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**AFFINITY MATURATION OF GLUCOSE TRANSPORTER
SLC2A1 ANTIBODY 6F12#33 BY PHAGE DISPLAY**

Master's thesis

30 EAP

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“AFFINITY MATURATION OF GLUCOSE TRANSPORTER SLC2A1 ANTIBODY 6F12#33 BY PHAGE DISPLAY”

Antibodies against glucose transporter SLC2A1 have high potential in cancer therapy. Thus, this master's thesis aimed to enhance the affinity of antibody 6F12#33 against glucose transporter SLC2A1, which can then be used to inhibit the glucose uptake in various cancers, essentially starving and killing the cancer cells. The lead antibody 6F12#33 was discovered at Icosagen, which was then used in affinity maturation. Antibody maturation libraries were designed and generated, leading to the screening of approximately 600 colonies using ELISA. From these 600 clones, eight promising clones were selected based on signal intensity and sequence similarity. Antibody sequences were cloned into human IgG1 format, produced in CHO cells, and purified. Comprehensive assays, including cell proliferation and various binding assays, were conducted to evaluate their efficacy. Three out of the eight affinity-matured antibodies improved binding affinity to SLC2A1 compared to the parental which translated to a significant enhancement of their inhibitory effects. The remaining five antibodies exhibited similar or decreased inhibitory effects. All eight affinity-matured antibodies displayed binding affinity towards SLC2A1 with varying specificity levels, demonstrating their potential for targeted therapies. This study successfully enhanced the affinity of antibody 6F12#33 through affinity maturation and phage display techniques, providing valuable insights for the development of glucose metabolism-related cancer treatments.

CERCS code: B500 Immunology, serology, transplantation

KEYWORDS: Phage display, affinity maturation, SLC2A1, monoclonal antibodies

“GLÜKOOSI TRANSPORTERI SLC2A1 VASTASE ANTIKEHA 6F12#33 AFIINSUSKÜPSEMINE KASUTADES FAAGI DISPLEI TEHNOLOOGIAT”

Glükoosi transporteri SLC2A1 vastastel antikehadel on kõrge potentsiaal kasvaja teraapias. Seetõttu oli magistritöö eesmärgiks oli suurendada antikeha 6F12#33 afiinsust glükoosi transpordivalgu SLC2A1 suhtes, mida saaks seejärel kasutada vähirakkudes glükoosi transpordi inhibeerimisel. Esialgne SLC2A1 vastane antikeha 6F12#33 avastati Icosagenis ning seejärel kasutati afiinsusküpsemisel. Disainiti ja loodi antikeha küpsemise raamatukogud, mille tulemusena skriiniti ELISA meetodil umbes 600 klooniat. Nendest 600st kloonist valiti välja kaheksa paljutõotavat kloon ELISA signaali intensiivsuse ja järjestuse sarnasuse alusel. Antikeha järjestused klooniti inimese IgG1 formaati, toodeti CHO-rakkudes ja puhastati. Põhjalikud analüüsid, sealhulgas rakkude paljunemise ja antikeha-antigeeni seondumise katsed, viidi läbi nende efektiivsuse hindamiseks. Kolm kaheksast afiinsusküpsenud antikehast parandasid seondumise afiinsust SLC2A1 suhtes võrreldes algse antikehaga, mis tõi kaasa inhibeeriva toime olulise suurenemise. Ülejäänud viis antikeha näitasid sarnast või vähenenud inhibeerivat toimet. Kõik kaheksa afiinsusküpsenud antikeha seondusid SLC2A1-ga erineva spetsiifilisusega, näidates nende potentsiaali sihitud teraapiate jaoks. Käesolevas magistritöös edukalt suurendati antikeha 6F12#33 afiinsust afiinsusküpsemise ja faagi displei tehnoloogia abil, pakkudes väärtuslikke teadmisi glükoosimetabolismiga seotud vähiravimite väljatöötamiseks.

CERCS kood: B500 Immunoloogia, seroloogia, transplantoloogia

Märksõnad: Faagi displei, afiinsusküpsemine, SLC2A1, monoklonaalsed antikehad

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USED ABBREVIATIONS

BCR – B-cell receptor

BSA – Bovine serum albumin

Carb – Carbenicillin

CDR – Complementarity determining region

CH – Variable heavy chain

CL – Constant light chain

Cmp – Chloramphenicol

ELISA – Enzyme linked immunosorbent assay

FACS – Fluorescence-activated cell sorting

Fab – Fragment antigen binding

Fc – Fragment crystallizable

Fv – Variable fragment

GLUT1 – Glucose transporter

GLUT1-DS - GLUT1 deficiency syndrome

Ig – Immunoglobulin

Kan – Kanamycin

PBS – Phosphate buffered saline

SLC2A1 – Solute carrier family 2 member 1

VH – Variable heavy chain

VL – Variable light chain

VLP – Virus like particle

mAb – Monoclonal antibody

scFv – Single chain variable fragment

INTRODUCTION

Monoclonal antibodies (mAbs) are one of the fastest-growing therapeutics in the pharmaceutical industry, with applications ranging from cancer to autoimmune diseases. They are derived from a single clone of B cells and recognize a specific epitope on a target antigen, providing targeted therapy with fewer side effects due to their high affinity for their target antigens. This increase of affinity and specificity is achieved by the immune system through an affinity maturation process called somatic hypermutation and clonal selection, which is crucial to effectively neutralize pathogens or recognize specific biomolecules.

This *in vivo* somatic hypermutation process to mature antibodies can be partially mimicked *in vitro* through several techniques. The most powerful and widely used technology in mAb engineering and drug discovery has been phage display. In phage display, a library of phages displaying billions of unique antibody variants, is constructed, and screened against a target antigen in a cyclic process. The phages that bind to the target antigen with high affinity are selected and amplified, and the corresponding antibody sequences are identified and characterized.

One of the advantages of phage display is its ability to quickly screen large libraries of antibody fragments against many different targets. For example, different proteins, peptides, bacteria, and viruses. This is particularly useful in the development of therapeutics for diseases with challenging targets that are difficult to produce in reasonable yields or to display in the natural conformation. For example, solute carrier family 2 member 1 (SLC2A1, also known as GLUT1) is a membrane protein that plays a critical role in glucose uptake in the brain. Mutations in SLC2A1 have been linked to a rare genetic disorder called GLUT1 deficiency syndrome (GLUT1-DS), which is characterized by seizures, developmental delay, and movement disorders. SLC2A1 also plays a crucial role in the cancer glycometabolism. The development of high-affinity mAbs against SLC2A1 could lead to the development of novel therapeutics for GLUT1-DS or various cancers. In this regard, the R&D department at Icosagen isolated a lead antibody from immunized chickens binding to SLC2A1 at a “reasonable” affinity and capable of slightly inhibiting the glucose uptake of cancer cells. We hypothesized that if we are able to increase the affinity of the lead antibody towards the same epitope, we would be able to significantly increase its biological efficacy.

By isolating phages with increased affinity for SLC2A1, and by generating new libraries through mutagenesis and cloning, we aim to develop mAbs that specifically bind to SLC2A1 with higher affinity to enhance its functional efficacy in cells. This could lead to the development of novel

therapeutics for cancers and potentially other diseases what require SLC2A1 inhibitions. This experimental part of this thesis was done in Icosagen Cell Factory, R&D department.

1. LITERATURE OVERVIEW

1.1. ANTIBODIES

Antibodies, also known as immunoglobulins (Ig), are glycoproteins that are produced by the immune system in response to the presence of foreign substances known as antigens. The primary function of the antibodies is to recognize and bind to specific antigens, marking them for destruction or removal by other components of the immune system. The antigens can be any kind of molecule, molecular structure, foreign substance, or pollen grain that can bind to antibody or T-cell receptor. Antibodies are produced by B-cells when they encounter foreign antigen. Upon binding antigen, B-cells undergo clonal expansion, and some differentiate into plasma cells, which produce large amounts of antibodies, while others differentiate into memory B-cells that can rapidly produce antibodies upon re-exposure to the same antigen. The produced antibodies display varied levels of specificity and form a vast repertoire of diverse antibodies capable of targeting virtually any antigen. It is estimated that the human naïve antibody diversity is in the range of 10^{12} unique clones (Alberts et al., 2002b), ensuring the presence of antibodies against most antigens, albeit with some displaying low affinity. Through a process known as affinity maturation, that occurs in animals, particularly in the immune system, to improve the binding strength or affinity of antibodies towards specific antigens. It is a crucial step in the development of a highly effective immune response. Affinity maturation occurs through a combination of natural selection and mutation, leading to the production of antibodies with increased affinity over time. In natural affinity maturation, the process primarily takes place within the germinal centres of secondary lymphoid organs, such as lymph nodes and spleen. When an antigen enters the body, B cells recognize and bind to the antigen using their surface receptors called B cell receptors (BCRs). This binding triggers the activation of the B cells and the formation of a germinal centre. Within the germinal centre, B cells undergo two key processes: somatic hypermutation and antigen-driven selection (Teng & Papavasiliou, 2007). Somatic hypermutation involves random mutations in the genes encoding the BCR, which results in a diverse pool of B cells with slightly different BCRs. These mutations introduce changes in the antigen-binding region, altering the affinity of the BCR for the antigen. The next step, antigen-driven selection, involves competition among B cells for antigen binding and subsequent survival signals. B cells with BCRs that possess higher affinity for the antigen are more likely to receive survival signals from helper T cells and undergo further development, while those with lower affinity may undergo cell death (Li et al., 2004). This process leads to the preferential expansion of B cell clones that produce antibodies with improved affinity

for the antigen. While the immune system produces a diverse range of antibodies, some may not be specific enough to target a particular antigen of interest. In such cases, antibodies can be produced using recombinant DNA technology or hybridoma technology (Dodd et al., 2018). Recombinant DNA technology involves inserting the genes encoding the antibody into a host cell, while hybridoma technology involves the fusion of B cells with cancer cells to produce hybrid cells that can continuously produce antibodies with a specific antigenic specificity.

1.1.1. ANTIBODY STRUCTURE

Humans have five different isotypes of antibodies, namely IgA, IgM, IgG, IgD and IgE, each with a specific function in an adaptive immune system. The antibodies are Y-shaped proteins composed of 4 polypeptide chains, two identical heavy chains and two identical light chains, which are held together by disulphide bonds (Figure 1A). Each chain consists of a variable and a constant domain. The light chain contains one variable (VL) domain and one constant (CL) domain and is classified into two functionally similar subtypes – κ and λ chains. In contrast, the heavy chain of IgA, IgG and IgD contains one variable domain (VH) and three constant (CH) domains, while IgM and IgE have one variable and four constant domains (Chiu et al., 2019).

Antibodies have two distinct functional components: two antigen binding fragments (Fab) and the fragment crystallizable (Fc), with hinge region connecting the two (Figure 1). The Fab region contains the VH-CH1 and VL-CL domains and is responsible for the specific recognition and binding of the antigen. On the other hand, the Fc region is composed of CH2 and CH3 domains and is responsible for the effector functions of the antibody, such as complement activation and antibody-dependant cell-mediated cytotoxicity. All the constant and variable domains have approximately 110 amino acid residues and adopt a characteristic immunoglobulin fold (Ig fold) tertiary structure. The Ig fold comprises two roughly parallel β -sheets, where one β -sheet contains four anti-parallel β -strands and the other contains 3 anti-parallel β -strands (Poljak et al., 1974). The β -sheets are linked by a disulfide bond between two conserved cysteines in two of the β -strands. Additionally, the variable domain contains three hypervariable loops that are responsible for specific recognition and binding of the antigen. These loops are called complementarity determining regions (CDRs) and there are three of them in the VH (CDRs H1, H2, H3) and VL (CDRs L1, L2, L3 domains (Kabat & Wu, 1971). In addition, the amino acid residues that are in contact with the antigen are called the specificity determining residues (Almagro, 2004). Out of all six CDRs, the H3 most commonly has the largest role in antigen binding, as it contributes around

25% of contacts in anti-protein recognitions (Jain et al., 2007, Almagro, 2004). Most of the variable domain is composed of non-hypervariable, highly conserved loops connecting the CDRs, called the framework regions.

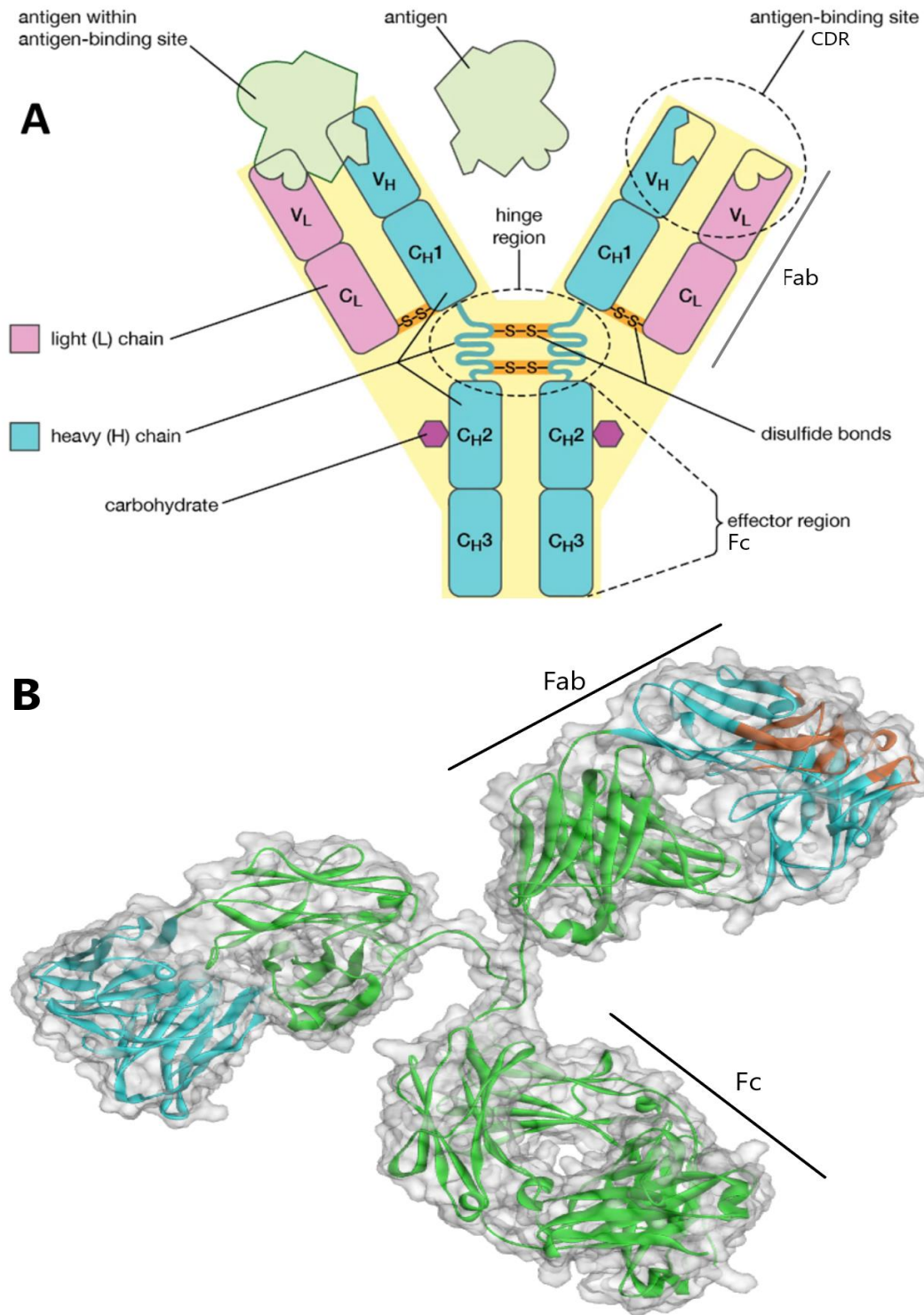


Figure 1. Overview of the IgG antibody structure. (a) Schematic representation of IgG structure, where the different parts of the antibody are highlighted. (b) IgG antibody structure representation in ribbons and a semi-transparent white surface. Constant domains are colored in green, variable domains in light blue and CDRs of one of the paratopes in orange (PDB entry: 1IGT).

