart origins. The wells were probed with purified 3RE2-10 scFv antibody. The antigen bound scFv were detected with anti-FLAG tag M2 antibody.

The selection from the naïve libraries and the panning protocols were established. The isolation of 3RE2-10 scFv from anti-yeast cyt *c* panning of ETH-2 library suggests that the panning protocols and the library are functioning. To obtain antibody fragments suitable for co-crystallization of membrane proteins, emphasis should be given to the presentation of target proteins in their native conformation during the process of library panning and selection.

2.2 Generation of streptavidin anchorage surface

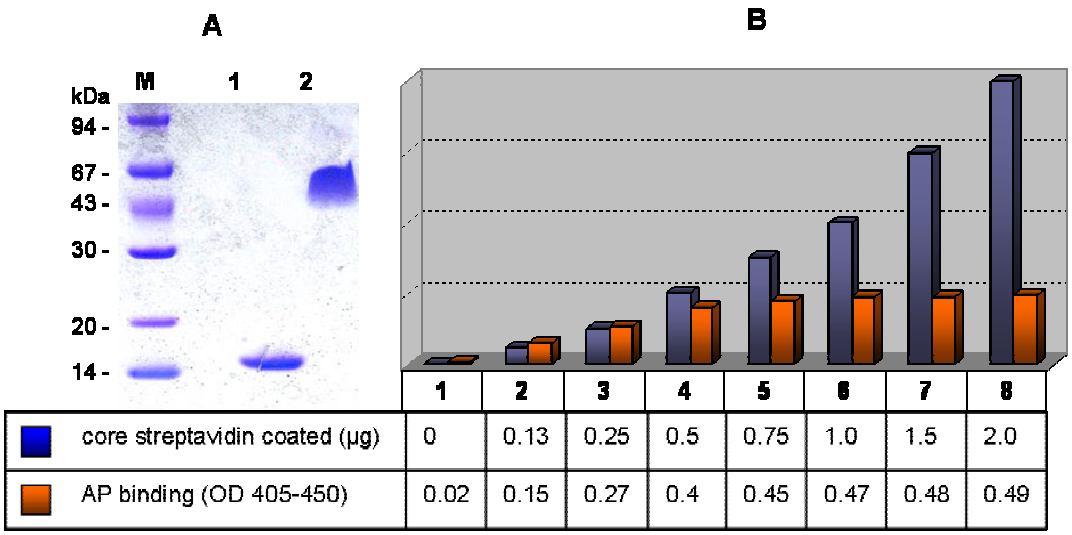
One of the requirements of antibody fragments suitable for co-crystallization is that they should bind to the target protein in its native conformation (Hunte and Michel 2002). To obtain such conformation specific antibodies by the phage display method, the presentation of the target membrane protein in its native conformation is required during the panning experiments. A strategy to immobilize the proteins on solid surfaces via their terminal affinity tags have been shown earlier to preserve the native conformation (Padan *et al.,* 1998, Ott *et al.,* 2005). Streptavidin coated magnetic beads have been used to anchor biotinylated-proteins for phage display library selections (Rothlisberger *et al.,* 2004). The extraordinary high affinity of biotin for streptavidin makes this system attractive, as intensive washing steps can be used during the panning process. Here, a streptavidin-MaxiSorp^{TM} surface was generated and optimized for the binding of target proteins.

2.2.1 Production and purification of core Streptavidin

Recombinant core streptavidin was produced, refolded and purified from inclusion bodies as previously described (Schmidt and Skerra 1994). The quality of the streptavidin was determined by SDS-PAGE analysis (Fig.13A). Pure and properly folded tetrameric streptavidin with a yield of ~ 40mg/ L culture was obtained.

2.2.2 Optimization of streptavidin-immobilization on MaxiSorp^{TM} surface

In order to optimize streptavidin coating, increasing amounts of purified core streptavidin were incubated overnight in microtiter plates (MaxiSorp^{TM}). The binding capacity of the coated surface was checked by an assay utilizing the streptavidin binding properties of biotinylated alkaline phosphatase (section 4.6.1). It was observed that streptavidin binding to the surface reaches the level of saturation when coated at ~ 10µg/ml protein concentration (Fig. 13B).



	1	2	3	4	5	6	7	8
core streptavidin coated (µg)	0	0.13	0.25	0.5	0.75	1.0	1.5	2.0
AP binding (OD 405-450)	0.02	0.15	0.27	0.4	0.45	0.47	0.48	0.49

Figure 13: Generation of streptavidin-maxisorp anchorage surface. A. Coomassie stained SDS-PAGE (section 4.5.5) showing homogeneity of core streptavidin purified from inclusion bodies, Lane 1: heat treated (70°C for 10 minutes) sample representing monomeric form of protein, Lane 2: sample prior to heat treatment representing the stable tetrameric form of streptavidin. **B.** Core streptavidin coating of polystyrene (MaxiSorp™) surface. Increasing amounts of purified core streptavidin were coated overnight in microtiter wells (100µl/well, in bi-carbonate buffer at 4°C. Unbound streptavidin was removed by PBST washing. The binding capacity was analyzed by using the binding of biotinylated alkaline phosphatase enzyme probed with pNPP.

2.2.3 Characterization of streptavidin-MaxiSorp™ surface

In order to validate the utility of the streptavidin-MaxiSorp™ surface for the specific immobilization of the target proteins, biotinylated-AP was incubated with increasing amounts of free excess biotin and allowed to bind the streptavidin-MaxiSorp™ surface (Fig. 14). Similar experiments were performed using a biotinylated membrane protein β_2-adrenergic receptor (β_2AR) binding in presence of free excess streptavidin or biotin (data not shown). Binding of the target protein was inhibited by excess of either free biotin or free streptavidin, indicating specific immobilization via Biotin-Streptavidin interaction.

In order to validate that the above generated streptavidin-MaxiSorp™ surface is suitable for immobilization of membrane proteins in their native conformation, β_2AR was immobilized via its C-terminal bio domain (section 4.4.7). In this experiment monomeric-avidin purified β_2AR (1µg/well in 100µl volume) was immobilized on freshly generated streptavidin-MaxiSorp™ surface. The activity of bound receptor was determined by incubating it with radioactive ligand (section 4.6.2). The non-specific activity was measured in presence of molar excess of antagonist (Reinhart *et al.,* 2003).

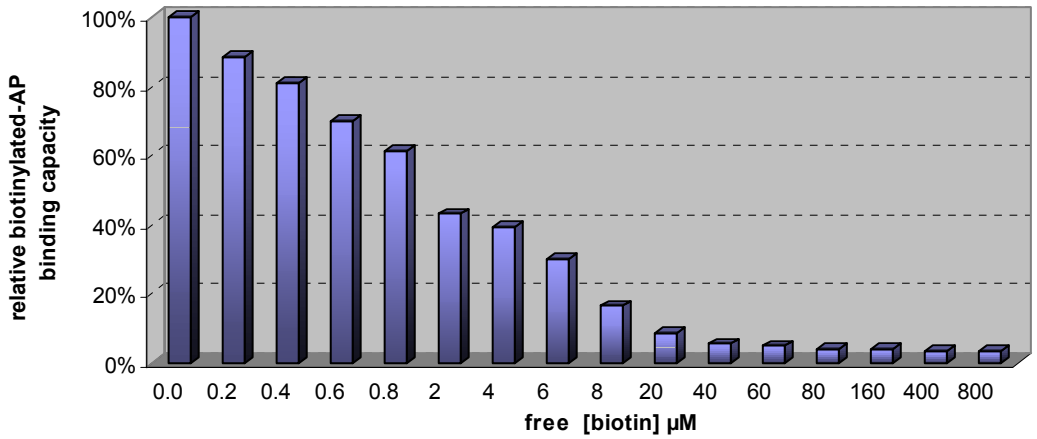


Figure 14: Competition with excess free biotin. To validate the functionality of streptavidin-maxisorp surface, biotinylated-alkaline phosphatase (AP) enzyme was incubated on the freshly generated streptavidin-coated MaxiSorp™ surface in presence of increasing concentrations of free biotin. After washing the surface of unbound protein, the bound fraction was detected by the alkaline phosphatase activity, measured by color development with pNPP substrate solution.

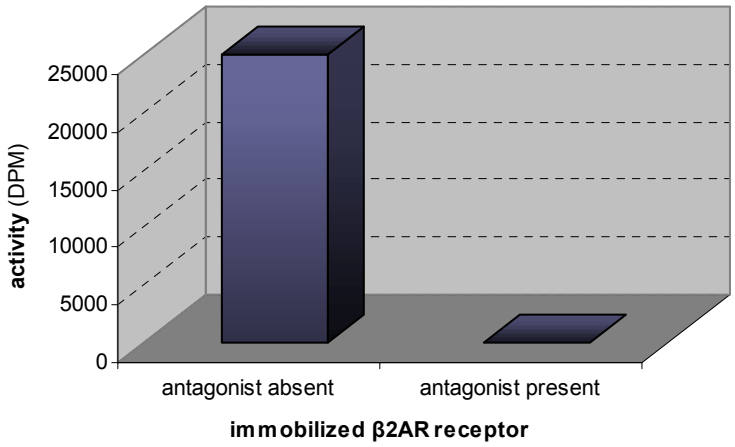


Figure 15: Immobilization of active β_2AR on streptavidin anchored surface. Purified β_2AR was immobilized on streptavidin-MaxiSorp™ surface. The activity of bound receptor protein was determined by binding of radioactive ligand (CGP-12177, [5,7-^{3}H]) in presence and absence of excess antagonist alprenolol. The experiment was done in collaboration with Dr. C. Reinhart.

The bound receptor was found to be in its active conformation as shown by the ligand binding assay (Fig. 15). The absence of relevant activity in presence of excess antagonist shows the specificity of activity results. The activity of 25000 DPM/ microtiter well was obtained. That corresponds to ~ 20ng of active β_2AR.

Both experiments indicate that the surface for immobilization of target proteins is suitable to enrich native *binders* during a phage-panning experiment.

2.3 Selection of antibody fragments from Tomlinson (I+J) scFv libraries

2.3.1 β_2AR as target membrane protein

The β_2 adrenergic receptor (β_2AR) is an integral membrane protein belonging to the family of so called serpentine receptors collectively known as G-protein coupled receptors (GPCRs). Like many other GPCRs, β_2AR is a potential target for many existing and developing drugs. Little is known about the structure-function relationship as till date no high resolution structure is available for these proteins. All these features make β_2AR a good target for antibody fragment-mediated crystallization attempts. The other reason to choose β_2AR as membrane protein candidate was that it was available in biotinylated form. And a ligand binding assay was available to check its activity (Reinhart *et al.,* 2003). One of the prerequisite to select the antibody fragments suitable for co-crystallization by phage display method is to immobilize the target membrane protein on a solid surface in its native conformation. We were successful to immobilize β_2AR (via its c-terminal bio-tag) on freshly generated streptavidin-MaxiSorp^{TM} surface in its active conformation (section 2.2.3). The purified β_2AR protein was kindly provided by Dr. C. Reinhart.

2.3.2 Anti-β_2AR panning of Tomlinson (I +J) scFv libraries

The quality of the previously used ETH-2 scFv libraries was compromised as a result of repeated propagation cycles, as indicated by a high ratio of insert-less clones (section 2.1.4). Therefore, a new library (Tomlinson I+J) was obtained. Tomlinson (I+J) is a phagemid library in scFv format based on stable human frameworks: V_H (V3-23/DP-47 and JH 4b) for heavy and V_K (O12/02/DP-K9 JK1) for light chain genes. The potential diversity of these libraries is higher as four CDRs (i.e. CDR-H2, H3, L2 and L3) have been randomized as compared to only two (i.e. CDR-H3 and L3) in ETH-2 library. In these libraries, the pIT2 phagemid vector has been used (appendix A-2).

The phages from I and J libraries were rescued and packaged by helper phage (KM13) supplied with the libraries, and biopanned against immobilized β_2AR (section 4.4.6). Two rounds of panning and selection were performed on streptavidin-MaxiSorp^{TM} surface. A third round of panning was performed with β_2AR immobilized on monomeric-avidin coupled resin.

2.3.3 Monitoring enrichment by polyclonal scFv ELISA analysis

Enrichment of anti-β_2AR specific *binders* across the panning rounds was analyzed with polyclonal soluble scFv ELISA (section 4.4.8). Moderate levels of antigen-specific enrichment were reported only in J sub-library after third round of panning, whereas no

significant enrichment was reported in I sub-library (Fig. 16). A relative increase of background signals, i.e. binding to streptavidin and binding to no-antigen surface, was also observed. Despite the high unspecific background, phages from second and third rounds of selection were screened for specific monoclonal scFv.

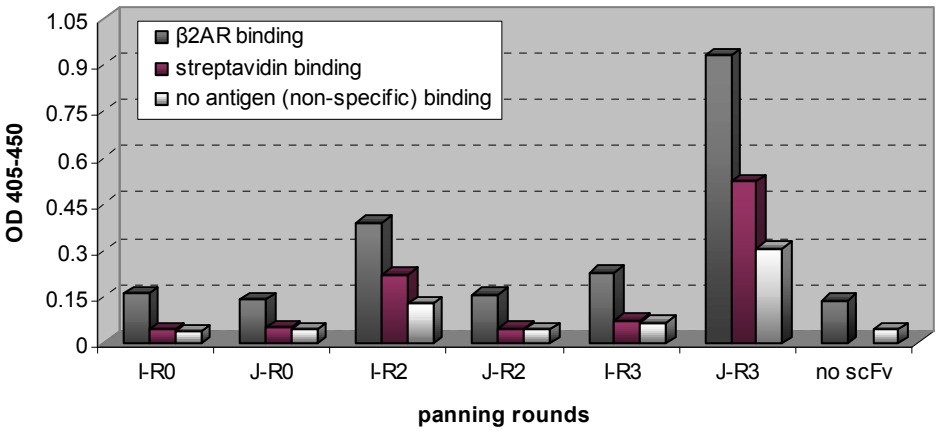


Figure 16: Analysis of anti-β_2AR enrichment by polyclonal scFv ELISA. The figure shows the enrichment across the panning rounds (R0, R2 and R3). Antigen β_2AR (5mg/ml) was immobilized on streptavidin-MaxiSorp^{TM} microtiter plates. In control wells only streptavidin or no antigen was coated for unspecific background measurement. The binding was checked with cleared supernatants of overnight cultures containing soluble scFvs. The bound scFvs were detected via their C-terminal myc-tag by anti-myc tag and anti-mouse alkaline phosphatase conjugate antibody. I and J: samples from respective (Tomlinson I and J) sub-libraries.

2.3.4 Isolation of β_2AR-specific monoclonal scFv

Single colonies were obtained by infecting phages eluted from second and third anti-β_2AR selection rounds directly into freshly grown non-suppressor strain of *E. coli* (HB2151). The infected cultures were plated in serial dilutions and grown to obtain single colonies. Well separated single colonies were carefully picked up and grown for expression of scFv (section 4.4.11). The cleared culture supernatants containing soluble scFvs were used for anti-β_2AR monoclonal ELISA binding assay (section 4.4.12).

The screening of more than 50 individual clones resulted in the isolation of only few scFv (C1, B2, and C2) with clear positive ELISA signals (Fig.17). Out of these clones, only the B2-scFv showed relevant anti-β_2AR binding signal with low background. The C1-scFv showed a high background signal. It turned out to be a clone with only half an insert. The clone C2-scFv showed a streptavidin-specific binding signal.

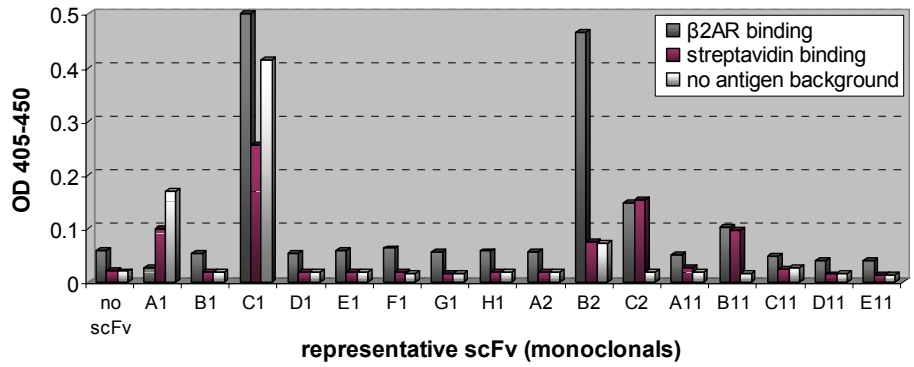


Figure 17: Anti-β_2AR binding analysis of individual clones. Monoclonal scFv ELISA analysis of representative scFvs obtained after 2nd and 3rd round of panning. β_2AR was immobilized on streptavidin-MaxiSorp™ microtiter wells. Only streptavidin or no antigen was coated for unspecific background measurement. The scFv binding was probed using cleared supernatants of overnight cultures containing soluble scFvs.

To analyze the antigen-specific binding properties of the selected clones, the ELISA binding assay was performed with periplasmic extracts of B2-scFv and C2-scFv. For B2-scFv the ELISA binding was also performed with purified scFv-protein samples (data not shown). In all ELISA experiments, B2-scFv always showed β_2AR specific binding properties with low background signals. C2-scFv was again showing streptavidin specific binding signals (Fig. 18).

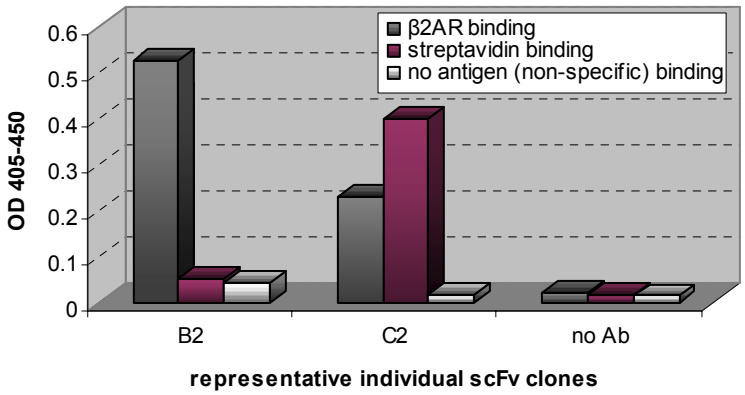


Figure 18: ELISA binding of scFvs from periplasmic preparations. Anti-β_2AR binding of scFvs identified from monoclonal ELISA. Periplasmic preparations of respective clones were checked for binding as described (section 4.4.12). The results indicate the β_2AR-specific binding nature of B2-scFv, and streptavidin-specific binding properties of C2-scFv.

2.3.5 Characterization by insert-check PCR

Phagemid preparations from the clones selected after anti-β_2AR panning were analyzed by PCR for the presence of scFv-DNA insert using flanking region primers (section 4.2.9). The PCR resulted in amplification of a ~960bp size product corresponding to clones that carry full length scFv insert ~750bp (Fig. 19), i.e. B2-scFv clone (lane 9), while the

clones like C2-scFv and A1-scFv did not carry full length scFv DNA inserts (lane 10 and 11). These clones had partial scFv inserts corresponding to either the V_H or the V_L chain. Again the clone that shows the high unspecific signal in ELISA, i.e. A1, turns out to be a partial-insert carrying clone. And the clone which was thought to be streptavidin-specific, i.e. C2 does also carry a partial insert.

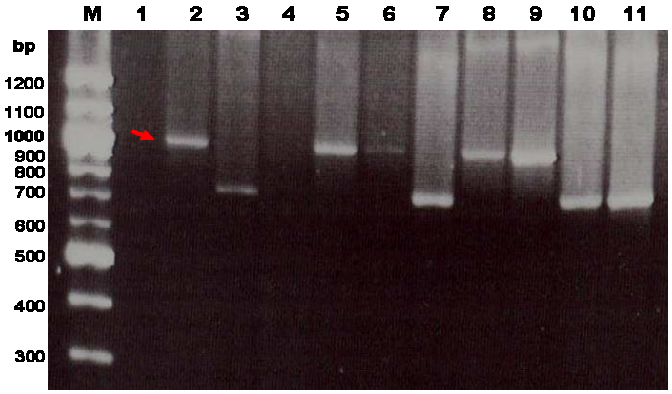


Figure 19: Characterization of the Tomlinson (I+J) library clones by insert-check PCR Analysis. Phagemid DNA samples (100 -200 ng) from respective clones were checked for the presence of scFv insert. The figure shows the agarose gel electrophoresis analysis of PCR samples. *Lane profile,* lane M: 100bp ladder (NEB), Lane 1: negative control, Lane 2: 3RE2-10 clone of ETH-2 library as positive control. Lane 2 to 11: clones selected after anti-β_2AR panning (i.e. lanes 9, 10 and 11 represent B2, C2 and A1 clones respectively).

On the basis of the presence of a full-length scFv insert and β_2AR-specific binding signals in ELISA, B2-scFv was explored for further characterization.

2.3.6 Periplasmic production and IMAC purification of B2-scFv

The scFv from the clone B2-scFv was produced and purified from periplasmic extracts of *E. coli* strain HB2151 (section 4.5.1). Single step IMAC purification resulted in a homogenous species of scFv-protein observed as a single band of ~29 kDa size on SDS-PAGE analyzed with silver staining (Fig. 20A). The anti-His tag Western-blot analysis showed small loss of scFv (lane 2) and small amounts of degradation products, which were not visible on silver-stained SDS-PAGE (Fig. 20B). Batch purification was established by scaling up the expression culture volume and by optimizing the imidazol concentration for the washing during IMAC (section 4.5.4). With 10mM imidazol, the yield of scFv was higher (~1 to 2mg/L), but the purity was compromised (lane 4, Fig. 20A). Such scFv protein preparations when used in subsequent Blue Native PAGE (BNP) binding assays with the target membrane protein were producing smearing aggregates. Increasing the imidazol concentration to 40mM, resulted in a highly pure scFv preparation at the cost of protein yield (0.2 ~ 0.5mg/L), but

such a preparation of scFv protein was necessary to improve the quality of the BNP binding assays.

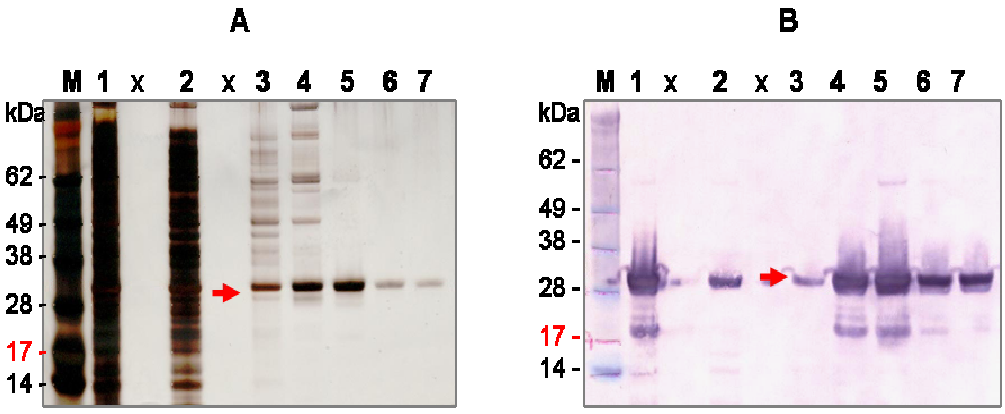


Figure 20: IMAC-purification of B2-scFv. A. The figure shows the silver stained SDS-PAGE (section 4.5.5) **B**. Anti-His tag western blot analysis. B2-scFv$_{HIS}$ protein was purified from respective HB2151 *E. coli* periplasmic extracts using Ni-NTA agarose resin. Lane 1: sample from periplasmic extract, Lane 2: flow through Lane 3: 10mM imidazol washing, Lane 4: 40mM imidazol washing, Lane 5, 6 and 7: elution samples with 70mM, 100mM and 400mM imidazol respectively. 5µl of 50ml samples are loaded in lane 1 and 2, whereas in lanes 3-7, 16µl of 12ml are loaded.

2.3.7 Blue Native-PAGE binding assay

Binding of B2-scFv with its target antigen β_2AR was analyzed on BNP gel shift assay (section 4.6.3). Purified β_2AR was mixed with purified B2-scFv in stoichiometric amounts and incubated for about 30 minutes on ice to allow formation of co-complex. The samples were loaded and resolved on a 6%-19% polyacrylamide gradient BNP gel. A shifted band was observed in response to the presence of scFv (Fig. 21).

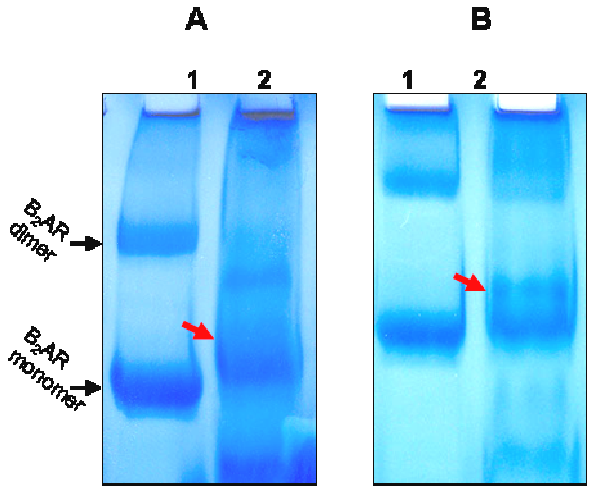


Figure 21: Blue Native PAGE gel shift assay. A. Lane 1: represents the β_2AR (5µg) alone and Lane 2: β_2AR in presence of B2-scFv antibody fragment. In response to the presence of B2-scFv, a band shift is observed (lane 2, indicated by red arrow). **B.** shows the improvement in quality when high purity B2-scFv preparations (obtained after 40mM imidazol washing) were used.

In order to prove that the band-shift observed in these BNP assays is occurring due the co-complex formation between the β_2AR and B2-scFv, a normal SDS-PAGE was performed as second dimension. In this experiment, after resolving the β_2AR bound to B2-scFv on BNP, the gel slices corresponding to the shifted bands were excised and transferred on high resolution SDS-PAGE (Fig. 22B).

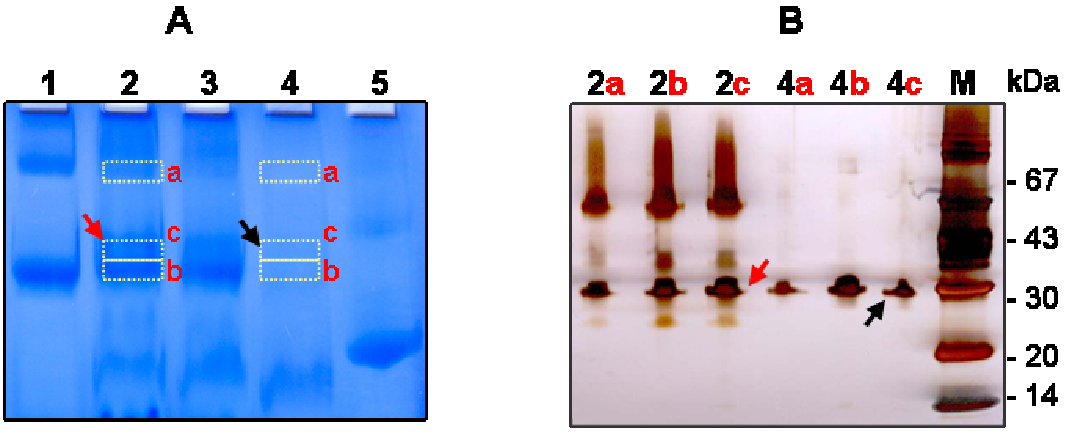


Figure 22: Evidence of co-complex formation between B2-scFv and β_2AR. A. Blue Native PAGE gel shift assay on BN-PAGE (6%-19% gradient). Lane 1: β_2AR alone (~3µg), Lane 2: B2-scFv + β_2AR (~3+3µg), and Lane 4: scFv alone (~3µg). Lane 5: BSA. **B.** Silver stained high resolution SDS-PAGE demonstrating second-dimensional resolution of excised bands from BNP gel.

The presence of two bands (~29kDa and ~58 kDa) in 2a, 2b and 2c corresponding respectively to B2-scFv and β_2AR proteins indicates the existence of a possible co-complex in these bands. The presence of scFv protein in the corresponding excised bands of bands of lane-4 (scFv only) was earlier thought to be due to the residual smearing affect. But later was found to be due the presence of scFv dimeric form. The considerable presence of scFv in 4b was representing the possible dimeric form of scFv. The presence of co-complex in the shifted band (2c) is indicated by the presence of equal amounts of scFv and receptor as seen in the second dimension SDS page. The comparative amount of scFv in this shifted-band (2c) was much higher than found around the same region in scFv-only lane (4c). This observation was an indication suggesting the accumulative behavior of B2-scFv in this region in response to presence of β_2AR, a possible feature of a week or moderate affinity *binder*.

2.3.8 B2-scFv exists in monomeric and dimeric forms

Analytical gel filtration of B2-scFv suggests the existence of two oligomeric states of the scFv (section 4.6.4). The tendency of some scFvs to form monomeric and dimeric forms has been reported earlier (Griffiths *et al.,* 1993, Schodin and Kranz 1993, Raag and Whitlow 1995, Lee *et al.,* 2002), and reviewed in detail (Arndt *et al.,* 1998). The elution profile of Ni-

NTA purified B2scFv on Superdex-200 column resolves two peaks at ~1.57ml and ~1.73 ml of elution volume corresponding to possible dimeric and monomeric forms (Fig. 23A). The sample fractions corresponding to both these peaks show a pure single band (~29 kDa) of scFv when resolved on SDS-PAGE (data not shown). When elution fractions from 1.57 ml or 1.73 ml peaks were again loaded on the Superdex200 column for a second run, single peaks were obtained corresponding to either monomer or dimer (Fig. 23B and C).

These results indicate that the IMAC purified B2-scFv exists in monomeric and dimeric forms that are fairly stable, as they remain in their respective states without inter-conversion at least for one week.

