

T.C.
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

**INVESTIGATION OF ENGINEERED ANTI-VEGF
ANTIBODIES FOR CANCER TREATMENT**

MURAT KARADAĞ

DEPARTMENT OF MOLECULAR BIOLOGY
AND GENETICS

DOCTOR OF PHILOSOPHY THESIS

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ABBREVIATIONS

Fab: Fragment antigen-binding

Fc: Fragment crystallizable

Fv: Variable fragment

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

IgG: Immunoglobulin G

scFv: Single-chain variable fragment

dsFv: Disulfide-stabilized Fv (variable) fragment

sc-dsFv: Single-chain disulfide-stabilized variable fragment

V_H: Heavy chain variable domain

V_L: Light chain variable domain

V/C interface: Variable-constant domain interface

CDR: Complementarity-determining region

HCDR: Heavy chain complementarity determining region

LCDR: Light chain complementarity determining region

C_H: Heavy constant domain

C_L: Light constant domain

mCRC: Metastatic colorectal cancer

NSCLC: Non-small-cell lung cancer

RCC: Renal cell carcinoma

mBC: Metastatic breast cancer

GBM: Glioblastoma multiforme

OC: Ovarian cancer

CC: Cervical cancer

GS Linker: Glycine-serin linker

T_m: Melting temperature

T_{1/2}: Transition mid-point

G: Gibbs free energy

SCs: Stabilization centers

Aga1p: a-agglutinin anchorage subunit (protein)

Aga2p: a-agglutinin adhesion subunit (protein)

HA: Hemagglutinin

His: Histidine

c-Myc: Cellular Myc

FACS: Fluorescence-activated cell sorting

MACS: Magnetic-activated cell sorting

TCRs: T cell receptors

scTCRs: single-chain TCRs (T cell receptors)

MHCs: Major histocompatibility complexes

GFP: Green fluorescent protein



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INVESTIGATION OF ENGINEERED ANTI-VEGF ANTIBODIES FOR CANCER TREATMENT

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ABSTRACT

Monoclonal antibodies (IgGs) are powerful therapeutics for many diseases, including cancer, due to their high specificity and other favorable properties. However, their large size limits their penetration into large masses of biological tissue, such as solid tumors. The single-chain variable fragments (scFvs), on the other hand, have better tissue penetration due to their smaller size. Yet, due to the absence of constant domains, scFvs are not as thermally stable as their parental IgGs or even Fab (fragment antigen-binding) fragments, which poses a challenge for therapeutic applications. Therefore, in this thesis, we aimed to develop a general method for increasing the thermal stability of scFvs.

Anti-angiogenic therapy has a critical importance in tumor-mediated angiogenesis, which is a hallmark of solid tumors. Bevacizumab (IgG) was the first therapeutic developed for anti-angiogenic therapy, and it is still used in the clinic for a variety of solid tumor types, mostly in combination with chemotherapy.

In this thesis, we developed anti-VEGF scFvs derived from bevacizumab and they demonstrated better anti-angiogenic efficacy compared to bevacizumab on a transgenic zebrafish model. Next, by utilizing rational design approaches including computational methods, and directed evolution techniques including yeast surface display we obtained thermally more stable scFv variants, the highest of which has around 10 °C higher transition mid-point ($T_{1/2}$) compared to wild type scFv.

This method described in this thesis can be used to improve the thermal stability of any kind of antibody or antibody fragment.

Keywords: Anti-VEGF, single-chain variable fragments (scFvs), thermal stability, yeast surface display

KANSER TEDAVİSİ İÇİN PROTEİN MÜHENDİSLİĞİ YAKLAŞIMLARI İLE GELİŞTİRİLMİŞ ANTI-VEGF ANTİKORLARININ ARAŞTIRILMASI

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ÖZET

Monoklonal antikorlar (IgG'ler), yüksek spesifiklikleri ve diğer olumlu özellikleri nedeniyle kanser de dahil olmak üzere birçok hastalık için güçlü terapötiklerdir. Bununla birlikte, büyük boyutları katı tümörler gibi büyük biyolojik doku kütlelerine penetrasyonlarını sınırlamaktadır. Tek zincirli değişken parçacıklar (scFv'ler) ise daha küçük boyutlarından dolayı daha iyi doku penetrasyonuna sahiptir. Yine de, sabit bölgelerin (domain) olmaması nedeniyle scFv'ler, ana IgG'leri ve hatta Fab parçacıkları kadar termal olarak kararlı değildir ve bu, terapötik uygulamalar için bir zorluk teşkil etmektedir. Bu nedenle, bu tezde, scFv'lerin termal stabilitelerini arttırmak üzere genel bir yöntem geliştirmeyi hedefledik.

Anti-anjiyojenik tedavi, katı tümörlerin ayırt edici özelliği olan tümör aracılı anjiyogeneze kritik bir öneme sahiptir. Bevacizumab (IgG), anti-anjiyojenik tedavi için geliştirilen ilk terapötiktir ve halen klinikte, çoğunlukla kemoterapi ile kombinasyon halinde çeşitli katı tümör tiplerinde kullanılmaktadır.

Bu tezde, bevacizumab'dan türetilen anti-VEGF scFv'leri geliştirdik ve bu scFv'ler transgenik zebra balığı modelinde bevacizumab ile karşılaştırıldığında daha iyi anti-anjiyogenik etkinlik gösterdiler. Ardından, hesaplamalı yöntemler dahil rasyonel tasarım yaklaşımları ve maya yüzeyi gösterimi dahil yönlendirilmiş evrim teknikleri kullanarak, termal olarak daha kararlı scFv varyantları elde ettik. Bunların en yükseği yabancı tip scFv ile karşılaştırıldığında yaklaşık 10 °C daha yüksek geçiş orta noktasına ($T_{1/2}$) sahiptir.

Bu tezde açıklanan bu yöntem, herhangi bir türde antikor veya antikor parçasının termal stabilitesini arttırmak için kullanılabilir.

Anahtar Kelimeler: Anti-VEGF, tek zincirli değişken parçacık (scFv), termal stabilite, maya yüzey gösterimi

1 INTRODUCTION AND AIM

Since Ehrlich's conception of monoclonal antibodies as "magic bullets" for targeted therapy in the late nineteenth century, they have been utilized in a variety of medical applications for both therapeutic and diagnostic purposes in the form of full-length antibodies as well as fragments, Fc (fragment crystallizable) fusion proteins, and antibody conjugates. Likewise, antibodies are still attractive for diagnosing and treating diseases, especially cancer, due to their high specificity and a multitude of other favorable properties [1-14].

Cancer cells are mainly differentiated from healthy cells by their distinctive features, which include a high demand for oxygen and nutrients due to their high metabolic rates, which ultimately contribute to tumor growth and progression [15, 16]. The uptake of these metabolic requirements and waste disposal in solid tumors depends on vascularization [15-17], and it can be triggered by angiogenesis called as the "angiogenic switch," which is a hallmark of solid tumors [15, 17].

Along with that, due to the critical importance of anti-angiogenic therapy in tumor-mediated angiogenesis, Vascular Endothelial Growth Factor (VEGF) has been extensively studied as a critical therapeutic target for inhibiting angiogenesis and normalizing tumor vasculature, and this strategy has been validated *in vivo* using anti-VEGF monoclonal antibodies to inhibit angiogenesis in various tumor models in the past decades [13, 15-20]. In this concept, bevacizumab, a humanized anti-VEGF monoclonal antibody (immunoglobulin G; IgG), was the first therapeutic agent developed for anti-angiogenic therapy [15, 16, 21], and it is still used in the clinic for a variety of tumor types, mostly in combination with chemotherapy [15].

On the other hand, the challenges associated with the pharmacokinetic properties of full-length antibodies became apparent early in the development of this new field of medicine, with one of the most significant discoveries being that the large size of antibody molecules (IgGs) precludes them from penetrating large masses of biological tissue, such as solid tumors [22, 23]. However, it was discovered that antibodies do not require their constant domains in order to bind and neutralize [2, 4, 7, 9, 10, 14, 24, 25]. This finding led to the improved development of antibody fragments that are smaller than their parental antibodies. Among the antibody fragments, the single-chain variable fragment (scFv) is the most widely used and popular format for recombinant antibodies [25]. The main advantage is that scFvs retain the

parental paratope and binding affinity unless the orientation of the variable domains is changed [26].

On the other hand, scFvs have some drawbacks in terms of their stability, which is essential especially for therapeutic applications [26-28]. While linker engineering is a critical parameter to consider when enhancing stability, it is not always sufficient to generate a stable scFv structure [28]. Numerous strategies have been employed to increase the stability and decrease the aggregation propensity of scFvs, including modification of the V_H - V_L interface [26, 28], decrease of hydrophobic patches on the surface or increasing hydrophobic amino acids at the interior sides [28-30], replacing the hydrophobic amino acids at the former V/C interface with the more polar ones [29], CDR shifting to known stable scFv frameworks [26, 27], and considering the previously made stability enhancing mutations carried out in the literature [26]. However, there is no widely accepted technique for determining the sites that contribute to poor stability or determining the mutations that increase stability, particularly thermodynamic stability, in a much simpler, faster, and more accurate manner. Although, there has been a significant effort in this direction in recent years, including the development of computational prediction methods [31-33] to minimize the laborious, time-consuming, and costly experiments, developing methods for predicting mutation-specific changes in the thermodynamic stability of proteins is still an active research area in computational biology. Due to the limitations of current methods, it is advisable to utilize multiple methods based on multiple different approaches to increase feasibility of obtaining the desired increase in stability [33].

In this thesis we aimed to address that issue with a reasonable solution, namely by combining rational and computational approaches with evolutionary techniques. In this context we believed that the generation of synthetic gene libraries by utilizing computational methods and natural antibody repertoires [34], and later the rapid and purposeful screening of these libraries would be an effective approach. Display techniques (e.g., phage display, yeast surface display) are the most powerful screening techniques for this purpose, as they enable very rapid screening of antibodies with desired characteristics, such as increased affinity and stability, from synthetic, semi-synthetic, or natural antibody libraries [35-42]. So far, two strategies have been already developed to obtain antibodies with improved stability [38]: (i) indirect stability screening based on protein expression level and (ii) direct stability screening based on protein thermal stabilities. Moreover, these techniques are open to being used for a

variety of purposes with a variety of modifications, including the use of a combination of different types of approaches, which would help us reach our goal.

Given the background information discussed in more detail below, the main objective of this thesis is as follows:

1. First, we aimed to develop a scFv format of bevacizumab due to the above-mentioned advantages of scFv fragments, namely improved tissue penetration due to their smaller size relative to their parental antibodies and their adaptability to any type of modification for further application in both therapeutic and diagnostic fields, and the importance of anti-angiogenic therapy in cancer treatment.
2. Given the disadvantages of scFvs in terms of their instability, which can be caused by a variety of factors, we first aimed to select a suitable linker candidate and determine whether this linker alters the antibody fragment's basic *in vivo* and *in vitro* characteristics, most notably its binding property, when compared to its full-length form. We chose the zebrafish model for the *in vivo* application because the transparency of zebrafish embryos and larvae allows direct observation of blood flow and vascular development without sophisticated instrumentation [43]. Furthermore, their robustness for micromanipulation, combined with their transparency, makes them an ideal *in vivo* testing model for anti-VEGF antibodies [44, 45].
3. It is important to develop a general method to increase the thermodynamic stability of scFvs, which are intrinsically less stable than full-length antibodies. It was intended to develop a standardized methodology for this purpose by combining various approaches, including rational design, computational methods, and directed evolution techniques. Finally, following the completion of studies on the linker's effect on scFv characteristics, we intended to use the anti-VEGF scFv antibodies that will be developed here to demonstrate the utility of this method in improving thermal stability.

2 BACKGROUND INFORMATION & LITERATURE

2.1 Angiogenesis

Cancer cells are mainly differentiated from healthy cells by their distinctive features, which include a high demand for oxygen and nutrients due to their high metabolic rates, which ultimately contribute to tumor growth and progression [15, 16]. The uptake of these metabolic requirements and waste disposal in solid tumors depends on vascularization [15-17]. Vascularization can be triggered by angiogenesis, which is the process of forming new blood vessels from pre-existing ones [15, 17]. Angiogenesis is a strictly regulated process and plays a vital role in embryonic development [15, 17]. While in adult mammals, its function is largely limited to wound healing and female menstrual cycle processes [15, 17], under pathophysiologic conditions other than wound healing, angiogenesis is also induced by solid tumors, which results in altered morphology of the vasculature. This process is called the "angiogenic switch," which is a hallmark of solid tumors [15, 17].

2.2 VEGF as a therapeutic target in cancer treatment

In 1968, experiments demonstrated that diffusible factors secreted by tumor cells mediated tumor angiogenesis, and in 1971 it was proposed by Folkman that inhibiting angiogenesis might be an effective way to treat cancer [46]. Subsequent research has demonstrated the importance of vascular endothelial growth factors (VEGFs), as critical tumor-secreted pro-angiogenic factors [15, 19].

VEGF-A, -B, -C, and -D, and placenta growth factor (PlGF) are members of the VEGF family [18, 20]. These factors bind to VEGF receptor tyrosine kinases (VEGFR1-3), and the neuropilin and heparan sulfate proteoglycan family of co-receptors [15, 20]. They activate VEGF signaling in endothelial cells via interacting with VEGFRs (VEGFR1-3), increasing endothelial cell survival and proliferation, vascular permeability, and eventually supporting tumor growth by providing metabolic supplies [15, 17, 47]. The major signaling event in angiogenesis is the binding of VEGF-A (hereinafter VEGF) to VEGFR2, and the activity of VEGF serves as a rate-limiting step in the formation of normal and pathological blood vessels [17, 18, 20, 47, 48]. Additionally, the vasculature is more vulnerable to VEGF signaling suppression in tumors where VEGF is frequently overexpressed compared to normal tissues. [49].

Due to this critical importance in tumor mediated angiogenesis, as a critical therapeutic target, VEGF has been extensively studied for inhibiting tumor mediated angiogenesis and normalizing tumor vasculature, and this strategy has been validated *in vivo* using anti-VEGF monoclonal antibodies to inhibit angiogenesis in various tumor models in the past decades [13, 15-20].

2.3 Bevacizumab

Bevacizumab, a humanized anti-VEGF monoclonal antibody (IgG), with the trade name Avastin® (Genentech, Inc. South San Francisco, CA), is the first therapeutic agent developed for anti-angiogenic therapy [15, 16, 21]. Bevacizumab is employed to block VEGF/VEGR signaling that leads to neovascularization by binding to VEGF isoforms and blocking their interaction with VEGFR [15, 17]. *In vivo* studies have shown that bevacizumab suppresses vessel formation and stimulates the regression of newly-created ones, and normalizes the abnormal vasculature, allowing cytotoxic chemotherapy to be delivered more effectively, as well as having direct effects on tumor cells [15, 18]. Therefore, clinical applications of bevacizumab have been focused on tumors induced by angiogenesis, and the clinical studies conducted on different types of cancers [16] were confirmed the clinical utility of bevacizumab, which is mainly used with chemotherapy, in metastatic colorectal cancer (mCRC), non-small-cell lung cancer (NSCLC), additionally in renal cell carcinoma (RCC), metastatic breast cancer (mBC), glioblastoma multiforme (GBM), ovarian cancer (OC), and cervical cancer (CC) [15].

2.4 Antibodies

Antibodies are essential components of the immune system as they recognize foreign antigens and recruit other cells and molecules to initiate an immune response against the bound antigen [50]. Inevitably, because of their high binding affinity and specificity, as well as their ability to be tailored to the intended use, recombinant antibodies have been used in a variety of applications since their earliest days. The development of hybridoma technology [51], followed by recombinant DNA technology, marked the beginning of the history of antibody-based therapies [12]. The challenges associated with the pharmacokinetic properties of antibodies became apparent very early in the development of this new field of medicine

and one of the most significant discoveries was that the large size of antibody molecules in IgG format (~150 kDa) prevents them from penetrating large masses of biological tissue, such as solid tumors [22] (Figure 1). Additionally, it was discovered that antibody molecules can be modified to include additional effector functions [52], and it was realized that antibodies do not require their constant domains for their neutralization function [2, 4, 7, 9, 10, 14, 24, 25] (Figure 1). These advancements would pave the way for the unavoidable advancement of antibody engineering, especially on recombinant Fab (~50 kDa) and Fv (variable fragment, ~25 kDa) fragments, because there has been a need to broaden their range of effector functions by replacing their constant domains with enzymes, toxins, and radionuclides for a variety of therapeutic purposes, such as in cancer treatment to eliminate side effects of chemotherapy by utilizing their high target specificity [2, 3, 8, 10, 11, 53, 54].

2.4.1 Basic antibody structure

Antibodies in the immunoglobulin G (IgG) format consist of two identical heavy chains and two identical light chains, denoted by the letters H and L, respectively [50] (Figure 1). Disulfide bonds link the light and heavy chains, creating two identical Fab (fragment antigen-binding) and one Fc (fragment crystallizable) fragment. The Fab fragment is a structurally distinct unit that contains the antigen-binding site (paratope). Each Fab fragment contains variable and constant domains that are associated with heavy and light chains. The abbreviations V_H for heavy chain and V_L for light chain are used to designate variable domains, whereas C_{H1} , C_{H2} , and C_{H3} for heavy chain and C_L for light chain are used to designate constant domains. The remaining constant domains of the heavy chain are comprised of the Fc region, which is expected to be involved in additional biological activities such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [55].

In antibodies, dimerization of V_H and V_L domains results in the formation of an Fv (variable fragment) unit, which has a “paratope”. In detail, each variable domain has three hypervariable loops called complementarity determining regions (HCDR1-3 for heavy chains and LCDR1-3 for light chains). These CDR regions are separated by conserved framework regions (FR1-4), and that framework regions form a core beta-sheet (β -sheet) structure which results a unique structure presenting CDR loops on the surface of variable domains. Dimerization of V_H and V_L assembles six hypervariable CDR loops into a structure called a

“paratope” (Figure 1). Thus, if the two domains are not reoriented, there is no difference between a Fab and a Fv fragment in terms of an antibody's antigen-binding ability to a specific epitope (a region of an antigen to which antibodies bind), as both retain their original paratope region [26, 50] (Figure 1).

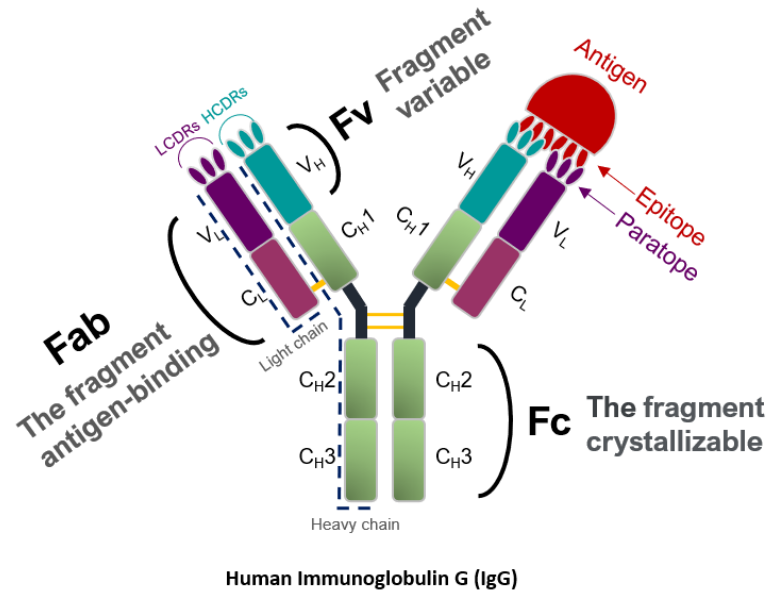


Figure 1. Human immunoglobulin G (IgG) structure. V_H and V_L designate heavy and light chain variable domains, respectively. Likewise, C_H1/2/3 and C_L designate heavy and light chain constant domains. The fragment antigen-binding is designated as Fab, variable fragment as Fv, fragment crystallizable as Fc, light chain complementarity determining regions and heavy chain complementarity determining regions as LCDRs and HCDRs, respectively. Yellow lines represent disulfide bonds. The figure was adapted from Karadag et al. [50].

2.5 Engineering of single-chain variable fragments (scFvs) for improved stability

Fv fragments, on the other hand, have significant drawbacks. The most critical one is that these constructs are less stable compared to their parental IgGs or even Fab fragments [56]. This is a significant disadvantage for a therapeutic molecule, which should possess a structure that is stable under the physiological conditions. As a result, a molecule that is an excellent therapeutic candidate with a high binding affinity may face significant limitations as an scFv due to this stability issue in therapeutic applications [27, 28]. The most important reasons of this instability are the lack of the constant domains that normally interact with the

variable domains and the lack of any covalent linking in between two variable domains to facilitate to association of the two variable domains. Therefore, the stability of the construct is mostly dependent on the hydrophobic interface between the V_H and V_L domains. Numerous modifications have been made thus far to prevent the dissociation of these two variable domains [57]. These include covalently binding these two domains via a flexible linker peptide (scFv) [58, 59], disulfide bonds (dsFv) [56, 60], or combining both approaches (sc-dsFv) [61] (Figure 2). Among them, scFv is the most widely used and popular format for recombinant antibodies [26]. One of the primary reasons for this is that scFvs completely involve the parental paratope region and maintain their original binding affinity if the variable domains can maintain their original orientation in the construct [26].

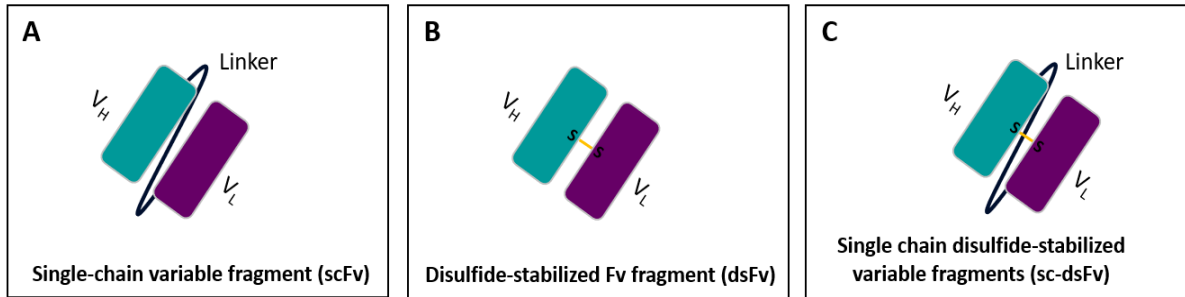


Figure 2. Recombinantly engineered Fv fragments by using a flexible linker (A), a disulfide bond (B), or both (C).

In this regard, linker structure has a critical effect on establishing an appropriate V_H - V_L antigen binding interface that ensures proper scFv functionality in *in vivo* and *in vitro* applications [26, 62-65]. It was previously stated that the length and sequence composition of the linker can affect the following properties of these scFv antibody fragments: their level of expression, affinity and specificity, stability, folding, oligomeric state, and *in vivo* activities [26, 65-68]. The most frequently used natural and synthetic linkers in fusion proteins can be divided into three categories: flexible, rigid, and cleavable [67-69]. Among these linkers, although they demonstrate some issues in PCR-based applications due to their repetitive character [70], the most preferred linkers in scFv constructs have been GS linkers (glycine-serine linkers) due to their flexible structure, and they have generally been used with $(G_3S)_n$ and $(G_4S)_n$ motifs [59, 62, 71-73]. Linkers can be designed in lengths of 5 to 35 amino acids to endow the designed scFvs with the desired properties [26, 65, 74]. Studies on scFvs have demonstrated that 12 residues and longer linkers enable scFvs to remain more monomeric

state and fold naturally. However, linkers shorter than 12 residues can cause scFvs to form multimeric structures, which are formed when two or more scFv molecules interact with each other [75, 76].