1.2. PHAGE DISPLAY

Phage display, one of many *in vitro* display technologies, is a powerful technique used in molecular biology and protein engineering to study protein-protein interactions, identify specific binding partners, and generate antibodies or peptides with desired properties. It was developed in 1985 by George P. Smith (Smith & Petrenko, 1997) and has been since widely employed in various fields, including drug discovery, diagnostics, and therapeutic antibody development. Phage display involves the use of bacteriophages, which are most commonly filamentous viruses that infect bacteria, as a means to express and display a large library of random or semi-random peptides or antibody fragments on their surface. The key idea behind phage display is that the genetic information encoding the displayed peptides or antibody fragments is linked to the DNA of the phage particles. This allows for the physical association of genotype (DNA) and phenotype (displayed protein), enabling the isolation and identification of desired binding molecules. (Valldorf et al., 2022)

1.2.1. PHAGE M13 OVERVIEW

Phage display technology relies on the crucial ability of filamentous phages to connect genotype and phenotype through the display of antibody fragments on the phage coat proteins. The predominant phage utilized in phage display experiments is M13, which belongs to the group of filamentous bacteriophages. M13 is classified as a non-lytic phage, exclusively infecting *Escherichia coli* (*E. coli*) strains expressing F pilus (F^+). The genome of M13 comprises a single-stranded DNA with a length of 6407 base pairs (bp). It encompasses nine genes responsible for encoding eleven proteins of which five are coat proteins, while the rest are essential for replication and assembly of new phages (Smeal et al., 2017).

The M13 consists of two types of capsid proteins: major capsid protein pVIII and minor capsid proteins pIII, pVI, pVII, and pIX. Among these, minor capsid proteins pIII and pVI are located at one end, while pVII and pIX are situated at the other end of the phage (Figure 2). Each minor capsid protein is present in five copies, while the major capsid protein pVIII is composed of approximately 2700 copies, dictating the length of the phages (Rondot et al., 2001). Notably, the minor capsid protein pIII plays a crucial role in recognizing the F pilus and initiating infection in host cells. Although all capsid proteins have been utilized for the display of diverse molecules and peptides, pIII has gained extensive usage due to its flexible protein structure, accommodating the

display of polypeptides of varying sizes (Lubkowski et al., 1998), having low copy number and is located only on one end of the phage. Additionally, fusion of molecules or peptides to pIII will not affect the function of pIII, allowing the phage to infect the cells.

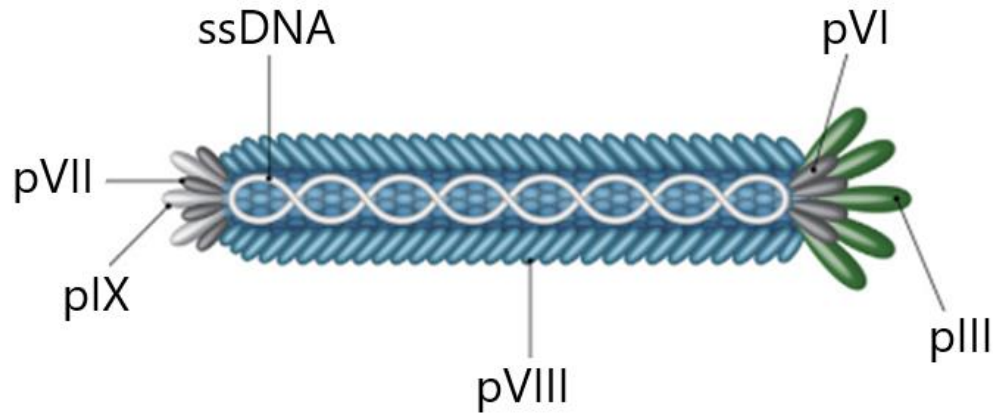


Figure 2. Overview of M13 structure. The M13 capsid proteins (pIII, pVI, pVII, pVIII, and pIX) and single-stranded DNA (ssDNA) are highlighted. Figure modified from Hentrich et al., 2018.

In the context of phage display, the utilization of M13, in conjunction with a phagemid, plays a crucial role. The phagemid represents a minimal viral plasmid encompassing essential components, including the plasmid origin of replication, an antibiotic resistance marker, specific regions from the M13 chromosome that are pivotal for the rolling circle amplification process, and the generation of the single-stranded DNA strand capable of being encapsulated within the virion. Additionally, the phagemid carries a gene encoding the pIII-fusion protein, which is responsible for the display of the desired peptides or antibody fragments on pIII protein. (Qi et al., 2012)

The incorporation of the phagemid in phage display experiments allows for the construction of a diverse phage library. This library is generated by introducing the desired DNA sequences encoding the peptides or antibody fragments into the phagemid's genome. Subsequently, the M13 helper phage is employed to facilitate the production of phages displaying the pIII-fusion protein. The phagemid acts as the vehicle for the construction and maintenance of the diverse library, while the M13 helper phage provides the necessary machinery for the replication and packaging of the phage particles displaying the pIII-fusion protein.

Overall, the integration of the phagemid and the M13 helper phage represents a fundamental aspect of phage display technology. Together, they enable the generation of a diverse phage library and the production of phages presenting the desired pIII-fusion protein, thereby facilitating the

subsequent screening and selection processes in the pursuit of identifying high-affinity binders and promoting affinity maturation.

1.2.2. PHAGE DISPLAY SELECTIONS

The basic steps involved in phage display include the following (Similar to affinity maturation workflow shown in Figure 3):

1. Construction of a phage display library: A diverse library of DNA sequences encoding the antibody fragments of interest is generated. This library is typically created by randomizing specific regions of the antibody variable domain allowing for the exploration of a vast sequence space.
2. Phage library screening: The phage library is exposed to a target antigen immobilized on a solid surface (e.g., a microtiter plate). Non-binding phages are washed away, while phages displaying antibody fragments with affinity towards the target antigen remain bound.
3. Phage recovery: The bound phages are eluted from the target antigen, thereby isolating the phage particles with desired binding properties.
4. Amplification and enrichment: The recovered phages are then amplified in bacterial hosts, typically *Escherichia coli*. This amplification step is crucial for increasing the number of phage particles carrying the desired binding sequences.
5. Iterative selection: Steps 2-4 are repeated for several rounds, with the goal of enriching the phage population for higher affinity binders.
6. Characterization and identification: After several rounds of selection, individual phage clones are analysed to determine their binding affinity and specificity. This can be achieved by techniques such as enzyme-linked immunosorbent assay (ELISA). Additionally, the phage clones are also sequenced.

Phage display offers several advantages over traditional antibody generation methods, such as hybridoma technology (Bradbury et al., 2011). It enables the screening of large and diverse libraries, allows for the identification of high-affinity binders, and facilitates the modification and optimization of antibody fragments for specific applications. Additionally, phage display allows far greater control over the selection and screening conditions, allowing to fine tune selections to direct the selections towards specific goals. Most common variables for selections are target antigen concentration and the number of washes. Reducing the amount of target antigen intensifies the binding competition among the antibody clones, while increasing the number of washes greatly

reduces the number on non-specific or weakly binding phages, resulting in a higher affinity population.

Furthermore, the inclusion of free antigen in the selection solution can elevate stringency (Brockmann et al., 2011). The presence of excess antigen in the solution competes with the phage-displayed antibodies for binding to the immobilized target antigen. This competition further favours the selection of antibodies with higher affinity, as only those with stronger binding affinity can effectively outcompete the excess soluble antigen and remain bound to the target antigen immobilized on the solid support. This approach promotes the isolation of antibodies with enhanced specificity and higher affinity for the target antigen.

Another way to fine tune the selections is the co-incubation of the target antigen with a competing antibody (Meulemans et al., 1994). The competing antibody, which binds to a different epitope on the target antigen, challenges the phage-displayed antibodies for binding to the target. This competition further enriches for antibodies that exhibit improved binding affinity and specificity, as only those with stronger binding capabilities can outcompete the competing antibody and remain bound to the target antigen.

Additionally, phage display selections can be conducted using two distinct methods: immobilization of the target antigen on microtiter plates or its retention in solution. While both approaches share similarities, the key distinction lies in the state of the target antigen. In immobilized selections, the target antigen is affixed to the surface of microtiter plates, while in solution-based selections, the target antigen remains unbound and freely floating in the solution. To facilitate the capture of phages bound to the target antigen in solution-based selections, streptavidin-coated magnetic beads are employed, necessitating prior biotinylation of the target antigen. (Hu et al., 2015)

Moreover, phage display has proven to be a valuable tool in generating antibodies against challenging targets, such as membrane proteins, which pose difficulties in traditional antibody production methods due to their extramembrane environment leading to loss of stability and functionality (Dodd et al., 2018). To overcome this challenge, researchers have explored incorporating membrane proteins into virion membranes, specifically virus-like particles (VLPs). For instance, Hötzel et al., (2011) successfully integrated mammalian multi-span membrane proteins like claudin-1 into baculovirus virions, employing these VLPs as targets in phage display experiments. In another study, van der Woning et al., (2016) employed VLPs expressing the G-protein coupled receptor (GPCR) glucagon receptor (GCGR) as the target in their phage display

investigations. These examples demonstrate the potential of utilizing VLPs to overcome the challenges associated with generating antibodies against membrane proteins, paving the way for further advancements in membrane protein research.

Overall, phage display technology is a versatile molecular technique that utilizes bacteriophages to present antibodies or antibody fragments on their surface. It enables the screening and identification of antibodies with high specificity for various targets, making it valuable in drug discovery. The technology's adaptability and integration with other methods further enhance its applications, making phage display a powerful tool for advancing biotechnology and biomedical research.

1.3. OTHER DISPLAY TECHNOLOGIES

In addition to phage display, various other types of display technologies have been developed for protein engineering and antibody discovery. These include ribosome display and cell surface display, such as bacterial and mammalian cell display and yeast surface display. Each of these techniques offers unique advantages and disadvantages, which are highlighted in Table 1, and can be employed based on specific experimental requirements.

Table 1. Comparison of different display systems.

Display system	Advantages	Disadvantages
Phage display	• Direct linkage of genotype and phenotype • Large library sizes (up to 10^9- 10^{11} variants) • Robust affinity selection and enrichment process • Ability to screen for specific binders or functional variants • Most established display system	• Restricted size and complexity of proteins that can be displayed • Relatively low throughput compared to some other display technologies • Requires phage propagation and amplification, which can be time-consuming and labour-intensive
Ribosome display	• Fully <i>in vitro</i> display system, independent of cellular expression systems • Direct linkage of genotype and phenotype through protein-ribosome-mRNA complex • Possibility for continuous affinity maturation during selections through error-prone PCR, DNA shuffling.	• Limited to proteins that can be synthesized <i>in vitro</i> • Requires specialized equipment and expertise for the synthesis and handling of ribosome-mRNA complexes

Bacterial surface display	• Very large library sizes (surpassing 10^{12}) • Production of toxic or proteolytically sensitive or unstable proteins	
	• Compatibility with FACS for efficient sorting and analysis of library candidates • Large library sizes (up to 10^{11} variants) • High transformation efficiency and ease of manipulation with <i>E. coli</i>	• Complexity of the secretion system • Many different display scaffolds
Yeast surface display	• Compatibility with FACS for efficient sorting and analysis of library candidates	• Smaller library sizes compared to some other display technologies (up to 10^8 variants) • More complex workflow and manipulation compared to phage or bacterial display

1.3.1. RIBOSOME DISPLAY

Ribosome display is a powerful and versatile technique for selecting proteins or peptides from large libraries in a cell-free environment, offering significant advantages over traditional cell-based display technologies. It operates by generating a protein-ribosome-mRNA complex, where the ribosome is stalled to maintain the attachment between the protein and its encoding mRNA (Hanes et al., 1998). This enables protein synthesis and folding, without the release of the nascent protein, thus connecting the genotype to the phenotype.

To initiate translation in prokaryotic systems, DNA constructs must contain a promoter and a translation initiation signal, such as Shine-Dalgarno (Shine & Dalgarno, 1975), while eukaryotic systems require constructs with a Kozak sequence (Kozak, 1999). These elements ensure efficient translation initiation and proper expression of the desired proteins. Additionally, including a spacer domain of approximately 20-30 amino acids is necessary in ribosome display to facilitate correct folding and exit of the displayed protein from the ribosome (He & Taussig, 2002; Plückthun, 2012). In addition to the spacer domain, the proper folding of the protein requires other folding enhancing factors, such as different molecular chaperones and ribosome-associated factor (Hoffmann et al., 2010; Hoffmann & Rinas, 2004).

Selection conditions for ribosome display can follow similar principles used in phage display. Following rounds of selection, it is crucial to capture the mRNA encoding the displayed protein. Prokaryotic systems can achieve this by releasing the mRNA from the ribosome complex using EDTA and then convert the mRNA into complementary DNA using reverse transcription polymerase chain reaction (RT-PCR). Meanwhile in eukaryotic systems a novel *in situ* RT-PCR protocol can be used to generate complementary DNA, without releasing mRNA from the protein-ribosome-mRNA complex. (He & Taussig, 2002)

One key advantage of ribosome display is its complete *in vitro* nature, eliminating the need for living cells in protein production. This feature enhances experimental flexibility and simplifies the setup, while also allowing the generation of much larger libraries compared to cell-based methods, surpassing 10^{12} in diversity (Hanes et al., 1998). The increased library size expands the search space and improves the chances of discovering rare and unique protein variants. Additional advantage of ribosome display is the ease of using PCR-based randomization techniques, which eliminates the need to transform each newly randomized library into cells, thus reducing workload (Plückthun, 2012).

Affinity maturation, an essential step in improving binding affinity, can be seamlessly incorporated throughout the selection process in ribosome display. By utilizing error-prone PCR or DNA shuffling, random mutations can be introduced into the coding sequences, resulting in the generation of protein variants with enhanced affinity (Zhao et al., 2009). This eliminates the need for creating a separate library for affinity maturation, streamlining the selection process, and saving time. Nevertheless, mutagenesis cannot be directed towards the CDR regions which are known to be mediating antigen interaction.

In summary, ribosome display offers a robust and flexible platform for protein selection, enabling the discovery of high-affinity binders and accelerating the development of novel therapeutics. Its *in vitro* nature, ability to generate large libraries and capacity for affinity maturation within the selection process make it a valuable tool in protein engineering and antibody discovery.

1.3.2. CELL SURFACE DISPLAY

BACTERIAL SURFACE DISPLAY

Bacterial surface display is a versatile technique that allows for the presentation of proteins on the outer membrane of bacterial cells, facilitating efficient screening and selection of desired protein variants. *E. coli* is a preferred choice for bacterial surface display due to its fast growth rate, ease

of physical and genetic manipulation, high transformation efficiency, and the ability to generate large libraries (up to 10^{11}) (Daugherty, 2007; Daugherty et al., 1998).

In this approach, the protein of interest, such as a single-chain variable fragment (scFv), is fused to a cell surface protein or its fragment. One widely used fusion involves linking the scFv to lipoprotein-outer membrane protein A (Lpp-OmpA), where Lpp acts as a transport signal and OmpA serves as an anchoring protein, enabling the display of the scFv on the *E. coli* surface (Francisco et al., 1993). Fluorescence-activated cell sorting (FACS) is a powerful tool used to rapidly and efficiently sort cells displaying the scFvs. FACS allows for real-time visualization and optimization of library screening, and it has the potential to directly sort high-affinity antibodies using low concentrations of fluorescently labelled haptens or receptors (Francisco et al., 1993).

When selecting a carrier protein (display scaffold), it is essential to consider certain criteria. The carrier protein should be monomeric, randomly distributed, and spatially separated on the bacterial surface (Lee et al., 2003). Additionally, high-level expression of the carrier protein should impose minimal metabolic burden on the host. Careful selection of the fusion site for the protein of interest is crucial, ensuring that it faces the surrounding medium and does not interfere with the function, stability, or specific activity of the carrier protein. Appropriate and sufficiently long spacer regions can be employed to achieve optimal display of protein of interest (Strauss & Götz, 1996).

While bacterial surface display offers advantages over other techniques with various disadvantages, such as magnetic-activated cell sorting selections that may lead to increased avidity interactions interfering with affinity screening, it is important to note some challenges (Daugherty, 2007). Screening for protein-specific antibodies can be complex, and the choice of the target protein and the host cell can influence the translocation process and final surface display. Additionally, high numbers of hydrophobic or charged amino acid residues in the protein of interest may result in inefficient secretion, making the display of protein of interest more challenging (Ngoc Nguyen et al., 1995).

YEAST SURFACE DISPLAY

Yeast surface display is a powerful technique for presenting proteins on the surface of yeast cells, offering numerous advantages in protein engineering and antibody discovery. One of the key advantages is that yeast, such as *Saccharomyces cerevisiae*, exhibits remarkable similarities to mammalian systems in terms of protein folding and secretory mechanisms (Boder & Wittrup, 1997). This resemblance allows for the accurate representation of proteins on the yeast cell surface.