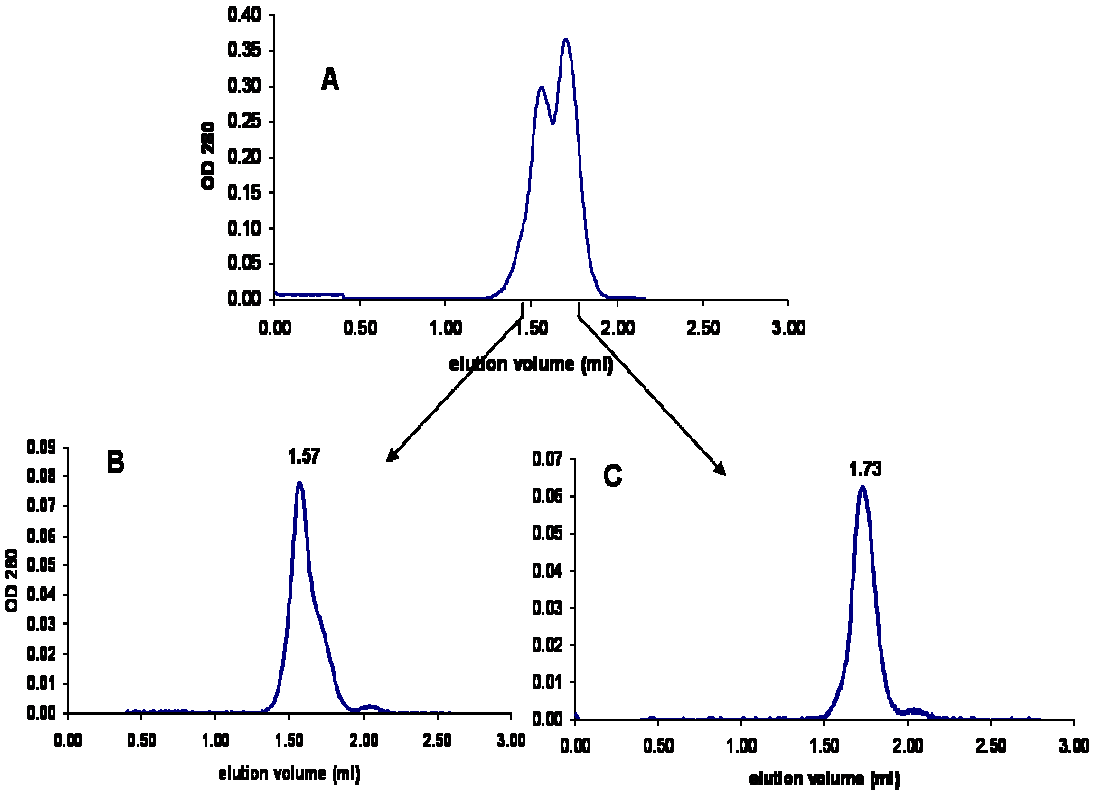


Figure 23: Analytical gel filtration profile of B2-scFv. Elution profile of Ni-NTA purified scFv on Superdex-200 PC 3.2 column (SMART system). **A.** IMAC purified B2-scFv (~100µg). **B.** reloaded elution fraction-12 of run-A (40µls/ of 80µl fraction). **C.** reloaded elution fraction-15 of run-A (40µls/ of 80µl fraction loaded). Fractions 12 and 15 correspond to the first and second elution peaks of run A.

2.3.9 BNP gel-shift binding assay using monomeric and dimeric B2-scFv

In the previous BN-PAGE binding experiments (Fig. 22), the presence of scFv close to the band of β_2AR could be partially attributed to the presence of scFv dimer or a smearing effect rather than its absolute binding to β_2AR. To probe specific binding, BNP gel-shift assays were performed using gel-filtration separated monomeric and dimeric forms of B2-scFv. After BNP gel separation was completed, the whole gel lanes were excised and resolved

in the second dimension on SDS-PAGE (Fig. 24). As seen from second dimension gel analysis, the dimeric scFv is present as a streak along the length of BNP gel even in absence of β_2AR (Fig. 24B, BNP lane 3). The similar but more intense streak is seen when β_2AR is present with it (BNP lane 2). In case of monomer scFv there is no such streaking effect, and instead scFv can be seen accumulating around β_2AR as indicated by red arrow in (BNP lane 4), an indication of specific but a weak binding.

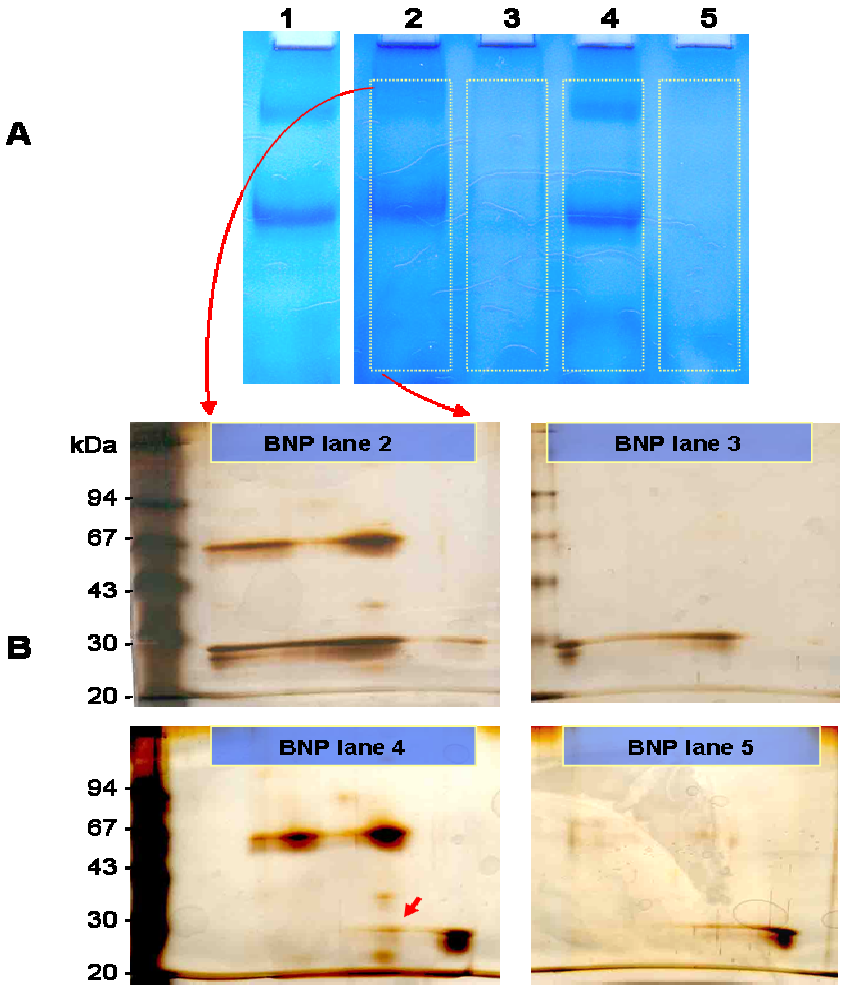


Figure 24: Binding of monomeric and dimeric B2-scFv to β_2AR. A. Blue Native PAGE gel shift binding analysis performed on BN-PAGE (6%-19% gradient). The samples were incubated 30 minutes on ice prior to loading Lane 1: β_2AR alone (~3µg). Lane 2: β_2AR + B2-scFv-dimer, Lane 3: dimeric-scFv alone. Lane 4: β_2AR + B2-scFv-monomer. Lane 5: monomeric scFv alone. **B.** Silver-stained laemmli gels (10% polyacrylamide), showing second dimensional high resolution SDS-PAGE (resolved in Tris-glycine buffer) of the excised BNP-gel lanes.

2.3.10 Gel filtration binding assay

In order to check the binding of B2-scFv to its target in solution, purified β_2AR was mixed with excess of IMAC and gel filtration purified monomeric B2-scFv (section 4.6.4). After 1 hour of incubation to allow co-complex formation, the mixture was loaded on Superdex-200 (SMART system). The elution fractions were collected and analyzed by high resolution SDS-PAGE (Fig. 25).

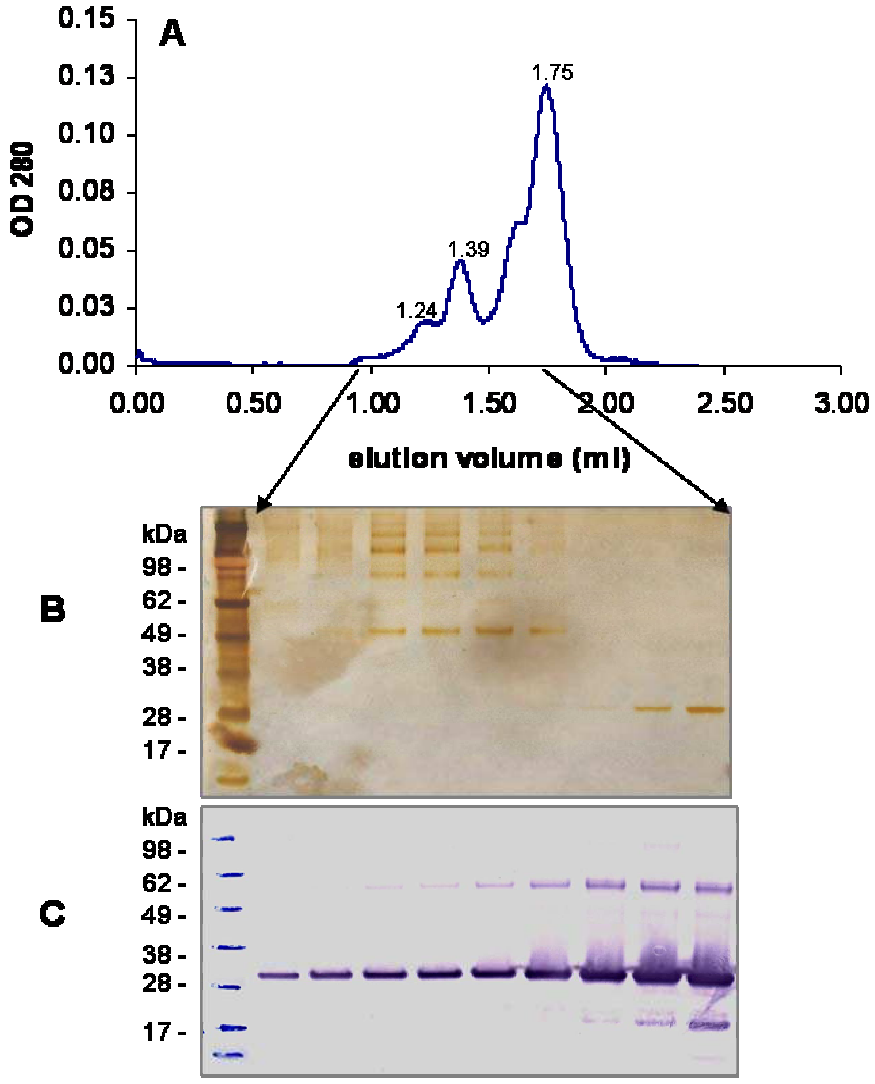


Figure 25: Gel-filtration binding analysis of B2-scFv. A. Elution profile of B2-scFv and β_2AR complex on Superdex-200 column (SMART). **B.** Silver-stained high resolution SDS-PAGE analysis of corresponding elution fractions. **C.** Western-blot analysis of the B developed by 9E10 anti-myc tag antibody.

The elution profile showed two main peaks at 1.39ml and 1.75 ml. These elution peaks correspond to free β_2AR and to free B2-scFv monomer respectively, an indication that most of these proteins were eluted separately. Another small elution peak at 1.24 ml was present,

likely due to the formation receptor-scFv co-complex. Such a shoulder or small peak was observed only when the amount of scFv was present in excess to the receptor fraction (Fig. 25A). The SDS-PAGE analysis of elution fractions corresponding to the 1.24 ml and 1.39 ml peaks did not show any significant presence of scFv protein (~ 29kDa) (Fig. 25B). But when the same gel was transferred on a PVDF membrane for Western-blot analysis, presence of scFv in both of these elution fractions was observed (Fig. 25C). When similar control experiments were performed using monomeric scFv alone or non relevant proteins BSA and NhaA from *E. coli* for binding with B2-scFv monomer, no such Western-blot signal was seen (data not shown). All these results and observations suggest the antigen-specific binding of B2-scFv, while being a moderate or low affinity *binder*.

2.3.11 Sequence analysis of B2-scFv

The DNA sequence of the B2-scFv was obtained from its phagemid, and aminoacid sequence corresponding to its heavy and light chain deciphered (appendix A-1). It carried the complete V_H and V_L gene segments as expected. The characteristic features include the comparative short CDR-H3 region with '**G_{95} A_{96} G_{97} S_{98} F D Y'** aminoacid sequence, possibly accounting for its low affinity binding. Therefore, there is a possibility for improving the affinity of B2-scFv by appending extra randomized residues in the CDR-H3 region.

2.4 Detergent effect on unspecific phage binding

2.4.1 Effect of detergents on the background binding of phages

During the work with the naïve phage libraries, it was observed that the phages show higher degree of non-specific background binding in the presence of detergents. This behavior often results in false enrichment, i.e. enrichment of non-specific *binders* during a panning experiment against a membrane protein target, where the presence of detergent is necessary to keep the target membrane protein in native conformation. Such a behavior of phages was clearly observed when various detergents were used with the aim to reduce the non-specific binding signal during phage ELISA. Surprisingly, it was found that most of these detergents influence the binding of these phages such that increased signals were obtained in antigen-coated as well as in no-antigen coated wells. This indicated that detergents increase phage binding to the surface in an unspecific manner.

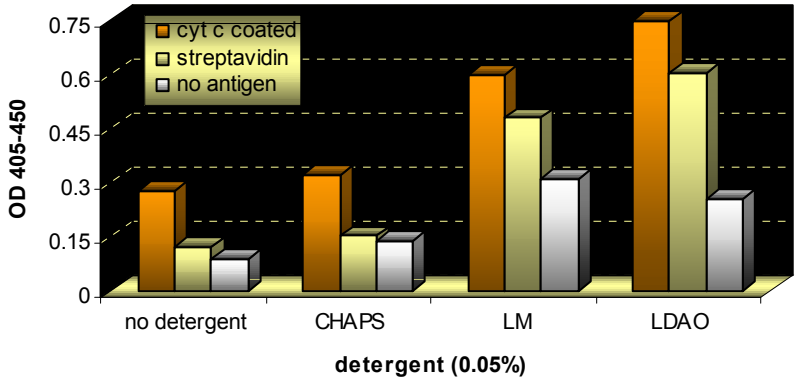


Figure 26: Effect of detergent on non-specific background binding of phages. The figure shows the binding of un-panned Tomlinson I+J libraries phages in presence of detergents. In this experiment microtiter wells (MaxiSorp™) were coated in duplets with 10µg/ml solutions of yeast cyt *c* or core streptavidin in bi-carbonate buffer. In control wells no antigen was coated. The surface was blocked with 2%MPBS, followed by incubation with pre-blocked phage (~10^{10}/well) from un-panned Tomlinson I+J libraries. Phage preparations were made in 2%MPBS in presence of respective detergent concentrations. The bound phages were detected with mouse anti-M13 antibody and anti-mouse alkaline-phosphatase conjugate. The ELISA was developed with pNPP substrate solution, and relative OD values were measured.

A series of experiments was performed, in which the effect of various detergents on non-specific binding of phages was checked. These phages were obtained from the ETH-2 or Tomlinson I+J scFv libraries. Detergents that are commonly used in purification and crystallization of membrane proteins such as LM, CHAPS, LDAO and others were tried. In these experiments, a fix number of phages were incubated on pre-blocked MaxiSorp™ surface to allow binding in presence of the respective detergents. The surface was briefly washed to remove the loosely bound phages. In order to find out the relative number of

phages that were still bound to surface in presence or absence of any detergent, two types of approaches were used. In a first ELISA-based approach, the bound phages were directly detected in the wells by anti-M13 antibody (Fig. 26). In the second approach, a TU-counting assay (section 4.4.5), the surface bound phages were eluted and titered by infecting XL1-blue *E. coli* (Fig. 27).

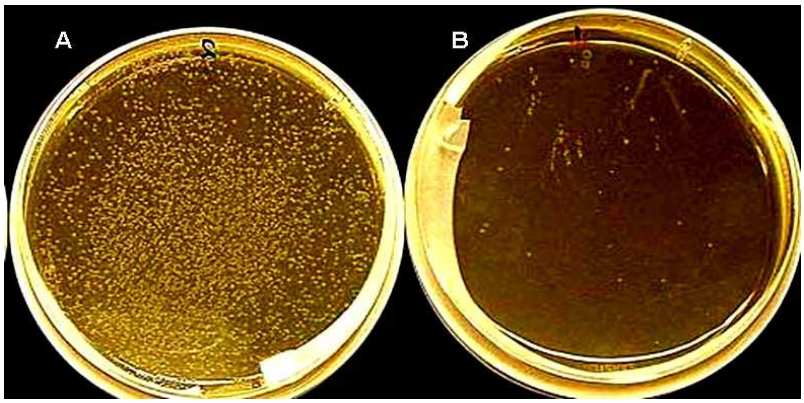


Figure 27: Influence of detergent LM on non-specific background binding of phages. The figure show the comparative presence of unspecifically bound phages **A.** in presence of detergent (0.02% LM) **B.** in absence of detergent. In this experiment equal number of phage particles from un-panned Tomlinson I+J were pre-blocked in presence and absence of the detergent. These phage preparations were allowed to bind the milk-blocked microtiter wells (MaxiSorp^{TM}) for an hour. The loosely bound phage particles were removed by washing, and bound phages were eluted and titered by infecting into XL1-blue *E. coli* cells (OD_{600}~0.5). The figure shows the comparative number of colonies (i.e. phage TU) obtained.

In both of these experimental approaches, it was observed that phages stick to the surface in higher numbers when detergents were present during the phage incubation step. In another experiment, a FF-phagemid (Fab displaying phage) was used and its non-specific binding to milk-blocked maxisorp-plastic surface was checked in presence and absence of detergent (0.02% LM) in an experimental setup similar to that shown in figure 27. Here also a great degree of non-specific phage retention on surface was observed in presence of 0.02% LM (data not shown).

2.5 Generation of chicken scFv phagemid library

The previous results showed the limitations of naïve scFv libraries to obtain high affinity recombinant antibody fragments suitable for co-crystallization of membrane proteins (Hunte and Michel 2002). High unspecific background binding caused by partial inserts and the required detergents appear to be the main obstacles. Highly diverse, excellent libraries would be needed to pursue this approach. As such libraries are difficult to obtain for academic research, an alternative strategy of constructing libraries from immunized animals was explored. Phage antibody libraries generated from the immunized rather than the naive sources should result in better selection of higher affinity *binders* (Burton 2001).

An immune antibody phagemid library was constructed in scFv format from the spleen and bone marrow of chicken. Antibody libraries from immunized chicken have been previously developed to isolate various useful *binders* (Andris-Widhopf *et al.,* 2000). Here, chicken were immunized with a mixture of two membrane proteins: the Na^+/H^+ antiporter STNhaA and the potassium exchanging cation proton transporter KefC. The scFv DNA library was constructed by joining V_L and V_H with two linker formats: one with a short linker (SL) region, and a second with a long linker (LL) region, coding for amino acid sequence "GQS_2RS_2" and "$GQS_2R(S_2G_4)_2S$" respectively (Whitlow *et al.,* 1993). The purpose of using SL and LL was to obtain dimeric and monomeric forms of scFv. It is known that the linker length and sequence affects the oligomeric state of the scFv (Tang *et al.,* 1996, Arndt *et al.,* 1998).

2.5.1 Anti-STNhaA/KefC–specific immune response in chicken

The selection of chicken as the host animals for immunization is advantageous primarily due to their unique immune system, which develops the B-lymphocyte diversity by "*gene conversion"*. Two white Leghorn female chickens identified as 'chicken-727' and 'chicken-728' were immunized with the antigen mixture of STNhaA and KefC (section 4.6.7). The immunization of chicken was carried out at the company PRF&L. To keep track of the antigen-specific immune response, the chickens were bled at regular intervals, and the anti-STNhaA (Fig. 28) and anti-KefC (Fig. 29) sera titer were determined by ELISA. An antigen specific sera titer of more than $5x10^4$ was obtained after 42 days of immunization schedule. The titer was determined as the highest dilution of sera at which the antigen specific ELISA signal was higher than the background signal (Fig. 30). As the titer dropped for chicken-728 (day-72), chicken-727 among the two was chosen for sacrifice at day 95 and total RNA was harvested from its spleen and bone marrow.

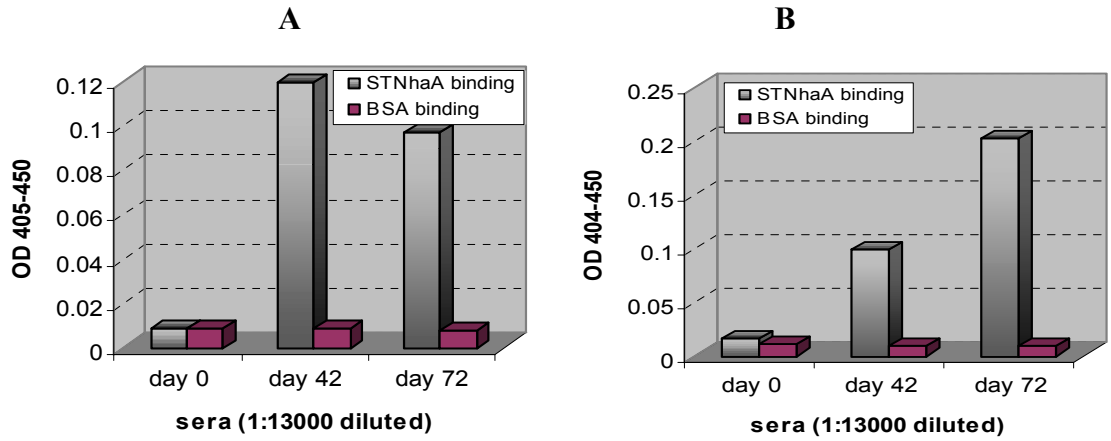


Figure 28: Analysis of anti-STNhaA immune response in chicken. A. Chicken-**728** and **B.** Chicken-**727**. The figures above represent the anti-STNhaA ELISA binding signals obtained with serum samples of the chicken drawn on different days of immunization schedule. For ELISA His-tagged STNhaA (10µg/ml) was immobilized on Ni-NTA chileate microtiter plates, BSA was coated as negative control (section 4.6.7). The plates were blocked with 2% milk in PBSLM, followed by incubation with the respective sera samples (diluted in 2% MPBS). The antigen-bound IgY were detected with anti-IgY alkaline phosphatase conjugate. ELISA was performed in duplets, and the results shown above are based on average OD value of two microtiter wells.

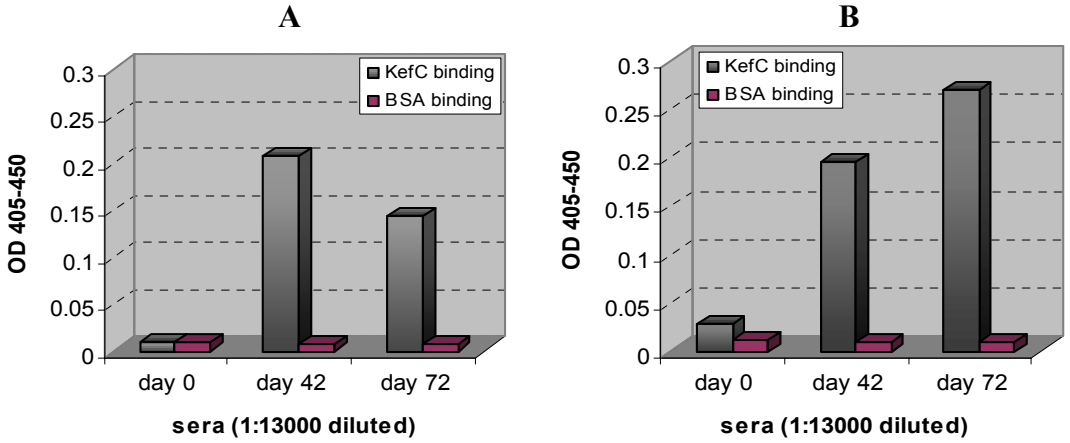


Figure 29: Analysis of anti-KefC immune response in chicken. A. Chicken-**728** and **B.** Chicken-**727**. The experiment was performed same as the previous one, except that binding to KefC was checked.

Antigen-specific immune response was generated in both of the chicken-727 and 728. The slight drop of antigen-specific anti-sera titer on day 72 was seen only in chicken-727. The generation of equally good immune response against both the STNhaA and KefC in each chicken demonstrates the feasibility of using more then one antigen in a mixture for immunization, thus saving cost and time.

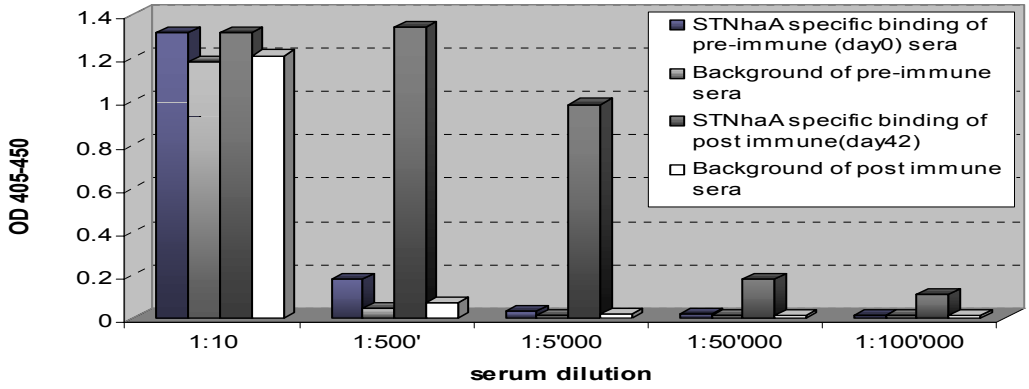


Figure 30: Anti-STNhaA specific titration of chicken-727 serum antibodies. In this experiment post-immune serum obtained on day 42nd of immunization was titered for the presence of antigen-specific antibodies while using pre-immune sera obtained on day 0 as reference. For ELISA analysis, His-tagged STNhaA was coated on Ni-NTA microtiter plates and BSA was coated for the analysis of non-specific background binding signal. The plates were blocked with 2% milk in PBSLM, followed by incubation with day 0 and day 42 sera dilutions prepared in 2% MPBSLM. The antigen-bound IgY were detected with anti-IgY alkaline phosphatase conjugate. The ELISA was performed in duplets, and the results shown above, are based on average OD value of two ELISA wells. The chicken in this case has a STNhaA antibody titer exceeding 50,000, determined as the highest dilution at which the STNhaA-specific signal is greater than the background anti-BSA signal.

2.6 Phage displayed antibody (scFv-phagemid) library construction

The construction of a good antibody scFv library involves the efficient PCR amplification of V_H and V_L antibody genes, followed by their joining in single construct scFv format. The scFv-DNA library is cloned in a suitable phagemid vector and transformed into compatible *E. coli* strain i.e. XL1-Blue. The outline of the scFv-phage library construction is shown below (Fig. 31).

2.6.1 Isolation of RNA

For isolation of RNA, a modified protocol based on (Chomczynski and Sacchi 1987) was followed. Immunized chicken-727 was sacrificed and total RNA was harvested from its spleen and one leg bone marrow (section 4.2.1). The final yield of purified total RNA was estimated as ~ 5mg from bone marrow, and around ~ 2mg from spleen.

2.6.2 First Strand (cDNA) synthesis

cDNA synthesis was done by RT-PCR using Superscript III First-Strand Synthesis system for RT-PCR with oligo dT_{20} primer and using 40 µg of total RNA as template (section 4.2.2). Since the 20mer dT primer anneals to poly-A tail of mRNA, all mRNA with poly-A tail was converted into corresponding cDNA. The quality of cDNA was later confirmed when antibody gene fragments were successfully amplified from it by V_H and V_L specific PCR.