On the other hand, conversion of many Fv molecules to the scFv format revealed that linker peptides do not always allow it to function properly. In other words, scFvs could still lose their natural folding ability and tend to aggregate [28] even though they have a proper linker. Therefore, this disadvantage has led to a lot of studies on improving scFv stability. One of the most important factors facilitating those studies was that, when compared to full-length antibodies and other fragments, scFvs had the greatest data with lighted crystal structures [26]. Furthermore, studying a protein structure like scFv, which consists of only two domains and an interface, was easier and more effective than studying other complex multi-domain proteins. In addition to allowing for the study of antibody stability, research on scFv stability provided insight into protein stability in general. As a result, scFvs has been a great model molecule for studying protein stability [26]. Due to their small size and lack of requirement for post-translational modifications, they can be expressed in a number of different hosts (e.g., bacteria, yeast, plants, and mammalian cells) and they are amenable to a variety of *in vitro* techniques due to their small size [6, 77-81]. All these advantages have allowed scFvs to find applications both in the medical field either as therapeutic or diagnostic agents and non-medical field such as in the environmental and food industries [2, 6, 7, 9, 10, 14, 73, 81-86].

As mentioned above, the use of scFvs in all these medical and non-medical fields made it essential for scFvs to have high stability. It was essential that scFvs, which will be used especially in the therapeutic and diagnostic field, should maintain their activity without precipitating or degrading in human sera for at least a few hours or days at 37 °C after injection, to be localized and enriched in the target site, such as, tumor tissue [26-28]. However, despite their high binding affinity, scFvs frequently lose their natural form and aggregate during the applications mentioned above. In this sense, many rational approaches have been developed to improve the stability of scFvs. The general characteristics of these approaches are as follows:

(i) **modification of the V_H-V_L interface**, particularly by increasing hydrophobic interactions, so that the domains interact more stably with each other. The interactions between the V_H and V_L are mostly hydrophobic in nature, and these hydrophobic interactions

mostly determine the stability of interface between the V_H and V_L . So, defining unusual hydrophilic amino acids and substitution of them with proper hydrophobic ones may increase the stability of the whole structure [26, 28].

(ii) **decreasing hydrophobic patches on the surface** of the molecule to minimize intermolecular aggregation [28-30] or increasing hydrophobic amino acids at the interior sides. Together with the first strategy, these applications were generally applied by using human antibody repertoires to determine unusual residues on the surface and/or interior regions of the molecule to substitute those unusual residues with the more proper ones depending on the environment [28, 29].

(iii) **detection of hydrophobic amino acids being solvent exposed at the former V/C interface** after removing the constant domains, and replacing them with more polar amino acids [29].

(iv) **CDR shifting** to known stable scFv frameworks. This strategy includes transferring CDR regions of an antibody into a stable framework scaffold of another antibody [26, 27]. This strategy may result somewhat affinity loss, but with some additional mutations they can regain their original affinities.

(v) Additionally, utilizing previously described stability-increasing mutations, since numerous structural features of scFv have been identified, and a substantial amount of data has been collected through detailed structural analyses and extensive research on the effects of single mutations [26].

However, there is no widely accepted technique for determining the stability-decreasing sites in protein structures, and/or for determining the stability-increasing mutations, particularly in thermodynamic stability, in a much simpler, faster, and accurate manner. On the other hand, there is a significant effort in this direction, including the development of computational prediction methods [31-33]. Frequently, experimental methods are laborious, time-consuming, and costly to directly assess the effect of numerous mutations on a protein's stability [32, 33]. As a result of this constraint, numerous computational methods for predicting the mutation-specific changes in thermodynamic stability have been developed over the years [33]. On the other hand, despite nearly three decades of work and a variety of different algorithms, developing methods for predicting the mutation-specific changes in thermodynamic stability of proteins is still a progressing research area in computational

biology. Due to the limitations of current models and predictions, research in this area often requires the utilization of multiple approaches in order to achieve successful increases in stability [33]. Those models utilized in this area are commonly based on the prediction of a difference in the free energy of unfolding [32]. Those computational methods predicting the difference in the free energy of unfolding between a wild type and mutant version of a protein can be classified into two main categories: first, those that utilize energy functions, which can be further classified into empirical potential approaches, statistical potential approaches, and physical potential approaches; and second, those that utilize machine-learning approaches [32]. Methods that developed by combination of these approaches are also available [87].

In addition to methods that predict thermodynamic stability of the protein, tools for predicting the melting temperature (T_m) of proteins are also available [88]. Thermal stability and thermodynamic stability are correlated to each other, even though they indicate different aspects of a protein's stability. The thermodynamic stability of a protein is defined as the value of the Gibbs free energy (G) at room temperature. Thermal stability is defined as the value of T_m , which is the temperature at which G equals zero and the protein is in equilibrium with its unfolded and folded forms [33]. Moreover, since three-dimensional structures of proteins are intimately related to their thermodynamic properties [89], utilizing the known three-dimensional structures of the proteins, tools have been developed to predict stabilization centers (SCs), i.e. amino acids involved in interactions that are critical for protein stability [90].

However, although the developed computational methods predict stability-enhancing or reducing mutations, they are still open to improvement in terms of their general reliability [33], and nevertheless, testing these predicted mutations one-by-one does not have the power to completely reduce the current labor, time, and cost. Therefore, combining rational and computational approaches, and combining evolutionary techniques with them may be a logical alternative. For example, the generation of synthetic gene libraries containing the desired mutations (for example, using the overlap-extension PCR technique [91, 92]) determined by these computational methods, and the rapid and purposeful screening of these libraries can be an example. At least, screening the effects of multiple alternative mutations (selected by utilizing natural antibody repertoires [34]) in the potential stability enhancing or reducing regions that are detected by these computational methods may facilitate achieving the desired goal.

Display techniques (e.g., phage display, yeast surface display) are the most powerful screening techniques for that kind of purposes, as they are enable very rapid screening of antibodies with the desired characteristics from synthetic/semi-synthetic or natural antibody libraries [35-42], which are used most often for affinity maturation. Although these techniques are relatively infrequently used for increasing antibody stability, recently, highly efficient approaches have been developed for screening antibodies with higher stability by utilizing phage and yeast surface display techniques [35, 37-40]. In those studies, a heat-stress step was added to the biopanning or sorting step of the display process to select the most stable antibody/antibody fusions.

2.6 Yeast surface display

Dane Wittrup's group pioneered yeast surface display by displaying antibody repertoires on *Saccharomyces cerevisiae* [93, 94]. Yeast surface display technique is a powerful tool for obtaining antibodies with the desired properties, such as affinity, stability and specificity [36]. Using yeast surface display, antibodies specific for a variety of antigens have been obtained [94-97]. Additionally, yeast display has been also used to obtain a variety of different types of proteins other than antibodies for a variety of different purposes [98].

Generally, the antibody displayed on the yeast surface (Figure 3) is in the scFv format [36] as a fusion protein with the a-agglutinin adhesion subunit Aga2p (scFv-Aga2p), and its expression is controlled by a galactose-inducible promoter included in a yeast surface display plasmid. On the other hand, a-agglutinin anchorage subunit Aga1p is expressed from a galactose-inducible expression cassette that is integrated in yeast chromosome. Thus, after induction of galactose-inducible promoters, expressed Aga2p forms disulfide bonds with Aga1p, which is bound to the yeast cell wall, and the Aga1p-(Aga2p-scFv) complex is displayed on the yeast surface. Then, scFv displayed on yeast can be identified using fluorescence-activated cell sorting (FACS) or flow cytometry [36, 93, 99].

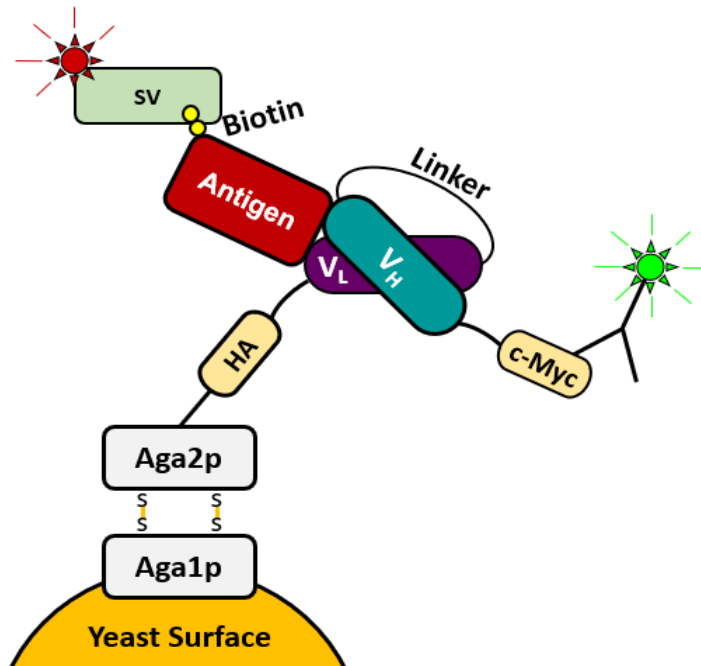


Figure 3. Yeast surface display system. The scFv (cyan and purple) is displayed on yeast as a scFv-Aga2 (light gray) fusion protein. The scFv expression is detected by using fluorophore conjugated antibodies targeting the epitope tags (e.g., HA and c-Myc) (yellow). The scFv binding to a biotinylated antigen (red) is detected by fluorophore conjugated streptavidin (SV) (green). HA, hemagglutinin; V_L, light chain variable domain; V_H, heavy chain variable domain. The figure is adapted from Chao et al. [36].

Typically, each yeast cell expresses between 1×10^4 and 1×10^5 scFvs on its surface, and it is possible to quantify the variation in the expression by using immunofluorescence labels targeting HA or c-Myc epitope tags fused to the N- or C-terminus of the scFv [36]. Similarly, the binding of an antibody to a specific soluble antigen can be determined, for example, by labeling the biotinylated antigen with a streptavidin-fluorophore conjugate [36, 92, 100, 101].

The largest yeast display library is around 1×10^8 - 10^9 variants due to the limitation in yeast transformation efficiency [102]. Therefore, the yeast surface display technique, which is generally used together with FACS, has a relatively low throughput compared to the other display techniques that utilize bio-panning based methods, such as phage display (up to 10^{11}) and mRNA-ribosome display (up to 10^{13}) [36, 41, 102]. Along with that, libraries are generally sampled at approximately tenfold higher coverage while using FACS, to ensure that every unique variant is scanned. Therefore, yeast display libraries with more than 10^8 variants are typically reduced in diversity using the magnetic-activated cell sorting (MACS) method

prior to screening with FACS [102]. Magnetic beads, which are typically coated with a soluble ligand, are used in this method. Following incubation the yeast library with the antigen-coated magnetic beads, antibody variants that do not bind to the magnetic beads are washed away, and the desired variants that bind to the target antigen are collected and used at the next sorting step [102] (Figure 4). This method allows for the removal of misfolded, truncated, as well as low affinity antibodies, allowing the library diversity to be reduced to a size that is more manageable for screening by FACS [36, 102].

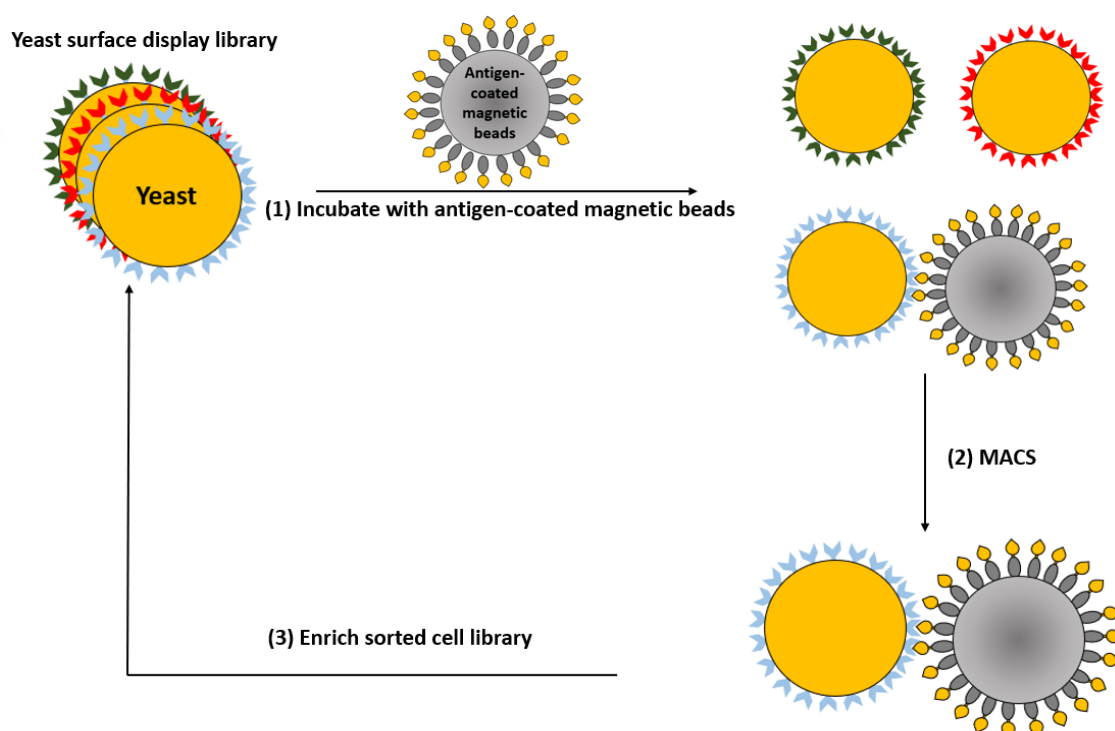


Figure 4. Magnetic activated cell sorting (MACS). Yeast surface display library is first incubated with antigen-coated magnetic beads. After incubation, magnetic beads are held by a suitable magnet apparatus, and other unbound yeasts are washed away. Finally, the sorted yeast cells are grown again and prepared for the next round of MACS or FACS.

There are many advantages of yeast surface display to directed evolution of proteins. First of all, the displayed proteins are folded in eukaryotic yeast cells' endoplasmic reticulum (ER), utilizing chaperones and the molecular quality control mechanism in the ER [36]. Next, it enables quantitative screening via FACS, which enables direct observation of the sample's equilibrium activity and statistics throughout the screening process [36, 92, 100, 101]. In

addition to that, the antigen-binding signal can be normalized by expression signal, thereby removing artefacts due to differences in expression levels between the cells and allowing for fine mutant differentiation [103]. Moreover, FACS is compatible with sorting for two properties at once, such as affinity and stability [36-40, 100].

2.6.1 Screening of antibodies for improved thermal stability using yeast surface display

The most important aspect of directed evolution research is establishing a link between genotype and phenotype. In this context, two different strategies have been used thus far for the thermal stability screening of yeast display libraries [40].

In the first strategy, the core assumption is that protein expression levels are correlated to their thermal stability, meaning candidates that are expressed at higher levels in yeast demonstrate higher thermal stability [104, 105]. This strategy has been successfully used to obtain a variety of proteins with higher thermal stability, including T cell receptors (TCRs), class I and II major histocompatibility complexes (MHCs) (refs [40]). However, a significant drawback of this strategy is that although some proteins are expressed at high levels in yeast, they could be in a misfolded form [106-108]. As a result, it was discovered that a conformationally specific ligand is required to monitor the proper folding of the target protein along with a tag that allows monitoring of expression level [40]. Furthermore, the expression level of proteins is not always directly correlated to their thermal stability. This strategy, for example, cannot be used to screen proteins that already have high basal thermal stability, such as IgGs. For instance, in a study, it was shown that the thermally stabilized IgG1-Fc variant (containing the K370L mutation) exhibits a level of expression comparable to that of the wild type which already exhibits a high level of basal thermal stability [38].

The second strategy is that screening proteins directly for thermal stability rather than screening them indirectly via their expression levels [40]. Thus, this approach eliminates those variants that are highly expressed but misfolded. In this strategy, after displaying the proteins on yeast, a heat shock step is added for a specified duration and temperature prior to the sorting process. Here, the duration and temperature of the heat shock should ensure that the wild type of protein of interest unfolds irreversibly. Thus, only those candidates that are still capable of binding their ligands are sorted from the library. This technique has been successfully used to stabilize proteins such as single-chain TCRs (scTCRs) [105, 109, 110] and a single-chain class I MHC molecule [111]. On the other hand, those proteins have

relatively low thermal stability and could be denatured in a range of temperatures between 40 and 46 °C. In other words, this strategy is ineffective for selecting proteins with high basal thermal stability which may require a heat shock at higher temperatures. On the other hand, the main factor that was limiting a heat shock at a higher temperature for that kind of proteins with a high basal thermal stability is that yeast cells almost lose their viability completely at 50 °C for 10 min [38]. As a result, library enrichment is impossible after a heat shock >50 °C, as yeast cells obtained during the sorting cannot be re-grown for library enrichment. However, this limitation has recently been overcome by adding a plasmid isolation and re-transformation step between the sorting steps [37, 38]. Although yeast cells lose their viability at around 50 °C, they still retain their cell integrity up to 85 °C, which is essential for proper sorting [38, 40]. Thus, rather than growing the cells following the sorting process after a heat shock at a high temperature above 50 °C, in this approach, plasmid isolation is carried out from the sorted cells. Subsequently, after amplifying those plasmids, they are used to reconstruct the yeast library by retransforming yeast cells with those plasmids. This method has been successfully applied to the optimization of a variety of protein candidates, including loop-inserted GFP (green fluorescent protein) scaffolds [37, 112] and IgG1-Fcs [38, 113]. Figure 5 illustrates the overall flow of these techniques for obtaining the most stable scFvs from libraries.

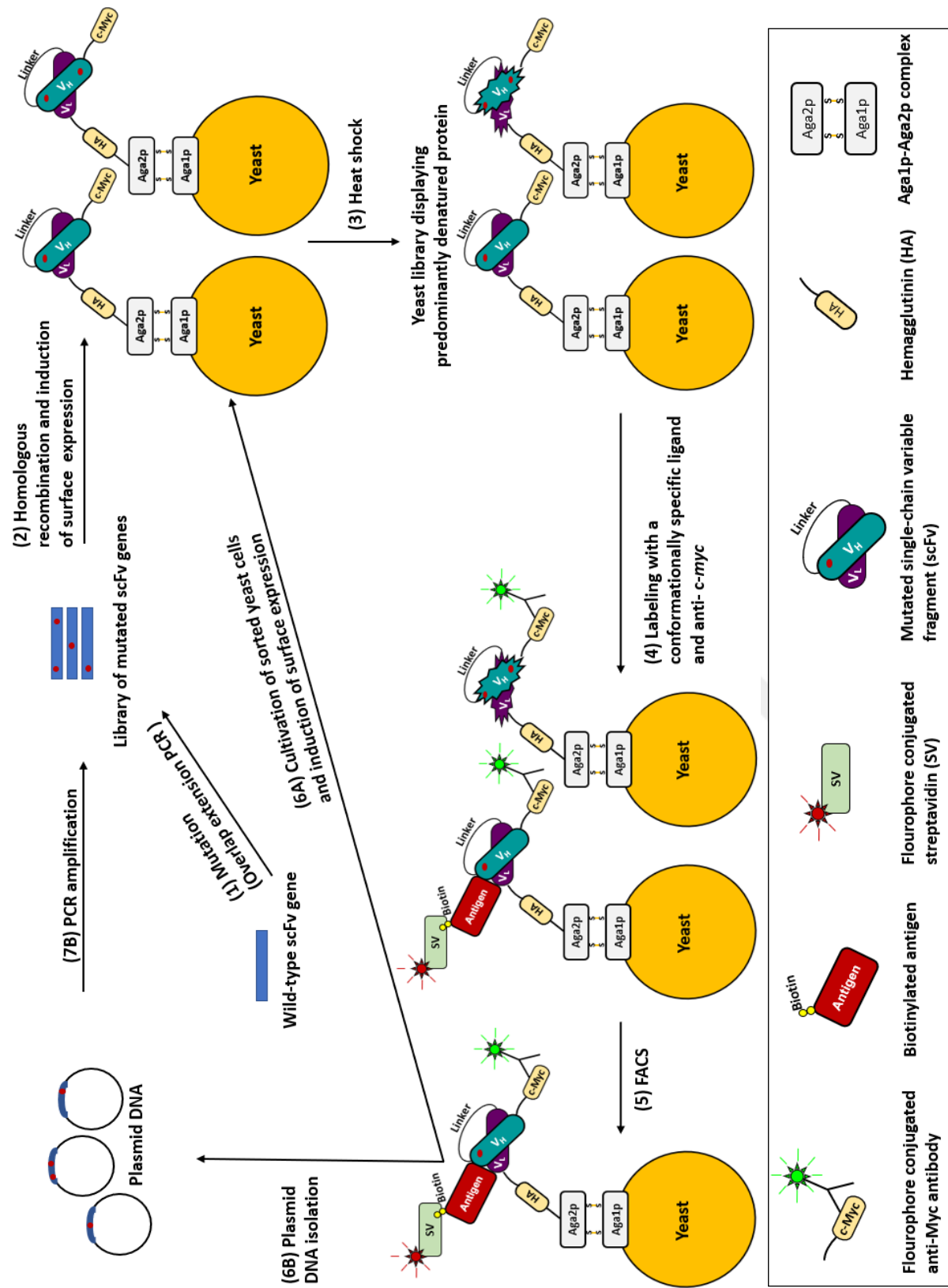


Figure 5. Thermal stability engineering through yeast surface display. A mutant scFv gene library is generated (red dots indicate mutation sites) from a wild type scFv gene (step 1) using proper PCR techniques, such as overlap extension PCR or error-prone PCR. Yeast display library is generated by homologous recombination by co-transformation of linearized backbone and mutated scFv genes (inserts) (step 2). The yeast display library is then induced for surface expression of scFvs (cyan and purple) and then subjected to a heat shock, which yields yeast cells with scFvs in denatured (irregular) or natural (smooth and regular) forms on their surface (step 3). Those scFv mutants that maintain their natural folding can still bind to their conformationally specific antigen labelled with a fluorescent label (here biotinylated antigen [red rectangle] and fluorophore conjugated streptavidin [green rectangle] couple is represented) (step 4) and can be sorted by FACS (step 5). Next, depending on the yeast viability, two ways could be followed: if the yeast cells are still viable after the heat shock, they can be regrown and be prepared for the next round of sorting (step 6A). If the yeast cells could not survive after the heat shock, plasmid DNA isolation (step 6B) and PCR amplification (step 7B) are applied, and the yeast display library is recovered by a re-transformation step (step 2). This cycle is followed several times until the desired antibody pool is obtained. The figure is adapted from Traxlmayr and Shusta [40].

3 MATERIALS AND METHODS

3.1 Type of the study

This study is based completely on experimental methods.

3.2 Place and time of the conducted study

This study was performed at two distinct institutions: Izmir Biomedicine and Genome Center (IBG)'s laboratories between February 2018 and April 2021; the University of Michigan's (USA) laboratories between April 2021 and March 2022. Zebrafish experiments were conducted at IBG zebrafish and optic imaging facilities.