The usual yeast surface display process typically begins with the identification of parent hybridoma clones, followed by the cloning of the VH chain and variable light VL chain genes into expression vectors to express for example single-chain variable fragments scFvs. The initial step involves characterizing the binding of the wild-type (WT) scFv to assess its functionality. To improve the affinity of the scFv, mutagenesis of the parent scFv plasmid is performed, followed by selections to identify clones with enhanced binding properties. The selected clones are then subjected to sequencing and thorough characterization. Subsequently, the VH and VL genes are recovered and cloned into the desired expression vectors for further analysis and applications. (Siegel, 2009)

The display of scFv on yeast cells is achieved by fusing it with a-agglutinin, a two-subunit cell surface receptor responsible for cell-to-cell adhesion, resulting in the formation of diploid cells. Importantly, the displayed proteins can be easily released from the yeast cell surface by employing reduction strategies such as using dithiothreitol to break the disulfide bonds between the two a-agglutinin subunits (Boder & Wittrup, 1997).

Yeast surface display offers several notable advantages. It is compatible with FACS, enabling efficient sorting and real-time analysis of library candidates (Valldorf et al., 2022). This compatibility empowers researchers to have precise control over the selection of desirable candidates. Additionally, yeast display allows for the screening of more complex molecules due to its sophisticated quality control mechanisms that ensure proper protein folding. Although yeast display libraries may be smaller compared to phage display libraries, yeast display supports the full display of IgG antibodies, preserving their glycosylation patterns, which is an advantage in antibody engineering and therapeutic antibody development (Almagro et al., 2019).

1.4. AFFINITY MATURATION USING PHAGE DISPLAY

Antibody libraries are typically designed to be as wide as possible so that they can be used to identify high affinity binders for as many antigens as possible independently of their size, conformation, and physicochemical properties and we can identify antibodies binding to different antigen epitopes. It is quite common to randomize the most observed CDR loops mediating antigen recognition (CDR L3 and H3, sometimes with H1 and H2 (Tsuchiya & Mizuguchi, 2016)) and to allow a wide variety of amino acid substitutions in each position (González-Muñoz et al., 2012). The disadvantage of such wide, naïve libraries is that the theoretical variability (position number to the power of amino acids allowed in each position) is impossible to achieve, since phage display

libraries show a diversity of 10^9 - 10^{11} unique antibody variants and most of the times <1% of the theoretical antibody variants are barely covered.

Mimicking natural affinity maturation in a laboratory setting can be achieved by using a lead antibody already known to bind the antigen of interest, and designing a library that would cover less positions and less amino acids to retain binding epitope but improve affinity.

These methods involve creating large libraries of antibody variants and subjecting them to multiple rounds of selection and amplification to enrich for higher affinity binders. General workflow of affinity maturation using phage display can be seen in Figure 3.

Affinity maturation, a complex process in antibody development, can be approached through two general methods: random mutagenesis and targeted mutagenesis (Tabasinezhad et al., 2019). However, both approaches face challenges due to the lack of precise knowledge regarding which specific amino acids should be replaced to enhance binding affinity. Simply replacing an amino acid residue within the CDR does not guarantee improved binding, and identifying the peripheral amino acids that could be mutated to enhance affinity also presents difficulties. Among the CDRs, CDR H3 holds particular importance in antigen recognition due to its high structural diversity (Kuroda et al., 2012), making it a crucial component in the binding interface between antibodies and their targets. Nevertheless, determining the specific amino acids within CDR H3 that should be mutated to enhance affinity remains a challenging task. Thus, the random and targeted mutagenesis approaches are adopted to introduce mutations at random positions or areas with the highest chance that the mutations increase the affinity.

Two additional parameters should be considered when designing affinity maturation libraries. Firstly, the selection of amino acids for randomization plays a pivotal role. The approach may involve either unbiased randomization, where all amino acids have an equal chance of mutation, or soft randomization, where each amino acid carries a distinct probability of mutation. For example, using degenerate oligonucleotides such that the first and second nucleotide in a codon was mixed with 70% of the wild type (wt) nucleotide and 10% of each of the other three nucleotides, whereas the third nucleotide was allowed to be only 50% T/G. Secondly, the extent of randomization across amino acid positions is a significant factor. Researchers can choose to randomize all positions simultaneously for a highly stringent approach, or alternatively, opt for soft randomization, wherein only a subset of positions is subjected to randomization. The careful consideration of these parameters empowers the generation of effective and tailored affinity maturation libraries to improve the binding characteristics of selected antibodies. (Shim, 2015)

RANDOM MUTAGENESIS

Random mutagenesis is an approach commonly used in *in vitro* affinity maturation to introduce diverse mutations in the antibody sequence. It involves the generation of libraries of antibody variants with random mutations introduced in the CDR regions. Two widely used methods for random mutagenesis are error-prone PCR and DNA shuffling. Error-prone PCR is a technique that introduces random errors during the PCR amplification process, resulting in a library of mutant antibody variants (Martineau, 2002). By using high error rates of the DNA polymerase, mutations are introduced at different positions within the antibody coding sequence. The degree of mutagenesis can be controlled by adjusting the error rate of the PCR reaction. This method enables the generation of a diverse population of antibody variants with a range of affinity-altering mutations. DNA shuffling, also known as DNA recombination, is another approach used for random mutagenesis (Patten et al., 1997). It involves the creation of chimeric libraries by recombining DNA fragments from multiple related antibody sequences. This technique capitalizes on the natural diversity present in a set of related sequences to generate a large number of antibody variants. Through enzymatic digestion and reassembly of the DNA fragments, the resulting chimeric library contains shuffled combinations of CDRs, leading to a diverse repertoire of antibody variants with potential affinity improvements. Both error-prone PCR and DNA shuffling allow for the generation of libraries with a wide range of mutations, exploring different regions and amino acid positions within the antibody. These methods increase the chances of finding antibody variants with improved binding affinity compared to the original antibody. However, it is important to note that single mutations introduced through random mutagenesis may not always lead to significant gains in affinity (Tabasinezhad et al., 2019). Cooperative effects between mutations or combinations of mutations may be necessary to achieve substantial improvements in binding affinity.

TARGETED MUTAGENESIS

Targeted mutagenesis is another approach used in *in vitro* affinity maturation to introduce specific mutations in the antibody sequence based on structural analysis and/or rational design. This method allows researchers to focus on specific amino acid residues or regions within the antibody that are predicted to play a crucial role in antigen binding and affinity. One commonly used technique for targeted mutagenesis is site-directed mutagenesis. This method enables precise and controlled

introduction of desired mutations at specific positions in the antibody sequence. Site-directed mutagenesis relies on the use of modified primers containing the desired mutation, which are introduced either by PCR-based methods or in the case of this thesis, using Kunkel's protocol for site-directed mutagenesis (Kunkel, 1985). By using site-directed mutagenesis, researchers can precisely introduce mutations at specific positions within the antibody sequence, including the CDR regions or other critical residues involved in antigen binding. This approach is usually guided by structural analysis (Kiyoshi et al., 2014), computational modelling (Cannon et al., 2019), or prior knowledge of residues important for binding interactions (Almagro, 2004). The rational design of mutations based on this information allows for a more targeted and focused exploration of potential affinity-enhancing mutations. Given the vast number of potential combinations of CDR mutant forms, it is impractical to experimentally produce all of them. *In vitro* affinity maturation methods typically involve mutagenesis, which can be achieved through random or targeted approaches.

IN SILICO APPROACH

Given the vast number of potential combinations of CDR mutant forms, it is impractical to experimentally produce all of them. To overcome the limitations of experimental methods, *in silico* affinity maturation provides an alternative approach. Through computational techniques, researchers can quickly and cost-effectively search for all potential combinations of mutations. *In silico* methods also facilitate the analysis of antibody-antigen interactions and enable structural analysis using various algorithms. This comprehensive exploration of potential mutations and their effects on affinity guides experimental design, reducing the number of iterations required in the laboratory. *In silico* affinity maturation also offers the advantage of speed and cost-effectiveness compared to experimental methods. While experimental approaches are limited by the number of variants that can be tested, computational methods can explore a vast number of antibody sequences and mutations (library with a theoretical diversity 10^{40}) in a short time, while experimentally screening a library with a diversity of 10^{10} can take several weeks (Mahajan et al., 2018). This accelerates the affinity maturation process and facilitates the identification of promising candidates for experimental validation. One notable advancement is the use of machine learning algorithms, such as generative language models like OpenAI's GPT-3, to predict and generate novel antibody sequences. In a recent article, Hie et al., (2023) used a general protein language model to evolve human antibodies by suggesting evolutionarily plausible mutations,

without providing the model with any information about the target antigen, binding specificity, or protein structure. The language model guided affinity maturation resulted in increased binding affinity in several already highly matured antibodies. By leveraging generative language models, researchers can explore a vast sequence space and predict antibody variants with improved binding affinity.

Overall, *in silico* affinity maturation, empowered by generative language models and computational techniques, provides a valuable tool for antibody engineering. These methods have the potential to streamline the development of antibodies with enhanced affinity and therapeutic efficacy. The synergy between computational and experimental approaches opens new possibilities in the design and optimization of antibody therapeutics for various diseases.

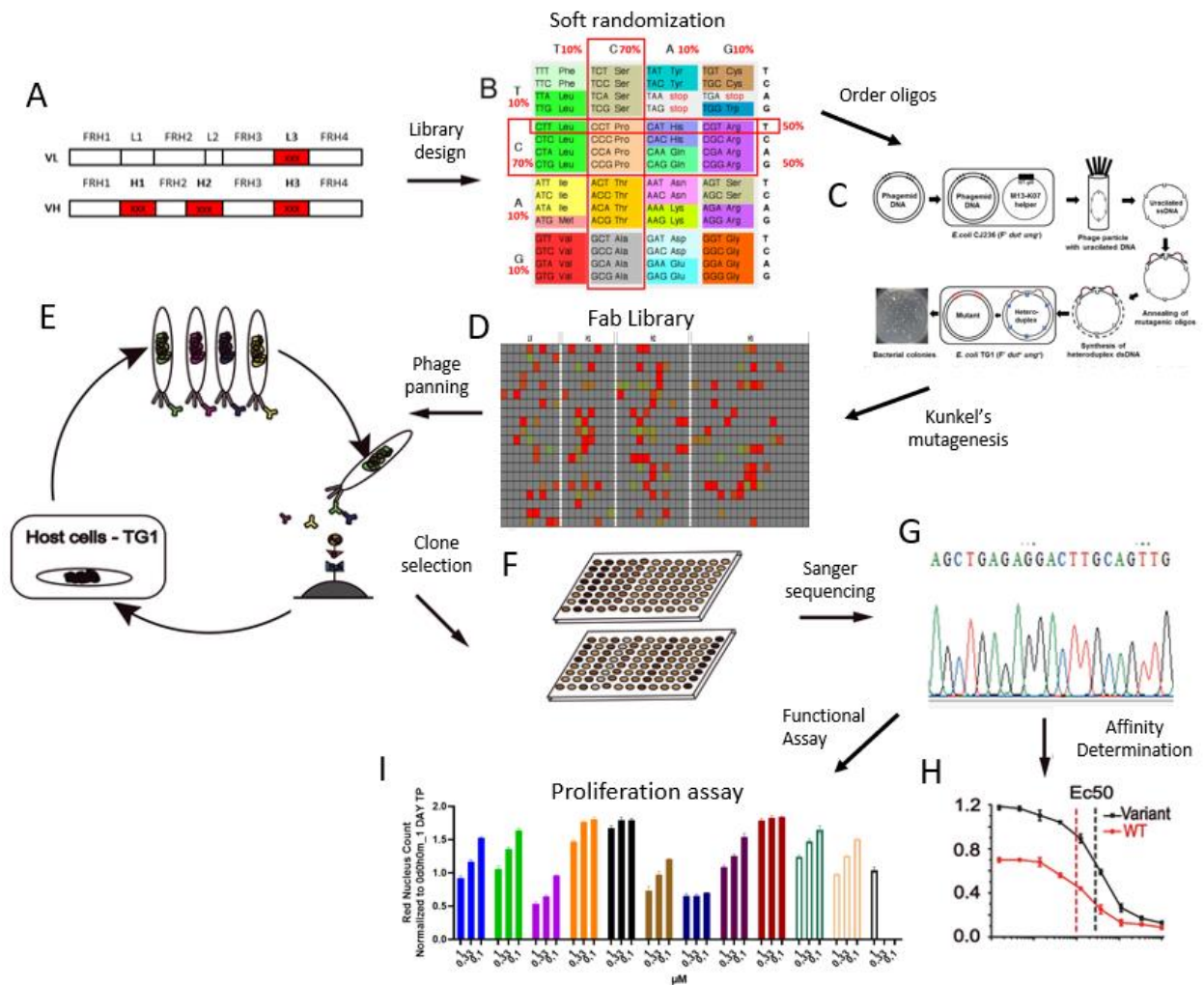


Figure 3. Affinity maturation workflow using phage display. (a) Acquisition of lead antibody. (b) Design of affinity maturation library (soft-randomization). (c) Library generation (site-directed mutagenesis). (d) Library quality control. (e) Phage display selections. (f) Screening of positive clones by ELISA. (g) Sequencing of binding clones. (h) Antibody binding affinity assays. (i) Cell/function assays.

1.5. TRANSMEMBRANE PROTEINS

Transmembrane proteins are a diverse class of proteins that span across the cellular membrane, playing crucial roles in various biological processes (Alberts et al., 2002a). They are characterized by their ability to traverse the lipid bilayer, with portions of the protein located both inside (cytoplasmic domains) and outside the cell (extracellular domains). There are three main types of transmembrane proteins based on their structural organization and orientation within the membrane (Alberts et al., 2002a). Single-pass transmembrane proteins: These proteins have a single transmembrane domain (TM) that crosses the lipid bilayer once. One portion of the protein resides

on the extracellular side, while the other resides on the cytoplasmic side of the membrane. Single-pass transmembrane proteins often act as receptors or transporters, transmitting signals or transporting molecules across the membrane. For example, glucose transporters transporting glucose (Hebert & Carruthers, 1992) or ligands related to Notch signalling pathway Delta-like and Jagged (Puigserver, 2018).

Multi-pass transmembrane proteins: These proteins contain multiple transmembrane domains that traverse the membrane multiple times (Alberts et al., 2002a). The number of transmembrane domains can vary, ranging from two to several dozen. Multi-pass transmembrane proteins can have different topologies, such as alpha helices or beta barrels. They fulfil diverse functions, including ion channels, transporters, G-protein-coupled receptors (GPCRs), and enzymes.

GPI-anchored proteins: Glycosylphosphatidylinositol (GPI)-anchored proteins are attached to the cell membrane through a GPI anchor, a complex lipid moiety (Lee et al., 2003). The protein portion of GPI-anchored proteins faces the extracellular side of the membrane, while the GPI anchor resides within the lipid bilayer. These proteins are involved in cell adhesion, signal transduction, and other cellular processes. Transmembrane proteins are essential for various physiological processes, including cell signalling, transport of molecules across the membrane, cell adhesion, and enzymatic reactions. Their structural and functional diversity makes them attractive targets for therapeutic intervention and drug development.

1.5.1. GLUCOSE TRANSPORTERS

The glucose transporter family, also called solute carrier family 2 (SLC2), is a group of transmembrane proteins that facilitate the transport of glucose across cell membranes. These transporters play a crucial role in maintaining glucose homeostasis and providing cells with the necessary energy source for various metabolic processes. These proteins have molecular size of 55kDa and a characteristic 12-transmembrane helix structure that spans the lipid bilayer, with both the amino and carboxyl termini located intracellularly (Deng et al., 2014; Mueckler et al., 1985). They contain highly conserved regions responsible for substrate binding and transport. The human genome encodes 14 members of the glucose transporter family, designated as GLUT1 to GLUT14 (Thorens & Mueckler, 2010). Each transporter exhibits distinct tissue distribution, substrate specificity, and kinetic properties. Some members, such as GLUT1, GLUT2, GLUT3, and GLUT4, are well-characterized and widely studied.

Glucose transporters have specific expression patterns across various tissues and organs, reflecting their specialized functions (Thorens & Mueckler, 2010). Here are a few examples:

GLUT1: Found in most tissues, GLUT1 provides basal glucose uptake and is essential for glucose supply to the brain and red blood cells. It is also expressed in the placenta and serves as a gateway for fetal glucose transport.