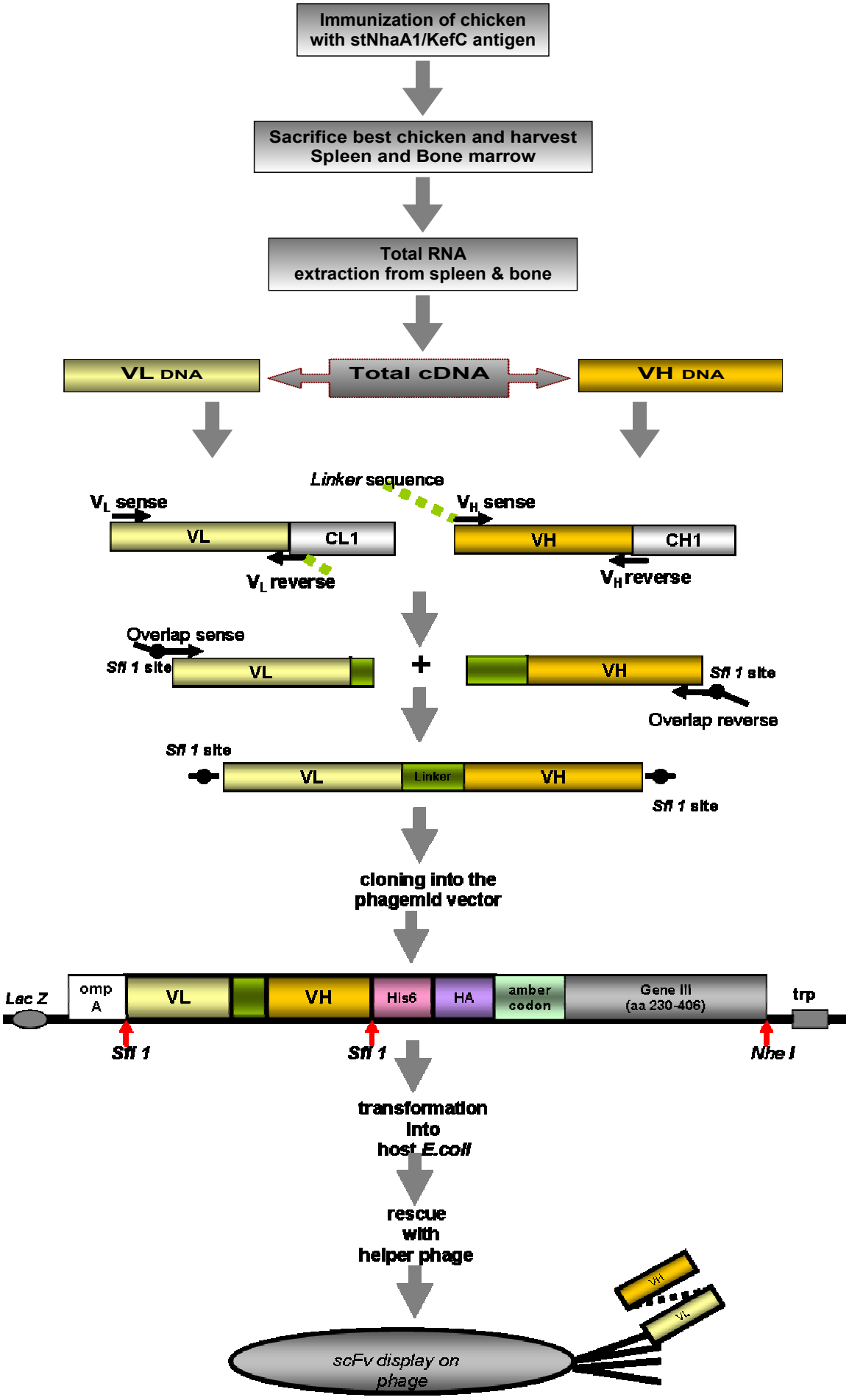

Immunization of chicken with stNhaA1/KefC antigen
Sacrifice best chicken and harvest Spleen and Bone marrow
Total RNA extraction from spleen & bone
VL DNA
Total cDNA
VH DNA
Linker sequence
VL sense
VH sense
VL
CL1
VH
CH1
VL reverse
VH reverse
Overlap sense
Sfi 1 site
VL
VH
Sfi 1 site
Overlap reverse
Sfi 1 site
VL
Linker
VH
Sfi 1 site
cloning into the phagemid vector
Lac Z
omp A
VL
VH
His6
HA
amber codon
Gene III (aa 230-406)
trp
Sfi 1
Sfi 1
Nhe I
transformation into host E.coli
rescue with helper phage
VH
VL
scFv display on phage

Figure 31: Outline for construction of phage displayed antibody-fragment library. The total RNA harvested from spleen and bone marrow of the immunized chicken is converted into cDNA by RT-PCR. Heavy and light chain variable gene pools are obtained from this cDNA by PCR using antibody gene specific primers. An overlap PCR is used to connect the V_H and V_L genes into the scFv format. The so obtained scFv DNA library is ligated into the phagemid (pComb3x) vector and transformed into host XL1-blue *E. coli* cells. Upon superinfection by helper phage, these host cells release the phagemid particles that carry the scFv-gene inside them, while display the scFv-antibody fragment on their surface.

2.6.3 Synthesis of V_H and V_L antibody genes by PCR

The antibody variable region genes were PCR-amplified with V_H and V_L gene specific primers, using the cDNA as template (section 4.2.7). The forward primers specific for the heavy chain V_H DNA were used to introduce sequences that code for the flexible linker region of the expressed scFv protein molecule. Two types of heavy chains were synthesized using two types of forward primers, SL-V_H: with a short linker, and LL-V_H: with a long linker, while only one type of light chains V_L was produced (Andris-Widhopf *et al.,* 2001).

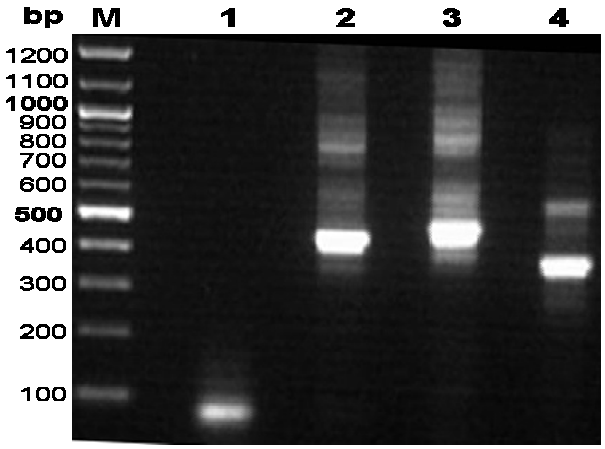


Figure 32: PCR synthesis of heavy (V_H) and light (V_L) chain genes. The figure shows the agarose electrophoresis profile of PCR amplified antibody gene products. Lane 1: control reaction in which negative control RT-PCR sample was used as the template, Lane 2: PCR amplified heavy chain gene with short linker (SL-V_H), Lane 3: same with long linker (LL-V_H), and Lane 4: amplified light chain gene segment (V_L). As expected no product was reported in the negative control, while the products of proper size (between 350~400 bp) were observed in other reactions. Each lane above corresponds to 4µl (out of 50 µl reaction) sample volume loaded on the gel.

The quality of the PCR was analyzed by agarose gel electrophoresis. PCR products of expected size for SL-V_H (~400 bp), LL-V_H (~450 bp) and V_L (~370 bp) were generated (Fig. 32). These gene products were synthesized only when cDNA was present in the template RT-PCR mixture (lane 2, 3 and 4). In the control reaction, in which negative control RT-PCR reaction mixture was used as template along with heavy chain specific primers, no amplification product (~V_H) was seen (lane 1). This was to verify that synthesized V_H and V_L gene products in lanes 2, 3 and 4 are not amplified from any DNA contamination, but from the corresponding cDNA made during the RT-PCR reaction. Once the proper size and quality of each chain was verified, the remaining PCR reactions were pooled together for each type of

chain, resolved on preparatory scale 2% Agarose gel electrophoresis, and purified from the gel slices. The yield of purified DNA determined by spectrophotometeric (OD_{260}) analysis was ~ 5 µg for each heavy and light chain.

2.6.4 Synthesis of scFv DNA libraries (SL-scFv and LL-scFv) by overlap-PCR

Overlap-PCR was performed to physically link the V_L and V_H chain DNA into scFv (Horton *et al.,* 1989). In addition of joining V_H and V_L chain genes, the overlap PCR was designed to introduce dissimilar *Sfi1* restriction sites flanking the ends of scFv (section 4.2.8). Upon digestion by *Sfi1*, two slightly different single strand overhang regions were generated that help in the directional cloning of such scFv inserts in pComb3x phagemid vector (Andris-Widhopf *et al.,* 2000). For the synthesis of the scFv DNA library, 1µg each from V_H and V_L DNA (gel purified) was used as template. Such high amounts of template DNA were used to preserve the diversity of the libraries so that every possible antibody gene was represented in the library. The quality of overlap-PCR products was analyzed on analytical agarose gel electrophoresis (Fig. 33).

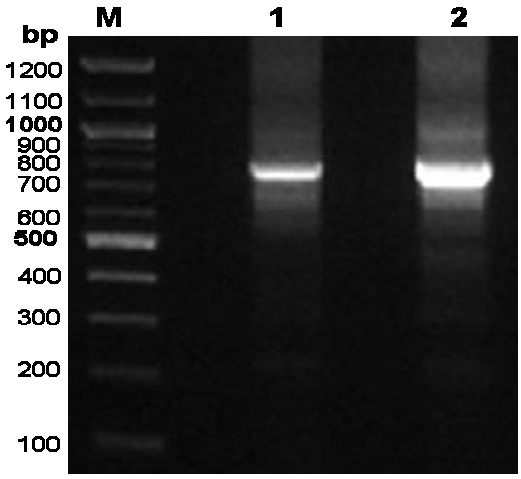


Figure 33: PCR synthesis of single-chain variable fragment (scFv) antibody fragment genes. The figure shows the electrophoresis profile of overlap-PCR amplified scFv DNA on 2% Agarose gel (run in 1x TAE buffer). Lane 1: sample from single chain variable fragment DNA with short linker (SL-scFv) and Lane 2: shows scFv DNA with long linker (LL-scFv). Both the lanes indicate the presence of amplification products of proper size (~750bp). Each lane above corresponds to 5µl (from of 100 µl reaction) loaded on the gel.

Once the proper size and quality of each scFv DNA was verified, the remaining PCR reactions were pooled together, resolved on preparatory scale agarose gel electrophoresis, and purified from the gel slices. Total purified yield of 20 µgs for SL-scFv and 29 µgs for LL-scFv was obtained.

2.6.5 Production of pComb3xSS phagemid vector DNA

For the construction of the phage display scFv library, the pComb3x phagemid vector system was used. This vector carries the ampicillin resistance gene for selection of transformants, and two different *Sfi1*-restriction endonuclease sites for directional cloning of scFv inserts (Andris-Widhopf *et al.,* 2000). The vector also carries genes for an intrinsic hemagglutinin (HA) tag (Wilson *et al.,* 1984) for immuno-detection, and for a 6x histidine tag for IMAC purification of soluble scFv proteins (Skerra *et al.,* 1991).

The pComb3x vector was produced in *E. coli* strain K12 ER2925. The *E. coli* cells were infected with pComb3xSS phages. A single cell culture of phage infected ER2925 was grown, and the phagemid vector DNA (pComb3xSS) was isolated from the harvested cells (section 4.2.3). A yield of 175 µg purified vector DNA was obtained from 300 ml of culture. The quality of the isolated vector DNA was assessed by *Sfi1* restriction analysis and by subsequent agarose gel electrophoresis (Fig. 34).

2.6.6 Quality control and preparation of vector and library DNA for cloning

For preparation of the vector pComb3xSS and scFv-library DNA, preparatory scale *Sfi1* restriction digestion was performed. Three parallel restriction digestion reactions, one each for vector, SL-scFv and LL-scFv DNA were performed (section 4.2.11).

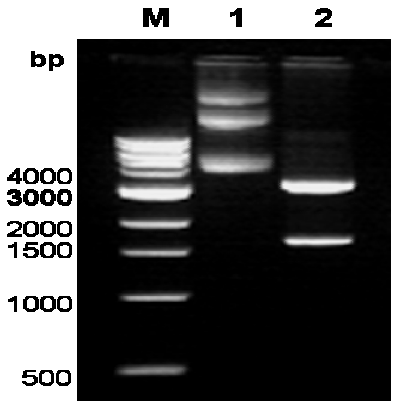


Figure 34: Restriction digestion analysis of phagemid vector DNA. The figure shows the electrophoresis profile of *Sfi*1-digested pComb3xSS vector DNA on 1.2% agarose gel. The vector contains a 1.6 kbp stuffer fragment (SS) flanked on both sides by two *Sfi* 1 restriction sites. Lane 1: no enzyme control showing uncut pComb3xSS DNA. Lane 2: shows same after digestion with *Sfi1*. The digested reaction shows two bands, the upper band corresponds to empty vector (~3.2 kbp) and the lower band corresponds to the dropped insert SS fragment (~1.6 kbp).

The quality of restriction digestion was analyzed by agarose gel electrophoresis. Two bands were seen at ~3.2kb corresponding to empty vector pComb3x, and at ~1.6 kb corresponding to the dropped stuffer fragment (SS) insert (Fig. 34). The presences of only two bands indicate the complete digestion of the pComb3xSS DNA.

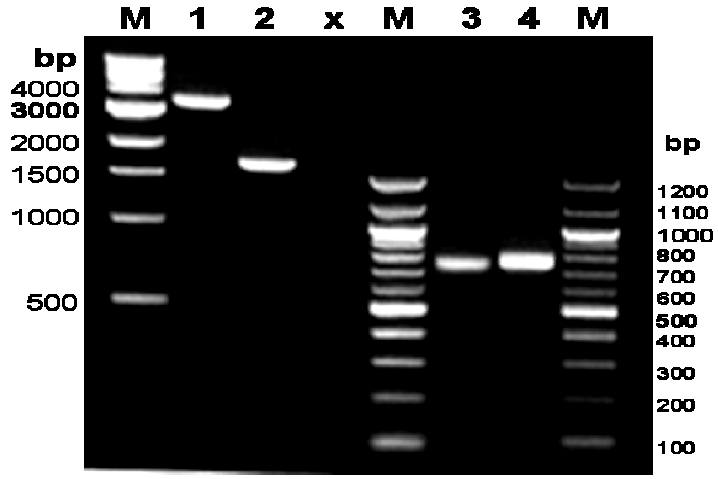


Figure 35: Quality control of *Sfi* 1 digested vector and insert DNA before ligation reaction. The figure shows the agarose gel electrophoresis of *Sfi* 1 digested and gel purified vector DNA pComb3x and SL-scFv and LL-scFv library DNA for comparative quantification with known amounts of maker DNA bands. Each of the lanes contains 5µl of respective purified *Sfi* 1-digested DNA samples. Lane 1: empty vector (~3.2 kbp), Lane 2: stuffer fragment (SS), and Lanes 3 and 4 show the respective samples from SL and LL scFv DNA library. For approximate quantification of DNA in respective lanes, the 1000 and 500 bp maker bands (extreme right lane) correspond to 95 and 97 ng of DNA respectively. And the marker band of 3000 bp (extreme left lane) corresponds to 125 ng of DNA.

Once the quality of restriction digestion was verified, the reaction mixtures were ethanol precipitated, resolved on preparatory scale agarose gel electrophoresis and purified from excised gel slices. Based on OD_{260} estimation, the total yields of *Sfi1*-digested purified DNA were estimated as; 7.5µg for SL-scFv, 9µg for LL-scFv, 8µg for pComb3x, and 3.6µg for stuffer fragment (SS). The DNA estimations obtained from comparison with NEB DNA-ladder in agarose gel electrophoresis (Fig. 35) are slightly lower and were considered for ligation and transformation experiments (table 3).

Table: 3 Comparative estimation of DNA yield

***Sfi*1-digested (gel purified) DNA**	**OD_{260} quantification**	**estimation from gel**
1. scFv (SL)	25 ng/µl	~ 12 ng/µl
2. scFv (LL)	30 ng/µl	~ 20 ng/µl
3. pComb3x (empty vector)	20 ng/µl	~ 17 ng/µl
4. Stuffer fragment (SS)	18 ng/µl	~ 17 ng/µl

2.6.7 Generation of pComb3x phagemid-scFv libraries

Two types of scFv-DNA libraries were generated by the respective ligation of *Sfi1*-digested SL-scFv and LL-scFv library DNA with the *Sfi1*-digested pComb3x phagemid DNA. The quality of an antibody library is influenced by various factors involved in its construction. One of these factors namely the size of the library depends upon the transformation efficiency and the quality of ligated library DNA. The ligation conditions were optimized, the ligation with T_4 DNA ligase resulted in good transformation efficiency (section 4.2.11). 5 x 10^7 transformants per µg of vector DNA for scFv-LL library and 1 x 10^8 for positive control stuffer fragment (SS) ligation. The negative control obtained from ligation vector without insert was limited to 1.7 x10^6, which is equal to 3.4% of the scFv-LL library. A background of ≤ 5% is considered as a standard for a good library construction (Andris-Widhopf *et al.,* 2001).

The second critical step during the construction of antibody library is the introduction of library vector DNA into the host *E. coli* cells. This step of library transformation very often puts the limitations on the size of a library. The transformation efficiency depends both upon the quality of ligated library DNA, as well as on quality of the competent host *E. coli* cells. Electroporation is the method of choice for large scale library transformation, as highest numbers of transformants are obtained by it. Electrocompetent XL1-blue cells were prepared in laboratory following a modified protocol (section 4.3.1). The library DNA was transformed (section 4.3.2.). Briefly, for each SL-scFv and LL-scFv library, 300µl of electrocompetent XL1-blue were transformed by electroporation with 10 µl of ligated library DNA (~ 700ng of vector DNA). The transformed cells were plated and incubated overnight. Next day the libraries were harvested, and stored as 1ml aliquots at -80^{o}C in 25% glycerol media.

Library size is determined as the total number of transformants i.e. XL1-Blue colonies obtained after library transformation. A library size of 9.1 x 10^7 was obtained for SL-scFv library, and 1.2 x 10^8 for the LL-scFv library. The library size of larger than 1 x 10^7 is considered sufficient for an immune antibody library, whereas for a naïve library the size of 1 x 10^{10} is required (Andris-Widhopf *et al.,* 2001). Based on the acquired size, both of the anti-NhaA1/anti-KefC chicken SL-scFv and LL-scFv libraries were considered of good quality, and panning experiments were started to select scFv *binders* for STNhaA.

2.7 Selection from immunized-chicken antibody library

2.7.1 Anti-STNhaA panning of SL and LL-scFv phage antibody libraries

To select anti-STNhaA scFv, both the SL and LL-scFv library versions were used. The phage-scFvs from the libraries were rescued and packaged with helper phage, and applied for anti-STNhaA panning (section 4.4.6). Three rounds of bio-panning were performed against STNhaA immobilized on microtiter plates (MaxiSorp^{TM}). A double recognition step was carried out after third round of selection. This was done by neutralizing the pH of third round pH eluted phage and applying them for fresh selection immediately. The phage obtained after such a double recognition step were considered as 4th round selected phage.

Table 4: Summary of anti-STNhaA panning of chicken libraries

Panning Round	Total input Phage (TU)	Total output Phage (TU)	Ratio of output/input titer x10^{-6}	No. of washing steps	Total duration of washing (minutes)
1st	$\geq 2x10^{10}$ for SL	$\geq 7x10^{4}$ for SL	3.5	7	30
	$\approx 2x10^{10}$ for LL	$\approx 9x10^{4}$ for LL	4.5		
2nd	$\approx 2x10^{11}$ for SL	$\approx 1x10^{8}$ for SL	500	13	45
	$\approx 2x10^{11}$ for LL	$\approx 1x10^{8}$ for LL	500		
3rd	ND	$\geq 2x10^{8}$ for SL	ND	19	45
	ND	$\geq 2x10^{8}$ for LL	ND		
4th	$\approx 1x10^{8}$ for SL	$\approx 1x10^{6}$ for SL	10000	10	30
	$\approx 1x10^{8}$ for LL	$\approx 1x10^{6}$ for LL	10000		

(SL and LL represent the respective scFv libraries with short and long linker region).

2.7.2 Enrichment of target-binding phage-scFv population

The first indication of the enrichment of specific binding phage-scFv population across the panning rounds was obtained by observing the increased ratio of output verses input phage-scFv titer in the second and later panning rounds (table 4).

2.7.3 Analysis of target-specific enrichment by polyclonal ELISA

In order to check for the anti-NhaA1 enrichment of phage-scFv population across the panning rounds, polyclonal phage-scFv and soluble scFv ELISA were performed. For phage-scFv ELISA, cleared culture supernatants obtained after respective selection rounds were tested for antigen binding. Whereas for the soluble-scFv ELISA, the supernatants obtained after the removal of phage by PEG/NaCl precipitation were used. The bound phage-scFvs were detected by anti-M13 antibody, whereas the bound soluble-scFvs were detected by their C-terminal 6xHis (histidine) or HA (hemagglutinin) tags using anti-His or anti-HA secondary antibodies (section 4.4.8 and 4.4.9).

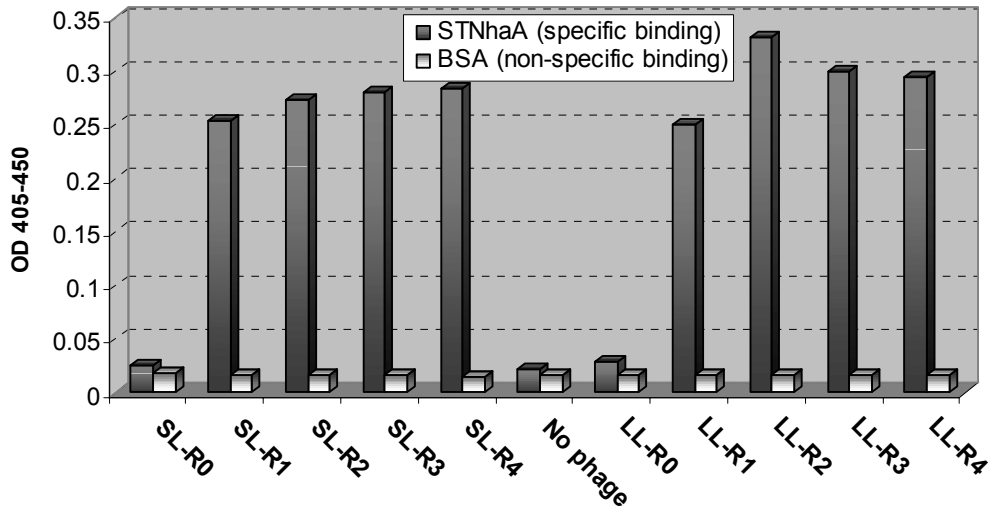


Figure 36: Enrichment of *binders* examined by polyclonal phage ELISA. The target antigen-specific enrichment of *binders* across the selection rounds is demonstrated. Briefly, polyclonal phage-scFvs from each round of selection were analyzed for specific binding to STNhaA and to BSA as background binding. Cleared supernatants from overnight amplifications of phage-scFv were used as the primary antibody. Antigen-bound phages were detected with anti-M13 antibody. SL and LL represent the respective short linker and long linker scFv antibody phage libraries, R0 – R4 demonstrate the respective biopanning rounds. 2xTY culture media was used as negative control (no phage) binding.

Polyclonal soluble-scFv ELISA provided the more relevant information about the antigen-specific enrichment across the panning rounds (Fig. 36), as it represents the enrichment of clones that are producing stable scFvs. Target-specific anti-STNhaA enrichment was observed in both forms of polyclonal ELISA experiments, i.e. phage-scFv as well as in soluble-scFv ELISA.

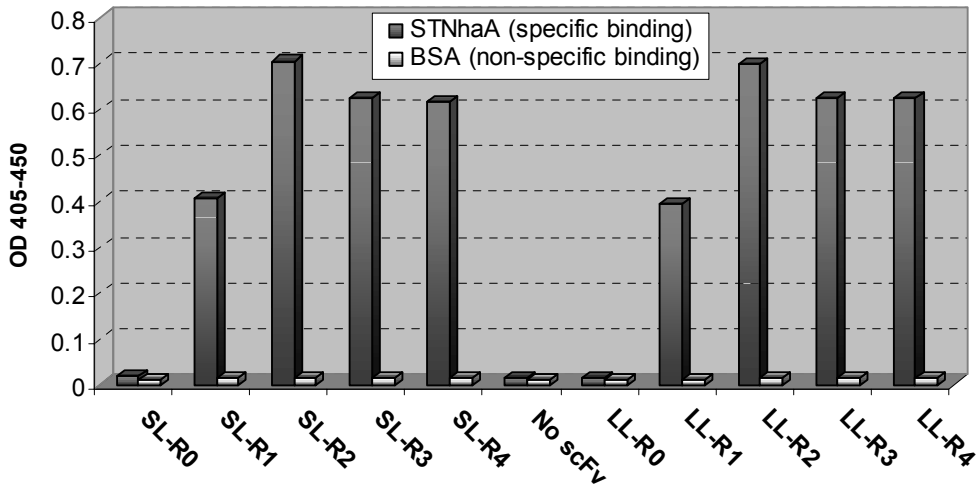
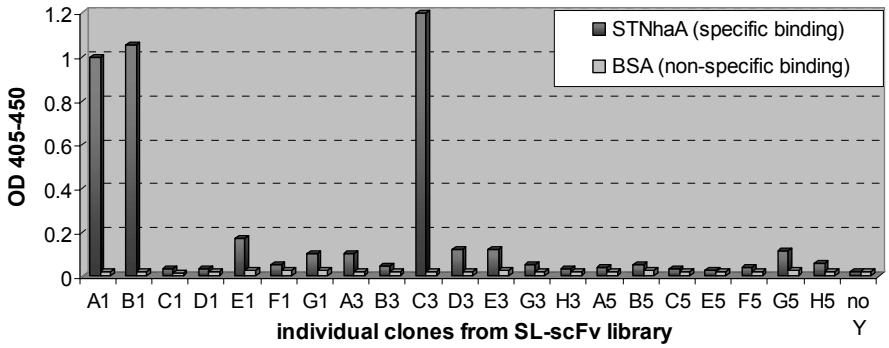


Figure 37: Enrichment of *binders* examined by polyclonal soluble-scFv ELISA. Here, the antigen bound scFv were detected via their C-terminal HA-tag using mouse anti-HA tag antibody (clone HA-7), followed by alkaline phosphatase conjugated rabbit anti-mouse antibody. R0 – R4 represent the respective bio-panning rounds and SL and LL represent the respective short linker and long linker scFv antibody libraries. Both the libraries reach the saturation of antigen specific enrichment by 2nd round of bio-panning.

In contrast to the results with the naïve libraries, the enrichment across the panning rounds was highly antigen-specific signal without enrichment of unspecific background signal. As observed from the polyclonal ELISA results (Fig. 36 and 37), the enrichment reached saturation at the 2nd round of panning indicated by the increased ELISA signals. The enrichment across the panning rounds was observed with both of the SL and LL-scFv antibody libraries.

2.7.4 Selection of antigen-specific monoclonal *binders*

In order to isolate high affinity monoclonal *binders* expressing stable scFv, second and third round selected and eluted phages were infected into non-suppressor strain of *E. coli* (HB2151), and serial dilutions plated to obtain single colonies (section 4.4.10).. Twenty one single colonies, representing the monoclonals from each SL and LL-scFv library were randomly picked and tested for anti-STNhaA binding with the monoclonal ELISA (section 4.4.12). Single colony cleared culture supernatants containing the scFv antibody fragments of the respective isolated scFv clone were used to test the binding against immobilized STNhaA. The target specific *binders* were identified by comparative analysis of specific (anti-STNhaA) and unspecific (anti-BSA) binding signals. The clones which showed specific binding signal ≥10 times stronger than the unspecific signal were considered as positive clones (Fig. 38).



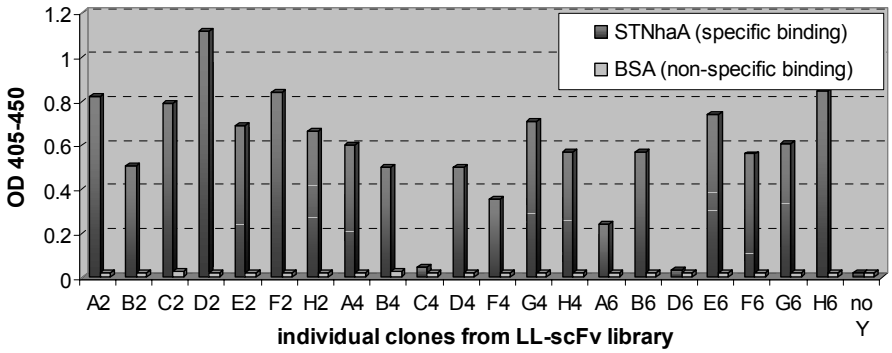


Figure 38: Identification of STNhaA specific *binders* by monoclonal ELISA. Single clones randomly picked from 2nd and 3rd rounds of anti-STNhaA of selection were grown (in HB2151 host *E. coli*) and induced for scFv production (section 4.4.11). Overnight culture supernatants containing soluble scFvs from each representative clone were tested for binding against STNhaA (*specific signal*) and against BSA (*non-specific signal*) (section 4.4.12). The bound scFvs were detected in a two step process with mouse anti-HA tag antibody (clone HA-7, sigma), followed by alkaline phosphatase conjugated rabbit anti-mouse antibody (sigma). For (no-scFv) negative control 2xTY liquid media was used. The clones with the specific OD ten times greater then the non-specific signals were considered as potential positive *binders*.

Out of 21 randomly picked clones from each SL-scFv and LL-scFv library, 19 clones from LL-scFv library showed target specific signals, while from the SL-scFv library fewer clones (i.e. A1, B1 and C3) showed such relevant binding signals. Further characterization of these selected anti-STNhaA ELISA positive clones was performed by DNA-sequence analysis, cross-reactive binding ELISA, and also by checking their ability to bind denatured STNhaA in western blot experiments.

2.7.5 Characterization of selected clones by cross-reactive ELISA

Anti-STNhaA selected scFv clones were analyzed for cross-reactivity, binding in respect to completely unrelated proteins such as BSA or to a closely related homologue i.e. NhaA from *E. coli*, and to an unrelated His-Tagged protein i.e. β_2AR_{His10}. The latter was to rule out that any of the scFvs are binding to the terminal His tag, as His-tagged antigenic proteins were used for immunization. None of the selected clones showed any cross-reactive binding to unrelated proteins such as BSA or β_2AR_{His10} as analyzed by ELISA (data not shown). An interesting observation is that all anti-STNhaA monoclonals except one, are binding to NhaA of *E. col*. NhaA from *Salmonella* species and *E. coli* show a high degree of homology and little variation in their amino acid sequence and composition (≥ 93% similarity). The only exceptional clone was the clone B2-scFv, which binds only to the *Salmonella* counterpart and not to its *E. coli* homologue. The *Salmonella*-specific scFv binds likely on region of STNhaA that is not present on its *E. coli* counterpart.

2.7.6 Characterization by insert-check PCR

Prior to DNA-sequencing of scFv inserts, PCR analysis was performed to verify the presence of full-length scFv insert in isolated phagemid DNA samples (section 4.2.9). The dual aim of the process was to find the percentage of clones that carry the complete scFv insert and to save time and resources, otherwise spend on sequencing of undesired clones. PCR amplification resulted in a ~ 950 bp size band only when complete scFv (~ 750bp) insert is present (Fig. 39).