3.3 Test systems

Zebrafish model was utilized as an *in vivo* test system in this study. They were housed and bred in IBG-Vivarium Zebrafish Facility.

3.4 Materials

3.4.1 Common reagents

The common reagents, chemicals and kits used in this study are listed in Table 1 and preparation of buffers and media are described in Table 2 and Table 3, respectively.

Table 1. The list of common reagents, chemicals, and kits

Reagent or Resource Name	Source	Identifier	Location
Antibodies			
Anti-c-myc chicken IgY antibody	Invitrogen	Cat# A-21281	UMICH
Bevacizumab (ALTUZAN 100 mg/4 mL)	Roche	n/a	IBG
Goat anti-mouse IgG AlexaFluor 488	Invitrogen	Cat# A11001	UMICH
Goat anti-Chicken IgY (H+L) Secondary Antibody, AlexaFluor 488	Invitrogen	Cat# A-11039	UMICH
Mouse anti-Myc-Tag antibody (9B11)	Cell Signaling Technology	Cat# 2276S	UMICH
Bacterial strains			
<i>E.coli</i> BL21(DE3)pLysS	Agilent	Cat# 200132	IBG
<i>E.coli</i> DH5α	Julian et al., 2019	n/a	UMICH

<i>E.coli</i> XL-1 Blue	Agilent	Cat# 200158	UMICH
<i>S. cerevisiae</i> EBY100	Julian et al., 2019	n/a	UMICH
Chemicals, peptides, and recombinant proteins			
Agar	Fisher Scientific	Cat# BP1423-2	UMICH
Agarose	Fisher Scientific	Cat#BP160-500	UMICH
Alpha-lactose monohydrate	Sigma-Aldrich	Cat# L2643	IBG
Ampicillin (AMP)	Bioshop Thermo Fisher Scientific	Cat# AMP201 Cat# BP1760-25	IBG UMICH
β-Mercaptoethanol (βME)	Sigma-Aldrich	Cat# M6250	IBG
Brilliant Violet 421 Streptavidin	BioLegend	Cat# 405226	UMICH
Bovine serum albumin (BSA)	Sigma-Aldrich Fisher Scientific	Cat# A2153 Cat# BP9706100	IBG UMICH
Calcium Chloride	Fisher Scientific	Cat# C77-500	UMICH
Calcium chloride dihydrate	Carlo Erba	Cat# 327607	IBG
Casamino acids (Acid casein peptone)	Fisher Scientific	Cat# BP1424-500	UMICH
Chloramphenicol	Bioshop	Cat# CLR201	IBG
Citric acid	Fisher Scientific	Cat# A940-500	UMICH
Dextrose (D-Glucose)	Fisher Scientific	Cat# D16-10	UMICH
Dimethyl sulfoxide (DMSO)	New England Biolabs	Cat# B0515	UMICH
1,4-Dithiothreitol (DTT)	Sigma-Aldrich	Cat# 10197777001	UMICH
DNA ladder (1kb)	Invitrogen	Cat# 10787018	UMICH
Drop-out Mix complete w/o yeast nitrogen base	Biological Life	Cat# D9515	UMICH
EDTA	Fisher Scientific	Mfr# O2793-500	UMICH
Ethanol	Scharlau Fisher Scientific	Cat# ET0005 Item# 04 355 223	IBG UMICH
Human VEGF-165 recombinant protein	Thermo Fisher Scientific	Cat# PHC9394	IBG
Human, VEGF165 Protein, recombinant (His & AVI Tag), biotinylated	SinoBiological	Cat# 11066-H27H-B	UMICH
iFluor647-Protein L	GenScript	Cat# M00922	UMICH
Magnesium chloride	Carl Roth	Cat# KK36	IBG
Magnesium sulfate	Merck	Cat# 106067	IBG
Magnesium sulfate heptahydrate	Sigma-Aldrich	Cat# M2773	IBG
Methanol	Tekkim	Cat# K.120320	IBG
Methylene blue	Merck	Cat# 1592700010	IBG
Galactose	Fisher Scientific	Cat# AC150610051	UMICH
Biotium GelGreen Nucleic Acid Gel Stain	Fisher Scientific	Cat# NC9728313	UMICH
Glucose	Biobasic	Cat# GB0219	IBG
Glycine	Isolab	Cat# 9270350	IBG
Glycerol	Tekkim Fisher Scientific	Cat# TK.070190 Cat# BP229-1	IBG UMICH
HBS-EP buffer 10x	Cytiva	Cat# BR100669	IBG
Hydrogen peroxide	Isolab	Cat# 932.122.2500	IBG
Imidazole	Sigma-Aldrich	Cat# I202	IBG
Kanamycin sulfate	Fisher Scientific	Cat# BP906-5	UMICH
Lithium acetate	Fisher Scientific	Cat# AC268640010	UMICH
LB-Agar	Biobasic	Cat# SD7003	IBG
LB Broth	Biobasic	Cat# SD7002	IBG

Ni-NTA sepharose resin	GE Life Sciences	Cat# 17531802	IBG
Paraformaldehyde	Merck	Cat# 104005	IBG
100X Penicillin-Streptomycin (P/S)	Gibco	Cat# 15140-122	UMICH
Peptone (Bacto peptone)	Fisher Scientific	Cat# DF0118-07-2	UMICH
Phenol red	Sigma-Aldrich	Cat# P0290	IBG
10X Phosphate buffered saline (PBS)	Fisher Scientific	Cat# BP39920	UMICH
Polyethylene glycol (PEG) (MW: 3350)	Sigma Aldrich	Cat# P4338-1KG	UMICH
Potassium chloride	Tekkim Merck	Cat# TK.150440 Cat# 104936	IBG
Potassium hydroxide	Merck	Cat# 105033	IBG
Potassium dihydrogen phosphate	Biobasic Merck	Cat# PB0445 Cat# 104873	IBG
Precision plus protein dual color standards	Biorad	Cat# 1610374	IBG
Single-stranded DNA	Sigma Aldrich	Cat# D7656-1ML	UMICH
Skim milk (Non-fat dry milk)	Bioshop Kroger	Cat# SKI40 Cat# 0001111008733	IBG UMICH
Sodium acetate	Sigma Aldrich	Cat# S2889-250G	UMICH
Sodium chloride	Isolab Merck Fisher Scientific	Cat# 969.033 Cat# 106404 Cat# BP358-10	IBG IBG UMICH
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat# L3771	IBG
Sodium citrate dihydrate	Fisher Scientific	Cat# S279-500	UMICH
Sodium hydroxide	Biobasic	Cat# SB6789	IBG
Sodium phosphate dibasic	Biobasic	Cat# S0404	IBG
Sodium phosphate dibasic dihydrate	Sigma-Aldrich Fisher Scientific	Cat# 04272 Cat# S472-500	IBG UMICH
Sodium phosphate monobasic monohydrate	Fisher Scientific	Cat# S369-500	UMICH
Sorbitol	Sigma-Aldrich	Cat# S1876	UMICH
Streptavidin AlexaFluor 647	Invitrogen	Cat# S32357	UMICH
SYPRO Orange Protein Gel Stain	Sigma-Aldrich	Cat# S5692	IBG
Tricaine methanesulfonate	Sigma-Aldrich	Cat# E1052	IBG
Tryptone	Oxoid Fisher Scientific	Cat# LP0042B Cat# BP1421-2	IBG UMICH
Tryptophan dropout media	Fisher Scientific	Cat# NC0931986	UMICH
Tris	Biobasic	Cat# TB0103	IBG
Yeast extract	Biobasic Fisher Scientific	Cat# G0961 Cat# BP1422-2	IBG UMICH
Yeast nitrogen base w/o amino acids	BD	Cat# DF0919	UMICH
4x Laemmli sample buffer	Biorad	Cat# 1610747	IBG
6X purple loading dye	New England Biolabs	Cat# B7024S	UMICH
50X TAE buffer	Fisher Scientific	Cat# BP1332-4	UMICH
Kits			
Mini plasmid isolation kit	BioBasic	Cat# BS614	IBG
Miniprep DNA isolation Kit	Qiagen	Cat# 27106	UMICH
QIAquick Gel extraction kit	Qiagen	Cat# 28704/28706	IBG UMICH
Novagen Pellet Paint NF Co-Precipitant Kit	Merck Millipore	Cat# 70748	UMICH

Zymoprep Yeast Plasmid Miniprep II	Zymo Research	Cat# D2004	UMICH
TGX FastCast Acrylamide Kit, 12%	Biorad	Cat# 1610175	IBG
QIAprep Spin Miniprep Kit	Qiagen	Cat# 27106	UMICH
QIAquick PCR Purification Kit	Qiagen	Cat# 28104	UMICH
PCR materials and reagents			
KOD Hot Start DNA Polymerase Kit	Sigma-Aldrich	Cat # 71086	IBG
<i>DpnI</i> digestion kit	New England Biolabs	Cat # R0176S	IBG
Phusion High-Fidelity PCR Kit	New England Biolabs	Cat # E0553S	UMICH
Q5 High-Fidelity DNA Polymerase	New England Biolabs	Cat# M0491L	UMICH
NheI-HF	New England Biolabs	Cat# R3131L	UMICH
XhoI	New England Biolabs	Cat# R0146S	UMICH
Quick CIP calf intestinal alkaline phosphatase	New England Biolabs	Cat# M0525L	UMICH
T4 DNA ligase	New England Biolabs	Cat# M0202L	UMICH
dNTPs	New England Biolabs	Cat# N0447L	UMICH
Other Materials			
HiTrap™ Protein L column	GE Life Sciences	Cat# 17547815	IBG
2 mm electroporation cuvettes	Fisher Scientific	Cat# FB102	UMICH
Nalgene 250 mL centrifuge bottles	Thermo Fisher Scientific	Cat# 3120-0250	IBG
10 ml Pierce centrifuge columns	Thermo Fisher Scientific	Cat# 89898	IBG
Amicon® Ultra-4 Centrifugal Filter Units with a MWCO of 10 kDa	Merck Millipore	Cat# UFC801024	IBG
CM4 chip	Cytiva	Cat# BR100534	IBG
35 mm Glass bottom dish with 14 mm micro-well	Cell Vis	Cat# D35-14-1.5N	IBG
96-well plates	Nest VWR	Cat# 402301 Cat# 650261	IBG UMICH
Streptavidin MicroBeads	Miltenyi Biotec	Cat# 130-048-101	UMICH
MACS MultiStand	Miltenyi Biotec	Cat# 130-042-303	UMICH
MidiMACS Separator	Miltenyi Biotec	Cat# 130-042-302	UMICH
MACS SmartStrainers (70 µm)	Miltenyi Biotec	Cat# 130-098-462	UMICH

Table 2. Preparation of common buffers and solutions (*serial tables*)

20X PBS (Phosphate Buffered Saline)		
Reagent	Final concentration	Amount
Sodium chloride	160 g/L	160 g
Potassium chloride	4 g/L	4 g
Potassium phosphate monobasic	4.8 g/L	4.8 g
Sodium phosphate dibasic	28.8 g/L	28.8 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L
Filter through a sterile 0.22 mm filter. Store at room temperature.		
10X PBS (Phosphate Buffered Saline) for Zebrafish studies		
Reagent	Final concentration	Amount
Sodium chloride	80 g/L	80 g
Potassium chloride	2 g/L	2 g
Potassium phosphate monobasic	2.4 g/L	2.4 g
Sodium phosphate dibasic	17.8 g/L	17.8 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L
Filter through a sterile 0.22 mm filter. Store at room temperature.		
1X PBS (Phosphate Buffered Saline)		
Reagent	Final concentration	Amount
20 X PBS solution (Phosphate Buffered Saline)	1X	50 mL
Double-distilled H ₂ O	n/a	950 mL
Total	n/a	1 L
Store at room temperature.		
10X Purification Buffer (PB)		
Reagent	Final concentration	Amount
Sodium phosphate dibasic	23.015 g/L	23.015 g
Potassium phosphate monobasic	4.56 g/L	4.56 g
Sodium chloride	292.2 g/L	292.2 g
Double-distilled H ₂ O	n/a	to 1 L
Total	n/a	1 L
Filter through a sterile 0.22 mm filter. Store at room temperature.		
1X Purification Buffer (PB)		
Reagent	Final concentration	Amount
10X Purification Buffer (PB)	1X	100 mL
Double-distilled H ₂ O	n/a	900 mL
Total	n/a	1 L
Store at room temperature.		

2 M Imidazole		
Reagent	Final concentration	Amount
Imidazole	2 M	34.04 g
Double-distilled H ₂ O	n/a	250 mL
Total	n/a	250 mL
Filter through a sterile 0.22 mm filter. Store at room temperature.		
Buffer A		
Reagent	Final concentration	Amount
2 M Imidazole solution	25 mM	3.125 mL
10X PB solution	1X	25 mL
Double-distilled H ₂ O	n/a	to 250 mL
Total	n/a	250 mL
Store at room temperature.		
Buffer B		
Reagent	Final concentration	Amount
2 M Imidazole solution	500 mM	12.5 mL
10X PB solution	1X	5 mL
Double-distilled H ₂ O	n/a	to 50 mL
Total	n/a	50 mL
Store at room temperature.		
Binding Buffer for HiTrap Protein L		
Reagent	Final concentration	Amount
Sodium phosphate dibasic	20 mM	4.333 g
Potassium phosphate monobasic		2.34 g
Sodium chloride	150 mM	21.915 g
Double-distilled H ₂ O	n/a	250 mL
Total	n/a	250 mL
Filter through a sterile 0.22 mm filter. Store at room temperature.		
Elution Buffer for HiTrap Protein L (pH 2.5-3)		
Reagent	Final concentration	Amount
Glycine	0.1 M	1.501 g
Double-distilled H ₂ O	n/a	250 mL
Total	n/a	250 mL
Filter through a sterile 0.22 mm filter. Store at room temperature.		
Neutralization Buffer for HiTrap Protein L (pH 8.5)		
Reagent	Final concentration	Amount
Tris-HCl	1 M	24.22 g
Double-distilled H ₂ O	n/a	200 mL
Total	n/a	200 mL
Filter through a sterile 0.22 mm filter. Store at room temperature.		

CIP (Clean in place) Buffer for HiTrap Protein L

Reagent	Final concentration	Amount
Sodium hydroxide	15 mM	1.2 g
Double-distilled H ₂ O	n/a	200 mL
Total	n/a	200 mL

Filter through a sterile 0.22 mm filter. Store at room temperature.

10X Tris/Glycine Transfer Buffer (for SDS-PAGE)

Reagent	Final concentration	Amount
Tris	30.285 g /L (25mM)	30.285 g
Glycine	144.1 g/L (192 mM)	144.1 g
Sodium Dodecyl Sulfate (SDS)	10 g/L (0.1 %)	10 g
Double-distilled H ₂ O	n/a	to 1 L
Total	n/a	1 L

Adjust pH to 8.3. Filter through a sterile 0.22 mm filter. Store at room temperature.

1X Tris/Glycine Transfer Buffer (for SDS-PAGE)

Reagent	Final concentration	Amount
10X Tris/Glycine Transfer Buffer	1X	100 mL
Double-distilled H ₂ O	n/a	900 mL
Total	n/a	1 L

Store at room temperature.

4X Laemmli Sample Buffer (for SDS-PAGE)

Reagent	Final concentration	Amount
4X Laemmli sample buffer	900 µL/mL	900 µL
0.142 M β-mercaptoethanol (βME) solution	100 µL/mL	100 µL
Double-distilled H ₂ O	n/a	1 mL
Total	n/a	1 mL

Store at -20 °C. Prepare the loading sample with a 3:1 ratio of sample and 4X Laemmli sample buffer.

Electroporation Buffer

Reagent	Final concentration	Amount
Sorbitol	1 M	182 g
Calcium chloride	1 mM	111 mg
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Prepare freshly prior to use and keep at 4°C.

1X TAE Buffer

Reagent	Final concentration	Amount
50X TAE buffer	1X	20 mL
Double-distilled H ₂ O	n/a	980 mL
Total	n/a	1 L

Store at room temperature.

PBSB		
Reagent	Final concentration	Amount
Bovine Serum Albumin (BSA)	1 g/L	1 g
10X PBS	1X	100 mL
Double-distilled H ₂ O	n/a	900 mL
Total	n/a	1 L

Filter through a sterile 0.22 mm filter. Store at 4°C for one month at max.

10% w/v Milk Solution in PBSB		
Reagent	Final concentration	Amount
Skim milk (Non-fat dry milk)	10% w/v	500 mg
PBSB	n/a	5 mL
Total	n/a	5 mL

Prepare freshly prior to use and keep at room temperature.

Plate Solution for Yeast Transformation (pH 7.5)		
Reagent	Final concentration	Amount
Polyethylene glycol (PEG) (MW: 3350)	40%	20 g
Lithium acetate	100 mM	330 mg
0.5 M EDTA solution	6.4 mM	40 µL
0.1 M Tris-HCl Buffer	10 mM	5 mL
Double-distilled H ₂ O	n/a	to 50 mL
Total	n/a	50 mL

Filter through a sterile 0.22 mm filter. Store at 4°C.

Conditioning Buffer		
Reagent	Final concentration	Amount
Lithium acetate	0.1 M	1.98 g
1,4-Dithiothreitol (DTT)	10 mM	462 mg
Double-distilled H ₂ O	n/a	300 mL
Total	n/a	300 mL

Prepare freshly prior to use and keep at room temperature.

0.142 M β-mercaptoethanol (βME)		
Reagent	Final concentration	Amount
Stock βME solution (14.2 M)	0.142 M	100 µL
Double-distilled H ₂ O	n/a	9.9 mL
Total	n/a	10 mL

Store at 4°C.

50 mg/mL Stock Tricaine Solution for Zebrafish studies		
Reagent	Final concentration	Amount
Tricaine methanesulfonate	50 mg/mL	2.5 g
Double-distilled H ₂ O	n/a	50 mL
Total	n/a	50 mL

Store at room temperature.

Pigment Discoloration Solution for Zebrafish studies		
Reagent	Final concentration	Amount
30 % Hydrogen peroxide	3 %	1 mL
5 % Potassium hydroxide	0.5 %	1 mL
Double-distilled H ₂ O	n/a	8 mL
Total	n/a	10 mL
Store at room temperature.		

Table 3. Preparation of common media and supplements

LB Media		
Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Sodium chloride	10 g/L (171 mM)	10 g
Yeast extract	5 g/L	5 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L
Sterilize by autoclave. Store at room temperature for two-three weeks at max.		
LB-Agar Plates (with ampicillin)		
Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Sodium chloride	10 g/L (171 mM)	10 g
Yeast extract	5 g/L	5 g
Agar	20 g/L	20 g
Double-distilled H ₂ O	n/a	1 L
Ampicillin (100 mg/mL)	100 µg/mL	1 mL
Total	n/a	1 L
Dissolve tryptone, sodium chloride, yeast extract, and agar in 1 L double-distilled H ₂ O. Sterilize by autoclave. As the agar solution has cooled down to ~ 40-50 °C add ampicillin and mix well. Store at 4 °C for one month at max.		
LB Broth Media (with ampicillin/chloramphenicol)		
Reagent	Final concentration	Amount
LB Broth	15.5 g/L	15.5 g
Double-distilled H ₂ O	n/a	1 L
Ampicillin (100 mg/mL)	100 µg/mL	1 mL
Chloramphenicol (25 mg/mL)	25 µg/mL	1 mL
Total	n/a	1 L
Dissolve LB Broth in 1 L double-distilled H ₂ O. Sterilize by autoclave. As the broth solution has cooled down to ~ 40-50 °C add antibiotics and mix well. Store at 4 °C for one month at max.		

LB-Agar Plates (with ampicillin/chloramphenicol)

Reagent	Final concentration	Amount
LB Agar	40 g/L	40 g
Double-distilled H ₂ O	n/a	1 L
Ampicillin (100 mg/mL)	100 µg/mL	1 mL
Chloramphenicol (25 mg/mL)	25 µg/mL	1 mL
Total	n/a	1 L

Dissolve LB Agar in 1 L double-distilled H₂O. Sterilize by autoclave. As the agar solution has cooled down to ~ 40-50 °C add antibiotics and mix well. Store at 4 °C for one month at max.

100 mg/mL Ampicillin (AMP) Stock Solution

Reagent	Final concentration	Amount
Ampicillin (AMP)	100 mg/mL	1 g
Double-distilled H ₂ O	n/a	10 mL
Total	n/a	10 mL

Filter through a sterile 0.22 mm filter. Store at -20 °C.

25 mg/mL Chloramphenicol (CAM) Stock Solution

Reagent	Final concentration	Amount
Chloramphenicol (CAM)	25 mg/mL	0.25 g
Ethanol	n/a	10 mL
Total	n/a	10 mL

Store at -20°C.

100 mg/mL Kanamycin (KAN) Stock Solution

Reagent	Final concentration	Amount
Kanamycin sulfate	100 mg/mL	1 g
Double-distilled H ₂ O	n/a	10 mL
Total	n/a	10 mL

Filter through a sterile 0.22 mm filter. Store at -20 °C.

SOC Medium

Reagent	Final concentration	Amount
Tryptone	2 g/100 mL	2 g
Yeast extract	0.5 g/100 mL	0.5 g
Sodium chloride	0.05 g/100 mL	0.05 g
250 mM Potassium chloride solution	2.5 mM	1 mL
Double-distilled H ₂ O	n/a	to 100 mL
2 M Magnesium chloride solution	10 mM	0.5 mL
1 M Glucose solution	20 mM	2 mL
Total	n/a	~100 mL

Dissolve tryptone, sodium chloride, and yeast extract in 100 mL double-distilled H₂O. Add 250 mM potassium chloride solution. Adjust pH to 7.0 with 5 N sodium hydroxide solution. Adjust the final volume to 100 mL with double-distilled H₂O. Sterilize by autoclaving. Prior to use add filter sterilized 2 M magnesium chloride and 1 M glucose solutions. Store at room temperature.

250 mM Potassium Chloride for SOC Medium

Reagent	Final concentration	Amount
Potassium chloride	1.86 g/100 mL	1.86 g
Double-distilled H ₂ O	n/a	100 mL
Total	n/a	100 mL

Filter through a sterile 0.22 mm filter. Store at room temperature.

2 M Magnesium Chloride for SOC Medium

Reagent	Final concentration	Amount
Magnesium chloride	19 g/100 mL	19 g
Double-distilled H ₂ O	n/a	100 mL
Total	n/a	100 mL

Filter through a sterile 0.22 mm filter. Store at room temperature.

1 M Glucose for SOC Medium

Reagent	Final concentration	Amount
Glucose	18 g/100 mL	18 g
Double-distilled H ₂ O	n/a	100 mL
Total	n/a	100 mL

Filter through a sterile 0.22 mm filter. Store at room temperature.

Auto-induction Media (pH:6.8)

Reagent	Final concentration	Amount
Tryptone	1 g/100 mL	3 g
Yeast extract	0.5 g/100 mL	1.5 g
Double-distilled H ₂ O	n/a	to 300 mL
20X NPS solution	1X	15 mL
50X 50/52 solution	1X	6 mL
1 M Magnesium sulfate solution	1 Mm	300 µL
Ampicillin (100 mg/mL)	100 µg/mL	300 µL
Chloramphenicol (25 mg/mL)	25 µg/mL	300 µL
Total	n/a	300 mL

Dissolve tryptone and yeast extract in 277.5 mL double-distilled H₂O. Sterilize by autoclaving. Prior to use add filter sterilized 20X NPS, 50X 50/52, 1 M Magnesium sulfate, 300 µL of ampicillin and 300 µL of chloramphenicol. Store at 4°C for one month at max.