GLUT2: Predominantly expressed in the liver, pancreatic beta cells, and renal tubules, GLUT2 plays a role in glucose sensing and transport in these tissues. It allows glucose uptake by the liver for glycogen storage and glucose release into the bloodstream. In pancreatic beta cells, GLUT2 contributes to glucose-stimulated insulin secretion.

GLUT3: Highly expressed in the brain, GLUT3 is responsible for glucose transport across the blood-brain barrier and glucose uptake by neurons, ensuring an adequate energy supply for brain function.

GLUT4: Primarily found in muscle and adipose tissue, GLUT4 is insulin-regulated and plays a vital role in glucose homeostasis. It facilitates glucose uptake in response to insulin stimulation, promoting glucose utilization and storage in these insulin-sensitive tissues.

The activity and cellular localization of glucose transporters are tightly regulated to meet the metabolic demands of different tissues and maintain glucose homeostasis. Several factors influence the expression and translocation of glucose transporters, including insulin, hormones, cellular energy status, and substrate availability. For instance, insulin stimulates the translocation of GLUT4 from intracellular vesicles to the cell surface in muscle and adipose tissue, thereby increasing glucose uptake. In contrast, hormones such as glucagon and epinephrine decrease GLUT4 translocation and inhibit glucose uptake (Sonksen & Sonksen, 2000).

Dysregulation or mutations in glucose transporters can lead to various metabolic disorders and diseases. For example:

GLUT1 deficiency syndrome, which is caused by mutations in the SLC2A1 gene encoding GLUT1. This disorder impairs glucose transport across the blood-brain barrier, resulting in developmental delay, epilepsy, and other neurological symptoms (Wang et al., 2000).

Insulin resistance and reduced GLUT4-mediated glucose uptake in target tissues are key features of type 2 diabetes. These abnormalities contribute to elevated blood glucose levels and impaired glucose utilization (Sonksen & Sonksen, 2000).

Altered glucose transporter expression is commonly observed in cancer cells, allowing increased glucose uptake and utilization for energy production and biomass synthesis (Min et al., 2021). This metabolic reprogramming supports the rapid growth and proliferation of tumour cells.

Studying the glucose transporter family and their roles in cellular glucose transport provides insights into normal physiology, metabolic disorders, and potential therapeutic targets. The development of drugs targeting glucose transporters is an active area of research, aiming to modulate glucose uptake and utilization for the treatment of metabolic diseases and certain types of cancer.

1.5.2. ACQUISITION OF ANTI-SLC2A1 ANTIBODY 6F12#33

The lead anti-SLC2A1 antibody was obtained through a multi-step process. Initially, the chickens were immunized with DNA encoding SLC2A1, which helped prime their immune system. Subsequently, the chickens received boosts using SLC2A1 pseudotyped virus-like particles (VLPs) to further enhance the antibody response. After the immunization process, antibodies were isolated from the chickens using hybridoma technology (Zhang, 2012), and screening assays were performed to identify potential lead antibodies.

From the screening assays, two lead antibodies were initially identified: 6F12#22 and 6F12#33. However, further characterization revealed that the clone 6F12#22 exhibited undesired properties. It showed cross-reactivity to mock VLPs (not expressing SLC2A1) and other members of the glucose transporter family, including SLC2A2, SLC2A3, and SLC2A4. Additionally, this clone caused side effects in mouse tests, which made it unsuitable for further development. In contrast, the clone 6F12#33 demonstrated highly specific binding to SLC2A1 and did not exhibit any side effects in mouse tests. This specificity and lack of side effects made it a promising candidate for further development including affinity maturation. The decision was made to exclude clone 6F12#22 from the affinity maturation process based on these findings. (Specific data not shown.) The specific characteristics of clone 6F12#33, including its high specificity to SLC2A1 and absence of side effects, make it a valuable starting point for the affinity maturation process. Thus, the antibody 6F12#33 was subsequently cloned into a phagemid vector and given to the author of this thesis for affinity maturation. Further optimization and refinement of this antibody may lead to improved affinity and efficacy, making it a potential candidate for therapeutic applications targeting SLC2A1-related disorders.

2. EXPERIMENTAL PART

2.1. AIM

The aim of this master's thesis was to utilize the phage display technology to improve the affinity and specificity of antibody 6F12#33 towards the glucose transporter SLC2A1.

2.2. MATERIALS AND METHODS

2.2.1. AFFINITY MATURATION LIBRARY AND OLIGO DESIGN

Affinity maturation was initiated by selecting the best clone from the previous selection, based on functional assay, namely SLC2A1 clone 6F12#33 (#33 henceforth). The design of the affinity maturation library was based on a structural analysis and rational approach. First, the structural model of the VL and VH of #33 (Table 2) was generated, using the online tool SAbPred antibody builder (Dunbar et al., 2016) to identify the CDR regions and the amino acids facing the solvent as potential regions to randomize in the library.

Table 2. The light and heavy chain amino acid sequence of SLC2A1 clone 6F12#33. CDR regions are highlighted in red, based on IMGT numbering format.

	Amino acid sequence
Light chain	ALTQPSSVSANPGETVKITCSGGSSSSYYGWYQQKSPGSAPVTLIYYND KRPSDIPSRFSGSKSGSTATLTITGVQVEDEAVYYCGTGDGSFAIFGAG TTLTVL
Heavy chain	AVTLDESGGGLQTPGGALSLVCKASGFTFSDHAIFWVRQAPDKGLEFV AAISSGTGSWTAYATVVKGRATISRDNQSTVRLQLNNLRAADTATY YCAKGAGSACCYAGSIDAWGHGTEVIVSS

Four oligonucleotides annealing to H1, H2, H3 and L3 CDR regions containing different number of randomized codons were synthesised (Integrated DNA Technologies; Figure 4 and Supplementary Table 1). The randomization was performed using non-equimolar degenerate oligonucleotides that combine mixture of nucleotides at specific positions during the DNA synthesis (Integrated DNA Technologies). The first and second nucleotide in a codon was mixed with 70% of the wild type (wt) nucleotide and 10% of each of the other three nucleotides, whereas the third nucleotide was allowed to be only 50% T/G, giving the lowest chance of mutating into a STOP codon while allowing the chance to mutate into other amino acids based on the proximity in the codon table, which is partially mimicking somatic hypermutations. The specific oligonucleotides ordered can be found in Supplementary Table 1. To force mutations and avoid obtaining the wt #33 Fab displayed on phage, two STOP codons (TAA and TGA) in tandem were introduced in the template phagemid in CDR H2 by Kunkel mutagenesis (explained below). These STOP codons substituted two amino acids in CDR H2 to generate a stop template, resulting in non-

functional Fabs. The STOP template also helps to reduce the interference of wt Fab clones during the phage display selections. The mutated oligonucleotides were then used to randomize CDR regions H1, H2, H3, and L3 simultaneously to generate fully functional binders.

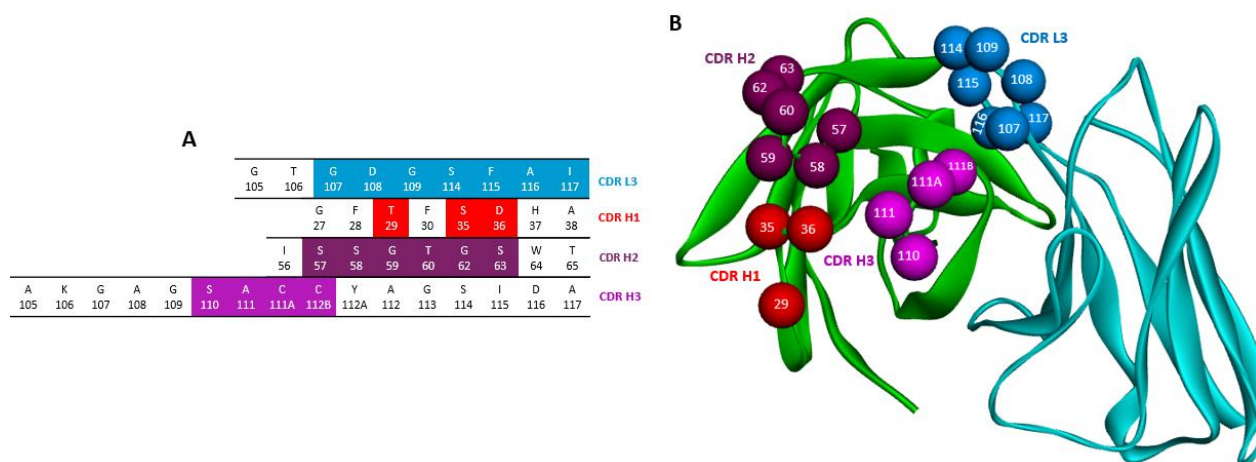


Figure 4. Randomization strategy employed for the affinity maturation library for Fab #33. (a) The amino acid and IMGT position for four CDR regions are shown, and the randomized amino acids are highlighted in different colors. (b) Homology modelling structure of clone #33 is shown in ribbons (VH in green and VL in blue) and the randomized amino acid positions are shown in spheres. The amino acid positions in CDR regions H1, H2, H3, and L3 are shown in red, purple, pink, and blue, respectively in both panels.

2.2.2. SITE-DIRECTED MUTAGENESIS AND LIBRARY GENERATION

The affinity maturation library was generated using Kunkel mutagenesis protocol (Kunkel, 1985), which is highly efficient and specific site-directed mutagenesis method that involves several steps. First, single-stranded DNA (ssDNA) template is generated from the viral phagemid DNA with the STOP template. This ssDNA template is then hybridized with a synthetic oligonucleotide that contains the desired mutations. The hybridized DNA is then used as a template for DNA polymerase, which extends the oligonucleotide and generates a covalently closed circular double-stranded DNA (ccc-dsDNA) molecule that contains the desired mutation. ccc-dsDNA is then electroporated into SS320 E. coli cells, grown overnight and the library is extracted by PEG/NaCl precipitation.

2.2.2.1. SINGLE-STRANDED DNA EXTRACTION

To generate the ssDNA for mutagenesis, the phagemid clone #33 was transformed into CJ236 cells which lack the dUTPase and uracil deglycosidase, which results in the incorporation of the uracil into the template ssDNA. Single colonies were picked and grown in 1 ml of 2xYT medium

supplemented with Carbenicillin (Carb, 25 µg/ml), Chloramphenicol (Cmp, 25 µg/ml) and M13KO7 (10⁹ CFU/ml, New England Biolabs) for 3 hours, followed by addition of Kanamycin (Kan, 30µg/ml) and continued growth for 5 hours. The culture of the three fastest growing single colonies was then transferred to 30 ml 2xYT media supplemented with Carb (25 µg/ml), Kan (30 µg/ml) and uridine (0.25 µg/ml) and grown for 20 hours at 37°C. After 20 hours, the cultures were centrifuged for 10 min at 8000rpm, 4°C using the F14-14x50cy rotor in Sorval Lynx 4000 centrifuge (Thermo Fisher Scientific, US). The resulting supernatant was then mixed with ~8 ml of PEG/NaCl and incubated on ice for 20 minutes, followed by centrifugation for 20 minutes at 12000 rpm and 4°C. The supernatant was discarded, and the phage pellet was resuspended in 1 ml of 1xPBS. The sample was incubated with MP (Qiagen) buffer and loaded onto a QiAprep spin column. After incubation with MLB buffer and cleaning with PE buffer, ssDNA was eluted in 50 µl of MilliQ water. We obtained about 5-15 µg of ssDNA per single colony based on Nanodrop (Thermo Fisher Scientific, US) measurements. The quality of ssDNA was confirmed by running 100 ng of ssDNA on a 0.8% TAE agarose gel for 30 minutes at 100V, along with 2 µl of 1 kb DNA ladder and 100 ng of dsDNA as a control. A single band lower than dsDNA indicates good ssDNA quality. Additionally, a uracil incorporation test was performed on 20 µl of phages prior to ssDNA extraction.

In order to assess the level of uracil incorporation, a titration analysis of phages was performed. Specifically, 10 µl of phages was serially diluted in 90 µl of 1xPBS for 12 dilutions. The diluted phages were then used to infect CJ236 and XL1 Blue cells for 30 minutes. Next, a 5 µl drop of infected cells was plated on 2xYT agar plates supplemented with Carb, Kan, or Tet and grown overnight.

2.2.2.2. LIBRARY GENERATION

Next step in the Kunkel's mutagenesis is to hybridize the ssDNA with the synthetic oligonucleotides. To achieve this, 20 µg of template ssDNA was mixed with 25 µl of 10xTM buffer, 1 µg of phosphorylated oligo and MilliQ water to reach a final reaction volume of 250 µl. The hybridization reaction was carried out sequentially at 90°C for 3 minutes, 50°C for 3 minutes and 20°C for 5 minutes. Subsequently, the reaction mixture was incubated with an enzyme mixture containing 25 µl of 10 mM dNTP (Thermo Fischer Scientific, US), 15 µl of 100 mM dithiothreitol (DTT, Thermo Fischer Scientific, US), 10 µl of 10 mM ATP (Thermo Fischer Scientific, US), 6 µl of T4 DNA ligase (New England Biolabs, US), and 3 µl of T7 DNA polymerase (New England

Biolabs, US) for 3 hours. To confirm the conversion of ssDNA to dsDNA, a 2 µl sample of the reaction mixture was loaded onto a 0.8% TAE agarose gel for 30 minutes at 100V, along with 2 µl of 1 kb DNA ladder and 100 ng of dsDNA as control. The presence of a shifted band height indicated the conversion of ssDNA to dsDNA. The DNA solution was then purified using QiaQuick PCR & gel clean-up kit (Qiagen). The sample was mixed with QG buffer and loaded to the QiaQuick Spin Column. After washing with PE buffer, the DNA was eluted in 35 µl of MillyQ water.

The cleaned-up DNA was then electroporated into SS320 E. coli cells, which have been pre-infected with helper phage and assure viral propagation of all clones. The following E. coli electroporation program was used: 2500V, 125Ω, 50µF and >0.4 picoseconds current. The cells were immediately rescued by mixing them 25 ml of SOC media, followed by incubation at 37°C for 30 minutes. SS320 E. coli cells contain enzymes that degrade DNA containing uracil and since mutated DNA strand does not contain uracil it will not be degraded; thus, the cells use the mutated DNA strand as a template and the mutations are successfully incorporated.

About 50 µl aliquot of the cells were taken before the cells were transferred to 250 ml of 2xYT media supplemented with Carb (25 µg/ml) and Kan (30 µg/ml) and incubated overnight at 37°C, 200 rpm to produce phages (now called library). The library was extracted from overnight culture by PEG/NaCl precipitation and was resuspended in 1 ml of 1xPBS and stored at 4°C. The library is then used to determine its concentration, to measure its display level and in selections.

The diversity of the library was determined by titration of the rescued cells. 10 µl of the rescued cells were serially diluted in 90 µl of 2xYT media for a total of 6 dilution and 5 µl of diluted cells were pipetted onto 2xYT agar plate containing either Carb, Tet, or Kan. The colonies were expected to appear in the 4th or 5th dilution on 2xYT, Carb plates, indicating the library diversity of 10⁸-10⁹ unique phages.

The concentration of the library was determined by the titration of the library. 10 µl of library was serially diluted in 90 µl of 1xPBS for a total of 12 dilutions. Dilution 7-12 were used to infect XL1 Blue cells for 30 minutes. 5 µl of infected cells were pipetted onto 2xYT agar plate containing either Carb, Tet, or Kan.

2.2.2.3. LIBRARY QUALITY CONTROL

To evaluate the quality of the generated libraries and the incorporation of synthetic oligos, the library display level was assessed using the FLAG tag at the C-terminal of the VL chain as a probe

for Fab display. Single colonies of the library were inoculated into 0.6 ml mini tubes in a 96-tube rack (Axygen) containing 450 µl of 2xYT media with Carb (50 µg/ml) and helper phage M13KO7 (New England Biolabs, US) and grown overnight at 37°C, 200 rpm. Meanwhile, a MaxiSorp™ high-capacity binding 384-well ELISA plate (Thermo Fisher Scientific, US) was coated overnight with 3 µg/ml anti-FLAG antibody (Sino Biological) and 0.25 µg/ml of anti-M13 antibody (Abcam) in 1xPBS. The overnight cultures were centrifuged at 4000 rpm for 10 min using Sorval Lynx 4000 centrifuge (Thermo Fisher Scientific, US) and the supernatant was used in the ELISA assay. The ELISA plate was washed 2x and blocked with 1% BSA in 1xPBS for 1 hour. After washing the plate 2x again, the supernatant was incubated on the ELISA plate for 1 hour, followed by a 20-minute incubation with anti-M13 horse radish peroxidase (HRP) conjugated antibody. The substrate used for HRP was TMB (3,3',5,5'-Tetramethylbenzidine) Solution X (Biopanda Diagnostics) and 0.5 M H₂SO₄ was used as a stop solution. After each step, the wells were washed with 0.05 % Tween 20 in PBS solution, and absorption at 450 nm was measured with the Thermo Scientific™ Multiskan™ FC Microplate Photometer. Good display levels of Fab on the phage was determined by a ratio of anti-FLAG to background (BSA) above 1.5, while a ratio below 1.5 indicated poor display.