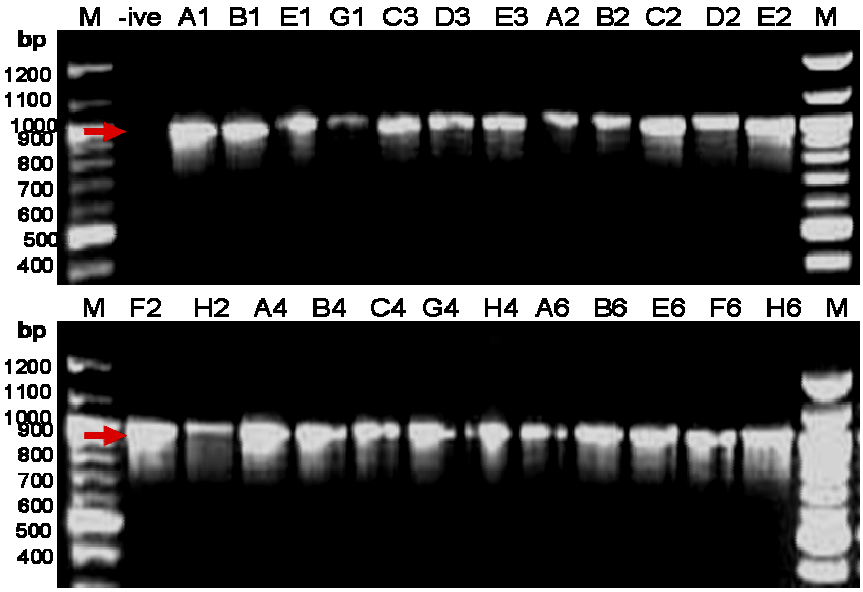


Figure 39: Insert-check PCR analysis of chicken library selected anti-STNhaA clones. The figure shows the agarose gel electrophoresis insert-check PCR samples. To check the presence of scFv-insert, PCR was performed with purified phagemid DNA as template using flanking region primers (section 4.2.9). The amplification of ~950 bp product (red arrow) indicates the presence of complete scFv-insert. A1, B1, E1… and so represent the respective anti-STNhaA clones. Each lane corresponds to 7µl /lane (out of 50 µl reaction) loaded on the gel. No template DNA was added in the –ive control.

As observed from agarose gel analysis, 100% of the selected clones carried the complete scFv inserts. The high percentage of clones carrying full length scFv inserts highlights the stability of the library format compared to the previous experiments with ETH-2 and Tomlinson I+J scFv libraries, in which partial or complete loss of scFv inserts in the clones was often observed.

2.7.7 Sequence analysis of selected clones

Once the anti-STNhaA selected clones were verified for the presence of complete scFv DNA insert, the respective phagemid-scFv DNA samples of 24 representative clones were sequenced (section 4.2.10). All sequences confirmed the presence of full-length scFv inserts. All clones carried proper scFv-inserts with complete V_H and V_L chain gene segments. The light and heavy chain gene segments were connected either by short or by long linker sequences corresponding to the scFv library (SL or LL) the clones had been selected from.

Amino acid sequences were compared to identify common and unique features of the anti-STNhaA clones. Amino acid sequences for respective selected-scFv were derived from the corresponding DNA sequences.

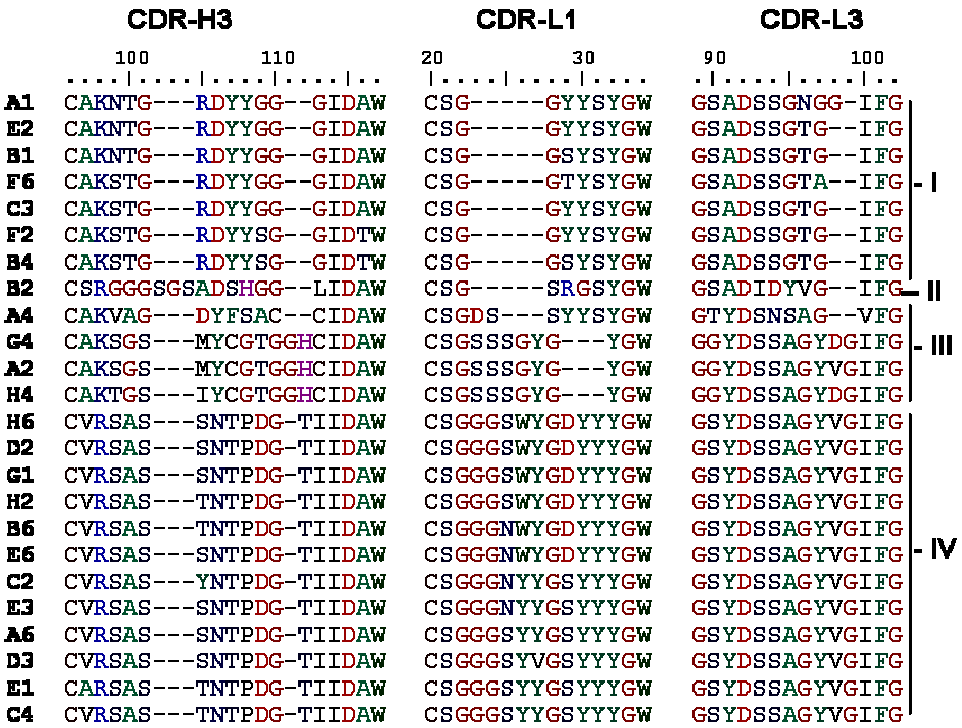


Figure 40: Amino acid sequence alignment representation. The sequence-alignment profile of representative CDR regions is shown here. The scFv DNA-sequence for each clone was obtained by HotShot sequencing. Based on sequence alignment of CDR3 from heavy chain, and CDR 1 and 3 from light chain, the selected clones could be categorized in four separate groups (I-IV). The numbering on the left represents the respective anti-STNhaA clone from chicken SL-scFv or LL-scFv library (The clones that end with even digit i.e. C2, C4, B6 etc are from LL-scFv library, while the ones that end with odd digit i.e. C1, E1, D3 etc were selected from SL-scFv library. The sequences were aligned by "*BioEdit Sequence Alignment Editor*" software program.

It was observed that the anti-STNhaA panning and selection had resulted in the enrichment of unique scFv clones. The 24 sequenced scFv clones could be grouped on the basis of similar CDR sequences in four groups (I-IV). Each group is represented by a unique set of enriched CDR (H3, L3 and L1) sequences (Fig. 40).

2.7.8 Identification of scFv binding to linear epitopes

Antibodies that are western blot positive i.e. binding to the denatured target protein of SDS-PAGE in Western-blot analysis, most likely bind to linear continuous epitopes on their target. These linear epitopes can be part of the flexible termini or loop-regions of a membrane protein, or may be only presented when the protein is denatured. For both of these cases, such antibodies are considered not suitable for co-crystallization of membrane proteins as flexible domains are created (Hunte and Michel 2002). To identify such candidates from the anti-STNhaA selected chicken scFvs, a series of Western-blots was performed, each probed by an individual scFv culture supernatant. The binding of representative clones from each group (CRD-H3 sequence based groups in (Fig. 40) was probed against denatured STNhaA on

Western-blots. It was observed that all clones from group-IV are western blot positive, whereas members of group I, II, and III are Western-blot negative. In total, out of 24 anti-STNhaA selected ELISA positive clones, 12 are western blot positive.

Figure 41 shows an example of the results: (B) represents a blot probed with a typical Western-blot negative scFv (A2-scFv), whereas (C) represents the blot probed with a typical Western-blot positive candidate (B6-scFv).

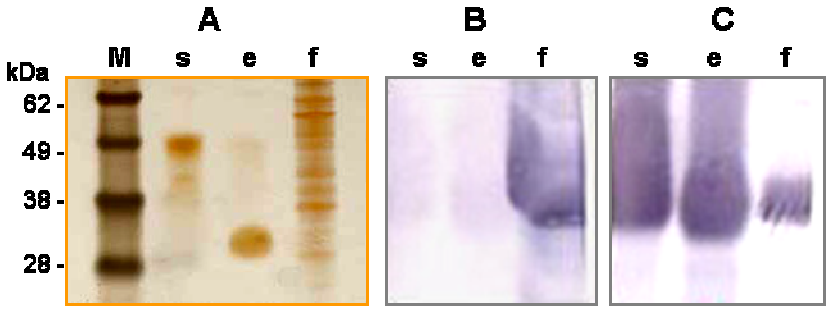


Figure 41: Identification of Western-blot positive STNhaA scFv clones. 3µg/lane of purified protein were resolved on SDS-PAGE and subsequently transferred on PVDF membrane. **A.** silver-stained SDS-PAGE gel partial transfer of proteins, **B** and **C.** Western-blots probed with respective scFv crude supernatants. In the figures **lane s:** *Salmonella* STNhaA, **lane e:** *E. coli* NhaA, **lane f:** positive control for HA-tag, i.e. crude scFv supernatant (40µl acetone concentrated) of respective clone that were used for binding. The bound scFv were detected with anti-HA tag antibody, which in turn was probed with anti-mouse alkaline phosphatase conjugate antibody.

2.7.9 Mapping of linear epitopes for scFv

The exact region on the target protein, to which an antibody binds can be located rather easily if the antibody in question binds a linear epitope. This information of the epitope of a given antibody is often beneficial for biochemical or structural characterization of a target protein. Antibodies recognizing native and linear epitopes of *E. coli* NhaA were produced by hybridoma technology, and the epitope was mapped for one of the selected antibodies (Venturi *et al.,* 2000).

In the current study, epitopes on STNhaA were mapped, that are recognized by the Western-blot positive scFvs generated from chicken libraries. A ready to use peptide microarray (Replitope^{TM} Microarray) derived from STNhaA peptide sequence, was custom-made by a commercial supplier. The peptide array consisted of 98 peptides which are 15 residues long, synthesized with 11 residue overlap, spotted on a microscopic glass slides. This microarray represents the complete amino acid sequence of STNhaA. These peptide microarrays were probed with two (C4 and H6) of the Western-blot positive scFv clones. It should be kept in mind that H6 and C4 scFv are the first and last members of the group-IV (Fig. 40).

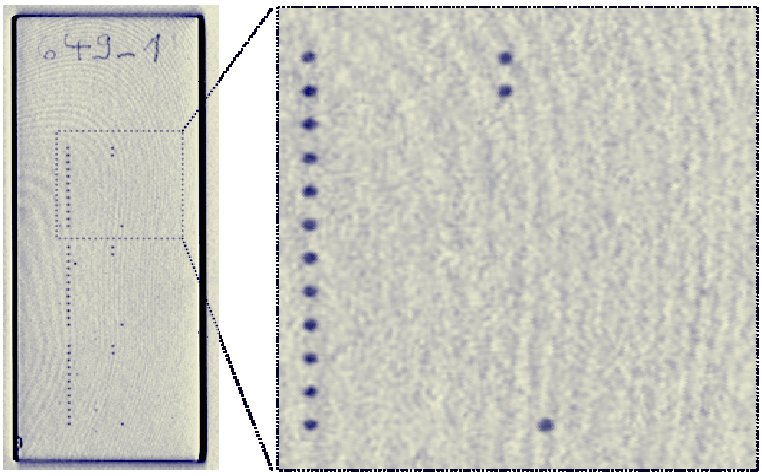


Figure 42: Epitope mapping for representative Western-blot positive clones. The figure shows the STNhaA Replitope™ Microarray glass slide 649-1 probed with a western blot positive chicken library clone H6-scFv (section 4.6.6). In figure the peptide microarray is present in triplets. The left lane of blue spots correspond to the positive control HA tag linear peptide. The other three positive spots present at position 59, 60 and 61 correspond to peptide sequences VGFFIPLKEKHGRSP, IPLKEKHGRSPAKRL and EKHGRSPAKRLEHVL respectively. These three peptides contain a seven amino acid common sequence corresponding to EKHGRSP as a target epitope sequence of H6-scFv.

Both the scFvs resulted in the identification of three positive peptide spots at position 59, 60 and 61 (Fig. 42). It was an indication that both these scFvs (and therefore all members of group-IV) bind to the same linear epitope. The peptide sequences corresponding to these positive spots were deciphered, and the epitope was identified as a common 7 amino acid sequence corresponding to 'EKHGRSP'. This epitope is present in the connecting loop of helix VIII and IX of STNhaA starting at position 241. To our knowledge, it is the first antibody against this epitope.

2.7.10 Analytical scale expression and purification of scFv proteins

Representative anti-STNhaA selected scFv (A1, E2, C3, F6, B4, A2, and B2) from different groups based on CDR sequence profile in figure-40 were produced in HB2151 *E. coli* cells (section 4.5.1). Briefly, each clone was grown in 250 ml cultures and induced overnight with IPTG. The cells were harvested and periplasmic extracts were obtained. The scFvs were purified via their C-terminal 6xHis tags using Ni-NTA agarose resin. The quality of purified scFv proteins was analyzed on SDS-PAGE and by gel filtration chromatography. The purified protein was seen as a ~29kDa single band corresponding to the size of a scFv monomer. Most of the scFvs proteins were purified to homogeneity by the single IMAC purification step (Fig. 43).

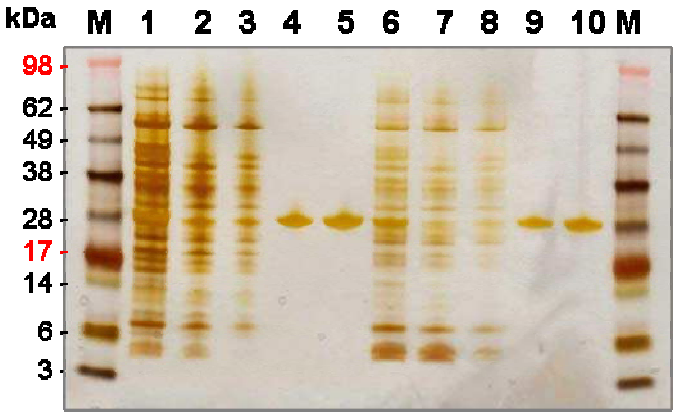


Figure 43: Purification of anti-STNhaA scFvs. The figure shows the silver stained SDS-PAGE gel. The $scFv_{HIS}$ proteins were purified from respective HB2151 *E. coli* periplasmic extracts by IMAC (section 4.5.4). Briefly, 5ml dialyzed periplasmic preparations were mixed with 2ml of Ni-NTA agarose resin and incubated for 2 hours at 4°C. The slurry was packed in analytical columns. The unbound protein was washed away with 50mM imidazol buffer. Bound protein was eluted with 250mM imidazol. The samples from respective fractions were analyzed. (Lanes 1-5 belong to **F6**-scFv and 6-10 to **B4**-scFv). **Lane 1 and 6**: show the respective periplasmic extract (35µls out of 5ml loaded/lane), **Lanes 2 and 7**: flow through (5µls/5ml), **Lanes 3 and 8**: washing fractions (35µls/5ml), **Lane 4**: eluted fraction of F6-scFv (~ 20µg protein) and **Lane 9**: eluted fraction of B4-scFv (~10µg protein). **Lanes 5 and 10:** respective F6 and B4 scFv purified samples after 1 week storage at 4°C.

In a second experimental setup, three different clones (B6, H6 and C4) were purified from their respective periplasmic extracts using high binding capacity His-Trap columns. The protein samples from various purification steps were analyzed on SDS-PAGE. All the three scFv could be purified to single band, but different individual purification profiles in response to imidazol concentrations were observed (Fig. 44).

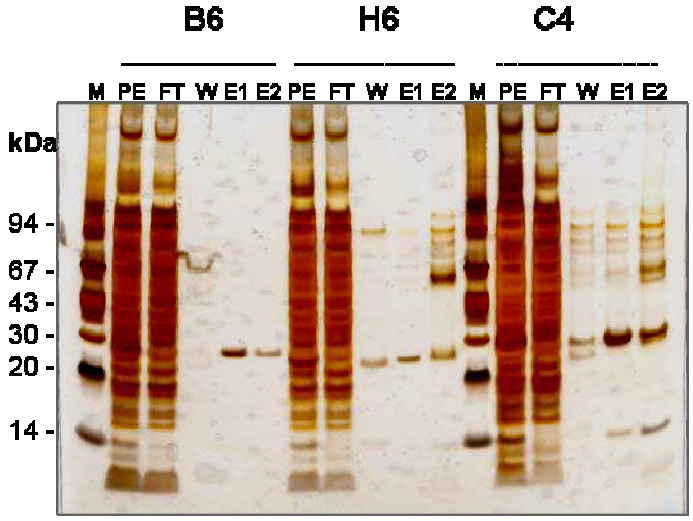


Figure 44: Comparative IMAC purification in 1ml His-Trap column. The silver stained SDS-PAGE gel showing the purification profile of B6, H6 and C4-scFv proteins from the periplasmic extracts. Gel lanes indicated by PE lanes show the samples from periplasmic extracts, FT lanes show the column flow-through containing unbound proteins in presence of 10mM imidazol. W lanes show washing with 90mM imidazol. And the lanes E1 and E2 show the respective 200mM and 500 mM elution fractions.

For example in case of B6-scFv, there was no loss of scFv protein in a washing step with 90mM imidazol, whereas the same was not true for H6 and C4 scFv clones. It was observed that not only the expression but also the purification profiles of different scFvs differ. One should keep in mind that all these scFvs are basically similar proteins with same basic structural features, and differ only in few selected amino acid residues mostly in CDR regions to which this difference can be attributed. These results indicate the need for the individual optimization of expression and purification protocols for each individual scFv.

The protein yield of the IMAC purified scFvs was determined by BCA. The overall yield per liter culture was different for individual clones ranging from 1.5 to 10 mg (table 5). Further optimization of individual scFv clones resulted in even higher amounts of purified scFv protein were obtained. For example, after optimization for F6-scFv, yields of 10 – 12 mg purified protein could be obtained per liter culture. The scFv protein obtained after analytical scale expression and purification were good enough in quality and sufficient in quantity to perform characterization experiments like gel-filtration binding assays.

Table 5: Protein Yield after single step IMAC (Ni-NTA agarose) purification

	Anti-STNhaA selected scFv clone	Library of origin	Yield of purified protein /L culture	Remarks
1.	A1-scFv	SL	nd	
2.	E2-scFv	LL	~ 2 mg	
3.	C3-scFv	SL	nd	Protein precipitating while concentrating
4.	F6-scFv	LL	~ 5 mg	≥ 10 mg after optimization*
5.	B4-scFv	LL	≥ 2 mg	
6.	A2-scFv	LL	~ 2 mg	
7.	B2-scFv	LL	≤ 2 mg	
8.	B6-scFv*	LL	~ 3.75 mg	
9.	H6-scFv*	LL	~ 2.25 mg	
10.	C4-scFv*	LL	~ 10 mg	

* His-Trap columns were used for IMAC purification.

2.7.11 Quality control of purified scFvs

IMAC purified anti-STNhaA scFv-proteins obtained from analytical scale expression were checked for stability maintaining the buffer conditions of the target protein STNhaA (20mM Bis-Tris Propane, 100mM NaCl, 0.02%LM, pH 7.25) to analyze their suitability for co-crystallization trials. Purified scFv-protein samples from the respective clones were loaded on Superose-6 gel-filtration column, and the elution profiles were observed. Following observations were made. Most of the scFvs analyzed were stable at 4^{o}C for more than a week under these conditions. All clones obtained from LL-scFv chicken library such as E2, B4, F6,

and B2 showed proper symmetrical elution peaks at a retention volume of 1.88 ml (expected for ~ 29 kDa monomeric scFv). Clone A2-scFv eluted at 2.2 ml (Fig. 45). Both the scFv clones tested from SL-scFv library, i.e. A1 and C3, were not stable under these buffer conditions. C3 was precipitating at 4°C after overnight storage. A3 shows the same behavior after 2 or 3 days of 4°C storage. Therefore, the latter two scFvs were not analyzed on gel-filtration column. It should be kept in mind that both of these clones are the scFvs with short linker regions, and thus are expected to be exclusively present in dimeric state. Screening of optimal buffer conditions for these scFvs is required. Based on this initial quality analysis, F6-scFv was chosen as the first candidate for large scale expression and subsequent co-crystallization trials.

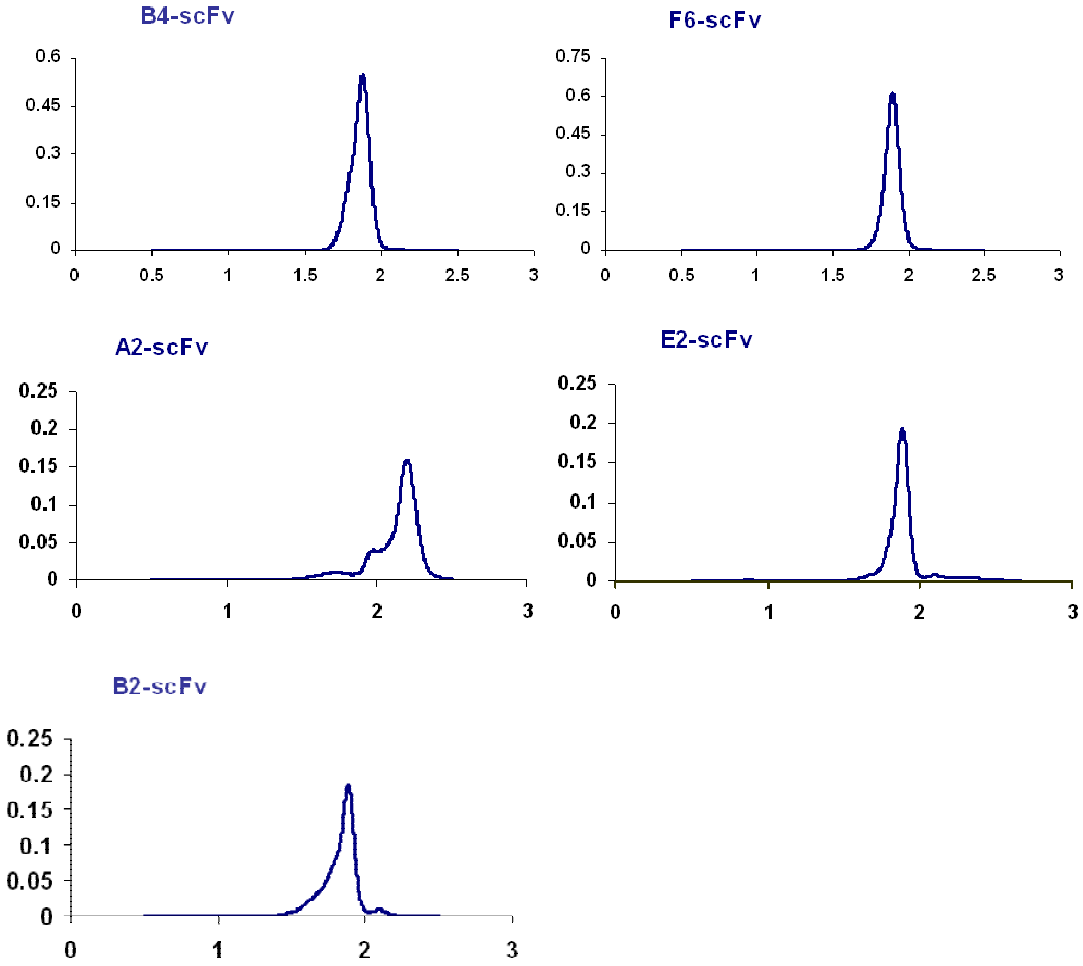


Figure 45: Quality control of IMAC purified representative scFv proteins. The analytical gel filtration profile (Superose-6 (PC3.0/ 3.2), SMART system) of representative anti-STNhaA selected chicken scFvs. ScFvs were obtained by single step IMAC purification. Filtered sample volumes of 50µl (20~50 µg) of purified scFv were resolved at 40µl/min. in 1xBTP running buffer. All the SMART runs were performed at 8°C temperature setup (x-axis in each figure represents the elution volume in ml, and y-axis is the relative absorbance at 280nm).

2.7.12 Gel filtration-binding analysis of representative scFvs

No data is available about the required affinities of antibodies suitable for the co-crystallization of membrane proteins (Hunte and Michel 2002). The criteria and type of quality control established by the workers is that the required antibody should form a complex with the membrane protein stable under gel-filtration conditions. Such co-complexes of a membrane proteins with their antibodies bound in stoichometric ratio were purified by size-exclusion chromatography, and directly used for co-crystallization experiments (Ostermeier *et al.,* 1995a, Hunte *et al.,* 2000).

To identify suitable anti-STNhaA scFvs selected from the chicken libraries, some of the representative scFvs where tested for binding in solution. Analytical scale IMAC-purified scFv samples were mixed with purified STNhaA to allow formation of the co-complex. This mixture was loaded on Superose-12 column (section 4.6.4). The elution profiles were recorded and compared with chromatographs of STNhaA and scFv alone. The binding of the antibodies was reported for F6-scFv as increase and shift (1.21ml to 1.18 ml) of STNhaA elution peak profile in response to presence of the antibody fragment (Fig. 46A).

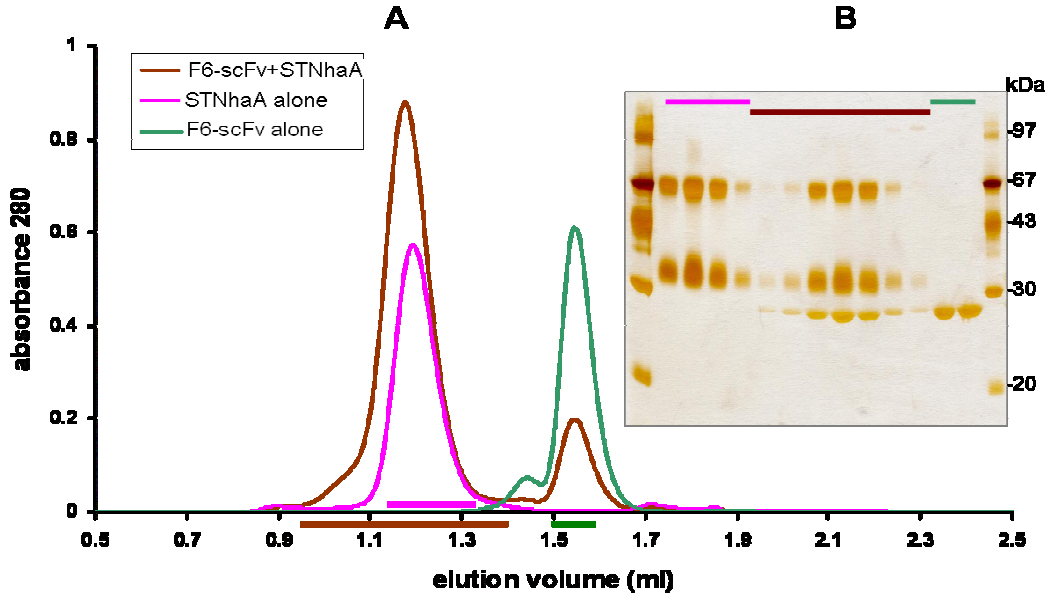


Figure 46: Gel filtration binding analysis of F6-scFv. Co-complex formation of representative anti-STNhaA selected chicken F6-scFv. IMAC purified F6-scFv (~60 µg) was mixed with IMAC purifies STNhaA (~60 µg) and incubated on ice for 50 minutes before loading on Superose-12 column (SMART system). A total sample volume of 50µl was injected. The column was run at 40µl/minute flow rate at ~ 8°C temperature in running buffer (200mM Bis-Tris Propane, 100mM NaCl, 0.02% LM, pH~7.25). For control runs STNhaA alone (~60 µg) and F6-scFv alone (~60 µg) were run separately under same conditions. **A.** The chromatographs showing gel-filtration elution profiles. The scFv-STNhaA co-complex elution profile is shown in brown. STNhaA alone in pink, and F6-scFv alone in green. **B.** Silver stained SDS-PAGE (NuPAGE 12% non-gradient, in 1x MOPS buffer) analysis of eluted peak fractions represented by the colored bars.

A similar peak shift was observed when STNhaA was incubated and loaded with B4-scFv (Fig. 47). To further verify the presence of co-complex, elution fractions from corresponding peaks were analyzed on high resolution SDS-PAGE. The later is required to separate the bands of STNhaA and scFv (Fig. 46B).

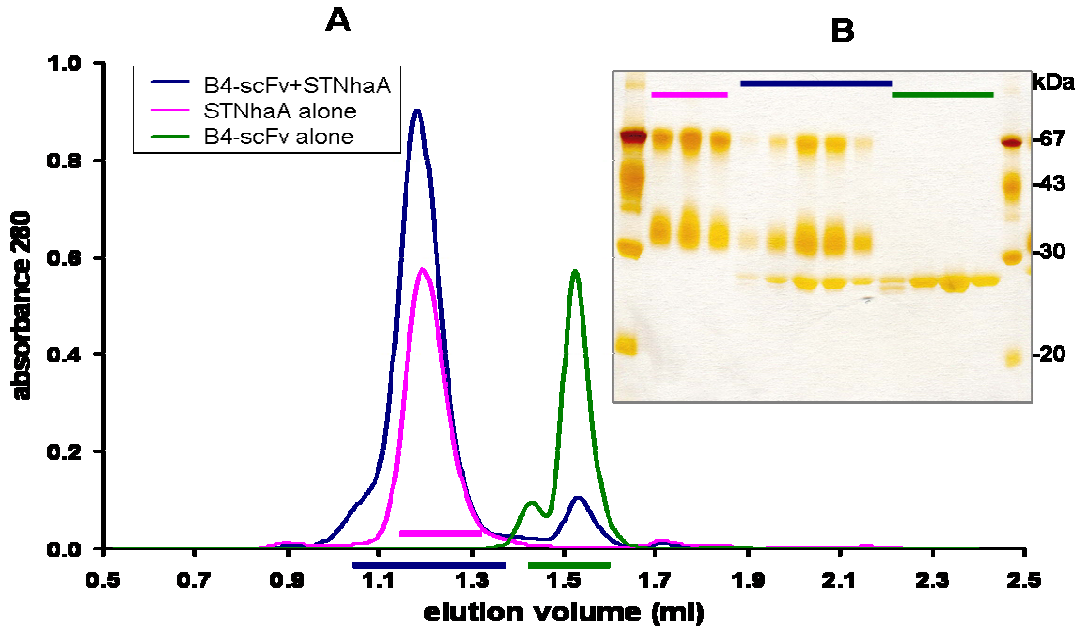


Figure 47: Gel filtration binding analysis of B4-scFv. Co-complex formation of representative anti-STNhaA selected chicken B4-scFv. IMAC purified B4-scFv (~60 µg) was mixed with IMAC purifies STNhaA (~60 µg) and incubated on ice for 50 minutes before loading on Superose-12 column. For control runs STNhaA alone (~60 µg) and B4-scFv alone (~60 µg) were run separately under same conditions. **A.** The chromatographs showing gel-filtration elution profiles. The scFv-STNhaA co-complex elution profile is shown in blue, STNhaA alone in pink and B4-scFv alone in green. **B.** Silver stained SDS-PAGE (NuPAGE 12% non-gradient, in 1x MOPS buffer) analysis of eluted peak fractions represented by the colored bars.