20 X NPS (pH 6.8) for Auto-induction Media

Reagent	Final concentration	Amount
Ammonium sulfate	6.6 g/100 mL	6.6 g
Potassium phosphate monobasic	13.6 g/100 mL	13.6 g
Sodium phosphate dibasic	14.2 g/100 mL	14.2 g
Double-distilled H ₂ O	n/a	100 mL
Total	n/a	100 mL

Adjust pH to 6.8. Filter through a sterile 0.22 mm filter. Store at room temperature.

50X 50/52 for Auto-induction Media

Reagent	Final concentration	Amount
Glycerol	25%	25 mL
Glucose	2.5 g/100 mL (2.5%)	2.5 g
Alpha-lactose monohydrate	10 g/100 mL (10%)	14.2 g
Double-distilled H ₂ O	n/a	to 100 mL
Total	n/a	100 mL

Filter through a sterile 0.22 mm filter. Store at room temperature.

1 M Magnesium Sulfate for Auto-induction Media

Reagent	Final concentration	Amount
Magnesium sulfate	1 M	6.02 g
Double-distilled H ₂ O	n/a	50 mL
Total	n/a	50 mL

Filter through a sterile 0.22 mm filter. Store at room temperature.

SDCAA

Reagent	Final concentration	Amount
Sodium citrate	16.75 g/L (57 mM)	16.75 g
Citric acid (anhydrous)	4 g/L (20.8 mM)	4 g
Yeast nitrogen base (without amino acids)	6.7 g/L	6.7 g
Acid casein peptone (casamino acids)	5 g/L	5 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Filter through a sterile 0.22 mm filter. Store at room temperature for two-three weeks at max.

SDGCAA

Reagent	Final concentration	Amount
Sodium phosphate dibasic dihydrate	6.76 g/L (38 mM)	6.76 g
Sodium phosphate monobasic monohydrate	8.56 g/L (62 mM)	8.56 g
Yeast nitrogen base (without amino acids)	6.7 g/L	6.7 g
Acid casein peptone (casamino acids)	5 g/L	5 g
Galactose	20 g/L (111 mM)	20 g
Dextrose (D-Glucose)	11.1 mM	2 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Filter through a sterile 0.22 mm filter. Store at room temperature for two-three weeks at max.

YPD Media

Reagent	Final concentration	Amount
Yeast extract	10 g/L	10 g
Bacto peptone	20 g/L	20 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Filter through a sterile 0.22 mm filter. Store at room temperature for two-three weeks at max.

YPD Agar Plate

Reagent	Final concentration	Amount
Yeast extract	10 g/L	10 g
Bacto peptone	20 g/L	20 g
Bacto agar	24 g/L	24 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Filter through a sterile 0.22 mm filter. Store at 4°C for one month at max.

Tryptophan Dropout Plates

Reagent	Final concentration	Amount
Agar	15 g/L	15 g
Dextrose (D-Glucose)	20 g/L (111 mM)	20 g
Yeast nitrogen base (without amino acids)	6.7 g/L	6.7 g
Tryptophan dropout media	3.8 g/L	3.8 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Dissolve the agar in 800 mL double-distilled H₂O. Sterilize by autoclaving. Dissolve dextrose, yeast nitrogen base (without amino acids), and tryptophan dropout media in 200 mL double-distilled H₂O and filter through a sterile 0.22 mm filter. Once the agar solution has cooled down to ~ 40-50 °C add 200 mL supplement solution and mix well. Store at 4°C for one month at max.

60X E3 medium for zebrafish studies

Reagent	Final concentration	Amount
Sodium chloride	17.2 g/L	17.2 g
Potassium chloride	0.76 g/L	0.76 g
Calcium chloride dihydrate	2.9 g/L	2.9 g
Magnesium sulfate heptahydrate	4.9 g/L	4.9 g
Double-distilled H ₂ O	n/a	1 L
Total	n/a	1 L

Sterilize by autoclaving.

1X E3 medium for zebrafish studies

Reagent	Final concentration	Amount
60X stock E3 Medium	1X	150 mL
0.3 % Methylene Blue	6x10 ⁻⁶ %	180 µL
Double-distilled H ₂ O	n/a	to 9 L
Total	n/a	9 L

3.4.2 Oligonucleotides

Primers were purchased from Eurofins Genomics or Integrated DNA Technologies (IDT).

Table 4. Primers for site-directed mutagenesis (purchased from Eurofins Genomics)

Primer ID	Sequence (5' → 3')
F(L83)E_Fwd	ATCGTTACAGCCCAGGACGAAGCTACATACTATTGCCAGC
F(L83)E_Rev	GCTGGCAATAGTATGTAGCTTCGTCCTCGGGCTGTAACGAT
L(H11)D_Fwd	GTTGAAAGTGGTGGAGGTGATGTTTCAGCCTGGTGGCTCG
L(H11)D_Rev	CGAGCCACCAGGCTGAACATCACCTCCACCACTTTCAAC
L(H108)D_Fwd	GTGGGGTCAGGGGACAGATGTAACCGTCTCTTCCG
L(H108)D_Rev	CGGAAGAGACGGTTACATCTGTCCCCTGACCCAC

Table 5. Primers for cloning scFv genes into yeast display plasmid (purchased from IDT)

Primer ID	Sequence (5' → 3')
scFv.FOR	GAGGAGGGGGTTCAGCTAGCGACATCCAGATGAC
scFv.REV	TTATCAGATCTCGAGCTATTACAAGTCCTCTTCAGAAATAAG CTTTTGTTTCGGAAGAGACGGTTACCAATG

Table 6. Primers for library generation (purchased from IDT)

Primer	Sequence (5' → 3')
pr1	CCATGGACTTTCGGGCAG
pr2	GTACGAGCTAAAAGTACA
newpr2	CGAGCTAAAAGTACAGTGGGAACAAAGTC
pr3	CAACAGAAACCGGGCAAGGCACCCAAGGTTCTTATTTATTTTCRNCAGCVVBT TGSMBTCTGGTGTTCRCRVBCGTTTCTCCGGGAGCGGCTCCGGAACCGATTTC ACCTTGACCATCTCATCGTTACAGCCCGAGGACGAGGCTRHCTACTATTGCC AGCAATATNNCACCGTGCCATGGACTTTCGGGCAG
pr4	GGAGGGGGTTCAGCTAGCVVBATCCAGATGACGCAGTCCCCAAGTTCTTTGT CTGCCTCTGTCGGTGATCGTGTTACAATCAVCTGCTCCGCCTCGCAAGATATC TCTAATTATCTTAATTGGTATCAACAGAAACCGGGCAAG
pr5	CATACGACGTTCCAGACTACGC
pr6	GCTAGCTGAACCCCTCC
pr7	GGGTCAGGGGACATTGGT
pr8	TACAAATGAACTCCTTGCGCGCGGAGGATACAGCTGTGTATTATTGTGCTAA GTACCCGCACTATTACGGTTCATCCCATTGGTATTTTCGATNHCTGGGGTCAG GGGACATTGGT
pr9	GTCCGCCAAGCGCCAGGGAAAGGATTAGAGTGGGTAGGGTGGATCAATACT TATACCGGCGAGCCACCTACGCTSHBGATTTCAAGRBCGTTTTACCDTBTC

	AVNAGACRVBTCCAAARRCACAKYBTACTTACAAATGAACTCCTTGC
pr10	GTCCGGTGGGGGATCTCAGSAAGTCCAGTTGGTTGAAAGTGGTGGAGGTCTG GTTTCAGCCTGGTGGCTCGCTGCGTTTAAGCTGTGCAGCATCAGGGTACACAT TTACAAATTATGGTATGNVCTGGGTCCGCCAAGCGCCAGGG
pr11	CTGAGATCCCCCACC GGAC

3.4.3 Plasmid DNA

Table 7. The list of plasmids

Plasmid	Selection	Tag	Tag Location	Source	Identifier
pET-17b	CAM	His7/FLAG	C-Term	GenScript	n/a
Yeast display plasmid	AMP	HA c-Myc	N-Term C-Term	Julian et al., 2019	n/a

3.5 Methods

3.5.1 Construction of anti-VEGF scFv plasmids for bacterial expression

The wild type anti-VEGF scFv gene sequence was derived from bevacizumab (PDB ID: 1BJ1) [114], and the variable heavy chain domain (V_H) and light chain domain (V_L) were fused using non-repetitive or repetitive linker sequences of “SPNSASHSGSAPQTSSAPGSQ” and $(G_3S)_4Q$, respectively. scFv genes with a non-repetitive linker and a repetitive linker (hereinafter scFv1 and scFv2, respectively) were cloned into pET-17b bacterial expression plasmid by GenScript (Figure 6 and 7, Table 7). An N-terminal PelB leader sequence was used to direct translated proteins to the periplasm of *E. coli* [115]. In addition, penta-histidine (His_7) and FLAG peptide tags were placed in the C-terminus of the genes for purification and detection purposes. To remove these affinity tags when desired, a protease sequence was inserted before the C-terminal tags. This sequence is “I-D/E-G-R” [116] for the Factor Xa protease and was used as “IEGR” in this design (Figure 6 and 7, Table 8).

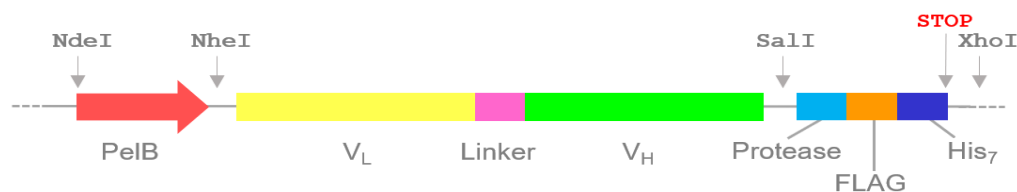


Figure 6. A schematic representation of the scFv gene structure in pET-17b. The PelB sequence was added for periplasmic protein expression and is removed by bacteria during translation. The linker sequence was added to fuse the two variable domains (V_L and V_H) of antibodies. The His₇ tag was added for purification, and the FLAG tag was added for detection purposes. Factor Xa protease sequence (IEGR) was added to remove the C-terminus affinity tags when desired.

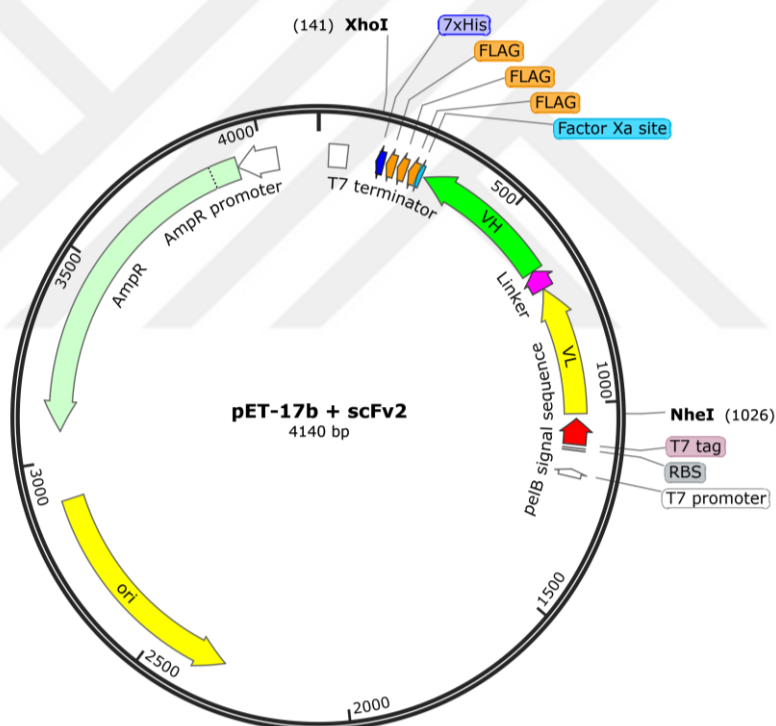


Figure 7. Plasmid map of the pET-17b bacterial expression plasmid. Generated by SnapGene® software (from Insightful Science; available at snapgene.com).

Table 8. Sequences of the components of the wild type scFv gene construct in pET-17b

Component	Sequence
PelB	KYLLPTAAAGLLLLAAQPAMAMS
V _L (light chain variable domain)	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKA PKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISLQPEDFATYYC QQYSTVPWTFGGGTKVEIKRT
V _H (heavy chain variable domain)	EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAP GKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQM NSLRAEDTAVYYCAKYPHYYGSSHWFYFDVWGQGTLLTVSS
Non-repetitive linker	SPNSASHSGSAPQTSSAPGSQ
Repetitive linker	GGGSGGGSGGGSGGGSQ
Xa protease	IEGR
FLAG	DYKDDDDK(AA)DYKDDDDK(AA)DYKDDDDK(AA)
His ₇	HHHHHHH

3.5.2 Design of anti-VEGF scFvs bearing single mutations

Hydrophobic amino acids were identified that become solvent exposed following the removal of the constant domains from the parental Fab fragment during scFv generation. For that, the percentage of solvent-accessible surface area for each residue in the Fab and scFv structures was calculated by GETAREA [117]. By setting a threshold value ($\geq 30\%$), those hydrophobic residues that become solvent exposed at the former V/C interface were identified. In turn, those hydrophobic residues (except Gly and Pro) were substituted with charged amino acids (Figure 8).

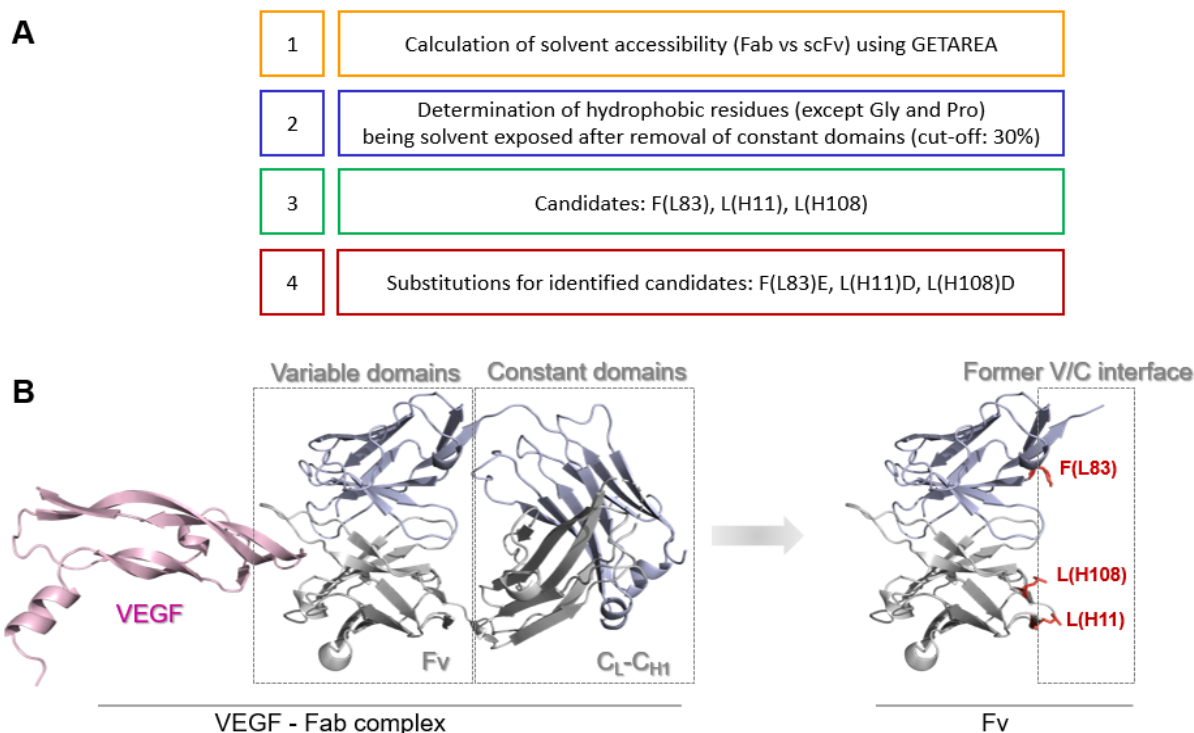


Figure 8. Engineering of the former variable-constant domain (V/C) interface. (A) The solvent-accessible surface area percentages of each residue in the Fab and scFv structures were calculated by GETAREA. By setting a cut-off value, three hydrophobic residues (except Gly and Pro) that are solvent exposed after the removal of constant domains were identified and substituted with charged amino acids. (B) Positions of mutable sites at the former V/C interface. Fv: variable fragment; C_L: light chain constant domain; C_{H1}: heavy chain constant domain 1; F(L83): Phenylalanine at the 83rd position on light chain according to Kabat numbering scheme; F(L83)E: substitution of glutamic acid for phenylalanine at the 83rd position on the light chain.

3.5.3 Generation of anti-VEGF scFvs bearing single mutations via site-directed mutagenesis

Single mutations were introduced into the scFv2 gene in pET17-b bacterial expression plasmid via site-directed mutagenesis method using a commercial KOD Hot Start DNA Polymerase Kit and appropriate primers specific to each mutant listed in Table 4. Two primers that have mismatch base pairs depending on the desired mutation were used for the generation of each variant. PCR reaction components and conditions for site-directed

mutagenesis are described in Table 9 and 10, respectively. All PCR reactions were applied using a SimpliAmp Thermal Cycler (Thermo Scientific).

Table 9. PCR reaction components for site-directed mutagenesis

Reagent	Volume (μL)
10X PCR Buffer for KOD Hot Start DNA Polymerase	2.5
Forward Primer (10 μM)	0.75
Reverse Primer (10 μM)	0.75
DNA Template (20 ng/ μL)	1
25 mM MgSO_4	1.5
KOD Hot Start DNA Polymerase (1.0 U/ μL)	0.5
dNTPs (2mM each)	2.5
Double-distilled H_2O	to 25

Table 10. PCR thermocycling conditions for site-directed mutagenesis

Cycle		Temperature ($^{\circ}\text{C}$)	Duration
1	Initial denaturation	95	2 min
30	Denaturation	95	10 sec
	Annealing	55	30 sec
	Extension	72	30 sec per 1 kb
1	Final Extension	72	5 min
1	Hold	4	∞

To remove cell-derived template plasmids from the PCR products, *DpnI* digestion, which is sensitive to (Adeno) methylated GA/TC sites, was applied. Reaction components and conditions for *DpnI* digestion are described in Table 11. Finally, a PCR clean-up procedure was applied using a commercial QIAquick PCR Cleanup Kit according to manufacturer's kit protocol. The DNA concentration was measured at 260 nm using NanoDrop 2000 (Thermo Scientific).

The scFv mutants that bear single mutations are listed in Table 12.

Table 11. Reaction components and conditions for *DpnI* digestion

Reagent	Volume (μL)
<i>DpnI</i> (2×10^4 units/mL)	0.6 μL
PCR Product	~ 25 μL
10 X NE Buffer	3 μL
Incubation Temperature / Time	37°C / 30 min

Table 12. ScFv mutants generated via site-directed mutagenesis

Template	Substitution	Variant Name
scFv2	F(L83)E	F(L83)E
scFv2	L(H11)D	L(H11)D
scFv2	L(H108)D	L(H108)D

3.5.4 Bacterial protein expression and purification

E. coli BL21(DE3)pLysS cells were used for bacterial protein expression. First, BL21 (DE3)pLysS cells were transformed with the pET-17b plasmids that bear scFv genes, as described following: (i) 50 μL cell and 2.1 μL 0.142 M β -mercaptoethanol were mixed and incubated on ice for 10 minutes by gently swirling every two minutes; (ii) Then 150 ng plasmid DNA was added to the mixture and kept on ice for 30 minutes by gently swirling every two minutes; (iii) The mixture was then subjected to a heat shock at 42 °C for 45 seconds in a water bath preheated to 42 °C, and immediately put on ice for two minutes; (iv) 500 μL of SOC medium was added to the mixture and incubated at 37 °C for one hour with agitation (250 rpm); (v) Finally, 100 μL of the mixture was plated onto LB-Agar plates supplemented with antibiotics (100 μg /mL AMP and 25 μg /mL CAM). The plates were incubated at 37 °C for 18 h.

For protein expression, single colonies were inoculated into LB broth media supplemented with antibiotics (100 μg /mL AMP and 25 μg /mL CAM) and grown overnight at 37 °C overnight with agitation at 250 rpm. These cells were then inoculated into 300 mL autoinduction-media in 1-L flask with an initial OD₆₀₀ (optic density) value of 0.05 and incubated at 18°C for 48 h with agitation at 250 rpm [118]. OD₆₀₀ (optic density) measurements were applied using a Varioskan Type 3001 Microplate Reader (Thermo Scientific).

Thereafter, cultures were centrifuged at 6500 x g and 4°C using an Avanti J-25i Centrifuge (Beckman Coulter) for 30 minutes using 250 mL centrifuge bottles. Supernatant was incubated with the Ni-NTA Sepharose resin (0.5 mL bed resin per 100 mL) at 4°C for at least two hours with gentle mixing. The mixture was loaded into 10 mL Pierce centrifuge columns and the supernatant was removed from the resin by using a vacuum manifold. The resin was then washed once with 100 mL 1X Buffer PB and once with 100 mL Buffer A, in order (see Table 2 for buffers). 1 mL Buffer B (Table 2) was used as the elution buffer and the eluent was collected into 3 mL 1X PBS. Membrane filtration was performed using Amicon® Ultra-4 Centrifugal Filter Units with a MWCO of 10 kDa to remove imidazole, and to exchange the buffer into 1X PBS (pH 7.4). As a second purification step, protein samples were then loaded into a HiTrap Protein L column for a better purity. The purity of the samples was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a vertical electrophoresis system (Mini-PROTEAN System, Bio-Rad). Protein concentration was measured at 280 nm using NanoDrop 2000 (Thermo Scientific).

3.5.5 Differential scanning fluorimetry (DSF)

The melting temperatures of the soluble scFvs were determined using differential scanning fluorimetry (DSF). The reaction solutions were prepared using 2 µM scFv and 2X SYPRO Orange dye. The scFv-dye mixtures were transferred to individual wells of a 96-well plate and analyzed using an ABI 7500 Fast RT-PCR instrument (Applied Biosystems). ROX channel was selected as the reporter. Thermal cycle parameters were chosen to examine changes in the fluorescence intensity at different temperatures, increasing at a rate of 0.05% between 25 and 99 °C. The melting temperatures (T_m ; the temperature at which the folded and unfolded forms of a protein are in equilibrium) were obtained from the thermogram data using the Hill1 equation fit in OriginPro 8.5 (OriginLab, Northampton, MA, USA).

3.5.6 Affinity analysis via Surface Plasmon Resonance (SPR)

A Biacore T200 instrument was used to perform the affinity measurement of the soluble scFvs. All experiments were conducted in a pH 7.4 HBS-EP buffer. The antigen was immobilized on a CM4 chip by running 1000 nM VEGF for one minute at a 10 µL/min flow rate. Following that, a serial dilution of scFvs (10 to 100 nM) and bevacizumab (10 to 50 nM)

were injected over to the VEGF immobilized surface with flow rate of 30 $\mu\text{L}/\text{min}$. Data correction was applied against two reference cells, a VEGF-free control cell and a buffer-injected cell. The manufacturer's software BiaEval 3.0 was used to evaluate the sensogram curves. The dissociation constant (K_D), the association rate constant (k_{on}), and the dissociation rate constant (k_{off}) were determined by fitting the kinetic association and dissociation curves to a 1:1 binding model.