The same colonies utilized in the ELISA assay were amplified by PCR for sequencing of the library. The PCR reaction mixture was prepared by mixing 14 µl of MilliQ water, 1.5 µl of supernatant (positive clone), 4 µl of HOT FIREpol Blend Master Mix (10mM MgCl₂) (Solis Biodyne), 0.25 µl each of forward and reverse primers. The mixture was amplified using the following thermocycler program: 5 min at 98 °C, 26 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C), 5 min at 72 °C. The amplification was confirmed by running 3 µl of the sample on a 0.8% TAE agarose gel at 100V for 20 minutes, along with 2 µl of 1kb DNA ladder as control. Samples showing a single band were selected for sequencing, which was performed at Institute of Genomics core Facility, University of Tartu. Primers used for amplification and sequencing are shown in Supplementary Table 1.

2.2.3. SLC2A1 SELECTIONS

In this thesis, three rounds of phage display selections were carried out to isolate high-affinity Fab binders against SLC2A1 displayed on in-house VLPs. The conditions used for the selection rounds are detailed in Table 3.

For the first round of selection, a Nunc ImmunoSorp 96-well ELISA plate (Thermo Fisher Scientific, US) was coated with 10ug/ml SLC2A1 VLP (Icosagen) in 1xPBS overnight to serve as the positive selection plate. The plate was then washed and blocked with 1% BSA, 0.05% Tween 20 in 1xPBS for 1 hour. A separate 96-well ELISA plate was blocked at the same time and used for negative BSA selection to remove the phages that bind to BSA. The library (100 µl) was first incubated on negative BSA selection plate for 1 hour, after which the library was transferred to the positive selection plate and incubated for an additional 1 hour. The plate was then washed 8 times to remove unbound, unspecific, and weakly binding phages. The target-bound phages were eluted by adding 100 µl of 0.1 M HCl per well and incubating for 10 min, followed by neutralisation with 20 µl of 1 M Tris pH 11 buffer per well. The eluted phages were then collected and transferred to a fresh 1.5 ml Eppendorf tube, which served as the output. From the output 20 µl of eluted phages were set aside for titration and the remaining phages were used for reinfection.

The XL1 Blue cells were grown in 2xYT media to 0.5-0.6 OD and infected with phage output and 10^{12} cfu/ml of helper phage M13KO7 (New England Biolabs, US) for 30 min. The infected cells were then transferred to 40 ml of 2xYT medium, containing Carb (50 µg/mL) and Kan (25 µg/mL) and grown overnight at 37°C, 200 rpm. The overnight culture was centrifuged at 8000 rpm for 10 min using the F14-14x50cy rotor in Sorval Lynx 4000 centrifuge (Thermo Fisher Scientific, US) to remove the cells. The supernatant was mixed with ~8-9 ml of PEG/NaCl and incubated on ice for 20 min. The supernatant was then centrifuged again at 12000 rpm for 20 min to precipitate the phages. The supernatant was aspirated and centrifuged again at 12000 rpm for 5 min to remove residual supernatant and the phage pellet was finally resuspended in 1 ml of 1% BSA, 0.05% Tween 20 in 1xPBS buffer, which served as the input for subsequent rounds.

Table 3. Selection conditions for direct immobilization of SLC2A1 VLP

Round	Target concentration (µg/ml)	Washes	Number of coated wells
1	10	8	8
2	10	10	4
3	10	12	2

For rounds 2 and 3 (Table 3), the similar conditions were used as for the first round of selection, except the number of washes was increased by 2, and the number of coated wells was reduced 2-fold between the rounds. The output from rounds 2 and 3 was stored at 4°C for positive clone screening.

The recovery of phages after each selection round was determined through titration analysis. The expected increase in phage output is approximately 10-fold per round. To quantify the phage

output, 10 µl of the output phages was serially diluted in 90 µl of 1xPBS for six dilutions. Then, 10 µl of the diluted phage was added to XL1 Blue cells and incubated for 30 min. Next, a 5 µl drop of the infected cells was pipetted onto a 2xYT agar plate containing Carb, Kan, or Tet. The input for each round was also titrated in 10-fold dilutions to measure the phage concentration.

2.2.4. CLONE SCREENING AND SEQUENCING

To identify positive clones from the selections, an ELISA-based binding assay was performed. Single colonies of output phages were inoculated into 0.6 ml mini tubes in a 96-tube rack (Axygen) containing 450 µl of 2xYT medium with Carb (50 µg/ml) and helper phage M13KO7 (New England Biolabs, US) and grown overnight at 37°C, 200 rpm. Meanwhile, a MaxiSorp™ high-capacity binding 384-well ELISA plate (Thermo Fisher Scientific, US) was coated overnight with 3 µg/ml SLC2A1 VLP (Icosagen) and 2 µg/ml mock VLP (not expressing SLC2A1, Icosagen) in 1xPBS. The overnight cultures were centrifuged at 4000 rpm for 10 min using Sorval Lynx 4000 centrifuge (Thermo Fisher Scientific, US) and the supernatant was used in the ELISA assay. The ELISA plate was washed 2x and blocked with 1% BSA in 1xPBS for 1 hour. After washing the plate 2x again, the supernatant was incubated on the ELISA plate for 1 hour, followed by a 20-minute incubation with anti-M13 horse radish peroxidase (HRP) conjugated antibody. The substrate used for HRP was TMB (3,3',5,5'-Tetramethylbenzidine) Solution X (Biopanda Diagnostics) and 0.5 M H₂SO₄ was used as a stop solution. After each step, the wells were washed with 0.05 % Tween 20 in PBS solution, and absorption at 450 nm was measured with the Thermo Scientific™ Multiskan™ FC Microplate Photometer. The positive clones were identified with a ratio of SLC2A1 binding signal to background binding (BSA) signal above 1.5.

The positives clones identified by ELISA were amplified by PCR for sequencing. The PCR reaction was prepared by mixing 14 µl of MilliQ water, 1.5 µl of supernatant (positive clone), 4 µl of HOT FIREpol Blend Master Mix (10mM MgCl₂) (Solis Biodyne), 0.25 µl each of forward and reverse primers. The mixture was amplified using the following thermocycler program: 5 min at 98 °C, 26 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C), 5 min at 72 °C. The amplification was confirmed by running 3 µl of the sample on a 0.8% TAE agarose gel at 100V for 20 minutes, along with 2 µl of 1kb DNA ladder as control. Samples showing a single band were selected for sequencing, which was performed at Institute of Genomics core Facility, University of Tartu. Primers used for amplification and sequencing are shown in Supplementary table 1.

2.2.5. ANTIBODY PRODUCTION AND PURIFICATION

Eight positive binding clones were selected based on binding signal intensity and sequence similarity and were subsequently cloned into human IgG1 format. The cloning, production, and purification of antibodies were conducted by various departments at Icosagen. The Hybrifree department performed the cloning and production of the antibody according to the company's standard protocols, while the Protein Purification department utilized the standard Protein A immunoprecipitation KingFisher protocol (as outlined in Table 4) for the purification of antibodies. The antibody light and heavy variable sequences were cloned into the human IgG1 format and produced transiently in Chinese Hamster Ovary (CHO) cells in 2 ml of HEK TF (Xell AG) media. The Cytiva Prot A beads kit was used for the antibody purification process. The antibodies were eluted in approximately 280 μ l of 0.1 M Na-Citrate buffer with a pH of 3.1 and neutralized with 1.5 M Tris buffer at a pH of 8.8. Finally, the antibodies were desalted using a PD MiniTrag G-25 column and eluted with 300 μ l of 1xPBS buffer at a pH of 7.4 before being stored at -20°C.

Table 4. KingFisher protocol for antibody purification

		KingFisher Protocol	Comments
1. Plate	24-Well Deep plate tips	Fitting tips to adapter	
2. Plate	Cytiva Prot A 150 μ l + 850 μ l 1xPBS	The binding of beads to the tips – 20 seconds	3x
3. Plate	2 ml 1xPBS	• Magnetic beads in solutions – 20 seconds • Mixing magnetic beads with the tips – 30 seconds	4x; Washing of magnetic beads
4. Plate	2 ml of cell culture supernatant	• Shaking magnetic beads in the supernatant, medium speed – 2:30 minutes • Shaking magnetic beads in the supernatant, slow speed – 2:30 minutes	12x; The binding of antibodies to the magnetic beads
5. Plate	2 ml of 1xPBS	• Washing magnetic beads – 5 minutes	1x; Washing of unspecific molecules
6. Plate	2 ml of 1xPBS	• Washing magnetic beads – 5 minutes	1x; Washing of unspecific molecules
7. Plate	300 μ l of 0.1M Na-citrate buffer (pH=3.1)	• Shaking magnetic beads - slow speed – 7 minutes	1x; Elution of antibodies

8. Plate	2 ml of 0.2M NaOH buffer	Shaking of beads – medium speed – 2 minutes	1x; Regeneration of magnetic beads, removal of all bound protein
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2.2.6. ANTIBODY BINDING ASSAYS

ELISA-based VLP binding assay

An ELISA based VLP binding assay was carried out to compare the EC₅₀ values of different clones and cross reactivity to different SLC2A1 proteins. MaxiSorp™ high-capacity binding 384-well ELISA plates (Thermo Fisher Scientific, US) were coated with 2 µg/ml of pseudotyped VLPs displaying human SLC2A1, mouse SLC2A1, human SLC2 family members (SLC2A2, SLC2A3 and SLC2A4), and mock VLP (not displaying SLC2A1) in PBS overnight. The plate was washed and blocked for 1h with 0,05% Tween 20, 1% BSA in PBS. Serial dilutions of antibodies were incubated on a washed plate for 1 hour, followed by a 20-minute incubation with goat anti-human IgG horse radish peroxidase (HRP) conjugated (Invitrogen) antibody. TMB (3,3',5,5'-Tetramethylbenzidine) Solution X (Biopanda Diagnostics) was used as a substrate for HRP and 0.5 M H₂SO₄ was used as stop solution after 10-minute incubation. After every step, the wells were washed with 0.05 % Tween 20 in PBS solution. Absorption at 450 nm was measured with Thermo Scientific™ Multiskan™ FC Microplate Photometer.

2.2.7. CELL BASED FUNCTIONAL ASSAYS

2.2.7.1. CELL PROLIFERATION ASSAY

To determine the inhibitory effect of affinity-matured anti-SLC2A1 antibodies in combination with Phenformin on cell proliferation, a MCF7 NuncLight Red cells (Icosagen) cell proliferation assay was conducted. Specifically, 1500 cells were grown in 20 µl of Plasmix (CancerTools) media supplemented with 2.5% Fetal Bovine Serum (FBS) in a 384-well cell culture microplate (Cat nr 781182, Greiner) for 24 hours at 37°C, 5% CO₂. The affinity-matured antibodies and Phenformin were diluted to a final concentration of 3 µM and 0.75 mM, respectively, and mixed. The mixture was added to the cells in three 3-fold dilutions, with the first dilution having a final concentration of 1 µM and 0.25 mM for the antibodies and Phenformin, respectively, and subsequent dilutions having 3-fold lower concentrations. A commercial SLC2A1 inhibitor, Bay 876 (Cayman Chemical Co), was used as a control at a final concentration of 1 µM. Additionally, anti-HEL (Hen Egg Lysozyme) Mouse IgG1 isotype (Icosagen) was used as a negative antibody control at a final

concentration of 1 μ M. The cells were then incubated at 37°C, 5% CO₂ in Incucyte (Agilent Technologies) and imaged at 4-hour intervals for 72 hours. Cell proliferation was analysed by normalizing the cell count to a starting timepoint of 0 hours and plotting the average cell count on a histogram.

2.2.7.2. FLOW CYTOMETRY-BASED CELL BINDING ASSAY

Flow cytometry-based HepG2 (hepatocellular cancer cell line) wild type (wt) and SLC2A1 knock-out (KO) cell (Abcam) binding assay was used to demonstrate the specific binding of the anti-SLC2A1 antibodies to SLC2A1 membrane protein. For this, 0.2×10^6 cells were stained with 100 nM anti-SLC2A1 antibody solutions in flow cytometry buffer (1% FBS, 1mM EDTA in PBS) on ice. This was followed by an incubation with a secondary goat anti-human IgG AF488 antibody (1:500 dilution) (Jackson Immuno Research). The mean fluorescent intensity (MFI) was measured in FITC channel with Becton, Dickinson and Company Accuri C6 Plus flow cytometer and MFI ratio was calculated by dividing the MFI of each antibody to anti-HEL isotype (Icosagen Cell Factory) control staining MFI. MFI ratio above 1.5 is considered as binding. All evaluated antibodies bound to HepG2 wt cells with MFI ratios well above 1.5. However, binding to HepG2 SLC2A1 KO cells was characterized by an MFI ratio below 1.5 for all antibodies indicating a specific binding to SLC2A1 membrane protein.

2.2.8. DATA ANALYSIS

All numerical data was analysed using Prism software (GraphPad, version 9) and Microsoft Excel while sequencing data was analysed using Geneious Prime software. The structures of SLC2A1 and antibodies were analysed using BIOVIA Discovery Studio software (2021 client).

During the writing process of this master's thesis, ChatGPT was used to enhance the clarity and coherence of my writing. To achieve this, a paragraph was first written and then submitted, along with the variations of the prompt "Rewrite the following to fit more to the thesis style," to ChatGPT. Then, the comparison of the output generated by ChatGPT with the original writing was done and any changes that improved the paragraph's structure and readability were incorporated. This involved using ChatGPT's responses in their entirety or selecting specific sentences that contributed to the improvement of the paragraph's overall quality.

2.3. RESULTS AND DISCUSSION

2.3.1. AFFINITY MATURED LIBRARY SHOWS HIGH VIABLE DIVERSITY

To enhance the affinity and specificity of antibody #33 towards the glucose transporter SLC2A1, we generated an affinity maturation library, named H2.

The first step was to model the structure of #33 Fab (Figure 4B) to identify the minimal number of CDR regions and the positions to randomize in our library. The CDR regions H1, H2, H3, and L3 were selected, since these are the loops that are more commonly observed to interact with the antigen in crystal structures (Almagro, 2004). In addition, the amino acids that faced solvent in the structural model were chosen for randomization to increase the chance of obtaining functional and higher affinity binders except for CDR H3. This loop is quite long within the chicken antibody repertoire and contains two cysteine residues that we suspect to form a disulfide bond restricting and constraining its conformation for optimal antigen recognition, which is quite common in chicken antibodies (Mallaby et al., 2022; Wu et al., 2012). Since CDR H3 has long been known to drive the recognition of the antigen and contribute to stabilizing binding interactions (Jain et al., 2007), we did not want to randomize it. However, unpaired cysteine residues can generate covalent bonds and create developability complications (Banks et al., 2008). Therefore, we decide to substitute 4 residues located at the tip of the loop including the two flanking cysteine residues and assess if the substitution of the cysteines was possible conserving or even improving binding to SLC2A1.

A total of 20 positions were chosen for randomization. Since our main aim is to keep the same binding site and mode and improve the affinity, we decided to have a soft randomization scheme in which about 70% of the wild type residues are retained, instead of randomizing all positions at once. This randomization consists of using degenerate oligonucleotides such that the first and second nucleotide in a codon was mixed with 70% of the wild type (wt) nucleotide and 10% of each of the other three nucleotides, whereas the third nucleotide was allowed to be only 50% T/G, greatly reducing the chance of mutating into STOP codon.

A total of 7 randomized oligonucleotides annealing to L3, H1, H2 and H3 DNA were incorporated into the ssDNA template with a Kunkle reaction (Kunkel, 1985). The libraries are expected to generate high diversities of unique antibody sequences. However, it will also contain a population of wt antibodies that would not have suffered any randomization and that would dominate the selections since it competes to single clones and already binds the target antigen. To avoid generating phages displaying wt Fabs due to no oligonucleotide incorporation, two tandem STOP

codons and a frame shift were incorporated into the VH region since it is the variant fused to the capsid pIII viral protein and it would remove the translation of the fusion assuring the generation of bold phage. The tandem STOP codons were incorporated into the CDR H2 region, which is the most randomized positions in the VH region and three oligonucleotides compete to anneal in this position. This will force the introduction of the randomized H2 oligonucleotide and assure the highest number of mutations. STOP codons were not incorporated in other loop regions since the number of non-viable clones in the library would increase until a point in which it would generate background noise during selections.