Both anti-STNhaA F6 and B4 -scFvs are binding to the target in solution and are forming highly stable co-complexes, indicating their suitability for co-crystallization experiments.

2.7.13 Surface plasmon resonance analysis

Surface plasmon resonance (SPR) experiments were performed to measure association (k_{on}) and dissociation (k_{off}) rate constants for selected anti-STNhaA chicken scFv with a Biacore-T100 system. These measurements were performed with the "kinetic-titration" approach (Karlsson *et al.*, 2006). The data was used to calculate the equilibrium dissociation constant (K_D) for the respective scFv clone. The experiments were performed with IMAC purified scFv protein samples. The protein concentration of these samples was determined by BCA method. Purified STNhaA was immobilized on a CM5 sensor chip by EDC/NHS chemistry, and 2000 to 2500 resonance units (RU) were obtained (section 4.6.5). Kinetic-

titration experiments were performed using a series of scFv concentrations from 0.1 to 62.5 nM (Fig. 48). The data obtained from the sensorgrams were kindly analyzed by Dr. Uwe Bierfreund and his colleagues (Biacore) to calculate the respective association and dissociation rate constants.

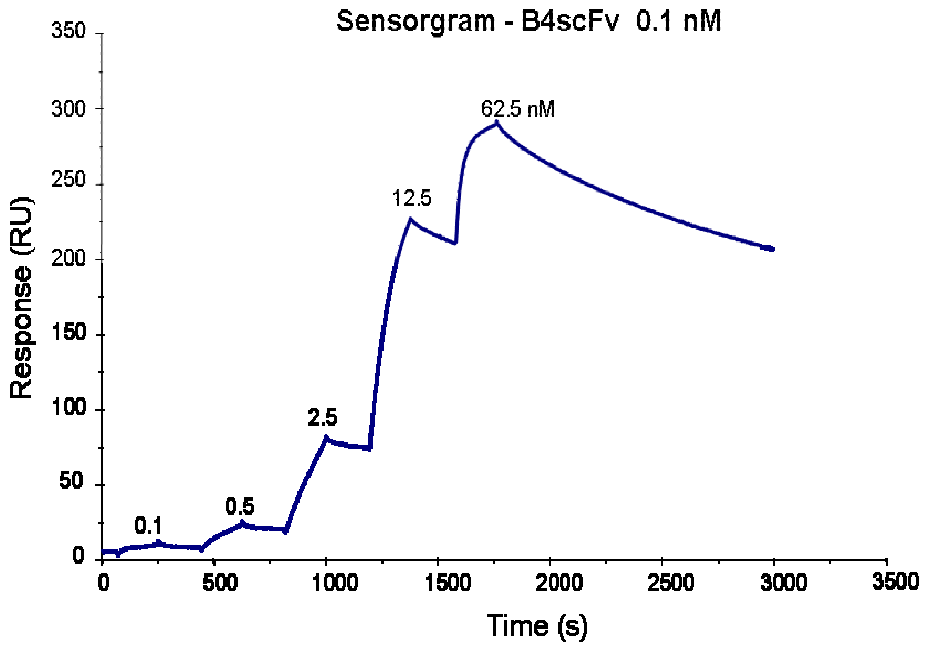


Figure 48: Kinetic-titration assay. The figure shows a typical sensorgram of kinetic titration simulated data. These data sets were obtained with B4-scFv concentrations of 0.1, 0.5, 2.5, 12.5 and 62.5 nM. In this experiment contact time of 180 seconds was used for both association and dissociation, while the dissociation phase for the last run was 20 minutes.

The equilibrium dissociation constant K_D for each scFv was calculated from the obtained k_{on} and k_{off} values. Most of the analyzed scFvs showed higher affinities as indicated by the lower K_D values (table 6). B4 and H4 scFv were found to be having sub-nanomolar affinities, while the F6-scFv is having a pico-molar range affinity as indicated by i.e. K_D ~7.0 x $10^{-11}M^{-1}$. The linear-epitope *binder* B6-scFv is also binding to the native STNhaA apparently with lower affinity (i.e. K_D ~3.4 x $10^{-9}M^{-1}$) as compared to the conformation-specific *binders* like F6 or H4-scFvs.

Table 6: Binding kinetics of anti-STNhaA scFvs obtained from chicken library

Anti-STNhaA scFv clone	*Association rate constant (k_{on})* x($10^5M^{-1}s^{-1}$)	*Dissociation rate constant (k_{off})* x($10^{-4}s^{-1}$)	*Dissociation constant (K_D)* x($10^{-10}M^{-1}$)
B4-scFv	6.7	4.5	2.02
H4-scFv	8.1	3.0	2.61
B6-scFv	7.6	0.26	34
F6-scFv	9.5	4.2	0.70

Experiments were done in collaboration with Marc Boehm, the kinetic-titration data was evaluated by Dr. Uwe Bierfreund (Biacore) using Biaevaluation software.

2.8 Large-scale expression and purification of anti-STNhaA F6scFv

On the basis of the best yield during analytical scale expression and purification screening and due to its best profile on analytical gel filtration chromatography (Fig. 45), F6-scFv was chosen as candidate for optimization of preparatory scale expression and purification. More importantly, its formation of stable in-solution complex with STNhaA, while being Western-blot negative, indicates its suitability for co-crystallization experiments.

Large scale production and purification of F6-scFv was established in the periplasmic expression system (section 4.5.2). IMAC-purification was optimized using a His-Trap column and the Akta prime system. Initially a continuous imidazol gradient (10 – 600 mM imidazol) was used to determine the best washing and elution parameters (Fig. 49). It finally led to the development of a step-gradient washing and elution program that is now routinely used for the preparatory scale F6-scFv purification. The program consists of following steps: Loading of dialyzed F6-scFv periplasmic preparation at 0.5 ml/minute in presence of 10 mM imidazol, 1st washing with 5 column volumes (CV) of 40mM imidazol, 2nd washing with 5CV of 100 mM imidazol, and the final elution step with 200mM imidazol (section 4.5.4).

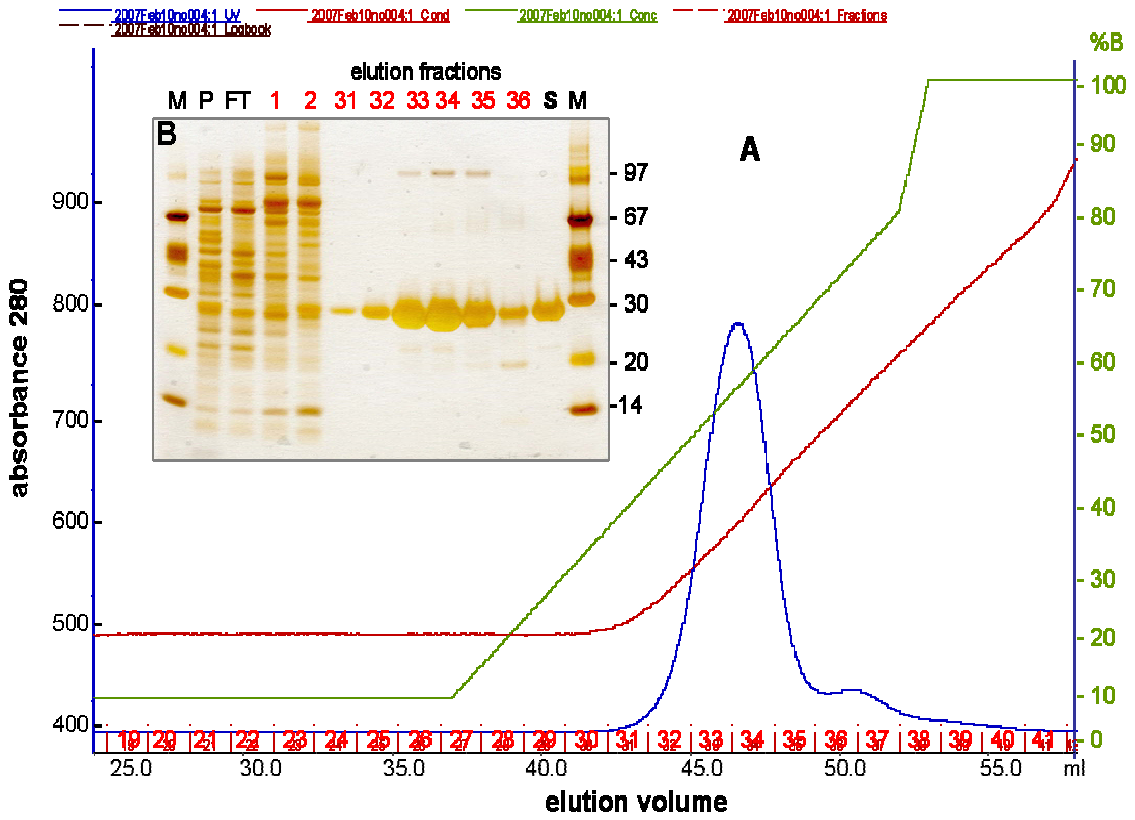


Figure 49: Optimization of large scale purification of F6-scFv protein. A. The IMAC profile of F6-scFv loaded on 1ml His Trap column using the Akta prime system. The chromatograph shows a nice symmetric elution peak (*blue curve*) in response to the (*green curve*) linear gradient 10-600mM imidazol concentration. **B.** Silver-stained high resolution SDS-PAGE analysis of elution fractions. P: sample from periplasmic extract, FT, 1 and 2: column flow-through, fractions 31 to 36 correspond to elution peak (10µl out of 1.5 ml fractions loaded). Lane S: sample from pooled and concentrated elution-peak (~ 9µg/lane).

Purified F6-scFv protein estimation: The elution-peak fractions were pooled together, concentrated and estimated for protein concentration by BCA. After optimization of expression and purification protocols, protein yields of 10~12 mg/L culture were obtained.

2.9 Co-crystallization of STNhaA with F6-scFv

The enlargement of the polar surface of a membrane protein by binding of the antibody fragments has been shown to produce high quality three-dimensional crystals for X-ray diffraction studies (Hunte and Michel 2002). Here, the co-complex containing STNhaA and F6-scFv in stoichiometric ratio was purified by gel-filtration chromatography, and used for crystallization screening. Co-complex crystals were obtained at 4^{o}C, pH 7.5 (Fig. 50). Under similar conditions neither scFv alone nor STNhaA alone produced any crystals.

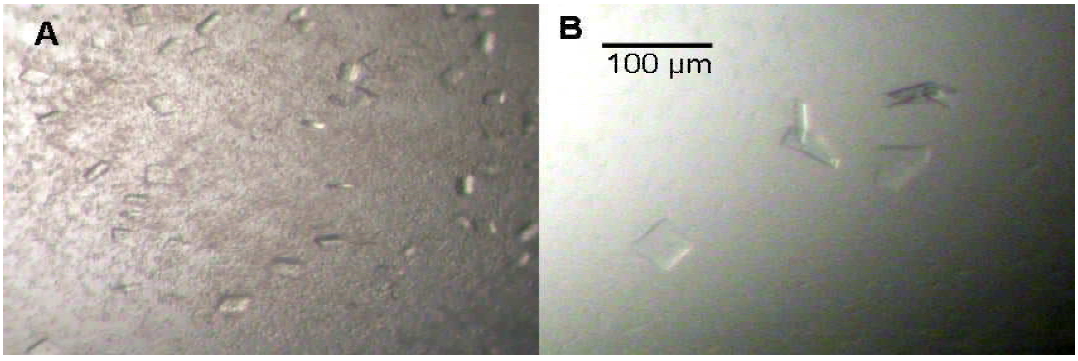


Figure 50: Crystals of STNhaA: F6-scFv co-complex. Purified stoichiometric co-complex was crystallized by vapor diffusion at 4^{o}C and pH 7.5. **A.** 2µl Hanging drop setup in 24-well Nextal^{TM}-plates. **B.** 1µl Sitting drop setup in 96-well Greiner plates. Photos kindly provided by Marc Boehm.

The presence of both the STNhaA and scFv proteins in the co-complex crystals was also observed. The importance of the crystals obtained at pH 7.5 is that NhaA is in up-regulated state under these pH conditions. All crystallization experiments were performed by Marc Boehm.

The co-crystallization of membrane protein mediated by antibody fragments is a promising approach to obtain quality crystals for structural studies. The technique has been so far underutilized, mainly because of the low availability of suitable antibody fragments for the purpose. Here we showed potential of the phage display technique using immunized chicken scFv libraries to provide the fast access to high quality antibody fragments suitable for co-crystallization. In this study STNhaA was used as a model protein, in principle the technique can be used to develop high affinity antibody fragments against any membrane protein.

3. Discussion

Integral membrane proteins are an essential class of biological molecules for all cellular living systems. They perform a wide variety of vital functions that are required for the integrity and viability of a cell. They are involved in generation and dissipation of energy, in cell-signaling pathways, and in transport of different molecules across the plasma membrane. A substantial part of the open reading frames (20%-30%) of an average genome belongs to them (Wallin and Heijne 1998). Membrane protein structure determination is a difficult task for a structural biologist for reasons outlined in the introduction. Obtaining high quality membrane protein crystals for X-ray diffraction studies is often the bottleneck. Antibody fragment mediated co-crystallization was shown as a promising approach to obtain high quality crystals for these proteins (Ostermeier *et al.,* 1995a, Hunte and Michel. 2002). But, the technique is impeded by the low availability of monoclonal antibody fragments suitable for co-crystallization. The traditional method of generating monoclonal antibodies by hybridoma technique is a long and tedious process. Therefore, from the late 1980s attempts were made to get antibodies directly by cloning of antibody genes, thus bypassing hybridomas (Winter and Milstein 1991). Antibody phage display has been used to obtain monoclonal antibodies against a wide variety of targets, most of them being soluble proteins (Bradbury and Marks 2004). Till now the success of the technique for selection of specific antibody fragments against integral membrane proteins targets was low. Here, we show that this technique can be used to generate high affinity and high quality monoclonal antibody fragments suitable for characterization, purification and co-crystallization of membrane proteins.

3.1 Antibody-mediated co-crystallization of membrane proteins

3.1.1 The use of antibody fragments for co-crystallization

Monoclonal antibody fragments such as Fv (*fragment variable*) and Fab (*fragment antigen binding*) have been successfully used to obtain or to improve the crystal quality of the membrane proteins. A number of high resolution membrane proteins structures obtained by this method have been reviewed (Hunte and Michel 2002). The cytochrome *c* oxidase (COX) from *Paracoccus denitrificans* was the first successful example showing the antibody mediated co-crystallization of a membrane protein (Ostermeier *et al.,* 1995a). The recombinant antibody fragment (7E2 Fv) used in this study was generated by cloning the heavy and light chain antibody gene segments derived from the parent hybridoma cell line and producing it in *Escherichia coli* (Ostermeier *et al.,* 1995b). Similarly the Fv fragment used in

the co-crystallization of cytochrome bc_1 complex (QCR) was also generated by recombinant methods from the monoclonal *mab*18E11 hybridoma cell line (Hunte *et al.,* 2000). Larger antibody fragments such as Fab were successfully used to solve or improve the structures of membrane proteins, such as the potassium channel KcsA (Zhou *et al.,* 2001) and the ClC chloride channel (Dutzler *et al.,* 2003). In both of these cases, the Fab fragments were obtained by proteolytic (papain) cleavage of parent monoclonal antibody preparations. The common feature of all the antibody fragments used in above successful examples is that, they all were directly or indirectly obtained from hybridoma technology (Kohler and Milstein 1975).

3.1.2 Quality control of antibody fragments suitable for co-crystallization

No published data is available about the general affinity requirements of antibody fragments suitable for co-crystallization of membrane proteins targets. Based on the experience gained in previous successful examples, the workers having expertise in the field have set up the following criteria (Hunte and Michel 2002):

i. The first criteria is that the antibody fragment should be conformation-specific, binding to a non-linear native epitope, a property that is usually associated with antibodies that are Western-blot negative for SDS-PAGE probed proteins. Since the Western-blot positive antibodies usually recognize linear continuous epitopes, the opposite is true for the other type.

ii. The antibody fragment in question should be able to form a stable co-complex in solution with its target membrane protein in stoichiometric ratio. Such co-complexes are generally separated and purified by gel-filtration chromatography and directly used for co-crystallization trails.

3.1.3 Generation of recombinant antibody fragments by phage display

Phage display antibody technique offers a promising alternative to the lengthy and tedious approach of monoclonal antibody generation by classical hybridoma technology. The antibody fragments isolated from phage display libraries have proven to be valuable tools as therapeutic and laboratory reagents (Sergeeva *et al.,* 2006). The technique is well established for the isolation of *binders* against soluble protein targets. Even though some reports of selection against membrane protein targets are available, its use against them is not so frequent, mainly due to the problematic nature of the membrane protein itself. Recombinant antibody fragments have been isolated from a Fab based synthetic naïve library (Griffiths *et*

al., 1994) against $p185^{erbB-2}$, a transmembrane glycoprotein (Sawyer *et al.,* 1997). An immune Fab based library generated from HIV-type1 seropositive individuals (Burton *et al.,* 1991) was used to isolate *binders* against detergent solubilised HIV-1 Env glycoprotein (Labrijn *et al.,* 2002). A scFv based synthetic phagemid library was panned against the detergent solubilised mitochondrial complex I, and a *binder* to its 51-kDa subunit was isolated. (Rubinstein *et al.,* 2003). In a different approach rather than using the whole membrane protein, antigen-mimicking cyclic peptides were used for selecting from a naïve scFv phagemid library. In this particular case *binders* against the G-protein coupled receptor CCR5 (HIV-1 coreceptor on macrophages) were obtained that were able to inhibit CCR5-mediated HIV infection (Zhang *et al.,* 2004). In a similar approach, a synthetic peptide corresponding to N-terminal 31 residues of C5aR, a chemoattractant GPCR receptor was used to select *binders* from an autoimmune Fab-based phage library (Huang *et al.,* 2005). But, the approach using linear antigen peptides appears non favorable for co-crystallization (Hunte and Michel 2002). In another example, an antibody Fab library was constructed with an aim to select *binders* suitable for the stabilization and crystallization of membrane proteins, and *binders* against citrate carrier CitS were isolated from it (Rothlisberger *et al.,* 2004). Further applicability of this library has not been reported since then. All these examples demonstrate the principal feasibility of using the phage display system to select antibody fragments against membrane protein targets. But these examples are very scarce when compared to thousands available for soluble proteins, where antibody fragments were selected by phage display. This is an indication that the technique against membrane proteins is still challenging and problematic. In the following sections, the problems faced and the solutions learned while facing these challenges will be discussed.

3.2 Phage displayed antibody libraries

3.2.1 Selection of specifically binding antibodies

When intending to select antibody fragments by phage display against a desired target membrane protein, two approaches are possible:

i. The first approach involves the use of a natural or diversified naïve antibody library.
ii. The second approach involves the use of an immune antibody library.

The former approach is easy to begin with, as various naïve natural and synthetic libraries are available from different sources at nominal costs. But the quality of these freely available libraries particularly against membrane protein targets is yet to be ascertained, as to the best of

my knowledge no published data record is available in this regard. The second option often results in the isolation of antibody with excellent quality suitable for any purpose including for co-crystallization of membrane proteins. The later has been demonstrated in this work by the successful generation of an immune chicken scFv library and isolation of *binders* against STNhaA from it that have produced crystals in complex with the target as discussed in detail below. Earlier immune libraries have been used successfully to generate useful antibodies against soluble protein targets of therapeutic importance (Clackson *et al.,* 1991, Burton and Barbas 1994).To our knowledge, this is the first time where an antibody fragment suitable for co-crystallization of an integral membrane protein has been produced by phage display using chicken immunization route. The drawback is the time investment for immunization and construction of individual antibody library for individual target membrane proteins. But on the other hand, immunization and construction of a library may be easier and faster than a facile selection directly from a large library followed by more time-consuming affinity improvement procedures (Burton 2001).

3.2.2 Selection from Naïve antibody libraries

The first library used in this study was the naïve ETH-2 library. This is a phagemid based human synthetic antibody library in scFv format (Pinni *et al.,* 1998). The library uses a single functional heavy chain gene (V_H –DP47) and two functional light chain genes: a single *kappa* (V_L –DPK22), and a single *lambda* (V_L –DPL16). These heavy and light chain genes were chosen as they are forming one of the most stable and highly expressing framework scaffolds (Kirkham *et al.,* 1992, Griffiths *et al.,* 1994). The diversity of the ETH-2 library has been generated by randomizing the CDR3 sites of the heavy (V_H) and light chain (V_L) genes. ETH-2 library has been used to isolate *binders* against various important targets such as SARS-CoV nucleocapsid protein, Hamster Prion protein (HaPrPsc) and CEA tumor marker protein (Ascione *et al.,* 2005, Flego *et al.,* 2005, Pavoni *et al.,* 2006). In our case, the library was used to select *binders* against yeast cyt *c*.

Yeast cyt *c* was chosen as the first target antigen, primarily to optimize the various experimental steps involved in library panning. Secondly this protein is used in structural studies of the cytochrome bc_1 complex (Hunte and Michel 2002), and antibodies against it are thus beneficial for such studies. The panning of the ETH-2 library against the immobilized yeast cyt *c* resulted in isolation of 3RE2-10 scFv against it. This scFv binds the target in highly specific manner in ELISA as well as in Western-blot experiments. It does not show any unspecific binding to unrelated proteins like BSA. When its binding was analyzed against cyt

c from a different species origin, namely horse heart, a pronounced lower binding was reported. 3RE2-10 scFv appears to have a species-specific binding mode. The antibody fragment was found to be stable during expression and could be purified to homogeneity. Gel filtration binding experiments were performed, but no significant binding was observed. This indicates that 3RE2-10 scFv is not able to form the co-complex with native yeast cyt c in solution or that it has a low affinity. As the scFv preferentially binds to cyt c renaturating Western-blots, one can conclude that 3RE2-scFv is likely a moderate or low affinity *binder* that most likely binds to a partially denatured epitope on its antigen.

3.2.3 Selection from Naïve libraries against membrane–protein targets

The quality of ETH-2 libraries at hand was compromised partly as a result of repeated propagation cycles, as indicated by high ratio of insert-less clones. Therefore, a new library (Tomlinson I+J) was obtained. Tomlinson (I + J) is also a human synthetic naïve antibody library based on single fold scFv format. This library was evaluated for its use and selection of antibody fragments against membrane protein targets. In this library single human framework for heavy chain V_H (V3-23/DP-47 and light chain J_H4b) and V_K (O12/02/DPK9 and J_K1) have been used (Cox *et. al.,* 1994). The diversity of the library has been generated by the randomization of seven aminoacid residues at CDR-H2 (50, 52, 52a, 53, 55, 56 and 58), four in CDR-H3 (95, 96, 97 and 98), two in CDR-L2 (50 and 53), while five residues in CDR-L3 (91, 92, 93, 94 and 96). These positions were selected on the basis their involvement in antigen binding process. The potential diversity of this library is higher as four CDRs i.e. H2, H3, L2 and L3 have been randomized as compared to only two CDRs i.e. H3 and L3 in the previous ETH-2 library.

3.2.4 Selection of β_2 adrenergic receptor as potential target

The β_2 adrenergic receptor (β_2AR) is an integral membrane protein belonging to the family of so called serpentine receptors collectively known as G-protein coupled receptors (GPCRs). This group of transmembrane proteins is essential for cells to respond to a wide variety of extracellular stimuli (Strader *et al.,* 1994). These proteins couple the external signals to intracellular response by activating the intracellular G-proteins in response to the binding of the specific ligands on their extracellular domain. The β_2AR is a member of the adrenergic receptor family. The binding of ligands, catecholamines, causes it to release the G_s subunit to activate the membrane-bound adenyl cyclase, resulting in the elevation of cytosolic cAMP levels (Cerione *et al.,* 1983). Like many other GPCRs, β_2AR is a potential target for

many existing and developing drugs. Little is known about the structure-function relationship as till date no high resolution structure is available for these proteins. All these features make β_2AR an introducing target for antibody fragment-mediated crystallization attempts. The other reason to choose β_2AR as the first membrane protein target was that it was available in biotinylated form. And a ligand binding assay was available to check its activity (Reinhart *et al.,* 2003).

3.2.5 Presentation of membrane protein (β_2AR) for biopanning in active form

One of the prerequisite to select the antibody fragments suitable for co-crystallization by phage display method is to immobilize the target membrane protein on a solid surface in its native conformation. Functional immobilization of GPCRs has been performed using terminal-tag specific antibody capture on protein-G coated magnetic beads (Ott *et al.,* 2005). We generated a core streptavidin-coated surface on microtiter 96x well plates. This streptavidin-MaxiSorp^{TM} surface was optimized for binding to biotinylated proteins. Successful immobilization of β_2AR via its c-terminal bio-tag on this surface was possible. The immobilized β_2AR was shown to be in its active conformation, as verified by the available ligand binding assay.

3.2.6 Selection of antibody fragments against β_2AR

The Tomlinson (I and J) libraries were used for selection against immobilized active β_2AR. Multiple rounds of panning and selection were performed, and the population of specific anti-β_2AR enriched phages was checked by polyclonal ELISA analysis. The panning experiments were repeated three times against β_2AR, each time using a different strategy and panning conditions. Most of the panning and selection rounds resulted in the enrichment of nonspecific background *binders*. Often the increase in specific β_2AR binding signal was accompanied by the similar increase in background binding signal. To reduce the population of background binding phages, an alternative selection approach was adopted (Lu and Sloan 1999). In this new approach, two rounds of anti-β_2AR panning and selection were performed on the streptavidin-maxisorp surface. In the third alternative round, monomeric-avidin coupled resin was used to capture the β_2AR bound phages. This approach resulted in the selection of a single β_2AR specific B2-scFv clone. Its specific binding mode was indicated by ELISA and Blue-Native PAGE gel shift binding assays.

The phagemid (pIT2) vector used in the Tomlinson I+J library contains the pelB leader sequence upstream of the scFv cloning site, which drives the transport of scFv to the periplasmic space of host bacterium, *E. coli* HB2151. B2-scFv could be purified to homogeneity from periplasmic extracts by a single step IMAC chromatography. A modest yield of 0.5-1mg purified scFv per liter culture was obtained. The low yield was most likely caused by stringent purification conditions required to remove a portion of the protein that tends to aggregate.

The gel filtration profile of IMAC purified B2-scFv preparations show two elution peaks corresponding to monomeric and dimeric forms. The occurrence of monomer-dimer conformations in scFvs is not unusual. The different factors influencing the monomer-dimer phenomenon in scFvs have been reviewed (Arndt *et al.,* 1998). The dimerization tendency of a scFv depends upon various factors. Primarily on the composition and length of linker region connecting V_H and V_L. Secondly, factors like pH and the aminoacid sequence of the scFv also influence this behavior. Sometimes the dimerization of scFv molecules is intentionally induced by shorting the linker region connecting V_H and V_L to increase their apparent binding affinities. The tendency of scFvs with same linker length and aminoacid composition to exist in monomeric and dimeric states is not unusual either (Griffiths *et al.,* 1993). Such dimers are often formed either by back-to-back association of two monomeric molecules, or by domain swapping where heavy chain of one scFv molecule pairs with the light chain of another. A single oligomeric species of scFv is required for binding experiments. In the present case of B2-scFv, it was advantageous that the two oligomeric states were stable and could be separated by gel filtration. These forms did not interconvert into each other at least for a week-long storage at 4^oC. It was necessary as the monomeric scFv was needed for the subsequent binding assays.

The binding of monomeric B2-scFv to native β_2AR was analyzed by gel-filtration binding analysis and subsequently by BN-PAGE gel-shift assays. A low level binding of B2-scFv to its target is observed in the former case, when used at three to five-fold molar excess, whereas in BNP assays, a β_2AR band-shift occurs in response to presence of scFv. Together with the positive ELISA binding analysis, these experiments indicate that B2-scFv is a weak but specific *binder*. To permit co-crystallization studies, affinity maturation procedures would be the required next step to improve its quality (Chowdhury and Pastan 1999, Boder *et al.,* 2000, Rajpal *et al.,* 2005). The improvement of B2-scFv affinity is likely, as it contains a short CDR-H3 with '**G_{95} A_{96} G_{97} S_{98} F D Y'** aminoacid sequence, which can be targeted for

enlargement and randomization. The latest approaches used for enhancement of antibody affinities along with the experimental data have been reviewed (Wark and Hudson 2006).

3.3 Factors affecting selection of membrane protein-specific antibodies from naïve libraries

The selection of β_2AR-specific B2-scFv in this work and a limited number of other studies (Rubinstein *et al.,* 2003, Rothlisberger *et al.,* 2004) demonstrate the possibility of isolating the specific *binders* from naïve antibody libraries against membrane protein targets. But the success rate of selection against membrane proteins from these naïve 'single-pot' libraries is far lower than when used against soluble protein targets. While selection of antibody fragments from naïve libraries is relatively easy, the down stream application in co-crystallization experiments is complicated by poor expression levels and low affinities. Such antibody fragments often fail to form stable in-solution co-complexes with their target as required for co-crystallization experiments.

The majority members in a naïve synthetic library are usually low or moderate affinity *binders* (i.e. $K_d \sim$ 10-2000 x10^{-9}M). The average diversity of the naïve synthetic libraries used in this study, i.e. ETH-2 and Tomlinson I + J, is expected to be 100 million candidates (Pini *et al.,* 1998, Griffiths *et al.,* 1994). A library of 10^7 to 10^8 members can yield *binders* with 10nM range affinities, whereas libraries having more than 10^{10} members can produce 0.1nM affinity *binders* (Hoogenboom 2005). Larger size naïve libraries consisting of billions (10^{10}) of members have been created from natural as well as synthetic repertoires, and sub-nanomolar affinity *binders* have been isolated from them (Sheets *et al.,* 1998, De Haard *et al.,* 1999, Kretzschmar and Ruden 2002). ETH-2 library has also been improved to ETH-2-Gold, consisting of three billion individual antibody clones (Silacci *et al.,* 2005). These good quality libraries (including ETH-2-Gold) have been patented, and are usually not freely available, or under immense cost. Secondly, the proclaimed high-affinity *binders* isolated from these libraries have been mostly selected against soluble protein targets (Ascione *et al.,* 2005, Davern *et al.,* 2005, Silacci *et al.,* 2005) Therefore, it is not possible to comment about their efficacy against membrane protein targets.