3.5.7 Zebrafish experiments

The zebrafishes employed in these experiments were provided by the Izmir Biomedicine and Genome Center's zebrafish facility. All procedures applied to zebrafishes were approved by the Izmir Biomedicine and Genome Center (IBG) Animal Experiments Local Ethics Committee (IBG-AELEC) in Izmir, Turkey. Adult zebrafish were maintained at the Izmir Biomedicine and Genome Center Zebrafish Facility under standard conditions of 28°C and a 14/10-hour light/dark cycle. The transgenic *flil:EGFP* line [119] was crossed with wild-type AB to obtain zebrafish embryos. The embryos were collected within 30 minutes of fertilization and incubated at 28°C in a dark incubator in E3 embryo medium (Table 2).

Microinjection and Fixation: 55 μM scFv1, scFv2 and 55 or 27.5 μM bevacizumab in PBS supplemented with 0.5% phenol red were placed on ice until microinjection. A stage micrometer was used to estimate the injection volume, and 50 nL of scFv1, scFv2, and bevacizumab were microinjected into the yolk of each anaesthetized zebrafish embryo using 0.02% tricaine at 2 days post fertilization (dpf). For control group, only PBS without antibodies was injected. More than 20 embryos were used for each condition. Microinjected larvae were housed at 28°C and the development of subintestinal vessels (SIV) was examined at one day post injection (dpi). Larvae were fixed in 4% formaldehyde and rinsed with PBS. Melanocyte decolorization was applied by using a pigment discoloration solution (Table 2).

Imaging and quantitative analysis: Larvae were lateral mounted in 1% low melting agarose in an imaging dish (Table 1) and imaged using a Zeiss LSM880 confocal microscope. The area of the SIV was determined using the ImageJ polygon tool [120], and statistical analysis was performed in Excel using a one-tail t-test with a 95 % confidence interval. Statistical significance was represented by stars to indicate a range of p-values: non-significant (ns) > 0.05, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. The results are expressed as average (AVG) $\pm$ standard error.

3.5.8 Cloning anti-VEGF scFv genes into yeast surface display plasmid

The wild type scFv2 and the scFv variant F(L83)E were amplified from pET-17b bacterial expression plasmid and a C-terminal Myc tag was added using the primers listed in Table 5. The scFv genes with N-terminal NheI and C-terminal XhoI restriction sites were then ligated with the orientation of Aga2-V_L-linker-V_H-Myc into an Aga2-based yeast surface display plasmid (Figure 3 and 9, Table 7) [121]. The ligation product was then transformed into *E. coli* DH5 α cells, and the sequence of the resulting isolated plasmid was verified by Sanger sequencing (Eurofins Genomics). In detailed, the following procedure was followed.

(i) *Insert amplification:* The wild type scFv2 and the scFv variant F(L83)E were amplified from pET-17b bacterial expression plasmid by adding a C-terminal Myc tag using appropriate primers listed in Table 5. All PCR reactions were applied using a ProFlex PCR System (Thermo Scientific). PCR reaction components and conditions are described in Table 13 and 14, respectively. Thereafter, gel electrophoresis and gel purification were applied to the PCR products. 1% Agarose Gel (in 1X TAE buffer) supplemented with GelGreen Nucleic Acid Stain (10 μ L 10.000 X stain for 100 mL agarose gel) was prepared. 1X TAE buffer was used as the running buffer. 10 μ L 1kb DNA ladder was loaded into the first lane of the agarose gel. For sample loading, 10 μ L 6X purple loading dye was added to each 50 μ L DNA sample and mixed well. Samples were then loaded into the other wells of the agarose gel. The gel was run at 135 V for about 50-60 min using an electrophoresis system (Thermo Scientific). Under the blue light, DNA fragments was visualized, and appropriate DNA fragments were cut from the gel. Gel purifications were applied using Qiagen Quick Gel Extraction Kit. A restriction digest was then performed on the amplified inserts as explained in Table 15 and reaction mixtures were purified using Qiagen Quick Gel Extraction Kit according to manufacturer's protocol. These final purified PCR products were used as inserts for the final ligation into digested yeast surface display plasmid (see backbone preparation and ligation below).

Table 13. PCR reaction components for insert amplification

Reagent	Volume (μL)	Final Concentration
5X Phusion HF Buffer	10	1X
Forward Primer (10 μM)	2.5	0.5 μM
Reverse Primer (10 μM)	2.5	0.5 μM
DNA Template	variable	~ 10 ng
Phusion High-Fidelity DNA Polymerase	0.5	1.0 units/50 μL PCR
dNTPs (10 mM for each nucleotide)	1	200 μM
DMSO	1.5	3%
Double-distilled H_2O	to 50	

Table 14. PCR thermocycling conditions for insert amplification

Cycle		Temperature ($^{\circ}\text{C}$)	Duration
1	Initial denaturation	98	30 sec
30	Denaturation	98	10 sec
	Annealing	53	30 sec
	Extension	72	1 min
1	Final Extension	72	10 min
1	Hold	4	∞

Table 15. Restriction digestion of DNA fragments

Reagent	Volume (μL)
10 X Cut Smart Buffer	5 μL
NheI-HF	1 μL (10 units)
XhoI	1 μL (10 units)
DNA Template	1 μg
Nuclease-free water	to 50 μL
Incubation Temperature / Time	37 $^{\circ}\text{C}$ / 20 min

(ii) *Backbone preparation*: For backbone preparation, first a sufficient amount of the yeast display plasmid was digested with the restriction enzymes as described in Table 15. After digestion, 1 μL CIP (calf-intestinal alkaline phosphatase) added into each reaction mixture and incubated at 37 $^{\circ}\text{C}$ for 15 minutes. Then, gel purification was applied as described previously.

(iii) *Ligation*: At this final step inserts and linearized backbone were ligated using T4 DNA ligase according to manufacturer's protocol (NEB) as described in Table 16.

Table 16. T4 DNA ligation reaction components and incubation conditions

Reagent	Volume (μL)
T4 DNA Ligase Buffer (10 X)	2 μL
Vector DNA (backbone)	~ 50 ng (0.020 pmol)
Insert DNA	~ 38 ng (0.060 pmol)
T4 DNA Ligase	1 μL
Nuclease-free water	to 20 μL
Incubation Temperature / Time	Room Temperature / 15 min

After T4 DNA ligation, *E.coli* DH5α cells were transformed with these ligation products. For that, simply, 5 μL from the ligation product was added into 50 μL *E.coli* DH5α cells (previously thawed on ice) and incubated on ice for 30 minutes. Then, heat shock was applied at 42 °C for 45 seconds. Then, cells were left on ice for two minutes and after that 500 μL SOC media was added and incubated at 37 °C for one hour. After the incubation, cells were pelleted by centrifugation at 800 x g for five minutes. 300 μL from the supernatant was removed and the pellet resuspended in the rest supernatant. 100 μL of the cell suspension was plated onto LB-Agar plates supplemented with AMP (100 μg /mL). The day after, single colonies from the plates were selected and inoculated into 5 mL LB media supplemented with AMP (100 μg /mL). The cultures were incubated at 37 °C overnight with agitation at 225 rpm. The plasmid isolation was carried out from the overnight incubated cultures using Qiagen Miniprep DNA Isolation Kit according to manufacturer's protocol and sequences were verified by Sanger sequencing (Eurofins Genomics).

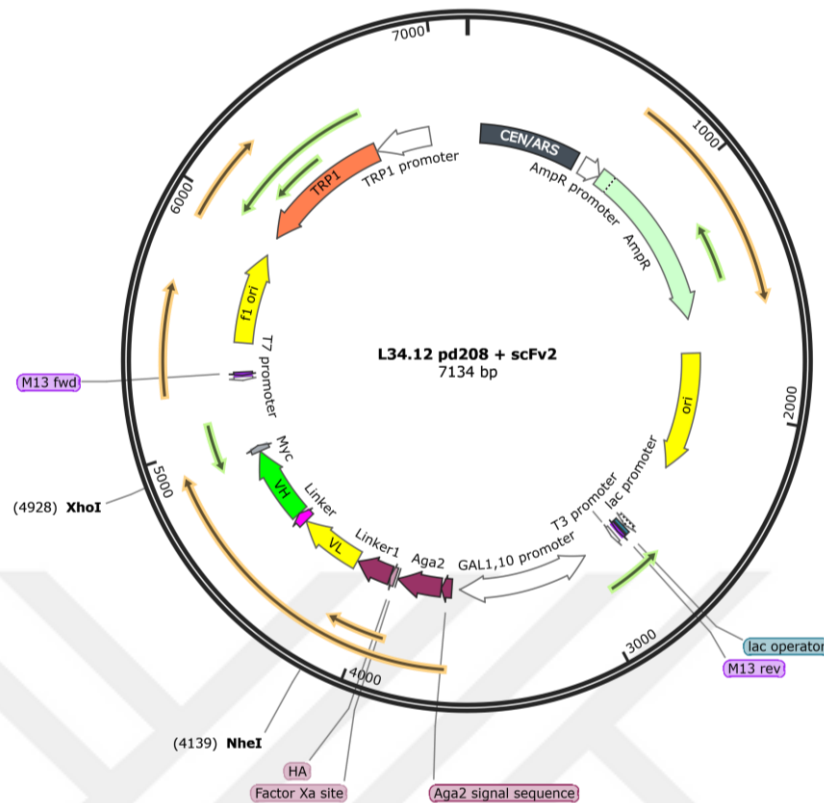


Figure 9. Plasmid map of the yeast surface display plasmid. Generated by SnapGene.

3.5.9 Yeast surface display

EBY100 (*Saccharomyces cerevisiae*) cell line was used for yeast surface display of antibody fragments. Yeast transformation with yeast display plasmids bearing scFv genes was applied as follows: One loop yeast cell from a freshly prepared YPD agar plate or 500 μ L cell culture in YPD media (both stored at 4 $^{\circ}$ C) were transferred to 5 mL YPD media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S), and incubated overnight at 30 $^{\circ}$ C with agitation at 225 rpm. For each transformation, 500 μ L liquid culture was taken from the overnight incubated yeast culture and pelleted at 16000 x g for one minute. The cell pellet was resuspended in 500 μ L of plate solution (Table 2). 10 μ L 10 mg/mL single-stranded DNA and 1-2 μ g plasmid DNA were added and first incubated at room temperature for 15 minutes, then incubated at 42 $^{\circ}$ C for 15 minutes. After centrifugation at 16000 x g for one minute, 400-450 μ L of supernatant was removed. The cell pellet was resuspended in the rest 50-100 μ L plate solution and plated onto dropout plates (Table 3). The plates were incubated at 30 $^{\circ}$ C for 2-3 days.

One-loop transformed EBY100 cells from dropout plates (or 500 μ L cell culture in SDCAA media stored at 4 °C for the later studies) were inoculated into 5 mL SDCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x dilution of P/S) and incubated overnight (~16-24 h) at 30 °C with agitation at 225 rpm. To induce the expression of Aga2-scFv fusion proteins, the media was then switched to SDGCAA media with an initial OD₆₀₀ of 1. The required volume of cell culture from the overnight incubated SDCAA culture was taken and pelleted at 2500 x g for five minutes. Then the cell pellet was resuspended in 5 mL SDGCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S), and incubated either at 30°C for 16-24 h or at 20 °C for 36-40 h with agitation at 225 rpm.

3.5.10 *Thermal stability measurement on yeast*

Thermal stability measurements on yeast were applied as described here unless otherwise stated. OD₆₀₀ of the SDGCAA culture was calculated and the corresponding cell titers were calculated (OD₆₀₀ 1 equals $\sim 3 \times 10^7$). Thereafter, required amount of cell was washed twice with PBSB. For each condition, 6×10^5 cells in 500 μ L PBSB were incubated at different temperatures (33-78 °C) for 10 minutes. After the heat shock, 1 mL ice-cold PBSB was immediately added to the cell suspension and cells were left on ice at least 30 minutes. 250 μ L from that cell suspension (contains 1×10^5 cells) was transferred to a 96-well plate with round-shaped well bottom. The plate was then centrifuged at 2500 x g for five minutes and supernatant was discarded. The cell pellet was then resuspended in a 200 μ L primary reagent solution (mouse anti-Myc tag antibody at a 1:1000 dilution, 1 nM biotinylated VEGF, and 1% milk). For control samples, antigen was not added to the primary reagent solution. The plate was incubated at 25 °C for three hours with mild agitation. After the incubation, cells were washed once with 200 μ L ice-cold PBSB and resuspended in 100 μ L secondary reagent solution (goat anti-mouse IgG AlexaFluor 488 conjugate at a 1:200 dilution and streptavidin AlexaFluor 647 conjugate at a 1:1000 dilution) and incubated on ice for four minutes with mild agitation. Samples were then washed with 200 μ L ice-cold PBSB and resuspended in 150 μ L ice-cold PBSB for flow cytometric analysis. 10000 events at minimum were recorded per sample using a Bio-Rad ZE5 cell analyzer.

3.5.11 *Affinity measurement on yeast*

Affinity measurements on yeast were applied as described here unless otherwise stated. OD₆₀₀ of the SDGCAA culture was calculated and the corresponding cell titers were calculated (OD₆₀₀ 1 equals $\sim 3 \times 10^7$). Sufficient amount of yeast cells was then washed twice with PBSB, and 1×10^5 cells for each condition were pelleted into each-well of a 96-well plate. After the final centrifugation at 2500 x g for five minutes, the supernatants were discarded. The cell pellets were then resuspended in 200 μ L of primary reagents solution (biotinylated VEGF over a range of concentrations (0.015 nM-100 nM), mouse anti-Myc tag antibody at a 1:1000 dilution, and 1% milk) and incubated at 25 °C for three hours with mild agitation. For control samples, antigen was not added to the primary reagent solution. After three hours of incubation with primary reagents, cells were washed once with 200 μ L PBSB and resuspended in 100 μ L PBSB secondary reagent solution (goat anti-mouse IgG AlexaFluor 488 conjugate at a 1:200 dilution and streptavidin AlexaFluor 647 at a 1:1000 dilution) and incubated on ice for four minutes with mild agitation. After the incubation with secondary reagents, samples were washed with 200 μ L ice-cold PBSB and resuspended in 150 μ L ice-cold PBSB for flow cytometric analysis. 10000 events at minimum were recorded per sample using a Bio-Rad ZE5 cell analyzer.

3.5.12 *Protein L binding analysis on yeast*

OD₆₀₀ of the SDGCAA culture was calculated and the corresponding cell titers were calculated (OD₆₀₀ 1 equals $\sim 3 \times 10^7$). Sufficient amount of yeast cells was then washed twice with PBSB, and 1×10^5 cells for each condition were pelleted into each-well of a 96-well plate. After the final centrifugation at 2500 x g for five minutes, the supernatants were discarded. The cell pellets were then resuspended in 200 μ L of primary reagents solution (iFluor647-Protein L conjugate over a range of concentrations (63 nM-2 μ M), chicken anti-Myc tag polyclonal IgY at a 1:1000 dilution, and 1% milk) at 25 °C for three hours with mild agitation. For control samples, Protein L was not added to the primary reagent solution. After three hours of incubation with primary reagents, cells were washed once with 200 μ L ice-cold PBSB and resuspended in 100 μ L secondary reagent solution (goat anti-chicken IgG AlexaFluor 488 conjugate at a 1:250 dilution) and incubated on ice for four minutes with mild

agitation. After the incubation with the secondary reagent solution samples were washed with 200 μ L ice-cold PBSB and resuspended in 150 μ L ice-cold PBSB for flow cytometric analysis. 10000 events at minimum were recorded per sample using a Bio-Rad ZE5 cell analyzer.

3.5.13 *Testing dual reporter system for use of thermal stability measurement*

OD₆₀₀ of the SDGCAA culture was calculated and the corresponding cell titers were calculated (OD₆₀₀ 1 equals $\sim 3 \times 10^7$). Sufficient amount of yeast cells was then washed twice with PBSB. 6×10^5 cells in 500 μ L PBSB for each condition were incubated at different temperatures (38-78 °C) for 10 minutes. After the heat shock, 1 mL ice-cold PBSB was immediately added to the cell suspension and cells were left on ice at least 30 minutes. 250 μ L from that cell suspension (contains 1×10^5 cells) was transferred to a 96-well plate with round-shaped well bottom. The plate was then centrifuged at 2500 x g for five minutes, and supernatant was discarded. The cell pellet was then resuspended in a 200 μ L primary reagent solution (chicken anti-Myc tag polyclonal IgY at a 1:1000 dilution, 50 nM i-Fluor647-Protein L conjugate, 1 nM or 5 nM biotinylated VEGF, and 1% milk). Control samples were incubated with primary reagent solutions prepared at three different conditions: (a) without Protein L, (b) without VEGF, (c) without both Protein L and VEGF. The plate was incubated at 25 °C for three hours with mild agitation. After the incubation, cells were washed once with 200 μ L ice-cold PBSB and resuspended in 100 μ L secondary reagent solution (goat anti-chicken IgG AlexaFluor 488 conjugate at a 1:250 dilution and streptavidin Brilliant Violet 421 conjugate at a 1:500 dilution) and incubated on ice for four minutes with mild agitation. Samples were then washed with 200 μ L ice-cold PBSB and resuspended in 150 μ L ice-cold PBSB for flow cytometric analysis. 10000 events at minimum were recorded per sample using a Bio-Rad ZE5 cell analyzer.

3.5.14 *Design of synthetic libraries for stability improvement of anti-VEGF scFvs*

The following methodology was applied for library design to develop thermally stable scFv antibodies (Table 18 and 19). The F(L83)E variant was used as the template for the library generation. Two libraries were designed, one for the V_L domain and one for the V_H domain. The other corresponding domain in each library was kept the same as the template.

Step 1. Selection of candidate mutable sites according to the following criteria:

(a) *The results of stability prediction methods:* One energy-based method (FoldX) [122], two knowledge-based methods (CUPSAT [123] and PoPMuSIC 2.1 [124]) and two machine learning methods (MAESTRO [125] and DeepDDG [126]) were used to predict $\Delta\Delta G$ values upon mutations between the wild type and the mutant scFvs. Additionally, a thermodynamics-based and knowledge-driven prediction method (HoTMuSIC) [127] was used to predict ΔT_m (K) upon mutations. Each single site was mutated to 19 other amino acids and $\Delta\Delta G$ and ΔT_m (K) values for each single mutation were recorded. Different thresholds were defined for each prediction method considering their accuracy and standard deviations (Table 17) to select the most significant mutations increasing thermodynamic/thermal stability. The potential mutations for each position that were predicted by each method were listed and the number of significant mutations for each residue were recorded. A residue was accepted as a candidate mutable site if at least one significant mutation (except Cys) was predicted for that site by at least two different methods.

Table 17. Defined thresholds for stability prediction tools

Method	Threshold	Calculation of the thresholds
FoldX	1.46 kcal/mol ($\Delta\Delta G$)	a single H-bond strength on average (1 kcal/mol) [128] + SD value of FoldX (0.46 kcal/mol) [122]
CUPSAT	2.15 kcal/mol ($\Delta\Delta G$)	a single H-bond strength on average (1 kcal/mol) [128] + SD value of CUPSAT for highest accuracy (1.15 kcal/mol) [123]
PoPMuSIC/MAESTRO/DeepDDG	0.45 kcal/mol ($\Delta\Delta G$)	
HoTMuSIC	0.45 ΔT_m (K)	

(b) *The frequency of framework residues in the natural repertoire:* Those framework residues in the template antibody sequence that have a frequency below 5% in the natural repertoire (based on the AbYsis database [34]) were also selected as candidate mutable sites and added to the list obtained at Step 1-a.

Step 2. Exclusion of those sites that:

(a) *are interacting and/or interfacing with the antigen:* PISA [129] was used to detect which sites are interacting and/or interfacing residue with the antigen.

(b) *has a frequency equal to or greater than 50% or has the highest frequency in the natural repertoire:* The AbYsis database was used to view amino acid frequencies for each site.

(c) *stabilizing residues:* SCide [90] was used to predict stabilizing residues in the template antibody structure.

Step 3. Selection of mutations for the selected mutable sites from the natural repertoire

The AbYsis Database was used to view amino acid frequencies for each site. Those amino acids that have $\geq 3\%$ frequency in a particular position in natural repertoire were selected for substitutions.

The three-dimensional (3D) structure of Fab fragment of bevacizumab interacting with VEGF was obtained from the RCSB Protein Data Bank [130] (PDB No: 1BJ1) and the scFv version was generated from that PDB file and used for the computational studies. PyMOL [131] was used for visualization of three-dimensional (3D) structure of antibodies.

Table 18. Library design for the light chain variable domain

Region	LFR1	LFR1	LCDR2	LCDR2	LCDR2	LCDR2	LFR3	LFR3	LFR3
Residue (Kabat Numbering)	L1	L22	L51	L53	L55	L60	L85	L92	
Residue Name	D1	T22	T51	S53	H55	S60	T85	S92	
Natural Repertoire ($\geq 3\%$)	Q (34 %) D (27 %) E (20 %) S (10 %) A (3 %)	S (56%) T (36%) N (4%)	A (42%) N (20%) D, V (11%) T (6%) G (4%)	N (22%) K, S (18%) T (15%) Q (8%) D (5%) E, R (4%)	P (42%) A (23%) Q (15%) E (10%)	D (51%) S (24%) A, E (9%) N (4%)	D (42%) V (27%) T (23%)	D (38%) Y (14%) N (11%) G (9%) T (6%) S (5%) A (4%) H, L (3%)	
Degenerate codon	VVB	AVC	RNC	VVB	SMB	RVB	RHC	NNC	
Amino acids encoded by degenerate codon	GAHPSTR KNQDE	STN	GAIVSTND	GAHPSTRK NQDE	AHPQDE	GASTRKN DE	AIVTND	GALIVHPF YSTCRND	
Coverage (%)	100%	100%	100%	100%	100%	100%	100%	100%	
Library Size	1.68 x 10 ⁷								

Table 19. Library design for the heavy chain variable domain

Region	HFR1	HCDR1	HCDR2	HCDR2	HFR3	HFR3	HFR3	HFR3	HFR3	HFR3	HFR3	HCDR3
Residue (Kabat Numbering)	H1	H35	H61	H65	H69	H71	H73	H76	H78	H102		
Residue Number	E1	N35	A62	R66	F70	L72	T74	S77	A79	V112		
Natural Repertoire ($\geq 3\%$)	Q (49%) E (44%)	S (32%) H (25%) N (12%) T (7%) G, Y (6%) A (5%)	D (%40) P (%25) Q (%17) A (%5) V (%3)	G (71%) S (21%)	I (76%) M (13%) L (5%) V (4%)	R (59%) V (14%) A (11%) K (4%) P, T (3%)	N (40%) T (35%) K (8%) D (5%) E (4%) R (3%)	N (66%) S (22%) D (3%)	L (41%) A (24%) F (19%) V (12%)	Y (33%) V (24%) I (9%) P (8%) L (7%) S (5%) H (4%) F (3%)		
Degenerate Codon	SAA	NVC	SHB	RGB	DTB	VNA	RVB	RRC	KYB	NHC		
Amino acids encoded by degenerate codon	QE	GAHPYS TCRND	ALVHP QDE	GSR	LIVMF	GALIVPT RKQE	GASTR KNDE	GSND	ALVFS	ALIVHPF YSTND		
Coverage (%)	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%		
Library Size	6.27 x 10 ⁷											

3.5.15 *Generation of synthetic antibody libraries using overlap extension PCR*

Two libraries, one for light chain variable domain and one for heavy chain variable domain were prepared by PCRs designed to introduce diversity through degenerate primers while minimizing the frequency of the wildtype sequence within the library. Specifications are shown in Tables 18 and 19. Briefly, DNA segments corresponding to the regions of the scFv which were held constant were first amplified. Desired mutations were then introduced at the selected sites using primers listed at the Table 6 containing degenerate codons by following the PCR steps described at the Table 20. The yeast surface display plasmid of F(L83)E variant was used as the template plasmid for the generation of the libraries. The first obtained fragment that was amplified from the template plasmid (e.g., PCR Number 1) was extended with the proper primers that have at least 18 bp complementary sequence (e.g., PCR Number 2-3) to the generated fragment. At the final step, two fragments obtained from the previous PCR steps were combined with a final overlap extension PCR (e.g., PCR Number 5). The components of the PCR reaction and the reaction conditions are given at the Table 21 and 22, respectively. For those PCR reactions at which the desired fragments were not obtained at a reliable specificity and sufficient amount, Touchdown PCR [132] was applied to increase the specificity and sensitivity. The thermocycling conditions for Touchdown PCR are specified at the Table 23. All PCR reactions were applied using a ProFlex PCR System (Thermo Scientific).