Overall, the H2 library that was generated revealed a diversity of 1.8×10^9 unique Fab variants and soft randomizes a maximum of four CDR regions and 20 amino acid positions. To assess the display levels of Fab on phage particles and analyse the incorporation rate and amino acid mutagenesis, we performed an ELISA and sequenced a total of 96 randomly selected clones. For the display ELISA, the anti-FLAG antibody was coated, and phage binding was detected using an anti-M13 antibody, as the light chain contained a C-terminal FLAG tag fusion peptide. Approximately 30% of the clones exhibited an anti-FLAG to background (BSA) ratio above 1.5, indicating acceptable display level. This finding is expected, considering that (1) the incorporation of H2 oligonucleotide is indispensable to remove the tandem STOP and frameshift that the template antibody contains, and (2) a total of 20 amino acid positions were simultaneously randomized, and the probability of a single mutation introducing a STOP codon was relatively high. Sequencing results further supported these limitations, as all clones displaying Fab randomized the H2 loop and contained viable codons. Moreover, we observed 7.3%, 16.6%, 29.2%, and 31.3% simultaneous oligonucleotide incorporation rates for clones with four, three, two, and one CDR loop, respectively. As expected, CDR2 incorporation was consistently observed in positive clones since the STOP template contained a STOP codon and a frame shift that prevented Fab display unless the corresponding oligonucleotide was incorporated.

Additionally, we evaluated the individual incorporation rates of each oligonucleotide targeting specific CDR loops for the 96 clones irrespectively of their display. Surprisingly, the L3, H1, H2 and H3 oligonucleotides were incorporated in 32%, 25% 65% and 24% of the clones respectively. Generally, the expected incorporation rate of a single oligonucleotide in Kunkel protocol is around 50-100%, but the more regions and oligonucleotides used simultaneously the lower the single oligonucleotide incorporation rate is (Firnberg & Ostermeier, 2012). Thus, these results are expected, and the disparity in incorporation rates between H2 and the other regions can be

explained by the fact that the H2 oligonucleotides had better annealing and the introduction of STOP codons in H2 loop, which force the loop to mutate.

Overall, the H2 affinity maturation library demonstrated successful generation and satisfactory Fab display levels. The incorporation of randomized oligonucleotides in specific CDR loops was observed, albeit with certain limitations. Based on the display levels obtained, we achieved a total diversity of 5.4×10^8 viable and unique Fabs. The unbiased distribution of individual oligonucleotide incorporations indicated the absence of preferential biases during library construction. These findings emphasize the quality and potential of the H2 library for selecting improved antibodies with enhanced binding properties and provide a solid foundation for subsequent affinity maturation experiments.

2.3.2. PHAGE DISPLAY SELECTIONS RESULTED IN MANY POSITIVE FAB BINDERS AGAINST SLC2A1 VLP

The H2 affinity maturation library was used to carry out phage display selections against SLC2A1 displayed on VLPs. The target was directly immobilized on plates and after conducting three rounds of phage display selections, SLC2A1 binding for about 600 individual colonies were examined from rounds two and three by ELISA. Results showed that about 250 clones bound the target antigen and showed no significant background signal to BSA or mock VLP based on ELISA. As expected, we observed a significant enrichment between round two and three, for which 12.5% and 73.6% binding success rate were observed, respectively (Table 5), suggesting a selection for tighter affinity-matured antibody binders and success of this phage display campaign. The DNA of all binding clones was PCR amplified and sent for Sanger sequencing to obtain the antibody amino acid composition in the randomized CDR positions.

The phage display selections encompassed several crucial variables that warranted careful consideration to achieve successful selection campaign. Key factors in these selections included the quantity of target antigen employed and the frequency of plate washing. To ensure simplicity and comparability throughout this thesis, a basic experimental condition was adopted. Specifically, a fixed antigen concentration of 10 $\mu\text{g/ml}$ was utilized for all selection rounds (Table 5), with the sole modification being a reduction in the number of coated wells, resulting in decreased amount of target antigen. Additionally, the number of plate washes was incrementally increased by two for each successive round. As a result, the selection stringency under these conditions was intentionally kept relatively low, resulting in about 250 positive binding clones.

Conversely, utilizing more stringent conditions, like reducing the amount of antigen between rounds and increasing the number of washes, can lead to the discovery of much higher affinity antibodies, but it can also heavily reduce the number of positive binding clones, leading to lower number of unique antibody clones.

Table 5. Phage display Selection conditions and clone screening results

Round	Target concentration (µg/ml)	Washes	Number of coated wells	Nr of clones picked	Nr of positive clones	Nr of positive clones sequenced	Nr of unique clones	% of unique clones
1	10	8	8	-	-	-	-	
2	10	10	4	288	36	36	12	33%
3	10	12	2	288	212	64	43	67%

2.3.3. SEQUENCE ANALYSIS OF UNIQUE AFFINITY MATURED ANTIBODY CLONES

Sequence analysis of 96 random positive binding clones allowed us to gain insights into the affinity maturation process. Among the 96 analysed clones, we identified 55 unique antibody sequences, which were subsequently aligned and sorted by sequence similarity into four groups (Figure 5). Notably, all the clones exhibited mutations in the CDR H2 loop, proving that the deliberate introduction of the two STOP codons in the CDR H2 loop of the template antibody in the phagemid is an effective strategy to force mutations in a desired region, since the introduction of the mutated oligonucleotide became necessary to generate functional Fabs. Otherwise, the STOP codons would interrupt the synthesis of the antibody heavy chain, resulting in non-functional antibodies incapable of binding. In addition, CDR H1 loop displayed mutations in 40% of the clones, while CDR L3 and CDR H3 loops showed the lowest mutation frequency, with 11% and 7.3% respectively.

Interestingly, none of the clones exhibited mutations in all four randomized CDR loops simultaneously. Among the analysed clones, 11% displayed mutations in three of the randomized loops, while 36.4% and 52.6% of the clones had mutations only in two and only one of the four randomized CDR loops respectively (Figure 5).

The analysis of the physicochemical properties of the amino acid mutations shows that the most frequent mutations involved the conversion of serine or threonine residues into arginine or lysine residues. Serine and threonine share similar properties, with the primary difference being the presence of methyl group in threonine, and their substitution to a much larger and positively

charged amino acids, lysine or arginine, significantly changes the local binding characteristics but also the global properties of the antibody, increasing the isoelectric point of the molecule. Notably, these mutations were predominantly observed at specific positions, such as CDRs L3:S114, H1:S35, H2:S63 and T65. The prevalence of these mutations at specific positions suggests that the introduction of a positive charge, along with an elongated side chain, may enhance antibody binding affinity compared to neutral charge or shorter side chains.

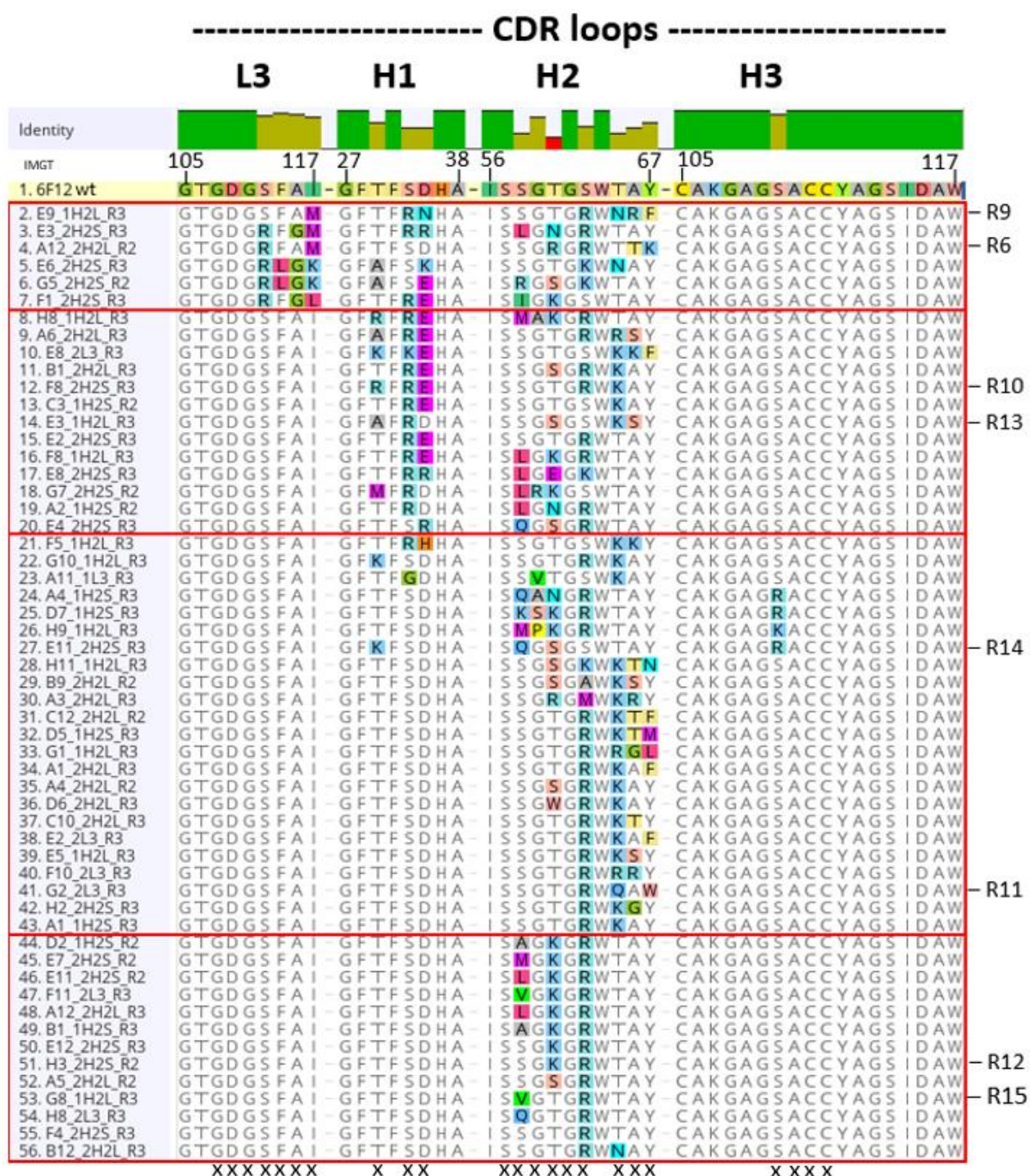


Figure 5. CDR sequences of unique Fabs discovered from the phage display selection campaign. The framework regions between the CDRs are substituted by dashes (-). The Fab CDR sequences are aligned and sorted by similarity, resulting in four distinct groups highlighted in red boxes. Amino acids that differ from the parent antibody in the first row are highlighted by different colors. The first and last amino acid in a CDR loop is indicated using the IMGT numbering scheme. The identity histogram provides an overview of the number of different amino acid residues observed at each position within the sequences. Antibodies selected for further evaluation are denoted by their antibody identifier (R#) at the right of their respective sequence. The X below the sequences denotes the amino acids that were randomized in the library.

In addition, other mutations within the CDR L3 and CDR H1 loops were primarily conserved. For instance, alanine at position 116 in the CDR L3 mutated into glycine, preserving the small size of the amino acid. Similarly, isoleucine at position 117 mutated into methionine, lysine, or leucine, thereby maintaining the size of the aliphatic carbons of the amino acid side chain. Similar patterns were observed in CDR H1 loop, where aspartic acid at position 36 primarily mutated into glutamic acid, leading to a longer side chain while retaining the negative charge properties. These conserved mutations indicate that specific amino acid properties at these positions are important for the stability and function of the antibody.

Conversely, we observed that certain positions within the randomized CDR loops exhibited minimal or no mutations despite being subjected to randomization. For example, CDR L3 positions G107, D108 and G109, as well as CDR H2 positions S57, G59 and G62, remained conserved across all analysed clones (Figure 5). That suggests that these positions play a crucial role in maintaining stability or functional characteristics of the antibody.

Surprisingly, the CDR H3 loop wild type sequence was selected for SLC2A1 recognition in almost all clones, with only four clones displaying a single point mutation from serine to arginine or lysine. Since the incorporation rate of the CDRH3 oligonucleotide in the library is comparable to the CDR loops H1 and L3, these results suggest a pivotal role of the CDR H3 loop in mediating the binding of antibody #33 to the target antigen. The minimal mutational diversity in this region suggests that the CDR H3 loop sequence in the initial antibody might already possess a favourable binding configuration, and further diversification was not required for increased affinity and/or specificity. To validate whether any of these clones exhibit higher binding affinity compared to the parent antibody, two representative clones were chosen from each of the four similarity groups (denoted by R# in Figure 5) guided by the ELISA signal intensity as an indicator of potential binding strength (data not shown). These eight representative clones capture a diverse range of mutations to assess their impact on the binding affinity to the target antigen, glucose transporter SLC2A1.

2.3.4. ALL AFFINITY MATURED ANTIBODIES EXHIBITED INCREASED AFFINITY

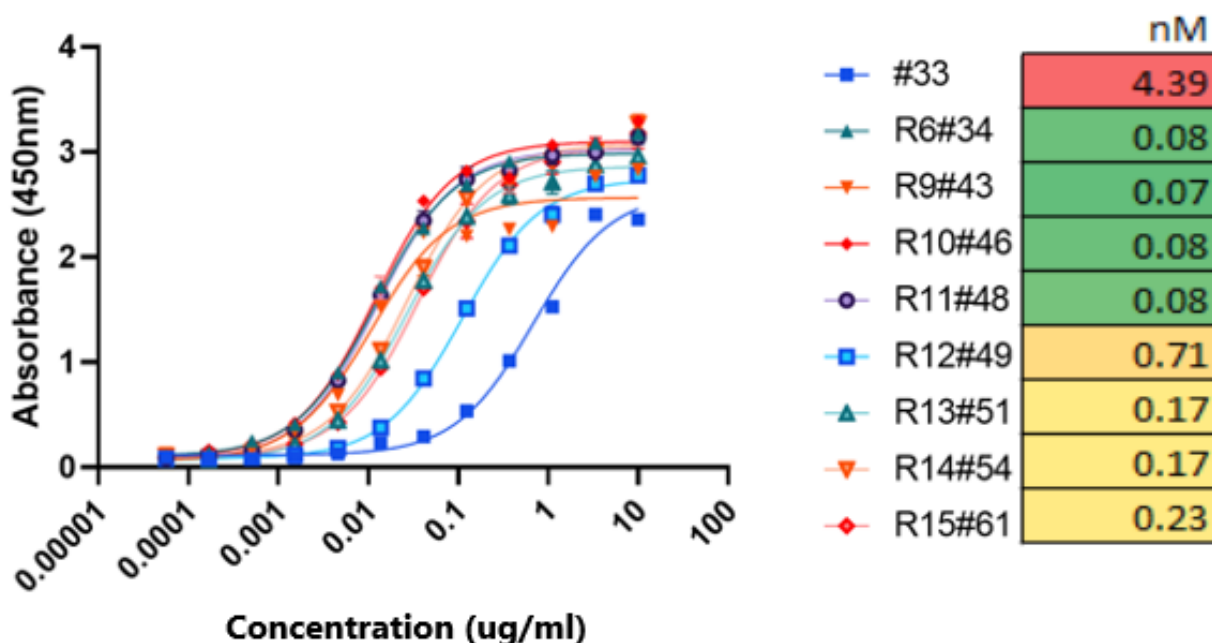


Figure 6. Binding affinity of matured antibodies by EC₅₀ ELISA-based assays. On the left, the concentration-dependent antibody binding curves to human SLC2A1 are shown for the parental (#33) and each affinity matured antibody (R#). On the right, the EC₅₀ values for each antibody are shown.

To assess the effectiveness of the affinity maturation process, eight representative unique clones were selected for further evaluation. The light and heavy chain sequences of these Fab clones were cloned into the human IgG1 isotype format and transiently produced in CHO cell lines using 2 ml of HEK TF media. Following expression, the affinity-matured antibodies were purified from the supernatant, resulting in a 300 μ l solution with antibody concentrations ranging from 0.5 to 1 mg/ml in 1xPBS buffer.