A limitation for membrane proteins is that most of them have scant hydrophilic surface area to which an antibody can bind. This scarcity of natural epitopes on membrane proteins can be a reason why the selection from a naïve library is not very effective against

them. In one of the recently reported study, a FLAG-tag epitope was inserted into a loop region of K^+ channel protein (KvPae), and anti-FLAG Fab fragment obtained from M2 monoclonal antibody was tried to co-crystallize it (Roosild *et al.,* 2006).

The presence of a detergent is essential to keep a membrane protein stable in solution outside its native membrane environment. Therefore, any analytical procedure involving a purified membrane protein requires detergents as default components in the buffers. The same is true when a purified membrane protein is used for the screening of a phage display antibody library. During the course of this study, it was observed that the presence of detergents during the selection process has an adverse effect on the desired enrichment process. It was found that phages show increased non-specific binding to the polystyrene surface in presence of detergents like LM (section 2.4). This increase in unspecific background phage binding was shown both in ELISA based experiments as well as in colony-counting assay. There are few published data available on the effect of detergents with respect to the panning of naïve phage antibody libraries. It was earlier reported that different detergents can have opposite effects on antigen-antibody interactions (Dimitriadis 1979). In that study it was shown that ionic detergents, e.g. 0.5% SDS, inhibit antigen antibody interaction, whereas the addition of a non-ionic detergent, in that case Triton X-100, has no negative effect. Detergents like Tween-20 and Triton X-100 are routinely used in ELISA for washing off loosely or non-specific bound reactants. In a study at Nunc Laboratories, a negative effect of detergents was seen when used with blocking agents like casein. It was recommended to avoid the detergent during the blocking and reactant incubation steps. Overall the combined use of blocking agent and detergent is a matter of considerable complexity (Esser 1990b). The situation will be more complex when a membrane protein is coated on surface and a library of phages with varied specificity is incubated over it.

In a study on characterization of plastic-binding phage isolated from a random peptide-displaying phage library, it was found that these phages were able to bind the milk-blocked plastic surface in response to the presence of non-ionic detergents like Tween 20. (Adey *et al.,* 1995). Most of these phages were displaying 22 or 36 residue peptides that were rich in Tyr and Trp residues. From the work in this thesis with naïve libraries, it was observed that most of the phages enriched in an unspecific manner during a panning experiment that show highest non-specific background binding lack most or a partial part of scFv inserts. For example, the clone-A1 isolated during anti-cytochrome *c* panning of the ETH-2 library, was carrying no insert, whereas the clones C1 and C2 isolated during anti-β_2AR of Tomlinson I+J

libraries carried partial scFv insert corresponding to either of heavy of light chain region. These partial inserts or detergent-induced miss-folded scFvs on the surface of the phage might be responsible for their stickiness. According to a suggestion by Prof. GP Smith, the detergent problem might be alleviated using a Fab-display rather than the scFv-display on the phage surface, as one expects a more stable antibody scaffold (personal communication). This suggestion was experimentally analyzed (section 2.4.). Here, also a great degree of non-specific phage retention on the surface was observed in presence of 0.02% LM. It was also observed that different detergents exert varied effects, i.e. 0.05% CHAPS was showing least effect, while the largest effect was shown by 0.05% LM. How the detergent induces nonspecific binding of phages, is not known. It is likely that this detergent-effect is a cumulative phenomenon as a result of multiple factors, such as the presence of partially denatured scFv inserts displayed on the phage, the presence of partially deleted scFv inserts, the low efficiency of milk-blocking due to the presence of detergent. Furthermore, the surface of filamentous phages is rich in basic amino acids. This highly charged surface of a phage particle might have some effect on the non-specific binding.

The screening of a large number of detergents is needed to find optimum panning conditions for a membrane protein, which balances the need of a stable target protein and a low background. Such a study would also help to understand the mechanism of such non-specific adsorption of phages. Another strategy could be to avoid the use of detergents during panning. This can be achieved by using the synthetic polymers ‘Amphipols’ to stabilize the membrane protein during selection (Tribet *et al.,* 1996), or the use of polymer-supported membranes to present the membrane protein for selection (Tanaka and Sackmann 2005). Competitive elution with excess amounts of free antigen can be used to elute the specific *binders* while letting the non-specific members stay back (Barbas *et al.,* 1992), but the availability of excess amounts of a membrane protein for competition is often an experimental hurdle.

With the limitations of the naïve libraries at hand, immune libraries provide an alternative option for the enrichment of high affinity *binders*. As shown in this study, the presence of high-affinity *binders*, i.e. with picomolar to sub-nanomolar range K_D values, in such libraries allows the selection under higher stringency panning conditions. The enrichment of high affinity specific-*binders* in these libraries overcomes the detergent induced non-specific background problems.

3.4 Generation and selection of chicken-immune libraries

3.4.1 Why an immune library?

The previous discussion outlined the limitations of naïve scFv libraries to obtain high affinity recombinant antibody fragments suitable for co-crystallization of membrane proteins (Hunte and Michel 2002). High unspecific background binding caused by partial inserts and the required detergents is the main obstacle. Highly diverse, excellent libraries would be needed to pursue this approach. As such libraries are difficult to obtain for academic research an alternative strategy of constructing libraries from immunized animals was explored. Phage antibody libraries generated from the immunized rather than the naive sources result in better selection of higher affinity *binders* (Burton 2001). Most of the antibody fragments that have been successfully used till date for co-crystallization and structure determination of membrane proteins invariably came from immunized animals via the hybridomas route. Considering all these factors along with the previously discussed limitations of the naïve libraries, the generation and use of an immune antibody library on the surface of phage was explored. A phagemid library in scFv format was constructed from the spleen and bone marrow of chicken immunized with a mixture of two target membrane proteins.

3.4.2 Why the chicken?

One of the limitations in obtaining high affinity *binders* against human membrane proteins of therapeutic and structural importance is the lack of immunogenic response produced by them in mice. This is because most of these membrane proteins are highly conserved across the mammalian kingdom. An attractive alternative to circumvent this problem is to use an animal host, which is more distant to humans. Phage displayed antibody libraries have been created by immunizing a wide variety of non-mammalian hosts such as chicken (Andris-Widhopf *et al.,* 2000) and domesticated nurse sharks (Dooley *et al.,* 2003). Birds are 200 million years apart from humans on the phylogenetic tree. Therefore, they might produce more potent immune response against human proteins, as compared to mice and rabbits (Davison 2003, Greunke *et al.,* 2006).

The second attractive advantage of using the chicken immune system is the ease of library-making out of it. The chicken immunoglobulin repertoire consists of only one type of functional variable gene segment (V_H1) for its heavy chain, and only one for its light chain

($V_\lambda 1$). Therefore, for each of the heavy and light chain, only one set of primers is needed for PCR-amplification of all the heavy and light chain genes (Andris-Widhopf *et al.,* 2000).

For the diversification of their immune repertoire, birds use a different mechanism than that of their mammalian counterparts. The mouse and human B-lymphocyte immune repertoire consists of 104 and 51 functional V_H gene segments respectively, which sets the basis for their antibody diversity of more than 10^8 specificities (Tonegawa 1983, deBono *et al.,* 2004). In contrast, the mature antibody gene repertoire of the chicken immune system comprises only a single functional V_H gene segment, and a single functional $V_\lambda 1$ gene segment for the development of entire B-lymphocyte army (Reynaud *et al.,* 1985, Reynaud *et al.,* 1989). The main mechanism used by mammals to generate the antibody diversity is *"somatic recombination",* in which the diversity arises from multiple sources. Primarily, due to the presence of the multiple functional copies of variable (V), junction (J) and diversity (D) gene segments. Second, by the *'combinatorial diversity'* derived from the rearrangement of these multiple variable V, J and D gene segments, and by the *'junctional site diversity'* arising due to the imprecise joining of ends at V-J, V-D and D-J junctions. Moreover, the junctional *'insertion diversity'* occurs at V-D and D-J junctions due to addition of one or more random nucleotides in template-independent fashion. And lastly, the accumulation of single nucleotide mutations in the re-arranged immunoglobulin genes by somatic hyper-mutation further diversifies these antibodies (Tonegawa 1983, Berek *et. al.,* 1985).

The avian immune system has evolved on an alternative path to generate the B-lymphocyte diversity. The avian B-cell immune repertoire is generated extensively by "*gene conversion*", a process that requires the unique environment provided by the bursa. Gene conversion events occur frequently during the bursal ontogeny to diversify variable domains of both heavy and light chain genes, by replacing short gene segments with the sequences from a large pool of pseudogenes present in the genome (McCormack *et al.,* 1991). The chicken heavy chain immunoglobulin loci contain ~ 80-100 (ΨV_H) pseudogenes 7 kb upstream of the functional $V_H 1$ gene. And similarly the light chain loci contain ~ 25 (ΨV_λ) pseudogenes 2.4 kb upstream of the functional $V_\lambda 1$ gene (Reynaud *et al.,* 1989). These pools of pseudogenes act as putative donors for the short stretches (12-100bp) of DNA sequences that get inserted in the functional V gene segments. A typical chicken B-cell encounters on an average 4-6 conversion events during their maturation phase. Gene conversion has also been reported to occur in some mammals like rabbits and sheep, reviewed in (Davison 2003).

3.5 Construction of anti-STNhaA/KefC scFv phagemid library

Monoclonal antibodies from chicken have been humanized, and are being explored for therapeutic purposes (Zhang 2003, Tsurushita *et al.,* 2004, Nishibori *et al.,* 2006). Chicken have been successfully used to generate immune antibody phagemid libraries. (Andris-Widhopf *et al.,* 2000). In this thesis, two chickens were immunized with a cocktail of two membrane proteins STNhaA and KefC from *Salmonella typhimurium.* The STNhaA is a homologue of *E. coli* sodium-proton antiporter NhaA (Hunte *et al.,* 2005), while KefC is a homologue of *E. coli* glutathione-gated potassium efflux system (Miller *et al.,* 2000). Antigen-specific immune response was generated in both of the chickens. The generation of strong immune response against both the STNhaA and KefC in each chicken demonstrates the feasibility of using more then one antigen in a mixture for immunization, thus saving cost and time. An immune antibody phagemid library was constructed in scFv format from the spleen and bone marrow of these chickens. The library construction was straight forward (section 2.6). The critical step during the construction of an antibody library is the introduction of library vector DNA into the host *E. coli* cells. This step of library transformation very often puts the limitations on the size of a library. The transformation efficiency depends both upon the quality of ligated library DNA, as well as on quality of the competent host *E. coli* cells. Electroporation is the method of choice for large scale library transformation, as highest numbers of transformants are obtained by it. Electrocompetent XL1-blue cells prepared in the laboratory and used freshly gave the best results.

The library size of larger than 1×10^7 is considered sufficient for an immune antibody library, whereas for a naïve library the size of 1×10^{10} is required (Andris-Widhopf *et al.,* 2001). In our case, the acquired size for the anti-NhaA1/anti-KefC chicken SL and LL-scFv libraries was ten times higher than the minimum standard. Therefore, these libraries were considered of good quality. This was later experimentally proven by the selection of a number of high affinity *binders* against STNhaA.

3.6 Selection of STNhaA-specific high affinity *binders*

Both, the quality of short linker SL-scFv and the long linker LL-scFv chicken library proved to be good, as they showed STNhaA-specific enrichment in polyclonal ELISA (section 2.7). It was observed that polyclonal ELISA serves as the important indicator of the enrichment quality across the panning rounds during a selection experiment. More precisely

polyclonal ELISA performed with soluble scFvs rather than phage-scFvs provided more relevant information about the antigen-specific enrichment across the panning rounds. It reflects the enrichment of clones that are producing stable scFvs. Here, unlike the earlier experiences with naïve libraries, there was relevant enrichment only in specific-binding signal without any increase in the background. The panning of these libraries against STNhaA resulted in the selection of a number of high affinity *binders*. More than 90% of monoclonals analyzed from the LL-scFv library were testing positive for STNhaA binding in monoclonal ELISA.

As reported earlier for co-crystallization of membrane proteins, conformation specific antibody fragments are required (Hunte and Michel 2002). 50% of the ELISA selected scFvs from both of the SL and LL-scFv libraries were Western-blot positive against SDS-PAGE separated target. Such a high proportion of *binders* recognizing the denatured antigen was not unexpected, as the selection was performed on STNhaA that was directly coated on polystyrene (MaxiSorp^{TM}) surface. Despite the presence of detergents, such a direct coating can cause partial denaturation of the antigen resulting in selection of *binders* that are not conformation-specific (Hunte and Kannt 2003). These results reflect the importance of presentation of antigen in native conformation during the panning experiments. Such a presentation has been achieved with *E. coli* NhaA by immobilizing on Ni-NTA plates via its short terminal His-tag (Padan *et al.,* 1998, Ott *et al.,* 2005). In the present case, it was not possible to use the above method for immobilization, as the chicken scFv library also carries the C-terminal His tag. An alternative strategy would be the use of streptavidin-MaxiSorp^{TM} surface that was successfully used to present β_2AR in its native conformation in previous experiments.

For future experiments, one of the strategies would be to remove the His-tag from the scFv library for the selection process. Alternatively, the His-tag from the antigen (STNhaA) can be removed and replaced by another tag. Such a strategy is already being explored. A strep-tag construct of STNhaA has been generated by Marc Boehm in our group. In future, the antigen obtained from such a construct can be used during panning, while capturing the scFv-phage bound antigen on streptavidin-MaxiSorp^{TM} surface or on streptavidin magnetic beads. Streptavidin coated magnetic beads have been used to anchor the biotinylated-proteins for phage display library selections (Rothlisberger *et al.,* 2004).

The insert-check PCR and subsequent DNA sequencing of scFvs showed 100% of clones carrying full-length scFv inserts, this was in contrast with earlier experiments with the naïve libraries where the ratio of insert-less or partial-insert carrying clones was high. The

sequencing of scFv for 24 representative clones revealed enrichment of specific CDR-sequences. The sequence of these clones based on the alignment of complementarity determining regions (CDR) revealed distinct features of enriched sequences (section 2.7.7). Based on the CDR-H3 alignment, the 24 scFv could be categorized in four groups. The largest group, group-IV comprises half of the sequenced clones. The group-I and III each comprises of seven and three members respectively, while group-II consists of two totally different members. It was an interesting observation that all the members of Group-I, II, and III are Western-blot negative *binders*, whereas all the members of Group-IV are Western-blot positive. Each group of clones is thought to bind a distinct and unique epitope on STNhaA.

It is advantageous for various structural and biochemical studies to know the exact region of the membrane protein where an antibody binds. Previously, a monoclonal antibody had been produced against *E. coli* NhaA that binds the N-terminus in a pH-dependent manner (Venturi *et al.,* 2000). In this thesis, the epitope region was mapped for the anti-STNhaA selected chicken H6 and C4 scFv. The epitope for these two antibodies was found to be same sequence "**EKHGRSP".** This epitope is present on the connecting cytoplasmic loop of helix VIII and IX of NhaA starting at position 241. These linear-epitope specific antibodies belong to the group-IV of the sequence alignment, but represent the large differences of this group. The observation that C4 and H6 bind to the same epitope suggesting that the rest of the Western-blot positive (group-IV) scFv clones bind to the same epitope.

During the analysis of the complete scFv DNA sequences, it was observed that some of the members in each group were having differences in their framework regions, although having a similar CDR-H3 sequence. This phenomenon implies that these genes may have evolved in multiple B-cell lineages as a result of different gene conversion events. And, that the antigen-specific panning has resulted in the enrichment of specific CDR-sequences i.e. enrichment for the antigen-binding pocket rather than just the enrichment of specific clones. It is possible that the enriched scFv have the natural pairing of light and heavy chain genes. In few cases, scFv were generated by random reshuffling of V_H and V_L genes during the library construction i.e. B6 and E6-scFvs have same V_L, but differ in their V_H sequences.

All of the anti-STNhaA selected clones of sequence group-IV bind also to NhaA homologue from *E. coli*. Therefore, it was not surprising that the epitope-region 'EKHGRSP' mapped for H6-scFv is conserved in both species. Like in group-IV, most of the clones from group-I and III also bind to NhaA from *E. coli* as well as from *Salmonella* species. The explanation for this phenomenon is the high degree of similarity between the two homologues of NhaA1. Secondly, the antigen binding sites of antibodies are known to have some degree

of conformational flexibility that allow them to adopt binding against slightly different epitopes (James *et al.,* 2003). A single clone, namely anti-STNhaA B2-scFv, was found to be *Salmonella*-specific. It binds only to STNhaA and not to its *E. coli* homolog. Interestingly, both the V_H and V_L chains of this scFv turned out to be unique, and where not represented in any other of the 24 sequenced clones. The potential of this scFv as diagnostic or analytic tool will be explored in future.

For any scFv to be suitable for co-crystallization experiments, it needs to be produced in sufficient quantities. A number of scFvs from the conformation specific sequence group-I, and few candidates from rest of the groups were produced in *E. coli* HB2151. The scFvs could be purified to homogeneity from periplasmic extracts by single step IMAC. In the initial trials of analytical scale of expression and purification, the yield of purified scFv-proteins was varying between 1.5mg – 5.0mg/liter of cultures. When the expression and purification was optimized with one of the selected clones (F6-scFv), the yield of 99% purified protein was increased from initial 5mg/L to 12mg/L. Therefore, it is expected that the yield of other selected scFvs can also be increased upon optimization of expression and purification conditions. The yield obtained for F6-scFv is 10 -20 times higher than the yield obtained with previous scFvs selected from naïve libraries in earlier experiments.

The quality and stability of purified scFv proteins was judged on gel-filtration chromatography. Most of the clones showed a single symmetric elution peak corresponding to the monomeric form of the respective scFv (~29kDa). Once the satisfactory quality and stability of these selected and purified scFvs was established, they were probed for binding to the target antigen in solution. In gel filtration binding experiments the co-complex formation between STNhaA and respective scFvs was checked. Conformation-specific clones like E2, B4 and F6-scFv showed stable co-complex formation with their target.

Binding affinities of selected scFvs were characterized. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were measured by surface plasmon resonance (SPR) in a BIAcore T-100 system using a kinetic-titration method (Karlsson 2006). The reason to use this method was that it doesn't require the repeated regeneration of surface during the experiment. Instead, a series of increasing antibody concentrations are consecutively passed over the antigen immobilized surface. This was necessary, because it was observed that STNhaA is very sensitive to the harsh regeneration steps and thus loses its native conformation. In that case, conformation specific antibodies such as F6 or B4-scFv no longer show any binding. Kinetic-titration is the method of choice to measure the affinities of conformation specific antibodies against delicate membrane proteins or membrane protein

complexes. The respective dissociation constants (K_D) values were calculated. F6-scFv was found to bind STNhaA with pico-molar affinity (K_D ~7 x10^{-11}M). This affinity is very good, considering the fact that the average affinities reported in literature for *binders* isolated from phage display libraries fall in micro to sub-nanomolar range. There are only few cases where pico-molar affinity antibodies have been reported. In most of these examples such high affinities were obtained by extensive affinity-maturation experiments (Chowdhury and Pastan 1999, Bradbury and Marks 2004, Wark and Hudson 2006).

3.7 The influence of phagemid-vector system on the outcome of selection

As observed from insert-check PCR assays, the incidence of loss or absence of scFv-DNA insert is quite frequent in clones randomly picked after the panning of the naïve libraries (i.e. ETH-2 and Tomlinson I +J).Whereas, this phenomenon was not seen during the panning of the anti-STNhaA/KefC chicken libraries. The primary difference between these two types of libraries is that while the former ones are naïve and are based on semi-synthetic variable chain genes, the later is immune and based on completely natural gene repertoires. The second difference is the type of phagemid vector that has been used in these two types of libraries. The phagemid vector pDN322 used in ETH-2 library and, pIT2 used in Tomlinson library are either based on or derived from the parent phagemid pHEN vector system (Hoogenboom *et al.,* 1991). The phagemid vector pCom3x that was used in the construction of chicken libraries is a derivative of the pComb system (Barbas *et al.,* 1991). In order to address the question, if the different types of phagemid vectors used in this study have any role to play in stability of the scFv inserts, that in the end may have affected the outcome of a selection, the respective phagemid sequences flanking the scFv-cloning site were compared. Some of these sequences were provided with the library literature, while others were obtained from the extended sequencing of scFv clones selected from these libraries. The main differences found between the two types of vector systems that may affect their functionality are following.

The gene-III protein sequence in pDN322 and pIT2 phagemid vectors start with (TVESCLA - - - - -), that is corresponding to first aminoacid residue of the mature gene-III protein. It implies that both of these vectors carry the complete gene-III sequence for the fusion and display of foreign scFv inserts. On the other hand the gene-III protein sequence in pComb3x vector starts with (EGGGSEGGGSEGGGSE - - - - -), that corresponds to the 248th amino acid residue. It implies that both of the amino-terminal N1 and N2 domains of gIIIp are absent (section 1.2). Since N1 and N2 domains of gIIIp are connected by a long glycine rich

linker region, whose presence might have been involved in the loss of scFv insert due to homologous recombination with sequence coding the glycine rich linker region of the scFv.

In a recent study, it was found that some of the present day phagemid-vectors possess a remnant 56 base pair *lac* Z'-like sequence (Soltes *et al.,* 2007). The following sequence *"lac*P-ATGACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGATACAGTCATA-scFv", is present between the *lac* promoter-region and the start codon. In the same study it was observed that, the removal of this fragment from the promoter region resulted in increased display level and increased phage production. Interestingly both the pDN322 and pIT2 phagemid vectors do carry this remnant sequence, while the pComb3x does not. Thus our observation that the pComb3x phagemid vector used in chicken library construction is more tolerant for the insertion of foreign DNA is likely true. But in order to validate this assumption, all the three vectors need to be checked in parallel for insertion and display of the same control scFv DNA.

3.8 Alternative scaffolds beyond *antibody fragments*

For the last two decades, antibody-fragment based combinatorial libraries displayed on phage or else have been extensively used to select binding molecules virtually against any target. Yet, the problems like low stability and lower expression levels occasionally encountered with these antibody fragments led the development of a new generation of alternative protein display scaffolds. Most of these compact scaffolds are stable in absence of the disulphide bonds, and show high levels of protein expression in *E. coli*.

The common feature of these scaffolds is the compact and stable framework of alpha-helices or beta-sheets having multiple exposed loop-regions which can be diversified by insertion of random aminoacid residues, without changing the overall structure and stability of the underlying scaffold. The alternative scaffold based libraries have been displayed and selected by phage or ribosome display systems. Some of these alternative scaffolds are Z-domain "*affibodies*", repeat proteins "*ankyrin repeat"* domains, compact proteins like "*cytochrome* b_{562}", "*PDZ domain"* of the Ras-binding protein AF-6, and other smaller domain proteins like 3kDa "*insect defensin A*" or 6.6 kDa "*kunitz domain inhibitors*". The characterization of these and various other available alternative scaffolds has been discussed in detail in reviews (Nygren and Skerra 2004, Hosse *et al.,* 2006).

Affibodies were the first non-immunoglobulin protein scaffolds used for library construction. They were derived from the Z-domain of staphylococcal protein A (Nilsson *et al.,* 1987). They are robust, comparatively small (~6kDa), highly soluble, resistant to

proteases, and have high expression levels in *E. coli* and are being used in a wide variety of applications, such as generation of phage displayed combinatorial libraries (Nord *et al.,* 1997). Affibodies have been shown to crystallize in co-complexes with the respective target protein (Wahlberg *et al.,* 2003). But their smaller size might not be suitable for the co-crystallization of the membrane proteins.

Repeat-motif proteins like "*ankyrin repeat domains*" have also been used for construction of designed libraries. They consist of repetitive 33 residue structural units stacked together. They are lacking disulphide bonds and are therefore well expressed in bacterial cytoplasm. The ankyrin repeats have been used to generate designed libraries. One of such library was used to select an AR-*binder* against maltose binding protein (MBP) that was later crystallized in complex with MBP (Binz *et al.,* 2004).

One of the interesting alternative scaffold proteins used for library construction is "*green fluorescent protein*" GFP. This beta-barrel scaffold was used in various studies to accommodate randomized residues, while in others to display a library of peptides (Abedi *et al.,* 1998). In such a library the selection of *binders* can be directly visualized without any need of secondary reagents (Hitoshi *et al.,* 2003).

Most of the alternative scaffolds that have been used for generation of libraries, and have been tried for selection experiments by phage or ribosome display are small domain proteins (~3 to 10 kDa). These scaffolds were actually aimed to design novel therapeutics, where their smaller size is advantageous for their pharmacokinetic properties (increased tissue penetration and rapid serum clearance). But as for their potential use in co-crystallization of membrane protein targets, the higher molecular weight domains like, multiple ankyrin repeats should be advantageous. This was earlier shown for the crystallization of the OmpC complexed with the antibacterial eukaryotic protein lactoferrin (Baalaji *et al.,* 2005), and has been recently demonstrated with the co-crystallization of the *E. coli* multidrug transport AcrB in complex with a designed ankyrin-repeat protein (Sennhauser *et al.,* 2007). These examples demonstrate the feasibility of alternative-scaffold binding proteins for use in co-crystallization experiments of membrane proteins. But antibodies and antibody-derived fragments have been, and will remain among the important biological molecules in biotechnology and biomedical research and at the forefront of the co-crystallization technique.

3.9 Conclusion

The successful isolation of antibody fragments suitable for co-crystallization of STNhaA can be attributed to the insights gained while working with naïve libraries, and ultimately to the use of a chicken immune-antibody library. It was observed that the selection from naïve libraries can yield specific *binders* against membrane protein targets, but these *binders* are most likely not directly suitable for the co-crystallization of membrane proteins because of their low affinities, low stabilities and sometimes even low expression levels. To make these antibodies suitable for co-crystallization time-consuming affinity maturation procedures are needed. In contrast, the use of immune libraries is more beneficial and practical, although some extra time and efforts are needed for their construction.

Here, we have shown that the chicken-immunization combined with phage-display antibody technique offers a very good perspective to obtain high-affinity *binders* against membrane proteins. Phage display is a method which can be adapted to high throughput technology. We propose the use of chicken as hosts for immunization to overcome the problem of low immunogenicity usually encountered in mice and rabbit against human membrane protein targets. A phagemid library based on scFv format was generated from the spleen and bone marrow of a chicken. The chicken was previously immunized with STNhaA/KefC membrane proteins. As a proof of principle, a number of quality *binders* against STNhaA were selected from this library having sub-nano to picomolar affinities. These antibodies form stable co-complexes with their target in solution, which can be separated by gel-filtration chromatography indicating their suitability for co-crystallization. One of these antibodies, F6-scFv has already been proven to produce co-complex crystals for X-ray diffraction studies.

To the best of my knowledge, it is the first time that the chicken immunization and phage display approach has been used to generate high-affinity antibodies against a full-length membrane protein and shown to be suitable for their co-crystallization. In principle, the strategy can be applied to other membrane protein targets to obtain fast access to high affinity *binders*.

4. Materials and Methods

4.1 Materials

4.1.1 Suppliers

Amersham Biosciences	(http://www.amershambiosciences.com)
Beckman Coulter	(http://www.beckman.com)
Bio-tek Instruments GmbH	(http://www.biotek.com)
Bio-Rad Laboratories GmbH	(http://www.bio-rad.com)
Biozym Scientific GmbH	(http://www.biozym.com)
Biometra	(http://www.biometra.de)
Biomol	(http://www.biomol.de/inhalt.php)
Calbiochem	(http://www.emdbiosciences.com/html/CBC/home.html)
Eppendorf	(http://www.eppendorf.com)
Fermentas	(http://www.fermentas.com)
Gerbu	(http://www.gerbu.de)
Gilson / ABIMED, Gilson	(http://www.gilson.com)
Invitrogen	(http://www.invitrogen.com)
Infors AG	(http://www.infors-ht.com)
JPT Peptide technologis GmbH	(http://www.jpt.com)
Merck	(http://www.merck.de)
Millipore	(http://www.millipore.com)
New England BioLabs GmbH	(http://www.neb.com)
Nunc GmbH	(http://www.nunc.de)
Pierce Biotechnology	(http://www.piercenet.com)
Pocono Rabbit farm and Lab	(http://www.prfal.com)
Promega	http://www.promega.com)
QIAgen	(http://www1.qiagen.com)
Roche	(http://www.roche.de)
Roth, Carl Roth GmbH	(http://www.carl-roth.de)
Seqlab	(http://www.seqlab.de)
Serva Electrophoresis GmbH	(http://www.serva.de)
Sigma-Aldrich and Fulka	(http://www.sigmaaldrich.com)
Strategene	(http://www.stratagene.com)
Thermohybrid	(http://www.thermo.com)
Vivascience	(http://www.vivascience.de)

4.1.2 Equipments

Akta Prime	Amersham pharmacia (Germany)
Analytical SMART station	Pharmacia Biotech (Germany)
Analytical Balance AE163	Mettler-Toledo (Germany)
Avanti J-20XPI (rotor JLA 8. 1000)	Beckman Coulter (USA)
Agarose gel imaging station	Bio-Rad
Balance PM4600	Mettler-Toledo (Germany)

Beakers, Shaking flasks, bottles	Schott AG (Germany)
BIAcore T100	Biacore
Amicon Ultra/ Centriprep (concentration devices)	Millipore
Digital pH meter (646)	Knick (Germany)
Electroporation Device (Gene Pulser)	Bio-Rad
ELISA reader (Power Wave X)	Bio-Tek Instruments
Eppendorf Centrifuge 5415R	Eppendorf
Electrophoresis Power Supply	Renner GmbH (Germany)
Freezer VIP^{TM} series -86^{o}C	Sanyo
Freezer (-20^{o}C) Premium	LIEBHERR
Ice machine Ziegra	Rauschenbach GmbH (Germany)
Infors-Shakers	Infors AG (Germany, Switzerland)
Millipore Ultrapure water system	Millipore (Germany)
NuPAGE Novex XCell Mini Cell	Invitrogen
Pipetts	Gilson (middleton, USA)
Scanner, hp scanjet 3500C	Hewlett-Packard (Palo Alto, USA)
Sigma 4K10 centrifuge	Sigma
Sorvall RC-5B (rotors GSA, GS3 and SS34)	Du Pont de Nemours GmbH
Speed vacuum (Concentrator 5301)	Eppendorf
Thermo mixer Comfort	Eppendorf
Thermocycler PTC-200	M J Research
Ultrospec2100 uv/visibile Spectrophotometer	Amersham Pharmacia
Vortex Genie 2 TM	Bender and Hohlbein AG (Zurich)
Western blot apparatus	“semi-dry” custom made system

4.1.3 Chemicals

All chemicals used in this work were of analytical grade and purchased from one of the companies (if not mentioned otherwise) listed in section 4.1.1.