Table 20. PCR steps performed for generating synthetic antibody libraries

Library	PCR Number	Fwd primer	Rev primer	Input	Output (bp)
V _L	1	pr1	newpr2	Plasmid	569
	2	pr3	newpr2	PCR1	743
	3	pr4	newpr2	PCR2	869
	4	pr5	pr6	Plasmid	206
	5	pr5	newpr2	PCR4 + PCR3	1057
V _H	6	pr7	newpr2	Plasmid	135
	7	pr8	newpr2	PCR6	232
	8	pr9	pr2	PCR7	365
	9	pr10	newpr2	PCR8	492
	10	pr5	pr11	Plasmid	584
	11	pr5	newpr2	PCR10 + PCR9	1057

Table 21. PCR reaction components for synthetic antibody library generation

Reagent	Volume (μL)	Final Concentration
5X Q5 Reaction Buffer (NEB)	10	1X
dNTPs (10 mM each)	1	200 μM
Forward Primer (10 mM)	2.5	0.5 μM
Reverse Primer (10 mM)	2.5	0.5 μM
DNA Template	...	10 ng/ 50 μL
Q5 High-Fidelity DNA Polymerase (NEB)	0.5	1.0 U/ 50 μL
DMSO	1.5	3 %
Double-distilled H ₂ O	to 50	

Table 22. PCR thermocycling conditions for synthetic antibody library generation

Cycle		Temperature (°C)	Duration
1	Initial denaturation	98	2 min
	Denaturation	98	10 sec
30	Annealing	50-55	30 sec
	Extension	72	1 min
1	Final Extension	72	10 min
1	Hold	4	∞

Table 23. PCR thermocycling conditions for Touchdown PCR

Cycle		Temperature (°C)	Duration
1	Initial denaturation	98	2 min
	Denaturation	98	10 sec
12	Annealing	62 (0.5 °C decrease per cycle)	30 sec
	Extension	72	30 sec
30	Denaturation	98	10 sec
	Annealing	55	30 sec
	Extension	72	30 sec
1	Final Extension	72	10 min
1	Hold	4	∞

3.5.16 *Generation of yeast display libraries by homologous recombination*

Yeast antibody libraries were prepared as previously described [133] with slight modifications.

1) One loop EBY100 yeast cells from freshly prepared YPD agar plate were inoculated into 5 mL YPD media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) and incubated overnight at 30 °C with agitation (225 rpm).

2) 1.5 mL of the overnight incubated culture was inoculated into a 50 mL YPD media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) and incubated overnight at 30 °C and 225 rpm.

3) A required volume of inoculum was inoculated into 100 mL YPD media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) from the overnight incubated culture for an initial OD₆₀₀ of 0.3. The culture was incubated until OD₆₀₀ value reaches to around 1.6 (generally 5-6 h).

4) Cells were then pelleted in 50 mL conical tubes by centrifugation at 2500 x g for five minutes.

5) Cell pellets were washed once with 25 mL ice-cold distilled water and once with 25 mL ice-cold electroporation buffer (Table 2).

6) Cell pellets then resuspended in 25 mL conditioning buffer (Table 2) incubated at 30 °C with agitation (225 rpm).

7) The cell mixture was then divided into two 50 mL conical tubes and pelleted by centrifugation at 2500 x g for five minutes.

8) Cells were then washed once with 25 mL ice-cold electroporation buffer and resuspended in electroporation buffer with a final volume of 400 µL and kept on ice until the electroporation.

9) 400 µL of cell suspension and the DNA pellet (prepared the day before by ethanol precipitation) were mixed until the DNA pellet is sufficiently solved in the cell suspension. For ethanol precipitation Novagen Pellet Paint® NF Co-Precipitant Kit was used and the manufacturer's protocol was followed. Briefly: (i) 12 µg of insert DNA fragment and 4 µg of linearized backbone was first mixed. 0.1 volume of Na Acetate (3 M, pH 5.2) (10 µL for 100 µL DNA mixture) and 2 µL Pellet Paint NF Co-Precipitant were then added and mixed well; (ii) 2 volume of 100% ethanol was added and vortexed. The mixture was incubated at room

temperature for two minutes; (iii) Sample was then centrifuged by using a tabletop microcentrifuge at a maximum speed for five minutes. At this step a dark blue colored pellet was obtained; (iv) Without disturbing the pellet, the mixture was washed once with 1 mL 70% ethanol and once with 1 mL 100% ethanol; (v) Finally as much as supernatant was removed and the rest solution was left in a fumehood overnight with the uncapped tube covered by aluminum foil to allow the liquid to evaporate.

10) Electroporation was applied by using an electroporation tube with a 0.2 cm electrode gap (Table 1) and the electroporation system (Bio-Rad Gene Pulser Xcell) with a voltage setting of 2.5 kV, the capacitance of 25 μ F and the resistance of 200 ohms.

11) After the electroporation, cells were immediately resuspended in 8 mL of 1:1 mixture of 1 M Sorbitol and YPD Media, then incubated at 30 °C with agitation at 225 rpm for one hour.

12) Cells were then collected by centrifugation at 2500 x g for five minutes and resuspended in 1 mL SDCAA media. To calculate the transformation efficiency 25 μ L sample from that solution was taken and 5-serial dilutions (10^1 - 10^2 - 10^3 - 10^4 - 10^5) were prepared with a final volume of 250 μ L for each. 200 μ L of sample from each dilution of 10^3 - 10^4 - 10^5 were plated onto dropout plates and incubated at 30 °C for 2 days in order to estimate the transformation efficiency. The rest of the cell mixture was inoculated into 200 mL SDCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) and incubated at 30 °C for 2 days with agitation (225 rpm). That culture was the yeast antibody library.

After library generation, plasmid DNA was isolated from 1.5 mL of yeast library culture using the Zymoprep Yeast Plasmid Miniprep II Kit. The isolated plasmids were then used for the transformation of *E.coli* XL-1 Blue electroporation competent cells. Yeast plasmid isolation and electroporation of *E.coli* XL-1 Blue electroporation competent cells were applied according to the manufacturer's protocols. Twelve colonies were sent for DNA sequencing (Eurofins Genomics) and the presence of selected mutations at intended sites in the libraries was verified.

3.5.17 *Magnetic activated cell sorting (MACS)*

Magnetic activated cell sorting (MACS) was applied mainly as previously described by Zupancic et. al [134]. MACS method was applied for two purposes: (i) for the removal of truncated, misfolded, and low affinity antibodies from the libraries, (ii) to reduce the diversity of the libraries to a size that is more manageable for screening by FACS. Two rounds of MACS were applied. At Sort 1 only affinity sorting was applied. At the second round of MACS, affinity and stability sorting was simultaneously applied.

Sort 1 (MACS for affinity sorting):

1. 30 mL of previously prepared library culture was transferred into 200 mL SDCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) and incubated overnight at 30 °C with agitation (225 rpm).
2. 30 mL of overnight incubated culture was transferred into a 50 mL conical tube and centrifuged at 2500 x g for five minutes at room temperature. Cell pellet was then resuspended in 200 mL SDGCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) and incubated for 2 days at 20 °C with agitation (225 rpm).
3. OD₆₀₀ value of SDGCAA culture was calculated. 1×10^9 cells transferred to a 50 mL conical tube and centrifuged at 2500 x g for five minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 25 mL PBSB. This step was repeated twice.
4. Cells were then resuspended in 30 mL antigen solution (5 nM biotinylated VEGF and 1% milk) and incubated by end-over-end mixing at room temperature for three hours.
5. Cells were then washed once with 25 mL ice-cold PBSB as previously described and resuspended in 40 mL ice-cold PBSB containing 750 μ L Miltenyi MACS streptavidin microbeads. The cells and beads were then incubated by end-over-end mixing at 4 °C for 30 minutes.
6. Cells were then washed once with 25 mL ice-cold PBSB and the cells passed through 70 μ m filter into MACS LS column placed on magnetic adapter (see manufacturer's protocol and Table 1 for details).
7. The captured beads were then washed once by passing 5 mL ice-cold PBSB through the MACS LS column that is still placed on magnetic adapter.

8. MACS LC column was then removed from the magnet and the beads were eluted by passing 5 mL SDCAA through the column.
9. The eluted beads were then transferred to a 50 mL SDCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S).
10. To calculate the number of cells collected, serial dilutions (10^1 , 10^2 , 10^3) from the 50 mL SDCAA culture were prepared and plated on dropout plates (generally 25 μ L sample was transferred into 225 μ L PBSB for each dilution and 200 μ L of each plated onto dropout plates).
11. The rest SDCAA culture and the dropout plates were incubated at 30 °C for 2 days (with agitation at 225 rpm for the liquid culture).
12. SDCAA culture was stored at 4 °C until the next round of sorting (can be stored for several weeks). For the next round of sorting, SDCAA media should be refreshed 18-24 h prior to induction in SDGCAA media.

Sort 2 (MACS for affinity and stability sorting):

1. 1×10^9 cells from the Sort 1 library was inoculated into 30 mL SDCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) and incubated overnight at 30 °C with agitation (225 rpm).
2. A required volume of inoculum was pelleted at 2500 x g for five minutes and resuspended in 50 mL SDGCAA media supplemented with antibiotics (100 mg/mL AMP, 100 mg/mL KAN, and 0.01x P/S) for an initial OD₆₀₀ of 1. SDGCAA culture was then incubated for 2 days at 20 °C with agitation (225 rpm).
3. OD₆₀₀ value of SDGCAA culture was calculated. 1×10^9 cells transferred to a 50 mL conical tube and centrifuged at 2500 x g for five minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 25 mL PBSB. This step was repeated twice.
4. Cells were then resuspended in 15 mL PBSB and incubated at 58 °C for 10 minutes using a water bath. Immediately, 15 mL ice-cold PBSB was added after the heat shock and left on ice for at least 30 minutes.
5. Cells were then pelleted at 2500 x g for five minutes and resuspended in 30 mL antigen solution (5 nM biotinylated VEGF and 1% milk) and incubated by end-over-end mixing at room temperature for three hours.

6. Cells were then washed once with 25 mL ice-cold PBSB as previously described and resuspended in 40 mL ice-cold PBSB containing 750 μ L Miltenyi MACS streptavidin microbeads. The cells and beads were then incubated by end-over-end mixing at 4 °C for 30 minutes.
7. Cells were then washed once with 25 mL ice-cold PBSB and the cells passed through 70 μ m filter into MACS LS column placed on magnetic adapter (see manufacturer's protocol and Table 1 for details).
8. The captured beads were then washed once by passing 5 mL ice-cold PBSB through the MACS LS column that is still placed on magnetic adapter.
9. MACS LC column was then removed from the magnet and the beads elute by passing 5 mL PBSB through the column.
10. Yeast cells are not viable after the heat shock at 58 °C. Therefore, to recover the sorted libraries, plasmid DNA isolation from the sorted yeast cells was first applied using a Zymo Yeast Miniprep Kit according to the manufacturer's protocol with some modifications as previously described [38], namely: the sorted cells were incubated at 37 °C for 60 minutes with a twice the recommended amount of zymolyase. After addition of the neutralization buffer, two-step centrifugation was applied for 10 minutes each. Finally, two-step elution with 10 μ L H₂O each were applied for a better yield.
11. Post plasmid DNA isolation, recovery PCR (using the primer sets for the PCR Number 5 and 11 in Table 6 to yield full-length inserts) and yeast transformation by homologous recombination were applied as described previously in Sections 3.5.15 and 3.5.16, respectively.

3.5.18 *Fluorescence activated cell sorting (FACS)*

Fluorescence-activated cell sorting (FACS) was applied mainly depending on the FACS protocol previously described by Zupancic et. al [134] with an additional heat shock step to sort the most thermally stable scFv variants. Therefore, at this step affinity and stability sorting were applied simultaneously.

1. Yeast libraries recovered after the previous sort by recovery PCR and re-transformation of EBY100 cells were induced in SDGCAA media as described in Steps 1-2 of Sort 2 (MACS).

2. OD₆₀₀ value of the SDGCAA culture was calculated. 1×10^8 cells transferred into a 2 mL microcentrifuge tube and washed twice with 1 mL PBSB. Cells were centrifuged at $2500 \times g$ for five minutes at room temperature. After the second wash, cells were resuspended in 0.5 mL PBSB and incubated at 58°C for 10 minutes using a water bath. Immediately, 1 mL ice-cold PBSB was added after the heat shock and the sample was left on ice for at least 30 minutes.
3. 150 μL (1×10^7 cells) of the cell suspension was then transferred into a new 1.5 mL microcentrifuge tube for control samples and kept at room temperature until the incubation with primary reagent solution. 750 μL (5×10^7 cells) of the cell suspension was transferred into antigen solution (1 nM biotinylated VEGF and 1% milk) at a final volume of 10 mL in a 15 mL conical tube and incubated by end-over-end mixing at room temperature for three h.
4. Post antigen incubation, both control and antigen positive samples were centrifuged at $2500 \times g$ for five minutes. The samples were then resuspended in 1 mL ice-cold PBSB and transferred to 1.5 mL microcentrifuge tubes. After centrifugation at $16,000 \times g$ for one minute at room temperature using a tabletop centrifuge, supernatants were discarded. The cell pellets were then resuspended in 200 μL primary reagent solution (mouse anti-Myc tag antibody at a 1:1000 dilution) and incubated on ice for 20 minutes.
5. Post primary reagent incubation, cells were washed once with 1 mL ice-cold PBSB. Centrifugation was applied at $16,000 \times g$ for one minute at room temperature.
6. Control samples and antigen positive samples were then resuspended in 200- and 500- μL secondary reagent solution (goat anti-mouse IgG AlexaFluor 488 conjugate at a 1:200 dilution and streptavidin AlexaFluor 647 conjugate at a 1:1000 dilution), respectively. Samples were incubated on ice for four minutes.
7. Following the secondary reagent incubation, samples were centrifuged at $16,000 \times g$ for one minute and supernatants were discarded. Samples were then immediately placed on ice and resuspended with 1-5 mL ice-cold PBSB prior to loading the cells to the cell sorter in order to prevent antibody-antigen dissociation.
8. The cell sorting was applied as previously described by Zupancic et. al [134] using a Beckman Coulter MoFlo Astrios under sterile conditions.
9. First, a plot of the forward scatter (FSC) and side scatter (SSC) of the yeast cells using

the 488 nm laser was created and the FSC and SSC voltages were adjusted for gating the main homogeneous cell population for excluding the cell debris and aggregates (see ref [134] for more details).

10. By gating this main cell population in the FSC-SSC plot, a subplot was created to examine the log are of the fluorophore signals corresponding the expression (AF488) on the x-axis and the antigen binding (AF647) on the y-axis.
11. First, the control samples were examined to evaluate the antibody expression and secondary reagent binding. The laser voltages were adjusted for the fluorophores that are used to detect expression and antigen binding signals (AF488 and AF647, respectively) to differentiate the antibody expression positive and negative cell populations.
12. After adjusting laser voltages, antigen positive samples were processed. To be able to sort the most thermally stable scFv variants, a collection gate was drawn for the top 1% of the expression and antigen binding positive cell population as previously described [37, 38]. Generally, $1-3 \times 10^7$ cells were processed and $0.9-1 \times 10^5$ cells were collected into 1-3 mL ice-cold PBSB.
13. A recovery PCR and re-transformation were applied, respectively as described in Steps 10-11 of Sort 2 (MACS for affinity and stability sorting).

3.5.19 Ethics

All animal studies performed in this thesis were approved by the Izmir Biomedicine and Genome Center (IBG) Animal Experiments Local Ethics Committee (IBG-AELEC) with the approval code of 2020-013 on March 11, 2020.

4 RESULTS

4.1 Generated wild type anti-VEGF scFvs demonstrate similar *in vitro* binding activity to bevacizumab but poor thermal stability

The anti-VEGF scFvs derived from bevacizumab (IgG) using two different linkers (Table 8) were produced in bacterial expression system. The purity of the produced scFvs was checked by SDS-PAGE (Figure 10A). Single bands were obtained around the expected molecular weights, which are 32.8 kDa and 31.9 kDa for scFv1 and scFv2, respectively.

The binding kinetics of the scFvs and bevacizumab against VEGF was measured by SPR. Considering the valency difference between bevacizumab (divalent) and scFvs (monovalent), it was observed that the generated anti-VEGF scFvs have an affinity for VEGF that is comparable to their parental antibody bevacizumab (Figure 10B).

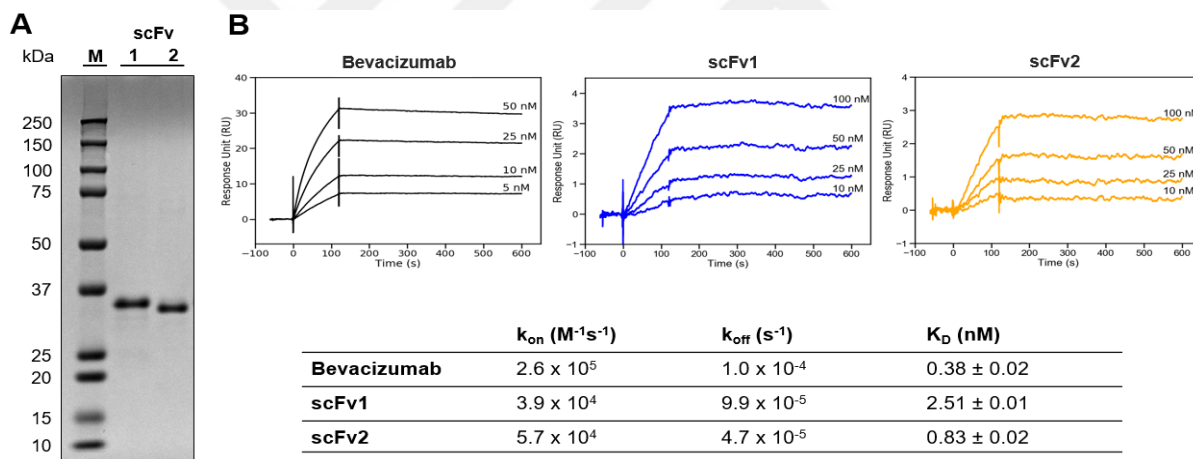


Figure 10. SDS-PAGE image and binding kinetics of scFv1, scFv2, and bevacizumab. (A) SDS-PAGE image of purified scFv1 and scFv2 antibodies. (B) Binding kinetics of scFv1 (blue), scFv2 (orange) and bevacizumab (black) obtained by surface plasmon resonance (SPR) analysis. M: Molecular weight marker.

The melting temperatures of scFv1 and scFv2 were measured using differential scanning fluorimetry (DSF) as an indicator of protein thermal stability. Both scFvs had significantly lower thermal stabilities (Figure 11) compared to previously developed thermally stable scFvs with T_m values of 60 °C or higher [135-137]. Therefore, this stability issue of the generated scFvs was further addressed as described in the following sections.

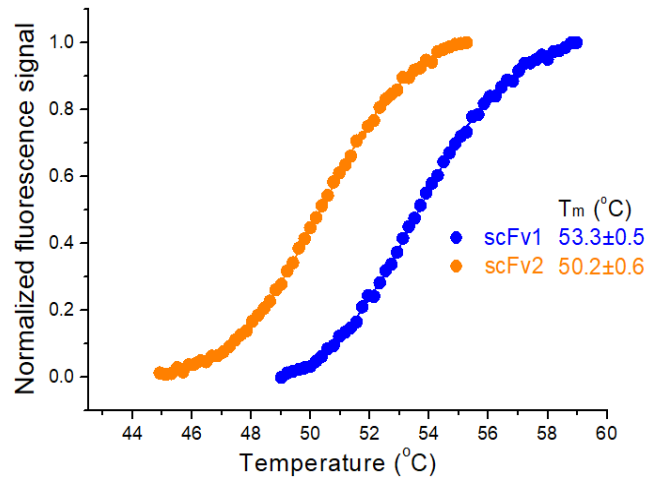


Figure 11. Thermal melting curves of scFv1 and scFv2. The thermal melting curves were obtained by differential scanning fluorimetry (DSF) analysis. T_m values (melting temperatures) of scFv1 (blue) and scFv2 (orange) were calculated from the thermal denaturation curves by Hill equation fit in OriginPro 8.5. The assay was repeated four times on different days and the results are expressed as AVG $\pm$ SD.

4.2 Generated wild type anti-VEGF scFvs demonstrate better *in vivo* efficacy on transgenic zebrafish model compared to bevacizumab

In vivo efficacy of the produced scFvs was tested using the zebrafish subintestinal vessel (SIV) assay, which employs the *flil:eGFP* transgenic zebrafish line that expresses green fluorescent protein (GFP) in endothelial cells [43]. PBS (negative control), scFv1 (55 μ M), scFv2 (55 μ M), and bevacizumab (27.5 μ M) were injected into the yolk of embryos at the initiation phase of SIV formation between 48- and 52-h post fertilisation (2dpf), and the anti-angiogenic effect of the antibodies was evaluated the following day by examining the SIV area of the zebrafish. Bevacizumab was used at half the concentration of scFvs in the experiments since it contains two Fab fragments involved in VEGF binding, whereas scFvs has only one paratope region responsible for antigen binding (Figure 1 and 2).

The SIVs of the zebrafishes treated with PBS were developed normally and intact in the yolk (Figure 12B), whereas scFv1 and scFv2 significantly inhibited vessel development in most embryos (Figure 12D and 12E). To quantify the inhibitory effect, the SIV area in each embryo was measured and expressed as the percent average area with respect to the control group treated with PBS (Figure 12F). In comparison to the PBS control, scFv1 and scFv2

reduced SIV area by 26% and 34.4%, respectively (Figure 12F). Bevacizumab, on the other hand, had no significant effect at 27.5 μ M. When the bevacizumab concentration increased to 55 μ M, a 35.6% reduction in SIV area was observed, comparable to that observed with scFv2 (data not shown).