The EC₅₀ is a useful characteristic for describing antibody binding to an antigen, which represents the concentration of a molecule to achieve a response that is half of the maximum achievable response. In the context of antibodies, EC₅₀ describes the concentration of antibody required to elicit a functional response (binding). The EC₅₀ value was measured for all eight affinity-matured antibodies and compared to the parental control #33, which presented an EC₅₀ value of 4.39 nM. As depicted in Figure 6, all affinity-matured antibodies demonstrated a significant increase in their binding affinity, which is indicated by their lower EC₅₀ values compared to the parent antibody #33. Specifically, the EC₅₀ values for the affinity-matured antibodies were 50-5-fold lower, indicating that a considerably lower amount of antibody is required to achieve the desired

functional response. All affinity-matured antibodies demonstrated very similar EC_{50} values, specifically 0.08-0.23 nM, except R12, which showed 0.71 nM. Additionally, R12, along with R15, had the least number of mutations, only two mutations compared to other affinity-matured antibodies, which had between 3-7 mutations. R12 and R15 are the only tested antibodies that have mutations only in CDR H2. This indicates that the mutations in CDR H2 are necessary to have higher binding affinity, but additional mutations in other positions may increase the affinity even more.

The observed increase in the binding affinity, denoted by lower EC_{50} values, highlights the successful enhancement of antibody affinity through our affinity maturation approach. The improved affinity, as reflected by the reduced EC_{50} values, is of paramount importance for therapeutic applications. Lower EC_{50} values indicate that the affinity-matured antibodies can achieve their functional response at much lower concentrations, potentially leading to reduced dosages and improved treatment efficacy. This finding underscores the success of our affinity maturation strategy in generating antibodies with enhanced functional activity.

2.3.5. SOME AFFINITY-MATURED ANTIBODIES RETAINED THEIR HIGH SPECIFICITY TOWARDS SLC2A1

SLC2A1 has three very close protein homologs, 2, 3, and 4, sharing a global sequence similarity of 55-65% (Figure 7D). At the extracellular part where the antibody recognizes the glucose transporter, the sequence similarity drops to 40-70% (Figure 7A, 7C) suggesting binding to different ligands and different biological functions. Having a specific antibody against SLC2A1 is of crucial importance. For example, SLC2A1 is related to various diseases, disorders, and certain cancers and having antibodies that are highly specific to SLC2A1, may help to treat these conditions without interfering with glucose transporter functions in healthy tissues (Zheng et al., 2022).

On the other hand, it is important to obtain a cross-reactive antibody that could be used to study glucose transport inhibition in mouse system. Importantly, mouse SLC2A1 is 97% globally identical to human SLC2A1, while the similarity drops to 85% in the extracellular domains, it still practically shares all residues in the extracellular part, dramatically increasing the chance of obtaining cross-reactive antibodies.

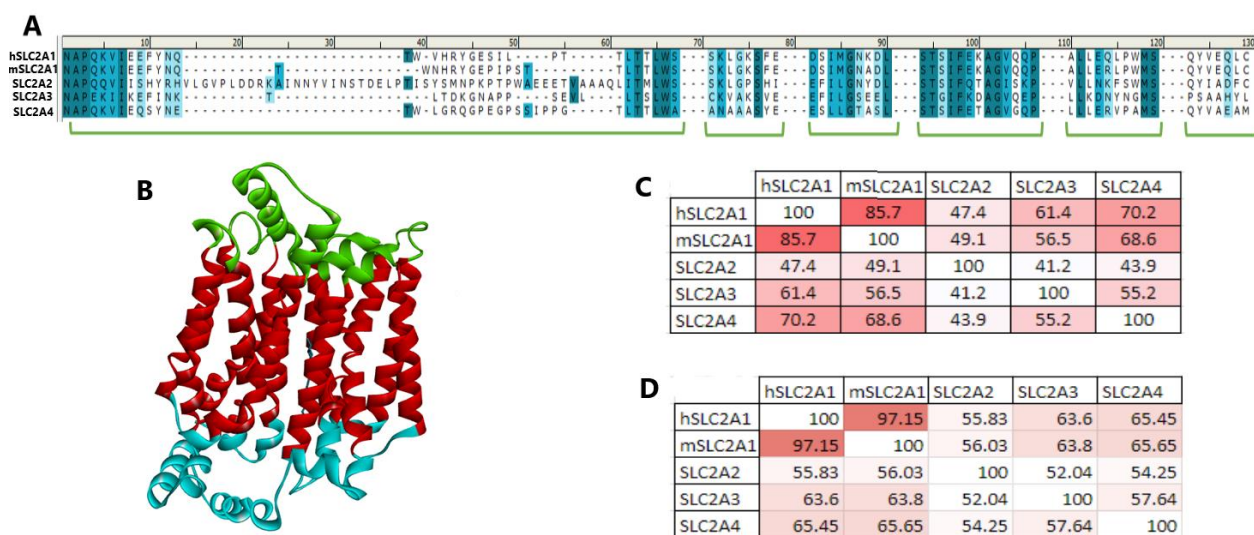


Figure 7. Similarity analysis of glucose transporter family members: human and mouse SLC2A1 and human SLC2A2, SLC2A3 and SLC2A4. (a) Amino acid sequences of SLC2A1 homolog's extracellular loops, shown in green in panel B. (b) SLC2A1 structure representation highlighting extracellular, transmembrane, and cytoplasmic domains in green, red, and blue respectively. (c) SLC2A1 homolog's similarity matrix of extracellular domains. (d) SLC2A1 homolog's global similarity matrix.

In this regard, the parent antibody, #33, already demonstrated high specificity towards human

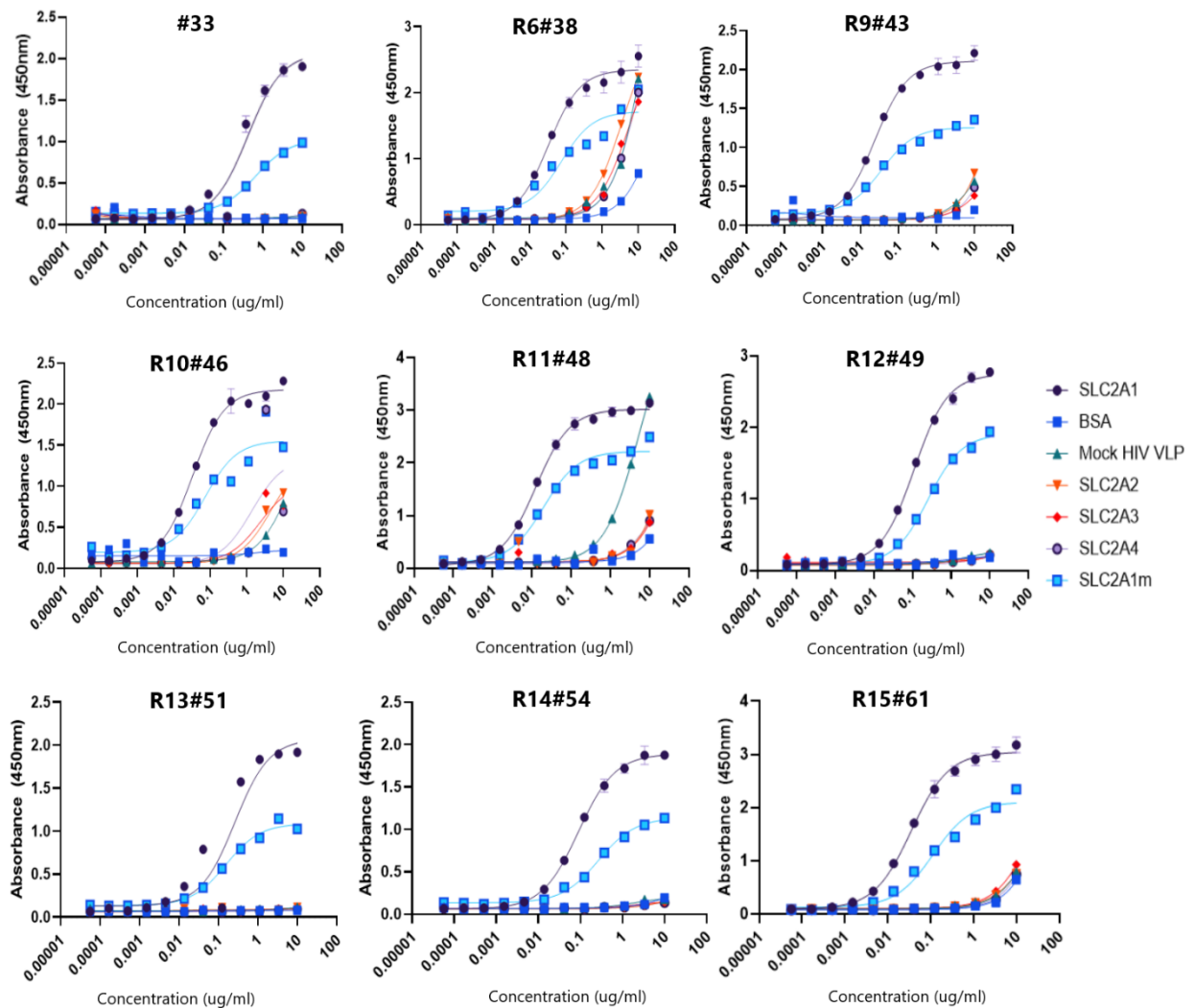


Figure 8. ELISA-based specificity binding assay. The specificity of the parental (#33) and each of the affinity-matured antibodies were tested against human SLC2A1, SLC2A2, SLC2A3, SLC2A4, and mouse SLC2A1 VLPs. The negative controls, BSA and mock VLP (not displaying SLCs) were also tested.

SLC2A1 over its homolog proteins and mouse SLC2A1 cross-reactivity (Figure 8) and we wanted to assess whether the affinity-matured antibodies maintained their binding specificity towards SLC2A1. An ELISA-based binding assay was conducted against the pseudotyped VLPs of human SLC2A1, mouse SLC2A1, and human SLC2A1 homologs (SLC2A2, SLC2A3, and SLC2A4). In addition to SLC2A1 and its homologs, the antibodies were also tested against BSA and mock VLP, that is not displaying SLCs. It is important to remember that, there is a chance that the antibodies are binding to common epitope on the VLP, since all the SLCs are displayed on the VLP and the

VLP was used in the selections. The same is true for BSA, as it was used as a blocking agent during the selection process and thus the antibodies might be binding to it.

The results of the binding assay revealed three groups of cross-reactive antibodies: no cross-reactivity (R12, R13, R14), low cross-reactivity (R9, R15), and high cross-reactivity (R6, R10, R11). Affinity-matured antibodies R12, R13 and R14 maintained the specificity exhibited by the parent antibody, including a tighter binding towards human and mouse SLC2A1 and a minimal to no binding to the homologous SLC proteins.

On the other hand, R9 and R15 presented some levels of cross-reactivity against SLC2A1 homologs and negative control mock VLP, which is known to display small levels of several SLC2A1 homologs, which also show some binding signal at high antibody concentrations.

Affinity-matured antibodies R6, R10, and R11 displayed the highest levels of cross-reactivity towards the negative control mock VLP, especially R11. While these antibodies are cross-reactive towards SLC2A1 homologs, they do not bind the second negative control BSA. In addition, all the cross-reactive affinity-matured antibodies show similar binding curves for SLC2A1 homologs and the mock VLP, suggesting that these antibodies found some common area or some residual SLC2A1 homologs on the VLP.

The reduced specificity observed in R10 may be attributed to a mutation at position 63 in CDR H2 (as shown in Figure 5), where R10 contains arginine while R13 and R14 possess serine. The mutations at other positions remain similar among the three antibodies.

To confirm whether the mutation at position 63 in CDR H2 is responsible for the altered specificity in R10, further investigations are required. Specifically, replacing the arginine mutation in R10 with serine, and vice versa for R13 and R14, followed by rerunning the binding assay, would provide valuable insights into the role of this specific mutation in determining antibody specificity. Further analysis is necessary to elucidate the exact contribution of each mutation to antibody specificity and understand the underlying molecular mechanisms.

In conclusion, the increased affinity combined with the retention of specificity of R13 and R14 affinity-matured antibodies is a promising outcome, as it ensures the antibodies remain targeted towards the intended antigen.

2.3.6. SOME AFFINITY MATURED CLONES SHOWED HIGHER INHIBITORY EFFECT THAN THE PARENTAL ANTIBODY

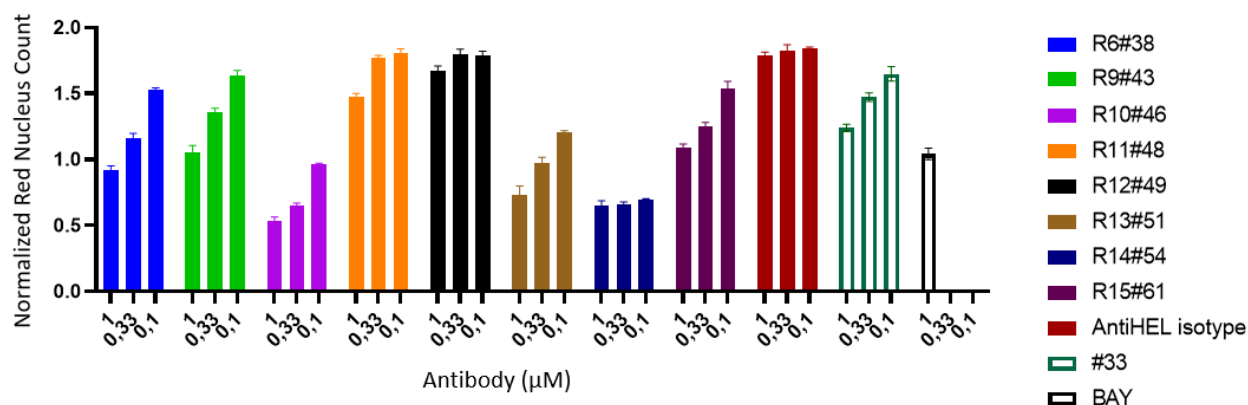


Figure 9. Antibody effects on cell-based proliferation assay. The MCF7 cells growth was evaluated in the presence of eight affinity matured antibodies, the parent antibody #33, AntiHEL isotype negative control, and the commercially available SLC2A1 inhibitor (BAY) in combination with phenformin. Antibodies were added at three different concentrations (1, 0.33 and 0.1 μM). Cell proliferation effect was measured by the cell count after 72 hours and normalized to cells counted at 0 hours.

To evaluate the functionality of the affinity-matured antibodies, we evaluated the effects of the antibodies on the MCF7 (breast cancer) cell proliferation in the presence of phenformin. This compound is a complex 1 inhibitor in the mitochondrial respiratory chain (García Rubiño et al., 2019). We have found that the combined co-incubation of phenformin with SLC2A1 inhibitory antibodies resulted in a synergistic effect, leading to a higher degree of cell proliferation inhibition (data not shown). The synergy observed between the anti-SLC2A1 antibody and phenformin may arise from their complementary mechanisms of action. While the antibody specifically targets the glucose transporter SLC2A1, phenformin acts at the mitochondrial level to impair cellular energy metabolism. The combination of these agents likely disrupts cancer cell survival and proliferation by simultaneously depriving the cells of glucose uptake and compromising their energy production. Thus, this assay provided insights into the inhibitory effects of the antibodies and allowed us to compare their performance to the parent antibody (#33; Figure 9).

After 72 hours after the addition of 1, 0.33 or 0.1 μM of each antibody to MCF7 cells in the presence of phenformin we observe a wide variety of effects in proliferation. The controls anti-HEL isotype (does not bind to SLC2A1) antibody indicates that the antibody has no effect of cell proliferation, while SLC2A1 inhibitor BAY allows to compare the effects of our antibodies to the commercially available chemical inhibitor. The inhibitory effect of antibodies depends on the concentration of antibodies added to the cells. At 1 μM concentration, the parental antibody, #33, is slightly worse

than the commercial control BAY, while at lower concentrations #33 still shows some inhibitory effect.

Interestingly, three affinity-matured antibodies, namely R10, R13, and R14, exhibited significantly greater inhibitory effects compared to the parent antibody. This outcome indicates that the affinity maturation process successfully enhanced the antibodies' ability to hinder the proliferation of MCF7 cells. These three antibodies share mutations in the H1 and H2 CDR loops although different mutation combinations are observed (Figure 5). Of particular note is the remarkable inhibitory glucose transport effect of affinity-matured antibody R14, even at low concentrations. This finding suggests that a much lower amount of this antibody is required to achieve the same inhibitory effect as the parent #33 antibody.