ABTS Buffer, 1112597	Roche
ABTS tablets, 1112422	Roche
BIS-TRIS-Propane	Biomol GmbH
Calcium chloride ($CaCl_2$)	Roche
DMF, dimethyl formamide	Fluka

pNPP AP substrate, 104.0	Sigma
EDTA, ethylene diamine tetra-acetic acid, di-sodium salt	Gerbu
Glycerol (99.5%)	Gerbu
HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid	Gerbu
Hydrochloric acid	Roth
Isopropanol	Roth
IPTG	Gerbu
Kanamycin	Gerbu
LM, n-dodecyl-β-D-maltoside	Glycon
Methanol (99.9%)	Roth
Milchpulver (blotting grade)	Roth
Polyethylene glycol (PEG 6000)	Roche
Polyethylene glycol (PEG 8000)	Sigma
Potassium hydroxide	Roth
Potassium sulfate (K_2SO_4)	Merck
Sodium-azide (NaN_3)	Merck
Sodium chloride (NaCl)	Gerbu
SDS, sodium dodecyl sulfate	Gerbu
Sodium hydroxide (NaOH)	Gerbu
Sulfuric acid (H_2SO_4)	Roth
Tris, tris-hydroxymethyl-9-aminomethane (99.9%)	Roth
Triton-X 100	Sigma
Tween 20	Sigma

4.1.4 Chromatography materials

His Trap^{TM} HP (5x1 ml)	Amersham Biosciences
Ni^{2+}-NTA Agarose	Qiagen
Superose 6 PC 3.2/30 (column for SMART^{TM} system)	Amersham Biosciences
Superose 12 PC 3.2/30 (column for SMART^{TM} system)	Amersham Biosciences
Superdex 200 PC 3.2/30 (column for SMART^{TM} system)	Amersham Biosciences

4.1.5 Other Materials and consumables

Bicinichoninic acid (BCA) Assay Reagent A and B	Uptima
NuPAGE® 4-12% Bis-Tris Gel	Invitrogen
IMMOBILIZER Ni-Chelate F96 CLEAR, 436024	Nunc
MAXISORP Loose F16 (Immuno modules), 469914	Nunc
Square-Well Blocks (96 wells), 19573	Qiagen GmbH
24-WellBlocks RB (4), 19583	Qiagen GmbH
Large petri-dishes (145/20 mm), 639102	Greiner bio-one GmbH
Disposable columns (10 ml), 29924	Pierce
PVDF membrane (Immobilon™ IPVH0010)	Millipore

4.1.6 Proteins and antibodies

Albumin from Bovine serum, A7906	Sigma, GmbH
Alkaline Phosphatase, P-5521	Sigma, GmbH
Anti-Chicken IgY -AP conjugate (Rabbit), A-9171	Sigma, GmbH
Anti-Flag® M2 Monoclonal Antibody, F3165	Sigma, GmbH
Anti-Mouse IgG-AP conjugate (Rabbit), A-4312	Sigma, GmbH
Anti-Mouse IgG-AP conjugate (Goat), A-5153	Sigma, GmbH
Anti-HA Tag Clone HA-7 (Mouse), H-9658	Sigma, GmbH
Anti-Histidine Tag Monoclonal antibody, H 1029	Sigma, GmbH
Anti-M13 monoclonal Antibody, 27-9420-01	Amersham-Pharmacia
Anti-M13 Monoclonal HRP-conjugate, 27-9421-01	Amersham-Pharmacia
Anti-myc-tag monoclonal antibody 9E10	hybridoma supernatant.
Biotin-c-Alkaline Phosphates, 8452 E01	Biomeda corp
Trypsin, TPCK treated from Bovine pancreas, T1426	Sigma, GmbH
See Blue Plus 2, pre-stained protein maker	Invitrogen

4.1.7 Molecular biology enzymes, reagents and kits

PerkinElmer AmpliTaq®DNA polymerase, A02060	Roche
Pfu DNA Polymerase , M7745	Promega
Sfi 1 restriction enzyme (20,000 U/ml), R0123S	New England *BioLabs*
SuperScript™ III First-Strand Synthesis kit, 18080-051	Invitrogen
T4 DNA Ligase, 15224-017	Invitrogen
Glycogen, R0561	Fermentas

Water (nuclease free), 1193	Promega
Water (nuclease free), R0581	Fermentas
100 bp DNA Ladder, N3231S	New England *BioLabs*
1 kb DNA Ladder, N3232S	New England *BioLabs*
RNase AWAY (250ml), 7000	Molecular BioProducts.Inc
QIAquick® Gel Extraction kit, 28704	Qiagen GmbH
QIAprep® spin miniprep kit, 27106	Qiagen GmbH
HiSpeed plasmid Maxi kit (25), 12663	Qiagen GmbH

4.1.8 Molecular biology consumables

Elektroporationskuvetten 2mm, 71-2020	PEQlab, biotech GmbH
Microfuge PCR Tubes (RNase-free), 12450	Ambion
SafeSeal Microcentrifuge tubes (1.7ml)/nuclease free, 11720	Sorenson
Nuclease free pippet tips (20µl), 14210	Sorenson
Nuclease free pippet tips (100-1000µl), 14200	Sorenson

4.1.9 Primers

Name	Sequence (in 5' to 3' direction)
P_1: VH_P-CSCVHo-F (forward) short linker	GGT CAG TCC TCT AGA TCT TCC GCC GTG ACG TTG GAC GAG
P_2: VHp-CSCVHo-FL (forward) long Linker	GGT CAG TCC TCT AGA TCT TCC GGC GGT GGT GGC AGC TCC GGT GGT GGC GGT TCC GCC GTG ACG TTG GAC GAG
P_3: VHp- CSCG-B (reverse)	CTG GCC GGC CTG GCC ACT AGT GGA GGA GAC GAT GAC TTC GGT CC
P_4: VLp- CSCVK (forward)	GTG GCC CAG GCG GCC CTG ACT CAG CCG TCC TCG GTG TC
P_5: VLp- CKJo-B (reverse)	GGA AGA TCT AGA GGA CTG ACC TAG GAC GGT CAG G
P_6: OEp- CSC-F (forward)	GAG GAG GAG GAG GAG GAG GTG GCC CAG GCG GCC CTG ACT CAG
P_7: OEp- CSC-B (reverse)	GAG GAG GAG GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT GGA GG
LMB3 (forward)	CAG GAA ACA GCT ATG AC
fd Seq (reverse)	GAA TTT TCT GTA TGA GG
pHEN Seq (reverse)	CTA TGC GGC GGC CCC ATT CA
Omp Seq (forward)	AAG ACA GCT ATC GCG ATT GCA G
g back (reverse)	GCC CCC TTA TTA GCG TTT GCC ATC

4.1.10 *E. coli* strains and phagemid vectors

E. coli K12 XL1-Blue Electroporation-Competent cells, 200228	Strategene
E. coli K12 TG1 (Viti *et al.,* 2000)	GeneService Ltd, UK
E. coli K12 ER2925, E4109S	NewEngland *BioLabs*
E. coli K12 HB2151, MRB-0027	Maxim Biotech
pIT2* -phagemid vector (supplied with Tomlinson library)	GeneService Ltd, UK
pDN322* -phagemid vector (Pini *et al.,* 1998)	Prof. Dr. D.Neri, Swiss Federal Institute of Technology, Zurich.
pCom3x*-phagemid vector (Andris-Widhopf *et al.,* 2000)	Prof. Dr. CF. Barbas, The Scripps Research Institute La Jolla, USA.

(* the maps of respective phagemids are provided in appendix A-2).

4.1.11 Buffers and Solutions

Biopanning buffers

- **Phage Dilution Buffer (PDB):1mM Tris/HCL pH 7.5, 2mM EDTA, 20mM NaCl, filter-sterilized and stored at 4^{o}C.**
- Phage elution buffer for low pH-elution: 0.1N HCl/glycine, pH 2.2.
- Phage neutralization buffer: 2M-Tris base, pH 8.0.
- Trypsin elution buffer: 1µg/ml trysin in PBS solution.
- Bi-carbonate buffer (antigen coating): 0.1N $NaHCO_3$, pH 8.5 to 9.5.
- PEG/NaCl solution (5x): 20% PEG-6000 or 8000 in 2.5M NaCl. autoclaved, and stored at RT.

General buffers

- TBS buffer: 25 mM Tris, 150 mM NaCl. pH 7.4, can be made as 20x stock, and autoclaved.
- TBST solution: 1xTBS + 0.05% Tween-20.
- PBS buffer: 50mM phosphate (i.e. 33mM Na_2HPO_4, 17mM), 100mM NaCl. pH 7.4, can be made as 10x stock, and autoclaved.
- PBST solution: 1xPBS + 0.05% Tween-20.
- MPBS: 2% milk powder in 1xPBS.
- BTP Buffer: 20mM BisTris Propane, 200mM NaCl, pH 7.2 at RT.

- HEPES buffer (HB): 20 mM HEPES, 100mM NaCl, 12mM $MgCl_2$, 5μM alprenolol, 0.02% NaN_3, pH 7.4.
- HBLM: 1xHB, 0.02%LM.
- Buffer A (for periplasmic extraction): 100mM Tris, 1mM EDTA, and 500mM Sucrose. pH 8.0 adjusted by HCl, filter-sterilized and stored at 4°C.
- pNPP-ELISA buffer for AP: 10% (v/v) diethanolamine, 0.5mM $MgCl_2$, pH 10 adjusted by HCl. Just before ELISA, add 5~10 mg pNPP sigma substrate solution to 10 ml of above buffer.

SDS-PAGE and Western blot buffers

- MES running buffer for SDS-PAGE: 50mM MES, 50mM Tris base, 0.1%SDS, 1mM EDTA, can be made as 20x stock, storage preferably at 4°C.
- Tris-glycine buffer: 25mM Tris, 250mM glycine, 10% SDS.
- LDS sample loading buffer (for SDS-PAGE): 3%LDS, 10% (w/v) glycerol, 50mM Tris, 0.075% Serva Blue-G250, 0.025% Phenol Red, 50mM DTT.
- Gel storage solution: 7% (v/v) acetic acid, 2% (w/v) glycerol.
- Blotting buffer (normal transfer): 20% (v/v) methanol in 1x MES.
- Renaturation Buffer 1x: 50 mM Tris/HC, 20% glycerol, pH 7.4.
- Transfer Buffer (basic): 10 mM $NaHCO_3$, 3mM Na_2CO_3, 20% Methanol. pH 10.
- AP Buffer for Western-blot (10x): 1M Tris base, 1M NaCl. pH 9.5, storage 4°C.
- BCIP stock solution: 50mg / ml in 100% DMF, store in aliquots at –20°C.
- NBT stock solution: 50mg / ml in 70% DMF, store in aliquots at –20°C.

SDS-PAGE staining solutions

- Fixation solution: 30% Ethanol, 10% Acetic acid.
- Conditioning solution: 0.5 M Na-acetate, 10mM $Na_2S_2O_3$, 0.125% (v/v) Glutaraldehyde, 30 %(v/v) Ethanol.
- Silver solution: 0.2% (w/v) $AgNO_3$, 0.02% Formaldehyde.
- Developing solution: 0.24 M Na_2CO_3, 0.04% Formaldehyde.
- Coomassie staining solution: 0.25% (w/v) Brilliant Blue-R250, 50% (v/v) methanol, 10% (v/v) acetic acid.
- Coomassie staining solution: 40% (v/v) methanol, 10% (v/v) acetic acid.

Blue Native PAGE solutions

- Deep Blue cathode Buffer: 50mM Trysin, 7.5mM Imidazol, and 0.02% CBB –G250.
- Light Blue cathode Buffer: 50mM Trysin, 7.5mM Imidazol, and 0.002% CBB –G250.

- Anode Buffer: 25mM imidazol pH adjusted to 7 by HCl.
- Gel buffer (6x): [3M Aminocaproic acid, 150mM Imidazol/HCl, pH-7.

Agarose gel electrophoresis Buffer

- TAE (50x) DNA/Agarose gel electrophoresis buffer: 24.2% (w/v) Tris base, 5.7% (w/v) glacial acetic acid, 50mM EDTA.
- Gel loading buffer (6x): 0.25% bromophenol blue, 0.25% Xylene cyanol FF, 30% glycerol.

4.1.12 Culture media

- 2xTY liquid media: 1.6% (w/v) Tryptone, 1.0% (w/v) Yeast extract, 0.5% (w/v) NaCl. pH 7.2, storage at RT after autoclaving.
- SOB liquid media: 2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.05% (w/v) NaCl, 2.5mM KCl, and 10mM $MgCl_2$. pH 7.0, autoclaved.
- SOC liquid media: To autoclaved SOB, filter-sterilized glucose was added (final 20mM).
- LB liquid media: 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) NaCl.
- TYA solid media for plates: 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.8% (w/v) NaCl, 1.5% Agar autoclaved and cooled to 40^{o}C prior to addition of antibiotics, poured in small or large petri dishes.
- Soft agar media: 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.8% (w/v) NaCl, and 0.75% Agar, autoclaved.

4.1.13 Antibiotics

- **Tet** Tetracycline (1000x) stock solution: 12.5 mg/ml in ethanol, filter sterilized and stored as 50% glycerol in dark at -20^{o}C.
- **Kan** Kanamycin (1000x) stock solution: 50 mg/ml in distilled water, filter sterilized and stored at -20^{o}C.
- **Amp** Ampicillin (1000x) stock solution: 100 mg/ml in distilled water, filter sterilized and stored at -20^{o}C.
- **Carb** Carbenicillin (1000x) stock solution: 100 mg/ml in distilled water, filter sterilized and stored as 50% glycerol at -20^{o}C.
- **Chl** Chloramphenicol (1000x) stock solution: 35 mg/ml in ethanol, filter sterilized and stored as 50% glycerol at -20^{o}C.
- **Nal** Nalidixic acid (1000x) stock solution: 15 mg/ml in distilled water, filter sterilized and stored at -20^{o}C.

4.2 Molecular biology methods

If not otherwise specified, standard protocols provided in *Molecular cloning: A laboratory manual* were followed (Sambrook *et.al.,*1989). All the molecular biology experiments were carried out at room temperature if not indicated otherwise. All experiments involving RNA were performed either on ice or at 4^{o}C.

4.2.1 RNA isolation

The RNA harvesting from chicken was carried out at the company PRF and L (Canadensis, PA 18325). For the isolation RNA, a modified protocol based on the original work of Chomczynski and Sacchi was followed (Chomczynski and Sacchi 1987). Briefly describing here, the chicken was sacrificed and its spleen and bone marrow of one leg was harvested. RNA was isolated separately from the two tissue types. RNA in isopropanol was shipped from the company. It was stored at -80^{o}C in 1 ml aliquots until use.

Extraction and purification of RNA from isopropanol

RNA-isopropanol suspensions were taken out of -80^{o}C, centrifuged at 13000g for 10 minutes. The RNA pellet obtained was washed once with cold (-20^{o}C) 70% ethanol (RNAse free), briefly air dried for 3-5 minutes, and finally resuspended in 100µl of nuclease free water. The purified RNA from spleen and bone marrow was pooled, and the concentration was determined at OD_{260} with a spectrophotometer.

4.2.2 RT-PCR

All RT-PCR experiments were performed with SuperScript^{TM} III RT-PCR kit, following the protocol and guidelines of manufacturer. Five reactions were prepared as shown below in nuclease free 0.2 ml PCR tubes as:

Reaction mix. A		**Reaction mix. B**	
Total RNA (6~7µg)	2µl	10x RT buffer	4µl
Oligo dT_{20} [50µM]	2µl	$MgCl_2$ [25mM]	8µl
dNTP mix.[10mM]	2µl	DTT [0.1M]	4µl
DEPC-H_2O	14µl	RNAse OUT [40 U/ µl]	2µl
Total volume	**20µl**	SuperScript^{TM}III [200 U/ µl]	2µl
		Total volume	**20µl**

(All reagents in the **A** and **B** (except the RNA) are provided and used from the kit).

The 20µls reactions (reaction mixture **A**) were incubated for 5 minutes at 65^{o}C, followed by a brief (2 minutes) chilling on ice. To each reaction mixture A: 20µl of reaction mixture B: containing the SuperScript^{TM} reverse transcriptase were added and gently mixed followed by a brief spin. The reactions were incubated at 50^{o}C for 50 minutes to allow the RT-PCR synthesis of cDNA. The reaction was stopped by a 5 minute incubation step at 85^{o}C, followed by 2 minute ice chilling. RNAse H was added (4 units/ 40µl reaction), and the reactions were incubated for another 20 minutes at 37^{o} to chop off the RNA strand. The cDNA so obtained from one of the RT-PCR reactions was used to amplify variable heavy (V_H) and light (V_L) chain antibody gene segments, and the rest of the reactions were stored at -20^{o}C.

4.2.3 DNA isolation

For all routine phagemid DNA preparations such as for sequencing, PCR and restriction analysis, QIAprep® spin miniprep kit was used following the protocol and guidelines of the manufacturer. Briefly describing, the desired phagemid clone was grown in host *E. coli* (XL1-blue or HB2151) until OD_{600} ~ 1.0. The cells from 2 to 4 ml culture were harvested, resuspended in 250µl of P1 buffer (RNAse added). 250µl of lysis buffer P2 were added and gently mixed. The reaction mixtures were neutralized by 350µl of N3 buffer. The mix was centrifuged (10000 g for 10 minutes) and supernatant applied to QIAprep column. After spinning the column (10000 g for 1 minutes), the flow through was discarded. The column washed with 250µl of PE buffer. The DNA was eluted with 50 µl of 2.5mM Tris-HCl, pH 8.5, and quantified by spectrophotometeric analysis.

For large scale production of pComb3xSS vector DNA, the phagemid was transformed in *E. coli* strain ER2925, and harvested from 300ml o/n culture. The DNA was isolated by Hi-speed plasmid Maxi-Kit (Qiagen), following the protocol and guidelines provided by the manufacturer. The final DNA elution step was carried out with EB (10mM Tris-pH 8.0).

4.2.4 Agarose gel electrophoresis

For routine analysis of DNA obtained after PCR or restriction analysis, 5µl reaction samples were mixed with loading buffer and loaded on 1.2% agarose gel made in 1xTAE buffer using a custom made electrophoresis apparatus. The analytical gel electrophoresis was run in 1x TAE at 80 V until the tracking dyes is resolved properly. The preparatory gel electrophoresis was carried at lower voltage (50 V) and extended duration in order to get better separation and sharp bands.

4.2.5 DNA purification

Extraction from agarose gel slices: The DNA samples were mixed with 1/5th volume of 6x DNA loading dye, and resolved on preparatory scale agarose gel electrophoresis. Once the corresponding DNA bands were resolved properly, they were cut out of the gel with clean and razor-sharp scalpel. The DNA from the gel slices was extracted by QIAquick® Gel Extraction kit following the protocol and guidelines provided by the manufacture. The final DNA elution step was carried out with 75µl/column of 2mM Tris pH-8.0.

NaOAc/Ethanol precipitation: The DNA sample were pooled and precipitated by adding 1/10th volume of 3M NaOAc pH-5.6 + 2 volumes of 100% ethanol + 1 µl glycogen. The reactions were properly mixed and incubated overnight at -20^{o}C. Next day the DNA pellets were obtained after spinning the samples at 15000g for 10 minutes, washing once with 70% ethanol (cold), briefly dried and finally resuspended in appropriate volume of nuclease free H_2O.

4.2.6 DNA/RNA quantification

All DNA or RNA quantification was performed by spectrophotometeric analysis using Ultrospec 2100$_{pro}$ UV/visible Spectrophotometer. An OD_{260} of 1 for ~ 50µg/ml of dsDNA and ~ 40µg/ml of RNA was considered. The Ultrospec 2100 has inbuilt software programs for analysis of DNA/RNA quantity and quality. During the chicken library construction, the relative amounts of DNA were checked also by resolving samples on agarose gel electrophoresis and comparing with known amounts of reference DNA (i.e. 1kb or 100bp standard DNA ladders).

4.2.7 PCR

For routine PCR, 50µl reactions were set up as:

H_2O	34.5 µl
template DNA (~100ng)	2.0 µl
10x buffer	5.0 µl
dNTP mix. [2mM each]	5.0 µl
Forward primer (~ 30 p mole)	1.5 µl
Backward primer (~ 30 p mole)	1.5 µl
Taq DNA Pol. [5 U/ µl]	0.5 µl
Total reaction volume	**50 µl**

The synthesis of chicken heavy and light chain genes was carried with following primer combinations cDNA as template:

- P_1 (*forward*) and P_3 (*reverse*) for V_H with short linker.

- P_2 (*forward*) and P_3 (*reverse*) for V_H with long linker.
- P_4 (forward) and P_5 (*reverse*) for V_L.

PCR was carried out in (PTC-200) thermocycler, programmed as: 94°C for 5 minutes, followed by 31 repeated cycles of denaturation (94°C for 45 seconds), annealing (55°C for 45 seconds) and extension (72°C for 1 minute). The 72°C extension step in the last PCR cycle was extended for 10 minutes. The quality of PCR amplified product was analyzed by agarose gel electrophoresis. The respective primer sequences are in section 4.1.9.

4.2.8 Overlap PCR

The overlap-PCR was performed to physically join the light (V_L) and heavy (V_H) chain with a linker region into a single-chain format. Overlap-PCR primers P_6 (forward) and P_7 (reverse) are used. These primers anneal to the 5`end of $V_L$ and 3`end of V_H respectively, and carry respective *Sfi1*-endonuclease restriction sites. The scFv DNA construct thus synthesized carry *Sfi1* sites on both terminals for the purpose of cloning in pComb3x vector.

The overlap-PCR was carried out with 100ng template DNA, 60 p mole forward and backward primer each, 3 U Pfu and 5 U Taq DNA polymerase in 100µl total reaction volume. The conditions are as described in section 4.2.7, except that the 72°C extension steps were prolonged to 2 minutes. The quality of PCR amplified scFv products was determined by agarose gel electrophoresis.

4.2.9 Colony/Insert-check PCR

To check the presence of scFv-inserts in colonies selected after panning, either the isolated phagemid DNA (100-200ng) or the bacterial colonies directly were used as template. The PCR was carried out as described in section 4.2.7, using following forward and reverse primers:

- *LMB3* and *fd Seq* for ETH-2 library clones.
- *LMB3* and *pHEN Seq* for Tomlinson library clones.
- *Omp Seq* and *g back* for chicken library clones.

Once the PCR-cycles were complete, the reaction samples were analyzed by agarose gel electrophoresis. The respective primer sequences are in section 4.1.9.

4.2.10 DNA sequencing

All DNA sequences were routinely obtained by HotShot sequencing (Seqlab). Phagemid-scFv DNA samples (100~150 ng) from respective clones were mixed with either of the forward or reverse primers as recommended by the company. For sequencing the respective insert-check PCR primers were used (section 4.2.9).

4.2.11 Digestion and Ligations

Restriction digestions and ligation reactions were carried out according to manufactures guidelines in a way that the glycerol content of the reaction was kept below 5%. For *Sfi*1 digestion of scFv inserts and pComb3xSS phagemid, the reaction mixtures were incubated at 50^{o}C for 6 ½ hours. For ligation, T4 DNA ligase was used.

For ligation of each SL and LL library, *Sfi1*-digested vector DNA was used with an insert to vector (~2:1) ratio. Stuffer fragment (SS) obtained during the *Sfi*1-digestion of pComb3xSS vector DNA was used as positive control for the ligation reaction. To check the extent of self annealing by the empty vector, a negative control ligation reaction was set up same as above minus insert DNA.

4.3 Library construction methods

If not otherwise specified, standard protocols are based or modified from *Phage Display: A laboratory manual* (Andris-Widhopf and Barbas 2001). All DNA samples used during the construction of library were gel-extracted and purified prior to use. Those include the DNA (i.e. for V_H, V_L and scFv) obtained after each PCR or overlap-PCR reaction, and also the *Sfi*1-digested scFv library and pComb3x vector.

4.3.1 Preparation of electrocompetent XL1-Blue *E. coli* cells

Electro-competent XL1-blue *E. coli* cells were prepared by a modified protocol adopted from Rader and Barbas (Rader *et al*., 2001). A well separated single colony was inoculated into 3ml of 2xTY supplemented with tetracycline and 1% glucose, and incubated overnight at 37^{o}C/225 rpm. A secondary culture of 250ml was raised from the culture, till it attained OD_{600} reached 0.8. The culture was chilled on ice for 20 minutes, and cells harvested by spinning at 3000g for 20 minutes at 4^{o}C. All the subsequent steps were carried out on ice. The pellet was gently washed twice with ice-cold sterile water and once in cold 10% glycerol, followed by final resuspension in 1.2 ml of chilled sterile 10% glycerol and divided into 0.3 ml aliquots. The aliquots were either used immediately or stored at -80^{o}C.

4.3.2 Transformation of XL1-Blue with scFv library DNA

Freshly prepared electrocompetent XL1-blue cells were used. For transformation, 10 µl of ligated library (~700ng of vector DNA) were added to 300µl of electrocompetent XL1-blue cells. Gently mixed, incubated for 2 minutes on ice and transferred in pre-chilled 0.2mm cuvettes. The electroproration was performed using Gene pulser (BioRad), set up as: 25µF, 2.5kV and 200Ω. The transformed libraries were immediately resuspended in 10 ml of pre warmed SOC media, and grown at 37^{o}C/225rpm for 1 hour, harvested at 2000g for 10 minutes, and resuspended in 500 µl of SOC and subsequently plated on large petri-dishes (145/20 mm) containing TYA supplemented with carbenecillin, tetracycline and 2% glucose. The plates were incubated overnight at 37^{o}C. Next day the libraries were harvested with 3ml/plate of 2xTY, pooled together, and stored in 1 ml aliquots at -80^{o}C as 25% glycerol stocks.

4.3.3 Library size determination

2µl of each 10ml library culture (section 4.3.2) were diluted to 2 ml (10^{-3} dilution), 10 and 50µl from this were plated on LB/carb/agar plates. The library size was determined as total number of transformants obtained after transformation.

4.4 Phage display methods

If not otherwise specified, standard phage display protocols from *Phage Display: A laboratory manual* (Andris-Widhopf *et al*., 2001) were used for panning and selection of Chicken libraries. For ETH-2 and Tomlinson I+J library panning, the standard protocols recommended by the manufacturer were followed. During the panning experiments against membrane protein targets i.e. β_2AR and STNhaA, detergent (~ 0.03%LM) was present in every buffer through out the experimental steps.

4.4.1 Preparation of KM*13* helper Phages

A well separated small plaque was picked and infected to 10 ml mid-log phase culture of XL1^{TetR} Blue (OD_{600nm} 0.4 ~ 0.5). The infection was carried out at 37^{o}C for 30 minutes without shaking and one hour at 180 rpm. After infection, the culture was transferred into 50 ml 2xTY+ Tet+ ½ Kan (i.e. 25µg/ml), and grown for another two hours at 37^{o} C/180rpm. ½ Kanamycin was added to allow the mild selection pressure. After two hours, the culture was transferred into 1L flask containing 500 ml 2xTY supplemented with Kan (50

μg/ml), at 30°C/180rpm shaking for 16 hours. Next day the helper phages were obtained from the cleared supernatants by PEG/NaCl purification (section 4.4.3), resuspended in 3 ml PDB, and stored in aliquots at 4°C.

4.4.2 Preparation of phages

The phage library was inoculated into 200 ml of 2xTY supplemented with 100μg/ml carbenecillin, 12.5 μg/ml tetracycline and 1% glucose and grown at 37°C / 235 rpm for 2 hours until OD_{600} ~ 0.5-0.8, followed by helper phage infection (~ $5x10^{11}$ pfu of KM13 or Hyperphage). Infection with helper phage was carried out at 37°C for 30 minutes without shaking, followed by 1 hour shaking at 180rpm. After infection the cultures were centrifuged at 3500g for 10 minutes, and the cell pellets resuspended in fresh 250ml of (pre-warmed) 2xTY supplemented with Ampicillin/Carbenicillin and Kanamycin. The cultures were grown overnight at 30°C/180 rpm for phage production. Next day, the phage-scFvs were purified from the culture supernatants by PEG/NaCl precipitation, and titer determined as described (section 4.4.5). Usually the phage-preparation was used for panning on the same day, and in parallel the titer of harvested phages determined.

4.4.3 PEG/NaCl purification of phages

The phages were purified from overnight culture supernatants as follows. Cultures were centrifuged at 8000g for 30 minutes, and to the supernatant 1/5th volume of precipitant solution (20% PEG_{8000} in 2.5 M NaCl) was added. Mixed properly and incubated on ice for two hours. Phages were harvested by spinning at 4000g for 30 minutes, and the pellet gently resuspended in the panning buffer (1x PDB or 1x TBS or 1x HB). Usually the pellet corresponding to 50 ml supernatant volume was resuspended in 1 ml volume. The phage suspension was cleared of the residual bacterial debris by spinning it at 10,000 g for 10 minutes, and was stored at 4°C until used for titration or panning.

4.4.4 Titration of helper phage: *Plaque forming-unit determination*

The titer of the helper phages is calculated as plaque forming units (pfu). Serial dilutions of the helper phage were made in PBS or 2xTY liquid media. To each phage dilution (~90 μl volume), 900 μl of freshly grown XL1 Blue (OD_{600nm} 0.4~0.5) was added, gently mixed and incubated in 37°C for 30 minutes without shaking to allow the infection. Meanwhile 5ml of soft agar was poured in 15ml falcon tubes, and cooled to 45°C. To these, 1 ml of the above infected XL1-blue cultures was added. After a homogenous gentle mixing, the mixture was immediately poured on petri plates (earlier a layer of agar (2% in ddw) had

been poured in these petri-plates to create an anchorage surface for the soft agar). The plates were kept at RT for 30 minutes to let the soft-agar solidify, and then transferred to 37^{o}C incubator for overnight incubation. Next day morning, the plates showing the well-separated plaques were used to calculate the pfu.