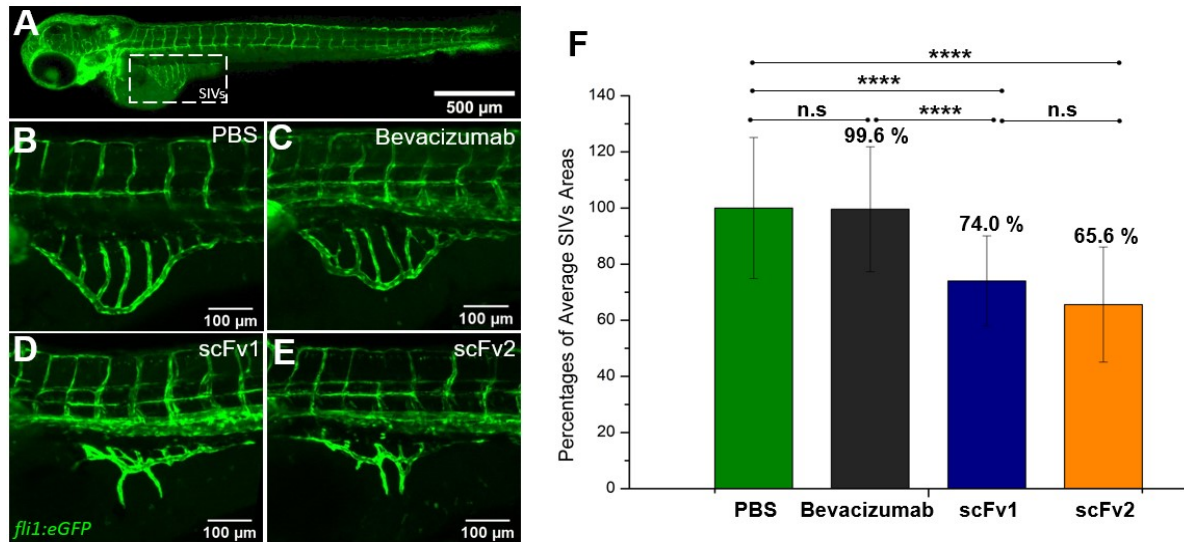


Figure 12. Anti-angiogenic effects of scFv1, scFv2, and bevacizumab on the transgenic zebrafish model. (A) Lateral view of *fli1:eGFP* transgenic zebrafish larvae's subintestinal vessels (SIVs) at 3 dpf. (B) PBS (negative control) (C) 27.5 μ M bevacizumab (D) 55 μ M scFv1, (E) 55 μ M scFv2 were injected into yolk of 2 dpf zebrafish embryos. At 3 dpf, SIVs were imaged by confocal microscopy. (F) SIV area of each zebrafish embryo was measured. The results of at least 23 repeats ($n_{\text{PBS}}=25$, $n_{\text{bevacizumab}}=24$, $n_{\text{scFv1}}=24$, $n_{\text{scFv2}}=23$) were expressed as $\text{AVG} \pm \text{SEM}$. The one tail t-test was used for statistical analysis and expressed as not significant (ns) if the p-value > 0.05 and significant if the p-value < 0.0001 (****).

As a conclusion, in this first part of the thesis, it was revealed that scFv1 and scFv2 retain almost their original affinity against VEGF. Moreover, they had a higher anti-angiogenesis activity in the transgenic zebrafish model compared to their parental antibody bevacizumab. On the other hand, they exhibited inadequate thermal stability for an antibody being developed for therapeutic purposes [26-28].

4.3 Engineering the former variable-constant domain (V/C) interface of the scFv2 results in a slight increase in thermal stability

Due to the low thermal stability of the generated anti-VEGF scFvs, one of the wild type scFvs was selected and further engineering studies were implemented to improve the thermal stability. Owing to its better binding activity compared to scFv1, scFv2 was selected for further engineering studies (Figure 10).

To begin with, the former interface between variable and constant domains (V/C interface) was focused on (Figure 8). It is known that hydrophobic patches at the V/C interface are significant contributors to the instability of scFvs [29]. Especially if a scFv molecule has a low thermodynamic stability, those hydrophobic residues may drive aggregation easily under stress conditions such as high concentration, elevated temperature, and long storage time [30]. Therefore, the hydrophobic residues at the former V/C interface being solvent exposed following the removal of constant domains were identified by comparing the solvent-accessible surface area percentages of each residue in the Fab and scFv structures. The 3D structures of the Fab and scFv molecules were derived from the crystal structure of bevacizumab (Figure 8). By setting a cut-off value of 30%, all hydrophobic residues (excluding Gly and Pro, which are generally critical for proper folding) that become solvent exposed following the removal of constant domains were identified, F(L83), L(H11), and L(H108). Following that, charged amino acids were substituted for these hydrophobic residues, and three scFv variants with single mutations were generated (Figure 8).

Following the generation and bacterial production of the scFv variants with single mutations at the former V/C interface, the affinity kinetics of these variants were determined by SPR analysis (Figure 13B). Among them, the variant F(L83)E demonstrated the highest affinity for VEGF by retaining the original K_D value of its parental scFv (scFv2) (Figure 13B).

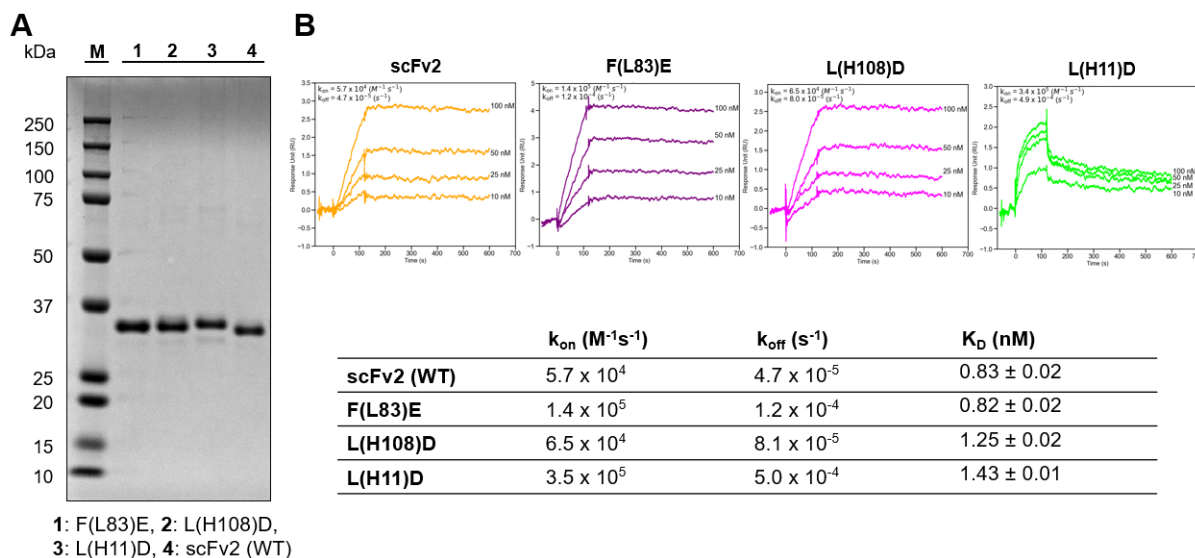


Figure 13. SDS-PAGE image and binding kinetics of scFv2, the scFv variants with single mutations, and bevacizumab. (A) SDS-PAGE image of purified wild type scFv2 and scFv variants bearing single mutations at the former V/C interface. (B) Binding kinetics of purified wild type scFv2 (orange), F(L83)E (purple), L(H108)D (pink) and L(H11)D (green) were obtained by surface plasmon resonance (SPR) analysis. M: Molecular weight marker. WT: Wild type.

The melting temperatures of the soluble scFv variants were determined as previously described and compared with the wild type scFv2 (Figure 14). Among them, the variant F(L83)E demonstrated only a slight but insignificant increase in thermal stability compared to the wild type scFv2, while the other variants demonstrated even lower thermal stability (Figure 14). All together, these findings revealed the need to apply different approaches to improve the thermal stability of the anti-VEGF scFvs.

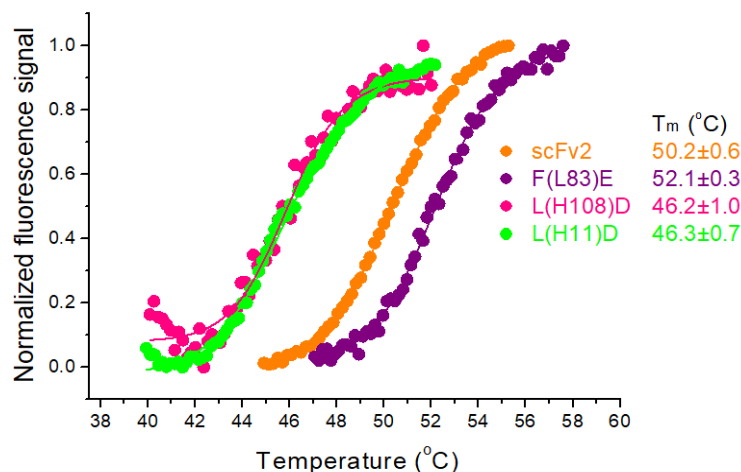


Figure 14. Thermal melting curves of scFv2 and the scFv variants with single mutations. The thermal melting curves were obtained by differential scanning fluorimetry (DSF) analysis. The T_m values (melting temperatures) of the wild type scFv2 (orange), F(L83)E (purple), L(H108)D (pink) and L(H11)D (green) were calculated from the thermal denaturation curves by Hill equation fit in OriginPro 8.5. The assay was repeated three times on different days and the results were expressed as AVG $\pm$ SD.

4.4 Anti-VEGF scFvs displayed on yeast maintain their affinity for VEGF

Due to the relatively low success on improving the thermal stability of scFv2 by introducing single mutations to the former V/C interface, it was decided to generate yeast display libraries and apply a stability sorting procedure using MACS and FACS to improve the thermal stability of the anti-VEGF scFvs.

To evaluate the applicability of the yeast surface display technique for anti-VEGF scFvs, expression and antigen binding activity of the scFvs on yeast were tested using scFv2 and F(L83)E. ScFv display level on the yeast cells was typically observed in a range of ~50-55% by calculation percentages of the two distinct yeast population depending on their antibody expression level (Figure 15). By comparing the antigen negative and positive samples, antigen binding ability of the scFvs were tested (Figure 16). As a results, the findings showed that the anti-VEGF scFvs are sufficiently displayed on yeast and maintain their affinities to VEGF.

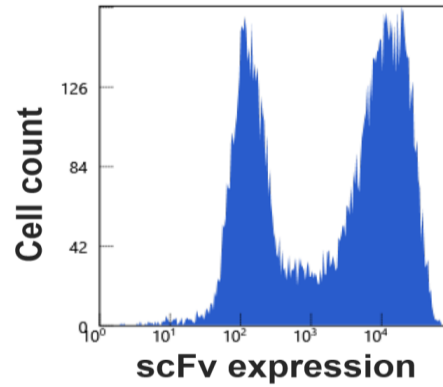


Figure 15. The scFv display level on the yeast surface. The yeast cells were induced for two days for scFv expression, and the scFv expression level was detected via mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488. See methods for details.

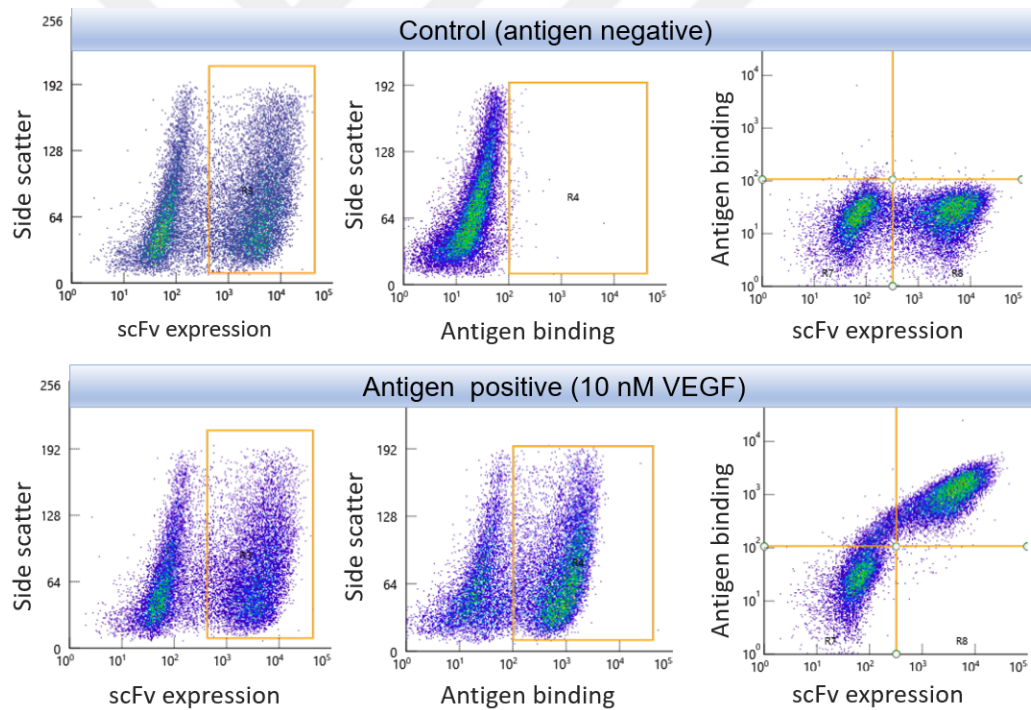


Figure 16. Antigen binding control of anti-VEGF scFvs displayed on yeast. Mouse anti-Myc primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin Brilliant Violet 421 couple was used to monitor antigen binding. Control samples were incubated with all reagents except biotinylated VEGF.

4.5 ScFv2 and F(L83)E show similar affinity and thermal stability characteristics on yeast

After confirming the utility of the yeast surface display system for anti-VEGF scFvs, wild type scFv2 and F(L83)E were tested in terms of their antigen binding affinity and thermal stability characteristics on the yeast. According to the results, scFv2 and F(L83)E demonstrated similar characteristics, with a slightly better apparent K_D and melting temperature values for F(L83)E (Figure 17 and 18).

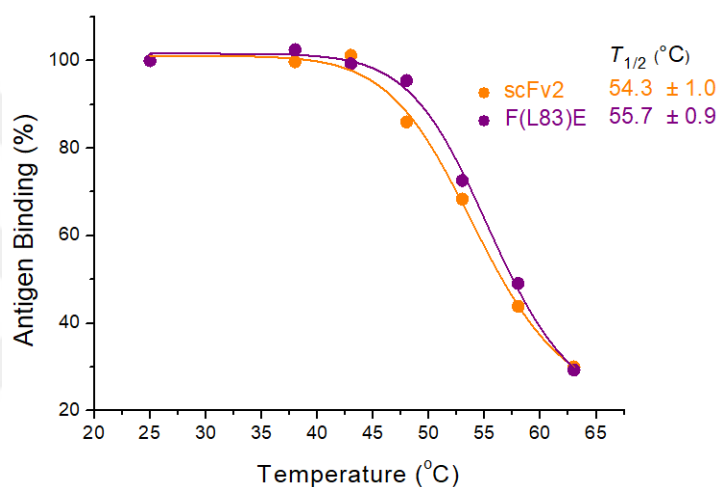


Figure 17. Thermal denaturation curves of scFv2 and F(L83)E displayed on yeast. Mouse anti-Myc primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF (10 nM) and streptavidin Brilliant Violet 421 couple was used to monitor antigen binding. Control samples were incubated with all reagents except biotinylated VEGF. The antigen-binding signal normalization was applied based on expression signal to remove the host expression bias. The $T_{1/2}$ values (transition mid-points) from the thermal denaturation curves were calculated by Hill1 equation fit in OriginPro 8.5.

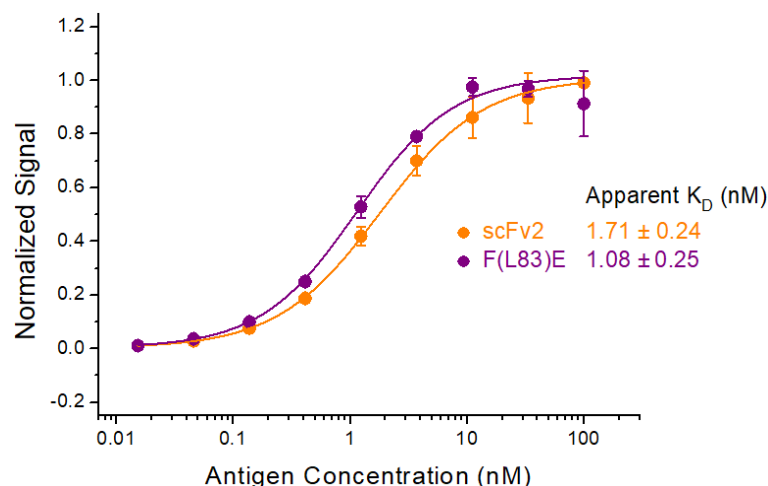


Figure 18. Antigen binding curves of scFv2 and F(L83)E displayed on yeast. Mouse anti-Myc primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin Brilliant Violet 421 couple was used to monitor antigen binding. Control samples were incubated with all reagents except biotinylated VEGF. The antigen-binding signal normalization was applied based on expression signal to remove the host expression bias. The apparent K_D values from the binding curves were calculated by Hill1 equation fit in OriginPro 8.5. The assay was repeated twice on different days, and the results were expressed as AVG $\pm$ SD.

4.6 VEGF is a more effective reporter than Protein L to monitor thermal stability of anti-VEGF scFvs on yeast

Next, it was investigated whether Protein L molecule could be used as a reporter (alone or in combination with VEGF) to monitor the folding stability of anti-VEGF scFvs and to sort thermally the most stable scFv variants from the yeast display libraries. Protein L is a conformational ligand binds to the kappa light chain of human immunoglobulins [138]. VEGF also has a conformational epitope that interacts entirely with the heavy chain variable domain of anti-VEGF scFv antibodies. Thus, Protein L and VEGF can be used simultaneously to assess the folding stability of the anti-VEGF scFvs. To that end, flow cytometry analysis was performed to assess the binding activity of Protein L and VEGF, both individually and in combination, against F(L83)E (Figure 19).

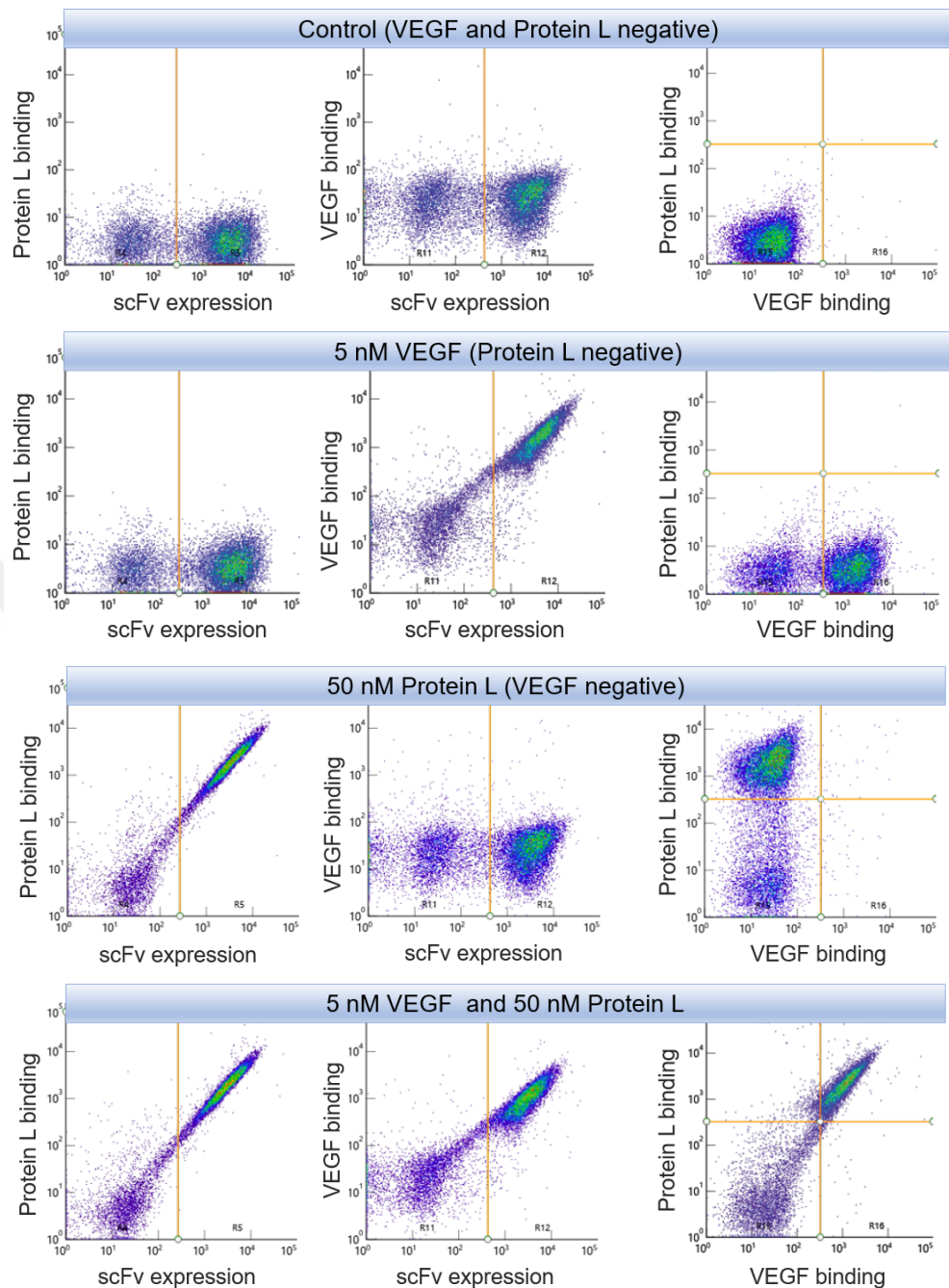


Figure 19. Protein L and VEGF binding of F(L83)E displayed on yeast. Chicken anti-Myc IgY primary antibody and goat anti-chicken secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin Brilliant Violet 421 couple was used to monitor VEGF binding. Protein L i-Flour 647 was used to monitor Protein L binding.

Encouragingly, the results showed that Protein L binds to F(L83)E displayed on yeast, and it is even possible to monitor both Protein L and VEGF binding to the anti-VEGF scFvs simultaneously (Figure19). To further test whether these two ligands can be used simultaneously to monitor folding stability of the anti-VEGF scFvs, a heat titration assay was performed. After subjecting the scFv displaying yeast cells to a heat shock step at different temperatures, Protein L and VEGF binding signals were evaluated by flow cytometry (Figure 20). The decrease in VEGF binding was well correlated with the increase in temperature. Although, Protein L binding was also well correlated with the temperature change, the VEGF binding capability of the scFvs was almost lost at the first significant decrease in Protein L binding. Since it was aimed to sort those scFv antibodies from the libraries that maintain their VEGF binding affinity while showing a better thermal stability, it was concluded that using VEGF alone for monitoring the folding stability is more efficient to sort thermally the most stable scFv variants from the yeast display libraries.