On the other hand, affinity-matured antibodies R6, R9, and R15 demonstrated inhibitory effects similar to that of the parent antibody. Although they did not exhibit a notable improvement in inhibitory activity, these antibodies still retained their functional capabilities, indicating that the affinity maturation process did not compromise their binding properties.

Conversely, affinity-matured antibodies R11 and R12 displayed a reduced inhibitory effect compared to the parent antibody. This observation suggests that the introduced mutations may have adversely affected their binding affinity or altered their interaction with the target antigen. These kinds of anomalies could also be due to the fact that the cloning from Fab to IgG1 may affect the properties of the antibody (Steinwand et al., 2014). The worst and averagely performing antibodies are discarded from further characterization, due to not showing sufficient inhibitory effect.

Taken together, three of the eight affinity-matured antibodies showed higher inhibitory effect compared to the parental antibody. In addition, using phenformin in combination with the antibodies showed much higher inhibitory effect than either alone. The consideration of combination therapy involving high-affinity and specificity anti-SLC2A1 antibodies, such as those obtained through affinity maturation, alongside phenformin holds significant promise in the fight against cancer. Such an approach has the potential to enhance therapeutic outcomes, maximize efficacy, and minimize the development of resistance.

2.3.7. AFFINITY-MATURED ANTIBODIES BIND TO CELL AT HIGH AFFINITY

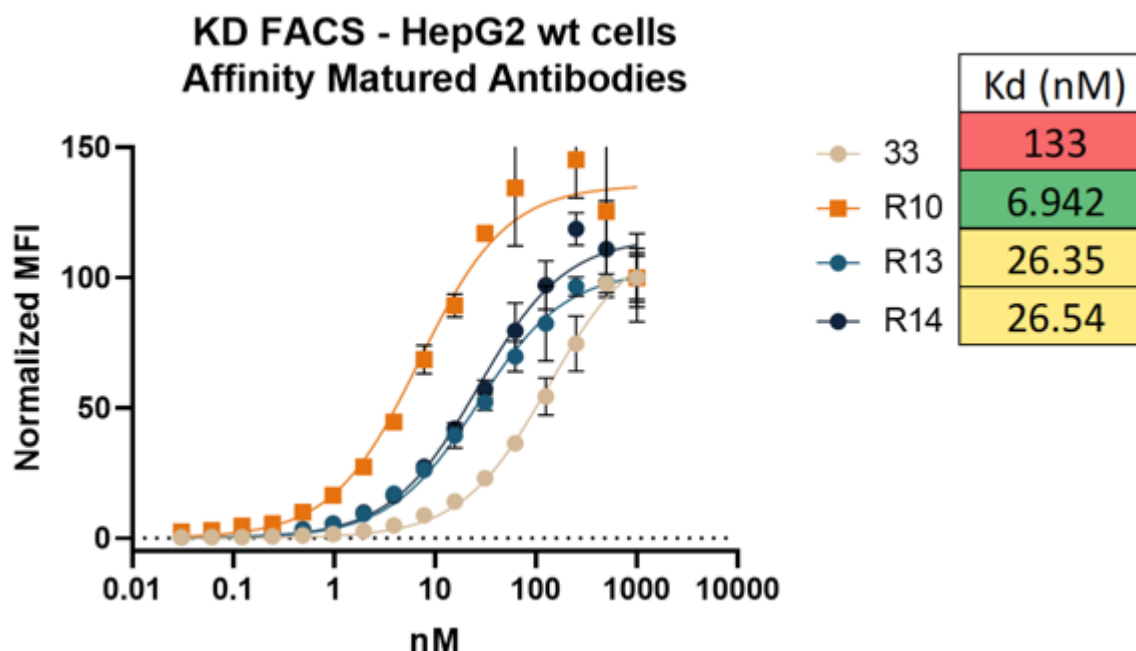


Figure 10. Antibody binding affinity to HepG2 cells based on fluorescence-activated cell sorting (FACS). On the left, the binding curves of the parental (#33) and the three best performing antibodies (R10, 13 and 14) towards HepG2 cells displaying SLC2A1 are shown. The graph shows the mean fluorescent intensity (MFI) normalized by dividing the MFI of each of the antibodies to anti-HEL isotype control staining MFI (y-axis) at multiple antibody concentrations (x-axis). On the right, the Kd values are shown for each antibody.

In order to validate the binding of affinity-matured antibodies to SLC2A1 displayed in natural environment, we carried out fluorescence-activated cell sorting (FACS) using hepatocellular (HepG2) cancer cells, which are known to overexpress the SLC2A1 (Peng et al., 2022). In addition to determining the EC50 values, the KD values were also determined for the three top-performing affinity-matured antibodies. The KD, a crucial parameter in characterizing antibody-antigen interactions, provides a quantitative measure of the binding affinity between the antibody and its target. The determination of KD values is of great significance, as it provides crucial insights into the binding affinity and stability of antibody-antigen complexes. Antibodies with lower KD values exhibit tighter and more favourable interactions, leading to increased specificity and potentially improved therapeutic efficacy. The KD was measured using the FACS based method.

To determine the KD values, the HepG2 cells were incubated with various concentrations of affinity-matured antibodies and the mean fluorescence intensity (MFI) was measured, resulting in the binding curve as depicted in Figure 10. Notably, the antibody R10 exhibited the highest affinity among the affinity-matured antibodies with KD value 6.9 nM, while antibodies R13 (KD = 26.4

nM) and R14 ($K_D=26.5$ nM) also exhibited higher affinity compared to the parental antibody. This is also confirmed by the EC_{50} values of the same antibodies.

The substantial decrease in K_D values for the affinity-matured antibodies suggests a significant enhancement in their binding affinity towards the target antigen. The improved K_D values reflect the successful outcomes of our affinity maturation strategy, where specific mutations were introduced to optimize the binding interface and strengthen the antibody-antigen interaction.

Of particular interest is the notably lower K_D value observed for R10. This antibody, with its 20-fold decrease in K_D , demonstrates the highest binding affinity among the affinity-matured antibodies evaluated. This finding highlights the effectiveness of the affinity maturation process in generating antibodies with superior binding capabilities.

The reduced K_D values for R13 and R14, though to a lesser extent compared to R10, indicate that these antibodies also exhibit enhanced binding affinity. These results support the notion that the affinity maturation approach effectively refines the antibody's binding properties, resulting in improved target recognition and interaction.

CONCLUSION

In this master's thesis, an affinity maturation library was meticulously designed and generated with the aim of enhancing the affinity and inhibitory activity of antibody 6F12#33 against glucose transporter SLC2A1, an overexpressed target in cancer cells. The library design process involved a comprehensive structural analysis of the antibody #33, coupled with a rational approach. These libraries were then subsequently employed in phage display selections, resulting in the screening of approximately 600 colonies using ELISA-based binding assay. Among the 600 clones screened, approximately 250 demonstrated positive binding, and upon further sequence analysis, approximately 50 unique clones were identified. From this subset, eight promising clones were chosen based on ELISA signal intensity and sequence similarity. These selected clones were then successfully cloned into human IgG format and produced in CHO cells and purified using Protein A immunoprecipitation for further characterization.

To evaluate the binding affinity and the functional efficacy of the purified antibodies, a series of comprehensive assays were conducted, including ELISA-based and Flow Cytometry-based binding assays as well as a cell proliferation assay. Strikingly, all eight affinity-matured clones exhibited substantially enhanced binding affinity towards SLC2A1 compared to the parental antibody, specifically 50-5-fold lower EC_{50} values than the parental antibody. The increased binding affinity was also confirmed in live hepatocellular cancer cells, HepG2, by fluorescence-activated cell sorting. Furthermore, the eight affinity-matured antibodies exhibited varying levels of specificity towards glucose transporter SLC2A1 and its homologs. Like the parental antibody, three of the affinity-matured antibodies were highly specific towards SLC2A1. While other five affinity-matured antibodies displayed cross-reactivity towards different SLCs and mock VLP (not displaying SLCs) at 10-100x higher concentrations. Importantly, three of the eight affinity-matured antibodies displayed greatly increased inhibitory effect in the cell proliferation assay, while the other five showed similar or worse inhibitory effect compared to the parental antibody.

In conclusion, by employing a combination of affinity maturation techniques and phage display technology, the properties of the antibody 6F12#33 towards the glucose transporter SLC2A1 were successfully enhanced. Three different antibodies bound 5-20-fold tighter to SLC2A1, retained the SLC2A1 specificity when tested against family members, and improved the SLC2A1 glucose uptake inhibitory effects in cancer cells when compared to the parental antibody. Moving forward, the forthcoming phase involves evaluating the functional effects of these antibodies on cancer-grafted mice (xenografts). The exceptional characteristics of these antibodies hold tremendous

potential in providing effective treatment options for cancer patients, highlighting a promising avenue for further research and clinical applications.

RESÜMEE

„GLÜKOOSI TRANSPORTERI SLC2A1 VASTASE ANTIKEHA 6F12#33 AFIINSUSKÜPSEMINE KASUTADES FAAGI DISPLEI TEHNOLOOGIAT“

Robin Pau

Resümee

Monoklonaalsed antikehad on ühed kiiremini kasvavad terapeutilised molekulid farmaatsiatööstuses, mille rakendused ulatuvad vähist autoimmuunhaigusteni. Nad on saadud ühest B-raku kloonist ning seonduvad spetsiifiliselt sihtmärk antigeeni epitoobiga ning pakuvad suunatud teraapiat vähemate kõrvaltoimetega tänu kõrgele afiinsusele. See suurenenud afiinsus saavutatakse immuunsüsteemi poolt läbi afiinsusküpsemise protsessi, mis osadeks on somaatiline hüpermutatsioon ja kloonaalne valik, mis on oluline patogeenide neutraliseerimiseks ja biomolekulide äratundmiseks. Afiinsusküpsemise protsessi saab samuti ka osaliselt *in vitro* jäljendada kasutades erinevaid tehnikaid. Faagi displei meetod on üks enam kasutatud ja võimsamaid tehnikaid monoklonaalsete antikehade tootmises ja ravimite avastamises. Faagi displei käigus konstrueeritakse miljardeid unikaalseid antikehavariante esitlevate faagide raamatukogu, mis seejärel skriinitakse tsüklilises protsessis sihtmärk antigeeni vastu. Seejärel valitakse välja faagid, mis seonduvad sihtmärk antigeeniga kõrge afiinsusega, ning need faagid kloonitakse ja vastavate antikehade järjestused tuvastatakse. Faagi displei abil saab kiiresti skriinida suuri antikeha fragmentide raamatukogusid paljude erinevate sihtmärkide vastu, näiteks valkude, peptiidide, bakterite ja viiruste. See on eriti kasulik haiguste ravi väljatöötamisel, kus sihtmärgid on keerulised ja raskesti toodetavad. Näiteks kõrge afiinsusega monoklonaalsete antikehade arendamine glükoosi transporter SLC2A1 (GLUT1) vastu võiks aidata ravida GLUT1 puudulikkuse sündroomi ja erinevaid vähi tüüpe, kus on SLC2A1 üleekspresseeritud.

Seetõttu Icosagen Cell Factory-s toodeti esialgu SLC2A1 vastased antikehad, mis seonduvad sihtmärgile piisava afiinsusega, et inhibeerida vähirakkude glükoosi ülevõttu. Seega me esitasime hüpoteesi, mille kohaselt kui suurendada selle antikeha afiinsust läbi afiinsusküpsemise, suurendame ka selle antikeha inhibeerimisvõimet.

Sellest tulenevalt oli selle magistritöö eesmärgiks suurendada glükoosi transportvalgu (SLC2A1) vastase antikeha 6F12#33 afiinsust, kasutades faagi displei tehnoloogiat. Selle jaoks disainiti ja loodi antikeha küpsemise raamatukogu, mille tulemusena skriinita ELISA meetodil umbes 600 klooniat. Nendest 600st kloonist valiti välja kaheksa paljutöötavat klooni ELISA signaali intensiivsuse ja järjestuse sarnasuse alusel. Antikeha järjestused klooniti inimese IgG1 formaati,

toodeti CHO-rakkudes ja puhastati. Viidi läbi põhjalikud analüüsid, sealhulgas hinnati antikehade efekti rakkude paljunemisel ning viidi läbi erinevad seondumise katsed, viidi läbi nende efektiivsuse hindamiseks. Kõik kaheksa afiinsusküpsenud antikeha parandasid seondumise afiinsust SLC2A1 suhtes võrreldes algse antikehaga. Kolm antikeha, lisaks afiinsuse suurendamisele, tõid kaasa ka olulise inhibeeriva toime suurenemise. Ülejäänud viis klooni näitasid sarnast või vähenenud inhibeerivat toimet. Kõik kaheksa afiinsusküpsenud antikeha seondusid SLC2A1-ga erineva spetsiifilisusega, näidates nende potentsiaali sihitud teraapiate jaoks. Käesolevas magistritöös suurendati edukalt antikeha 6F12#33 afiinsust ja spetsiifilisust afiinsusküpsemise ja faagi displei tehnika abil, pakkudes väärtuslikke teadmisi glükoosimetabolismiga seotud vähiravimite väljatöötamiseks.

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WEB PAGES

SAbPred antibody builder (Visited 12.07.2022) -

<https://opig.stats.ox.ac.uk/webapps/newsabdab/sabpred/abodybuilder/>

OpenAI. (2021). ChatGPT (March 23 Version) [Computer software]. - <https://chat.openai.com/>

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SUPPLEMENTARY MATERIALS

Supplementary table 1 – Oligonucleotides that were used in library generation and sequencing are ordered from Integrated DNA Technologies (IDT):

Name	Sequence	Purpose
AM_6F12_H2_Stop_Short	CTGGAATTTCGTCGCAGCTATT TAATGATGGA CAGCCTACGCGAC	STOP oligonucleotide
AM_6F12_H2_Stop_Long	GCTATTAGCAGTGGTACAGGT TAATGAACAG CCTACGCGACGGTG	STOP oligonucleotide
AM_6F12_LibF_H1_3	CAAGGCCTCCGGGTTC(N2:70101010)(N3:10701010)KTTC(N2)(N1:10107010)K(N1)(N2)K CATGCCATATTCTGGG	Mutagenic oligonucleotide
AM_6F12_LibF_H2_6	CGTCGCAGCTATT(N2:70101010)(N1:10107010)K(N2)(N1)(K)(N1)(N1)K(N2)(N3:10701010)K(N1)(N1)K(N2)(N1)KTGGACAGCCTACGCGAC	Mutagenic oligonucleotide
AM_6F12_LibF_H2_58_7	CGTCGCAGCTATT(N2:70101010)(N1:10107010)K(N2)(N1)KNNK(N1)(N1)K(N2)(N3:10701010)K(N1)(N1)K(N2)(N1)KTGGACAGCCTACGCGAC	Mutagenic oligonucleotide
AM_6F12_LibF_H2_63_7	CGTCGCAGCTATT(N2:70101010)(N1:10107010)K(N2)(N1)K(N1)(N1)K(N2)(N3:10701010)K(N1)(N1)K(N2)(N1)KNNKTGGACAGCCTACGCGAC	Mutagenic oligonucleotide
AM_6F12_LibF_H2_58_67_8	GTCGCAGCTATTAGCAGT(N1:10107010)(N1)K(N2:70101010)(N3:10701010)K(N1)(N1)K(N2)(N1)K(N4:10101070)(N1)K(N2)(N3)K(N1)(N3)K(N4)(N2)KGCGACGGTGGTGAAG	Mutagenic oligonucleotide
AM_6F12_LibF_H3_4	CTACTGCGCCAAGGGTGCTGGT(N2:70101010)(N1:10107010)K(N1)(N3:10701010)K(N4:10101070)(N1)K(N4)(N1)KTATGCTGGTAGTATCGACGC	Mutagenic oligonucleotide
AM_6F12_LibF_L3_7	GGCTGTCTATTACTGTGGGACT(N1:10107010)(N1)K(N1)(N2:70101010)K(N1)(N1)K(N2)(N1)K(N4:10101070)(N4)K(N1)(N3:10701010)K(N2)(N4)KTTTGGGGCCGGGACAAC	Mutagenic oligonucleotide
Forward PCR	TGTAAACGACGGCCAGT CTATTGCTACAAATGCCTATGCATCC	PCR amplification of VL chain
Reverse PCR	CAGGAAACAGCTATGACCCACCGGTT CGGGGAAGTAG	PCR amplification of VH chain
Forward sequencing	TGTAAACGACGGCCAGT	Sequencing of VL chain
Reverse sequencing	CAGGAAACAGCTATGACC	Sequencing of VH chain

LICENCE

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