4.4.5 Titration of library phage: *Transforming-unit determination*

The titer of the library phages is calculated as transforming units (TU). All the phagemids used in this thesis contain amp^{R} selection marker that allows the *E. coli* cells infected by them to grow on Ampicillin or Carbenicillin agar plates. Serial dilutions of the phage obtained after PEG/NaCl purification made in PBS or 2xTY liquid media were infected to freshly grown XL1 Blue (OD_{600nm} 0.4~0.5). The infection was carried out at 37^{o}C for 30 minutes. The samples were plated on TYA supplemented with 100µg/ml Ampicillin or Carbenicillin. The plates were overnight incubated at 37^{o}C. Next day morning, the plates showing the well-separated colonies were used to calculate the TU.

4.4.6 Panning of phage libraries

Phages obtained in section 4.4.3 were used for panning against immobilized targets. The phages were resuspended in respective panning buffers (1xTBSLM for STNhaA, 1xPBS for yeast cyt. *c*, and 1xHBLM for β_2AR). MaxiSorp^{TM} microtiter wells were directly coated with the antigen (except for β_2AR, section 4.4.7). For each library, 4 wells were coated for 1st round, and only 2 wells for the subsequent panning rounds. The coating was done for 2 hours with 100µl /well of antigen (70µg/ml STNhaA in TBSLM, or 20µg/ml yeast cyt. *c* in 0.1 N $NaHCO_3$, pH-9.8, or 20µg/ml of β_2AR in HBLM). The wells were briefly washed with respective panning buffers and blocked with 4% BSA or 2% milk for 2 hours at RT, followed by incubating with the phage preparations (10^{10}-10^{11} TU/well) for 90 minutes at RT. This was followed by the multiple washing with respective panning buffers to remove unspecifically bound phages. The washing was performed by adding 400µls of TBSLM or else to each well and mildly shaking it for 5 minutes.

Elution of specific phages: the antigen bound phages were eluted with respective elution buffers (glycine-HCl, pH-2.2 or trypsin solution). The pH-eluted phages were neutralized immediately with 2M Tris pH-8 (25 µl of 2M Tris were used to neutralize each elution volume of 100 µl). The eluted phages were pooled for each library.

Amplification of eluted phages: Half of eluted phage were immediately infected into 3 ml of freshly grown XL1-Blue cells (OD_{600}- 0.5), and the other half was stored at 4^{o}C for

backup stock. The phages were rescued by helper phage superinfection as described in section 4.4.2. Next day the phage-scFvs were harvested from the supernatants followed by PEG/NaCl purification. These purified phages were used as input for the next round of anti-STNhaA selection. The subsequent panning rounds were performed with increased stringency by increasing steps.

4.4.7 Immobilization of β_2 AR for panning

β_2AR was immobilized on streptavidin coated surface one hour prior to panning. The streptavidin surface was generated by coating microtiter wells (MaxiSorp^{TM}) with purified core streptavidin (20µg/ml) in 0.1N $NaHCO_3$ pH-9.6 (100µl/well). The streptavidin coating was usually performed overnight at 4^{o}C (2 to 3 hours at RT was also found to be sufficient). Streptavidin-coated wells were washed once with HBLM, followed by incubation with monomeric avidin purified of β_2AR (10 mg/ml) in HBLM (100µl/well). β_2AR incubation step was performed at 4^{o}C for 90 minutes.

4.4.8 Polyclonal phage-ELISA

Polyclonal phage ELISA was performed to check the specific enrichment of *binders* across the panning rounds. The target antigens were immobilized as done during panning using MaxiSorp^{TM} microtiter ELISA plate. BSA or no-antigen was coated in the control wells. For each sample ELISA was performed in duplets or triples. The unbound antigen solution was discarded, the ELISA plate gently tapped on a paper towel to remove the remaining liquid. Blocking was performed with 3%BSA or 2% Milk for in respective panning buffers. The blocking solution was discarded, and the wells incubated with the polyclonal phages (10^8-10^9 phages/well) obtained after the elution/amplification steps. The binding was carried out at RT for 75 minutes, followed by 5x 30-second washing. The bound phages were probed with anti-M13 antibody. The incubation with anti-M13 was carried out at RT for 1 hour, followed washing as done in previous step. Anti-M13 antibody was in turn detected by anti-mouse alkaline phosphate conjugate of rabbit origin. The ELISA was developed by adding pNPP-substrate solution to each well, and was read at OD405-450 after 5, 15 and 45 minutes in the Power Wave $_X$ ELISA reader. Each incubation step was carried with 100µl/well volume, washing and blocking steps with 400µl/well.

4.4.9 Polyclonal soluble-scFv ELISA

The basic experimental setup is similar to section 4.4.7, except that binding of soluble-scFv (i.e. not fused to pIII) rather than phage-scFvs was tested. Overnight culture cleared supernatants obtained from phage infected HB-2151, or XL1-Blue (in this case after PEG/NaCl precipitation of the phage) were used for binding. The bound scFvs were detected via their affinity tags by using anti-tag secondary antibodies (anti-HA tag mAb for chicken, anti-FLAG tag M2 mAb for ETH-2 and anti-myc tag 9E10 for Tomlinson library clones)

4.4.10 Isolation and propagation of monoclonal scFvs antibodies

For analysis of antigen-specific monoclonals after the panning experiments, the eluted polyclonal phages (usually from the rounds that gave highest enrichment signals in polyclonal ELISA) were infected to HB-2151, and serial dilutions were plated to obtain single colonies. Next day, well separated single colonies were picked up with sterile yellow (200µl) tips, and inoculated into 96x format deep well blocks (Qiagen) containing 0.5 ml/well of 2xTy/Nal./Carb./1.5%glucose. The blocks were incubated overnight at 37^{o}C/225 rpm. These single colony cultures were replicated on TYA/Carb and the original cultures (master-plate) stored at -80^{o}C as 25% glycerol stock.

4.4.11 Expression of monoclonal soluble-scFvs

For expression of monoclonal soluble-scFvs, single colonies picked from 96x well plate (section 4.3.10) were inoculated in 24x deep-well plate containing 1ml/well of 2xTY supplemented with Ampicillin or Carbenecillin and 1% glucose (1% glucose was omitted during the expression of chicken library clones). The 24xwell block was incubated at 37^{o}C/225 rpm for about 4 hours. The induction was carried out by adding 1ml/well of 2xTY containing the required antibiotic and 2mM IPTG, followed by overnight growth at 30^{o}C/225 rpm. Next day the cultures from 24xwell block were transferred into 2ml eppendoff tubes, and supernatants harvested by spinning at 12,000 g for 15 minutes in a table-top centrifuge. The cell pellets were stored at -20^{o}C for DNA analysis, and the supernatants containing soluble-scFvs were stored at 4^{o}C, or used for monoclonal ELISA.

4.4.12 Monoclonal ELISA

The general set up of monoclonal-ELISA experiments was similar to that of polyclonal-ELISA experiments (section 4.4.8 and 4.4.9). Here, monoclonal phage-scFv or monoclonal soluble-scFv supernatants (section 4.3.11) were used to check the binding.

4.5 Protein biochemistry methods

All protein biochemistry methods were carried out at 4^{o}C if not specified otherwise. All scFvs were produced and purified from periplasmic extracts of HB-2152 *E. coli* strain. And all the phagemid vectors used in this study (i.e. pDN322, pIT2 and pComb3x) place a 6x His-tag on the C-termini of scFvs for IMAC purification.

4.5.1 Production of scFv: *analytical scale*

E. coli harboring the respective scFv-phagemid clone, were directly inoculated from -80^{o}C glycerol stock into 10 ml of 2xTY/Amp/Nal/1.5% glucose. The cultures were grown at 37^{o}C/201 rpm shaking until OD_{600} ~ 0.5 was reached. The glucose was removed by spinning the cells at 4000g for 10 minutes and resuspend the pellet in fresh 200 ml of 2xTY/Amp, and grown till OD_{600} ~ 0.5, followed by induction with 1mM IPTG. Sucrose (3 grams/200 ml culture) was also added along increase the yield of periplasmic protein expression. Induction was carried out overnight at 30^{o}C/200 rpm, and next morning the cells were harvested by spinning at 6000g for 30 minutes to obtain periplasmic extracts (section 4.5.3). During the production of scFv from chicken library selected clones, Ampicillin was replaced by Carbenecillin and glucose was omitted from the beginning and the shaking was increased from 200rpm to 235rpm.

4.5.2 Production of scFv: *preparatory scale*

Large scale expression and production of selected scFvs similarly performed as above (section 4.5.1). The respective pre-culture volumes were scaled up. Based on the requirement of protein amount, the final culture volume was scaled up to 2 or 12 liters. 50µls of the -80^{o}C glycerol stock were inoculated in 12 ml of 2xTY/Carb/Nal., and grown at 37^{o}C/ 235 rpm till OD_{600} ~ 0.8, overnight growth of pre-cultures was always avoided to minimize the loss of insert by host cells. 6 x 5L flasks each containing 2 liters of pre-warmed 2xTY/Carb were inoculated with 2 ml of the above culture, and were incubated at 37^{o}C/ 211 rpm till OD_{600} 0.4~ 0.6. At this stage, the cultures were induced and grown overnight as above. Next morning the cultures were chilled on ice, and cells harvesting at 8000g for 20 minutes.

4.5.3 Periplasmic preparation

The cell pellet harvested from section 4.5.1 and 4.5.2 was resuspended in periplasmic extraction buffer i.e. Buffer A (100mM Tris, 1mM EDTA, and 500mM Sucrose, pH 8.0). 25 ml of chilled Buffer A were used to resuspend the pellet corresponding to each 1L culture. The suspensions were incubated on ice for 40 to 50 minutes, followed by spinning at 10000g for 30 minutes. The supernatant containing periplasmic extract (PPext) was frozen at -20^{o}C until use.

4.5.4 ScFv purification by IMAC

The periplasmic extracts (~25ml/L culture) obtained in section 4.5.3 were thawed on ice, and dialyzed in 10 kDa cut-off membrane to get rid of the EDTA. The dialysis was carried out at 4^{o}C, first against 1L of 200 mM NaCl for two hours, followed by overnight dialysis against 1L of loading buffer LB. (The LB: 20mM Bis-Tris Propane, 200mM NaCl, 10mM imidazol, pH~7.6 was used for chicken scFvs, and phosphate and HEPES buffers, pH~8.0 were respectively used for the anti-cyt *c* 3RE2-10 scFv and anti-β_2AR B2-scFv purification). The scFvs from overnight dialyzed samples were purified either with Ni-NTA agarose (Qiagen), 1ml His-Trap columns (Amersham).

Ni-NTA agarose purification: Ni-NTA agarose (5ml resin for 25ml PPExt) was twice washed with 5 column volume (CV) of H_2O, followed by equilibration with 5CV of LB. To this equilibrated resin, overnight dialyzed PPExts were added, and incubated for 2 hours on a slow overturn rotor wheel to allow binding. The slurry was loaded in vertical columns, and allowed to settle down slowly. The column flow-though was collected for SDS PAGE analysis. Each column was washed by 10CV of washing buffer i.e. LB + 40 mM imidazol, pH~7.6. The bound scFv was eluted with 2CV of elution buffer i.e. LB + 250mM imidazol, pH~7.2, and concentrated to ~ 250 µl in 10 kDa cutoff devices (Amicon). The excess imidazol was removed by diluting these samples with 5 ml of scFv storage buffer (20mM Bis-Tris Propane, 100mM NaCl, pH~7.25, for chicken scFvs, and 1xPBS/TBS or 1xHEPES buffer for other library clones) followed by re-concentration. These dilution/concentration steps were repeated usually until the leftover imidazol concentration remains ≤ 10 mM. The protein concentration was determined by BCA, and the quality of purification was analyzed by SDS-PAGE and gel filtration chromatography.

IMAC purification on Akta prime with 1 ml His-Trap column: Overnight dialyzed PPExts were loaded on equilibrated His-Trap columns using the *Akta prime* system. All the steps were carried out with 0.5ml/minute flow rate. The column was washed with 5 CV of loading buffer (20mM Bis-Tris Propane, 200mM NaCl, 10mM imidazol, pH~7.6), followed by a second wash with 10CV of 10%B (The 100%B corresponds to 600 mM imidazol). At this stage a linear-gradient of increasing imidazol concentration was applied, so that 80% of B was achieved within 15CV of elution volume. The samples corresponding to elution peak and flow-through were checked by SDS-PAGE, and the fractions carrying purified scFv were pooled together and concentrated as done above.

4.5.5 SDS-PAGE Analysis

For routine SDS-PAGE experiments, 4-12% Bis-Tris NuPAGE readymade gels (Invitrogen) were used in 1xMES running buffer (if not mentioned otherwise). Samples were prepared in LDS sample buffer and incubation at 95^{o}C for 10 minutes. Heating was omitted when STNhaA or eNhaA1 was to be resolved. The PAGE was run at 90 -125 mA constant current per gel for the time period, i.e. until the lower loading dye had reached the bottom of the gel. The gel was extracted out of the plastic-cassette either transferred onto a PVDF membrane or stained by silver or Coomassie staining procedure.

Coomassie staining: For Coomassie staining, the gels were incubated in the staining solution for two hours at RT, followed by incubation in de-staining solution. The de-staining solution was replaced once or twice until the background color diminished and protein bands became prominent.

Silver staining: The gel was incubated for 16 minutes in fixing solution, followed by 16 minutes in conditioning solution. The conditioned gel was washed trice in DDW each time for 5 minutes, followed by 20 minute incubation in silver solution. Excess silver was washed (3x 10 second) with DDW. The gel was transferred into the developing solution; as soon as the respective protein bands were visible, the reaction was stopped with 1 ml of acetic acid. The gel was washed with DDW, scanned, and dried after overnight incubation in storage buffer (7% (v/v) acetic acid, 2% (v/v) glycerol.

4.5.6 Western-blot analysis

Normal protocol: once the proteins were resolved on SDS-PAGE, the gel was taken out of the plastic-cassette. The protein was transferred onto a PVDF membrane at 50mAmp for 60 minutes at 4^{o}C, using a custom-made semi-dry transfer apparatus. The membranes were blocked with 3% BSA-TBST or 2% Milk in TBS either overnight at 4^{o}C, or 2 hours at 37^{o}C. The blocked membranes were rinsed once with TBST, and incubated for 1 hour at RT with respective probing primary antibody, followed by detection with secondary antibody conjugated to alkaline phosphatase. The blot was finally developed with NBT/BCIP* substrate solution. (* To 10 ml of AP buffer, add 66µl of NBT, 33 µl of BCIP and 50 µl of 1 M $MgCl_2$).

Western blot-Renaturation protocol (transfer at basic pH): The protocol is based on (Hunte 1993). Here, the SDS-PAGE resolved gel was incubated at 4^{o}C in renaturation buffer (3x10minutes), each time with fresh buffer. This step was performed to allow the partial refolding of proteins. Alkaline pH transfer onto PVDF* membrane was performed at 50mA for 2 hours in cold room. (* PVDF membrane was pretreated, 5 minutes in methanol, 10 minutes in DDW, and 2 minutes in transfer buffer). The rest of the blocking and detection steps were similar to that of normal Western-blot protocol.

4.6 Analytical biochemistry methods

4.6.1 Generation of MaxiSorp^{TM}-streptavidin surface

Recombinant core streptavidin was produced, refolded and purified from inclusion bodies as previously described (Schmidt and Skerra 1994). The quality was checked by analyzing the heat-treated (70^{o}C for 10 minutes) and un-treated samples on SDS-PAGE. For generation of MaxiSorp^{TM}-streptavidin, the coating of streptavidin was optimized by incubating increasing amounts of purified protein on MaxiSorp^{TM} surface in 0.1N $NaHCO_3$, pH-9.6 buffer overnight at 4^{o}C. The plate was washed thrice with PBST (0.05% tween) followed by 2 hours blocking step with 2%MPBS at RT. The blocked surface was once washed with PBST, followed by incubation with biotinylated Alkaline Phosphatase (AP) (1:5000 diluted in PBST) for 1 hour at RT. The unbound materials were removed by washing three times with PBST. The bound AP was detected by developing the ELISA with pNPP-substrate solution.

4.6.2 β_2AR activity assay

Monomeric avidin purified β_2AR (10µg/ml in 1x HBLM containing 0.05% LM) solution was added (100µl/well) to streptavidin-MaxiSorp^{TM} and incubated for two hours at 4^{o}C. The unbound protein was washed three times with 1x HBLM. The activity of bound receptor protein was determined by incubation with 5nM radioactive ligand (CGP-12177, [5, 7-^{3}H]) (Staehelin *et al.,* 1983) following the protocol described previously (Reinhart *et al.,* 2003). Unspecific activity was determined in the presence of excess antagonist 2mM alprenolol. After washing the surface, the receptor bound hot ligand was eluted by 6M urea protein denaturation, and was counted in scintillation counter (the experiment was done in collaboration with Dr. C. Reinhart).

4.6.3 Blue Native PAGE binding assays

Blue Native polyacrylamide gel electrophoresis (BN-PAGE) was performed based on method described by Schagger. BN-PAGE gradient gels were cast following protocol provided in (Schagger 2003). As per our requirement, 6%-19% gradient gels were prepared. The respective solutions were prepared as shown below. After casting the gels at room temperature, they were shifted to 4^{o}C, and usually left for overnight to polymerize. Next day the gels were either used immediately when required or stored at 4^{o}C in a moist chamber for future use.

	6% T	**1%T**
Acrylamide (bis) 30%solution	2.8 ml	7.55 ml
Gel Buffer [6x]	2.33 ml	2.0 ml
Glycerol [80%]	-	2.4 ml
H_2O {ddw}	8.8 ml	-
TEMED	7.5 µl	5 µl
APS [10%]	75 µl	50 µl
Total volume	**14.01 ml**	**12.05 ml**

The electrophoresis chambers were filled with respective cathode (deep blue) and anode buffers. Samples were prepared by adding 5-10% (w/v) glycerol. After the samples were loaded in respective wells, the electrophoresis was carried out initially at 50mV (setting the current at 12mA constant) to allow the samples enter into the gel.

At this stage the deep blue cathode buffer was replaced by slightly blue cathode buffer and the voltage was increased to 250 mV (while keeping the current setting still at max. 12mA). Such a setting allows uniform rate of migration of protein bands. The changing of cathode buffer from deep blue to slightly blue allows the real time visual analysis of migrating bands. The electrophoresis was carried out at 4^{o}C to avoid the heat denaturation of samples. Once the samples were resolved properly, the gel was taken out and de-stained, or the corresponding bands excised out and resolved for second dimension on SDS-PAGE.

4.6.4 Analytical gel-filtration binding assays

To check the in solution binding of antibody fragments with their respective cognate membrane protein targets, gel filtration binding assays were performed. IMAC purified scFv samples were mixed in stoichiometric amounts with the target protein, incubated on ice for 30 to 50 minutes to allow the co-complex formation. The 50µl of these were loaded on analytical gel filtration columns (SMART system). To check the STNhaA binding of chicken library isolated clones, equal amounts (~ 60 µg) of each i.e. purified scFv and STNhaA were incubated in 60µl volume of running buffer(20 mM BisTris propane, 150mM NaCl & 0.03% LM) incubated on ice for 50 minutes, filtered and injected on Superose-12 PC 3.2/3.0 column (SMART system). The column was previously washed with 2CV of water and equilibrated with running buffer. Column was run at flow rate of 40 µl/ minute at 9^{o}C. For B2-scFv and β_2AR binding in solution, purified β_2AR (~10µg) was incubated with excess of monomeric B2-scFv (~30µg) for one hour in a total volume of 50µl, and loaded on superdex-200 gel filtration column (SMART system). The column was run at 40µl/minute flow rate at ~5^{o}C temperature in 1x HBLM running buffer. The elution profile was recorded, and elution fractions were analyzed by SDS-PAGE and also by Western-blotting when required.

4.6.5 Kinetic-titration experiments using BIAcore-T100

Surface plasmon resonance (SPR) experiments were performed with IMAC purified scFv protein samples. The protein concentration of these samples was determined by BCA method. Purified antigen (STNhaA) was immobilized on CM5 sensor chip using NHS-EDC chemistry.

The STNhaA (12µg/ml in 20mM sodium acetate, 100mM NaCl, 0.03% ά-LM, pH 4.0 buffer) was coupled at a flow-rate of (10µl/minute) and 90 seconds contact time to obtain

approximately ~ 2000 to 2500 resonance units (RU). This amount of coupled STNhaA resulted in scFv RU_{max} of ~ 100 to 200 RU. Kinetic-titration (Karlsson *et al.,* 2006) was performed by injecting sequentially a range of scFv concentrations (i.e. 0.1nM, 0.5nM, 2.5nM, 12.5nM, and 62.5nM) without any regeneration steps. Each experiment was performed with 2 startups, 2 buffer injections to stabilize the baseline, followed by actual sample injection. The binding was performed in running buffer (20mM BisTris Propane, 150mM NaCl, 0.03% LM pH 7.2) at 25^{o}C (for both sample and flow cell). A continuous flow of 30µl/minute was used with 180 second contact time for binding and 180 second for dissociation.

The Kinetic-titration data was kindly evaluated by Dr. Uwe Bierfreund, and his colleagues at Biacore AB facility, Uppsala, Sweden.

4.6.6 Epitope mapping of anti-STNhaA Western-blot positive scFv

Replitope^{TM} Microarrays, a custom made peptide microarray derived from STNhaA amino acid sequence, were synthesized at JPT Peptide Technologies GmbH, Berlin. The peptide array consists of 15 residue long 98 peptides with 11 residue overlap region, spotted on a microscopic glass slides. This microarray represents the complete amino acid sequence of STNhaA. These peptide microarrays were probed with Western-blot positive scFv clones. The slide was incubated with the H6 or C4 scFv preparation to let the antibody fragment binding to its corresponding epitope. The bound scFv was probed with anti-HA tag antibody and anti-mouse alkaline phosphate conjugate. The spots were developed with BCIP-NBT substrate solution.

4.6.7 Titration of anti-STNhaA/ KefC chicken sera

Two white Leghorn female chickens (727 & 728) were immunized with mixture of purified antigen. The antigen mixture contained two proteins: STNhaA & KefC (1:1 w/w ratio), both produced separately in heterologous *E. coli* expression system and purified to homogeneity by Ni-NTA IMAC system. STNhaA and KefC were provided by Marc Boehm. Immunization was performed at company PFR &L. A long term immunization protocol was followed with intramuscular injections of 100µg protein mixture with Freund's adjuvant on days 0, 14, 28, and 56. Blood samples were drawn 10 to 14 days after each immunization, and blood sera samples were shipped on dry ice.

Titration of chicken sera was performed by coating His-tagged antigen (STNhaA or KefC) (10µg/ml in 1x PBS+ 0.02%LM, pH 7.8) on Ni-NTA chileate microtiter plates (Nunc). BSA was coated for negative control binding signal measurement. The plates were blocked with 2% milk in PBSLM, followed by incubation with the respective sera samples (diluted in 1% MPBSLM). The antigen-bound IgY were detected with anti-IgY alkaline phosphatase conjugate (1:1000 diluted). ELISA was performed in duplets, and the results were calculated as an average OD value of two microtiter wells. The antibody titer was determined as the highest dilution at which the antigen-binding signal was higher than the background.

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Abbreviations

AP	Alkaline phosphatase
Å	Angstrom
β_2AR	β_2-adrenergic receptor
BCA	Bicinchoninic acid
Bis-Tris	(bis(2hydroxyethyl)tris(hydroxymethyl)methane)
BNP	Blue Native PAGE
BSA	Bovine serum albumin
C_H	Heavy chain constant domain
C_L	Light chain constant domain
CDR	Complementarity determining region
CFA	Complete Freund's adjuvant
cyt *c*	Cytochrome *c*
CV	Column volumes
DDW	Double distilled water
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
E. coli	*Escherichia coli*
Fab	Fragment antigen-binding
Fc	Fragment crystalline
Fv	Fragment variable
g	Force of gravity
GPCR	G-protein coupled receptor
HA	Hemagglutinin
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IgY	Immunoglobulin (yolk)
IM	Intra-muscular
IMAC	Immobilized metal affinity chromatography
KefC	*Salmonella typhimurium* Potassium anti-porter
K_D	Equilibrium dissociation constant
KDa	Kilo Dalton
L	Liter

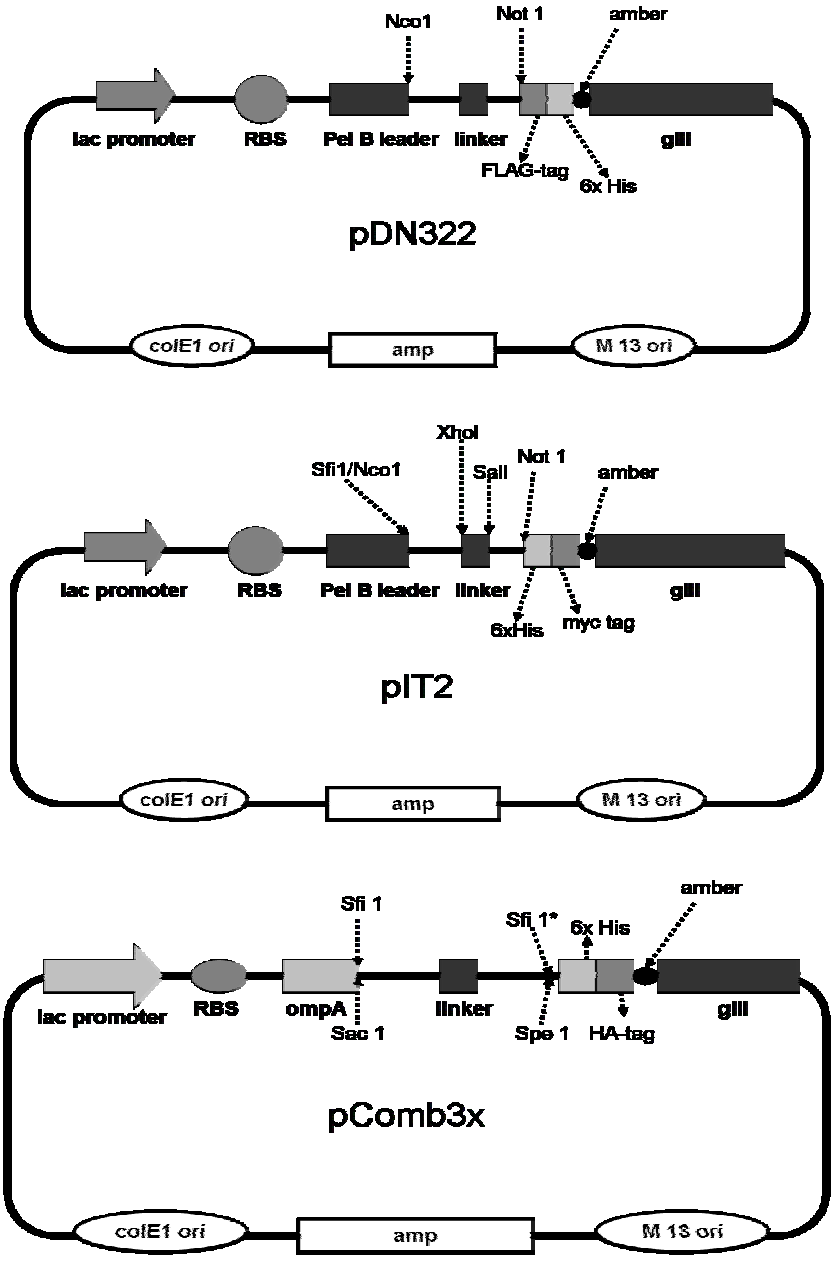
LL	Long linker
LM (ddm)	n-Dodecyl-β-D-maltoside
MOPS	Morpholinepropanesulfonic acid
MPBS	Milk in PBS
MPBSLM	Milk in PBSLM
NhaA	Sodium proton antiporter A
Ni^{2+}-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
o/n	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBSLM	PBS + LM
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pfu	Plaque forming unit
pNPP	para-nitrophenol phosphate
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
RT	Room temperature
RU	Resonance unit
scFv	Single chain variable fragment
SDS	Sodium dodecyl sulfate
SDS- PAGE	SDS- polyacrylamide gel electrophoresis
SL	Short linker
SPR	Surface plasmon resonance
STNhaA	*Salmonella typhimurium* NhaA
TAE	Tris-acetate-EDTA
TU	Transforming unit
V	volt
V_H	Heavy chain variable domain
V_L	Light chain variable domain
(v/v)	Volume per volume
(w/v)	Wieght per volume

Appendix

A-1 scFv-sequences

	V_H chain (CDR3)	V_L chain (CDR3)
anti-cyt *c* (3RE2-10 scFv)	**S_{95} W H P H N** F D Y	N_{91} S S H A R Q P L VV
anti-β_2AR (B2-scFv)	**G_{95} A G S - -** F D Y	Q_{91} Q A S A Y P T

A-2 Phagemid vector maps



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Curriculum vitae

Syed Hussain Mir

Date of birth: 3rd Jan. 1974

Academic Profile:

Year	Degree	Institute
2002-2007	Ph.D	Department of Biochemistry at JWG Universität and Max-Planck Institute für Biophysik Frankfurt, Germany
1999-2002	CSIR-JRF	Department of Biochemistry, All India Institute of Medical Sciences (AIIMS) New Delhi, India
1997-1999	M.Sc.	Department of Biochemistry, Hamdard University, New Delhi, India
1993-1996	B.Sc.	Science faculty, Lucknow Christian Degree College Lucknow, (University of Lucknow), India
1991-1993	Sr. School (12th)	Lucknow Christian Inter College Lucknow, India
1980-1990	Sec. School (10th)	J & K Board of School Education Srinagar, Kashmir, India

Awards and Fellowships

- Qualified joint UGC-CSIR (NET) Junior Research Fellowship (JRF) held on June 25th, 2000, conducted jointly by University Grants Commission (UGC) and Council of Science & Industrial Research (CSIR), Govt. of India.

Selected courses attended

- Phage display of peptides and proteins (Nov.7-21, 2004) at Cold Spring Harbor Laboratory, New York, USA.