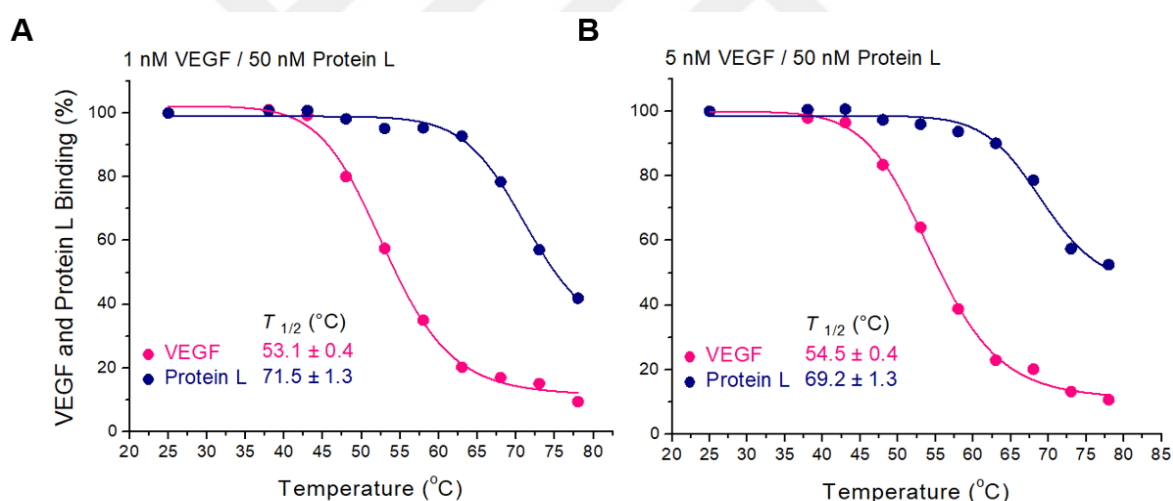


Figure 20. Thermal denaturation curves of F(L83)E displayed on yeast determined by using Protein L and VEGF simultaneously. Chicken anti-Myc IgY primary antibody and goat anti-chicken secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin Brilliant Violet 421 couple was used to monitor VEGF binding. Protein L i-Flour 647 was used to monitor Protein L binding. VEGF binding was monitored at two different concentration, 1 nM (A) and 5 nM (B). Protein L binding was monitored at 50 nM. The ligand-binding signal normalization was applied based on expression signal to remove the host expression bias.

4.7 Stability sorting procedure used in this study enables to sort thermally stable scFvs from the generated synthetic libraries

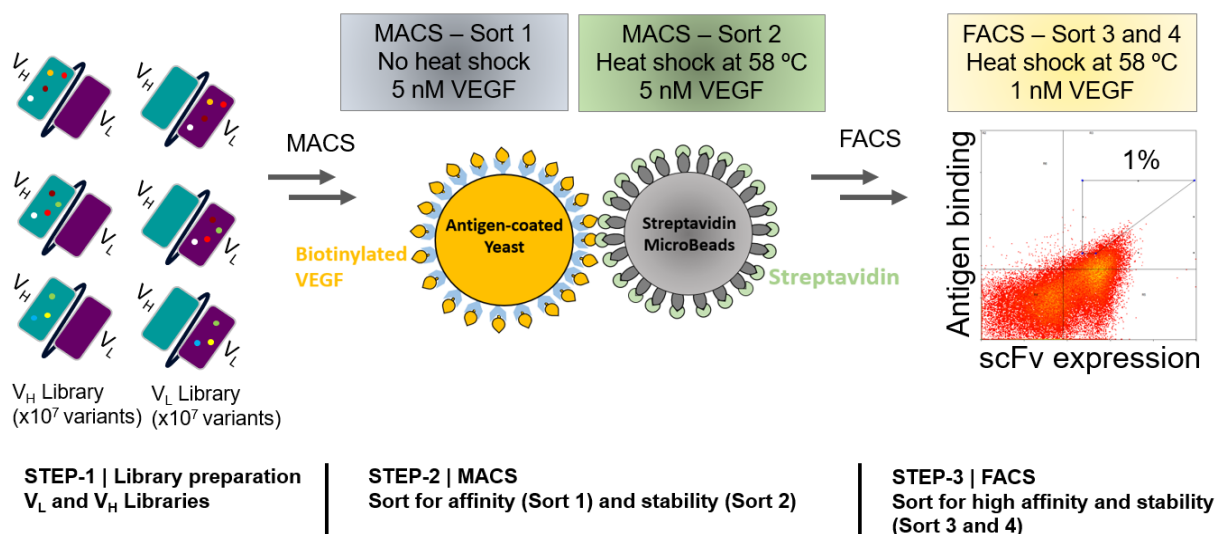


Figure 21. Summary of the thermally stable scFv discovery process using yeast surface display.

Thereafter, a series of sorting steps were conducted to obtain thermally stable anti-VEGF scFvs from the generated yeast display libraries (Figure 21). First, to remove truncated, misfolded, and low affinity scFvs from the generated scFv libraries (V_L and V_H libraries), affinity sorting at a relatively high antigen concentration was applied by MACS (Sort 1). Next, to enrich the thermally stable scFv variants in the libraries, a stability sort was performed by MACS (Sort 2) after conducting a heat titration assay at a range of 38-78 °C to determine an appropriate heat shock temperature (Figure 22 and 23). At this experiment, the two yeast libraries (V_L and V_H) were exposed to a heat shock step at a serial different temperature. Then, the yeast libraries were incubated with VEGF at two different concentration (1 and 5 nM) and the rest of the flow cytometric assay procedure was followed as previously performed. Accordingly, 58 °C was selected as the heat shock temperature to sort both scFv libraries (V_L and V_H). It was observed that, at this temperature, both libraries lose more than half their binding to VEGF (Figure 23) and maintain the triangular shape of the antigen-binding positive cell population (Figure 22) which is critical for the stability sorting process by FACS. Additionally, 1 nM VEGF was chosen as the antigen concentration for the subsequent rounds of FACS because it may allow selection of scFv variants with a

similar affinity value to scFv2 and F(L83)E for which apparent K_D values were calculated around 1 nM (Figure 18).

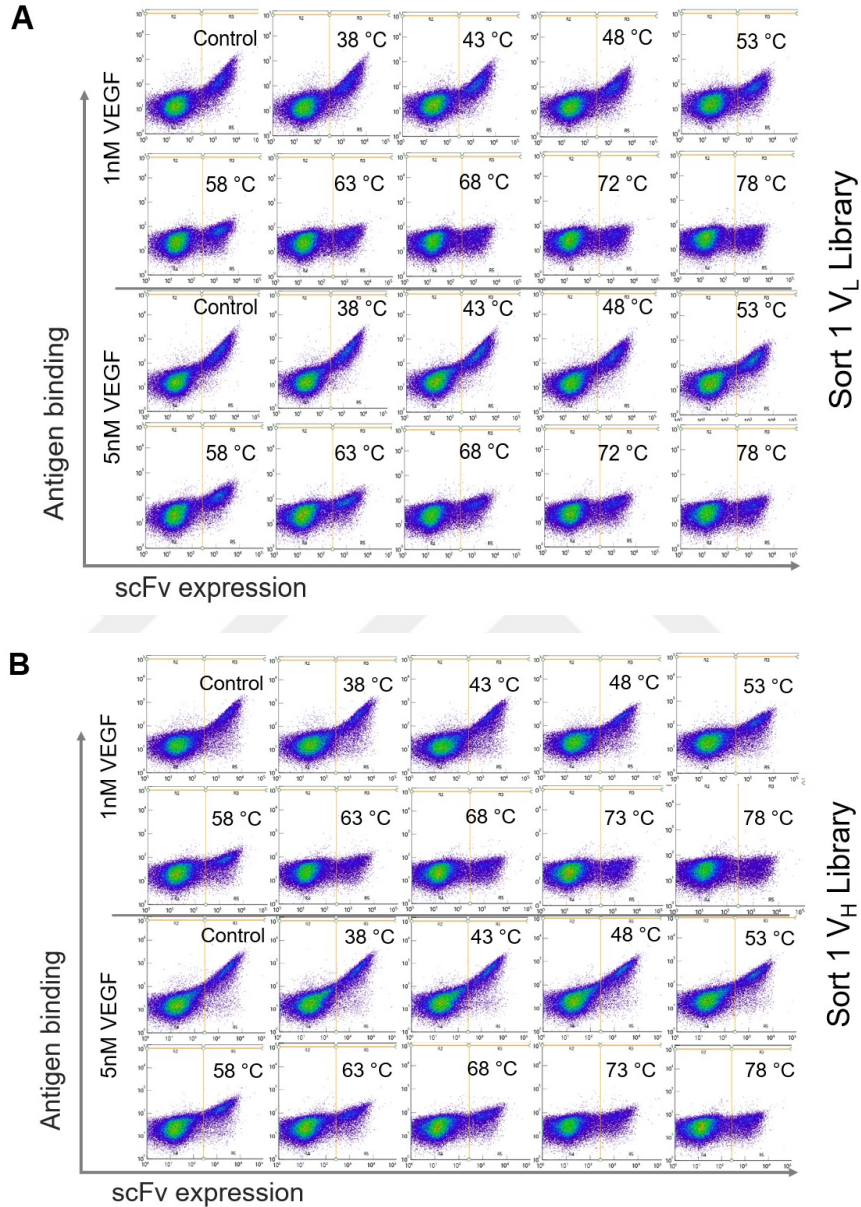


Figure 22. Heat titration results of Sort 1 V_L and V_H libraries. (A) Heat titration results of the Sort 1 V_L library and (B) Sort 1 V_H library. Mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin AlexaFluor 647 couple was used to monitor VEGF binding. VEGF binding was monitored at two different concentration, 1 nM and 5 nM.

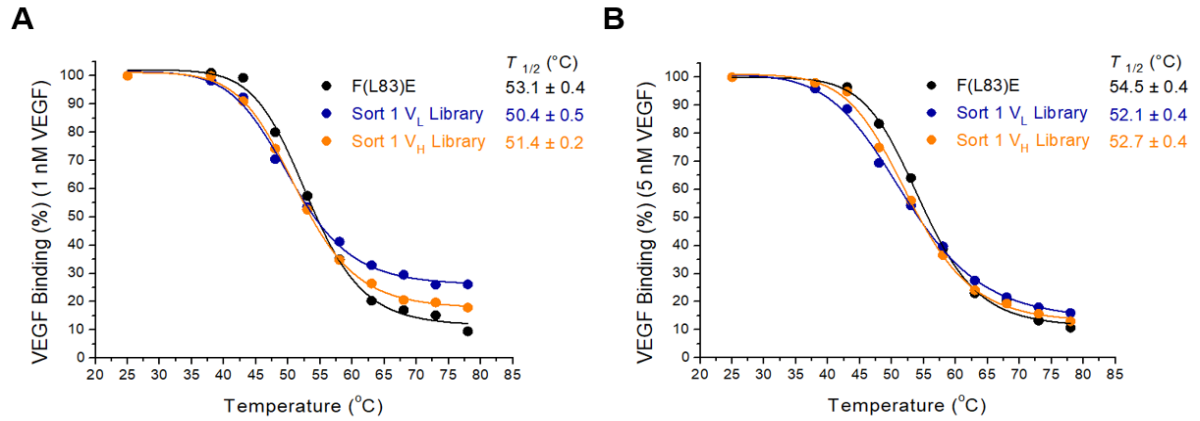


Figure 23. Heat titration curves of Sort 1 V_L and V_H libraries. Mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin AlexaFluor 647 couple was used to monitor VEGF binding. VEGF binding was monitored at two different concentration, 1 nM (A) and 5 nM (B). The antigen-binding signal normalization was applied based on expression signal to remove the host expression bias.

After one round of affinity sorting at a relatively high VEGF concentration (5 nM) by MACS (Sort 1), one round of MACS with an additional heat shock step at 58 °C was performed to enrich the thermally stable variants in the libraries (Sort 2). After the stability sorting by MACS, which yields non-viable yeast cells due to the heat shock at high temperature, the sorted libraries were recovered as discussed in the methods. Briefly, applying plasmid isolation, PCR amplification, and yeast re-transformation steps.

The recovered Sort 2 libraries were further sorted by FACS with a heat shock step at 58 °C and 1 nM VEGF (Sort 3) (Figure 23). During the FACS, $\sim 1-3 \times 10^7$ cells were processed and the top 1% population ($0.9-1 \times 10^5$ cells) that maintaining VEGF binding were collected (Figure 21).

After one round of stability sorting by FACS (Sort 3), all sorted V_L and V_H libraries (Sort1-3) and F(L83)E were compared to each other in terms of their thermal stabilities by flow cytometric analysis to see if there is an enriched distinct population that possesses a higher thermal stability (Figure 24). As a result, for the V_L library a distinct population that showed a better thermal stability was not observed at the end of the Sort 3 (Figure 24). Although there was an enrichment in Sort 2 V_L library compared to Sort 1 V_L library, it was lost in Sort 3 V_L library.

On the other hand, a distinct stable population was consistently enriched at each sorting step for V_H library (Figure 24). Moreover, that distinct population was not observed in the F(L83)E population, which means a distinct and stable population was enriched at Sort 3 V_H library. After that, Sort 3 V_H library was sorted with one more round of stability sorting by FACS at 58 °C and 1 nM VEGF again (Sort 4). After the library recovery process for Sort 3 and Sort 4 V_H libraries, a number of clones were sent for sequencing, and 25 unique variants from Sort 3 and Sort 4 V_H libraries were taken into further investigation.

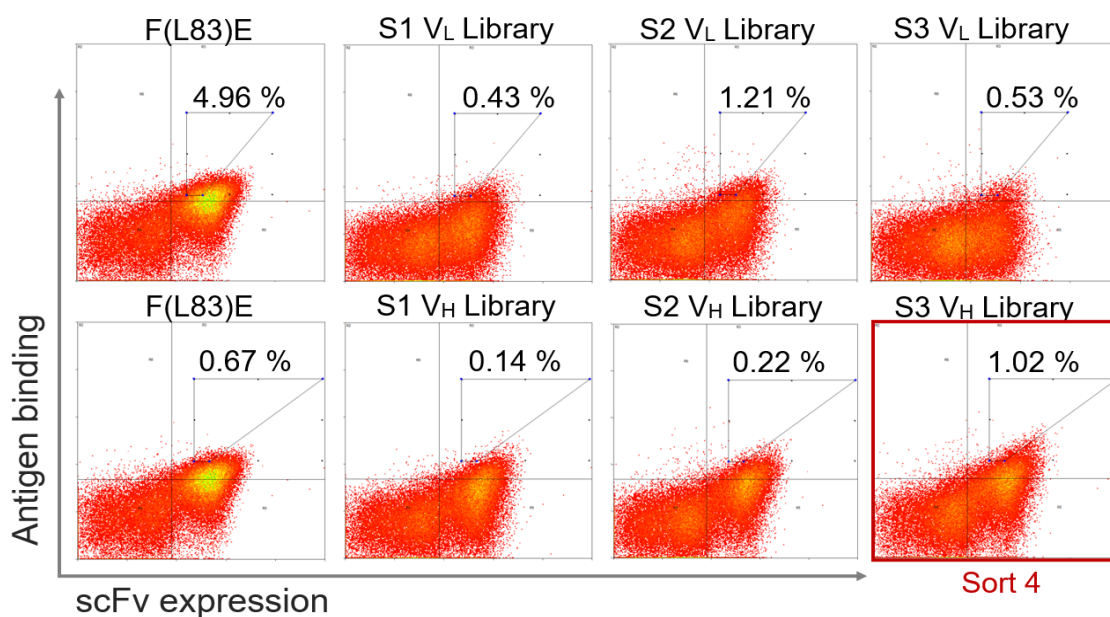


Figure 24. A distinct thermally stable population was enriched in the V_H library. Mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin AlexaFluor 647 couple was used to monitor VEGF binding. S1-3: Sort 1-3.

A stability screening assay was conducted for the 25 unique variants obtained from the V_H libraries to select the thermally most stable variants among them. Their antigen binding signals without heat shock and after a heat shock step at 58 °C were compared by a flow cytometric analysis. Those seven variants that maintain their 60% or higher antigen binding signal after the heat shock were selected as promising thermally stable variants (Figure 25 and 26).

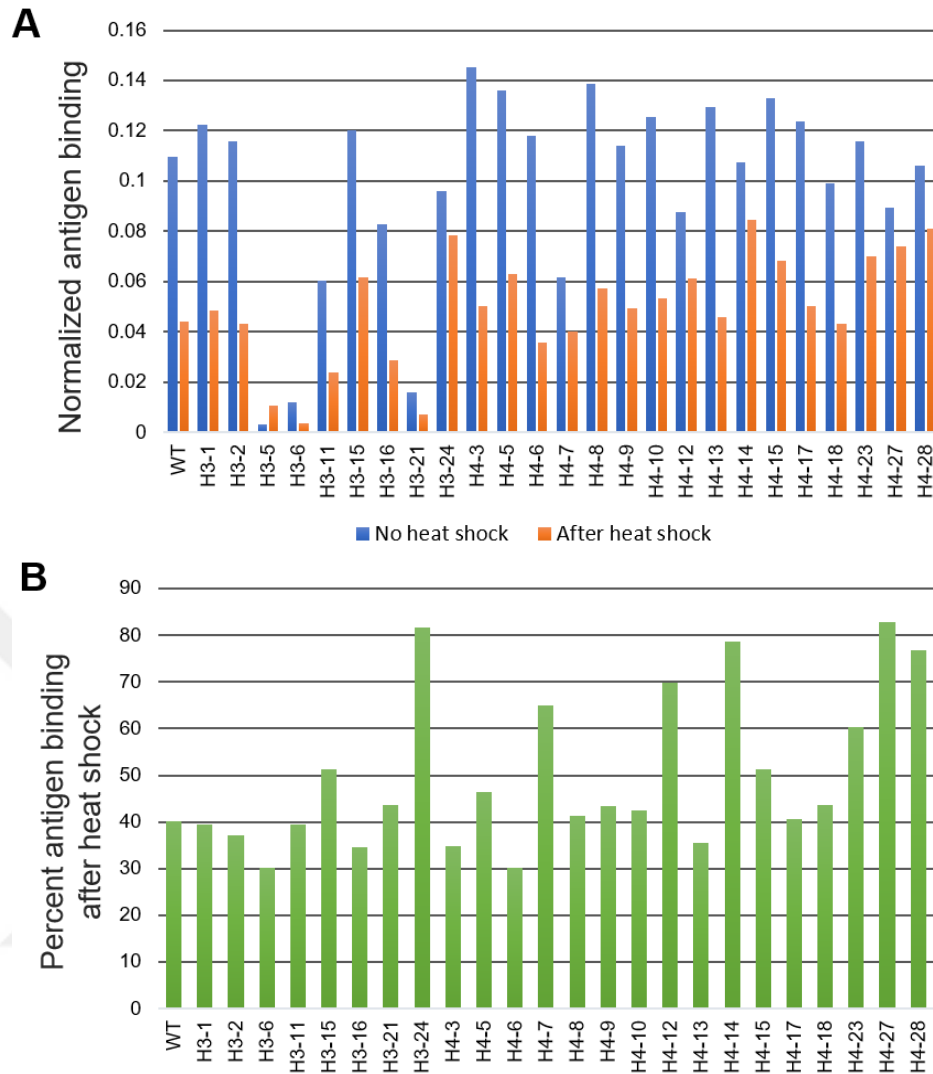


Figure 25. Stability screening results of the 25 variants obtained from the V_H library. The antigen binding signals of the variants without heat shock and after a heat shock step at 58 °C were compared by flow cytometric analysis (A), and their percent antigen binding maintained after the heat shock were calculated and represented in a bar plot (B). H3-5 was excluded in bar plot in panel B. Mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin AlexaFluor 647 couple was used to monitor VEGF binding. The antigen-binding signal normalization was applied based on expression signal to remove the host expression bias. H3 and H4 abbreviations were used to define the variants obtained from Sort 3 and Sort 4 V_H libraries, respectively. WT: F(L83)E.

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WT      EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H3-24   EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H4-7     EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H4-28    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H4-23    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H4-14    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H4-12    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
H4-27    EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTY 60
: *****

WT      AADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H3-24   AADFKGRFTMSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H4-7     AEDFKGRFTISQDESKGTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H4-28    AADFKGRFTMSQDESKNTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H4-23    AADFKGRFTMSVDRSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H4-14    AADFKSRFTMSVDASKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H4-12    AADFKGRFTISVDASKNTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
H4-27    AADFKGRFTISVDASKNTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWFVDVWGQGLTVTVSS 123
* * * * *

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Figure 26. Heavy chain variable domain sequences of the seven stable variants obtained from the V_H library. Mutational sites with wild type residues are highlighted in grey and written in bold. Mutations are highlighted in yellow and written in red. H4-7 and H4-28 variants had single unintentional mutations (R and V, respectively) in their GS linker sequences, and H4-23 had a 4-amino acid shorter linker compared to F(L83)E (data not shown). H3 and H4 abbreviations were used to define the variants obtained from Sort 3 and Sort 4 V_H Libraries, respectively. WT: F(L83)E.

Following that, a flow cytometric analysis was performed to obtain the thermal denaturation curves of the seven stable variants and F(L83)E (Figure 27). All variants showed better thermal stability compared to F(L83)E. The H3-24 variant showed around a 10 °C higher transition mid-point ($T_{1/2}$) value compared to F(L83)E. Additionally, it was further verified whether the high thermal stability of the variants was due to their high affinity, as stability and affinity sorting were performed simultaneously by FACS. To verify this, a flow cytometric analysis was performed to obtain the antigen binding curves of all variants (Figure 28). The binding affinities of all variants for VEGF were found to be very similar, with the highest value of 4.48 ± 0.48 for H4-7. It was confirmed that all seven variants obtained from the V_H library possess better thermal stability compared to F(L83)E.

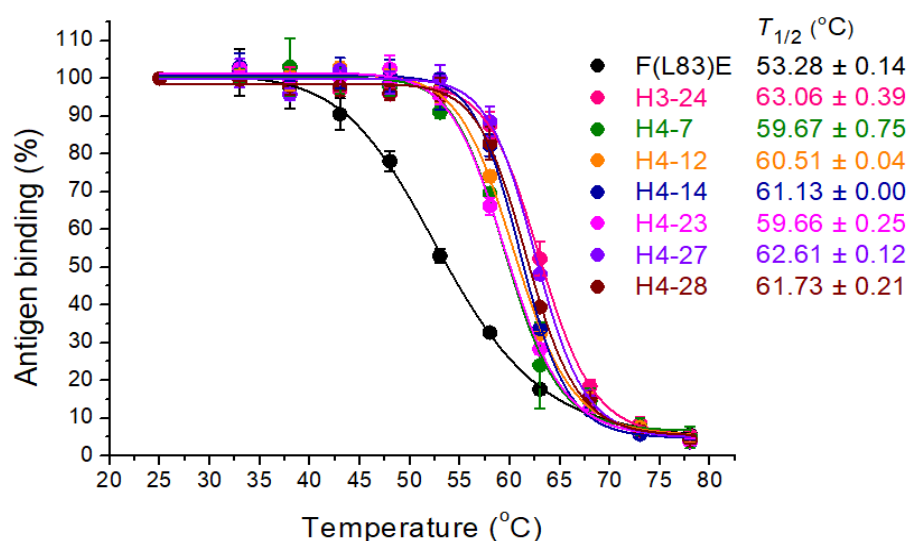


Figure 27. Thermal denaturation curves of F(L83)E and the seven stable variants obtained from V_H library. The thermal denaturation curves of the scFvs displayed on yeast were obtained by flow cytometry. Mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin AlexaFluor 647 couple was used to monitor VEGF binding. The antigen-binding signal normalization was applied based on expression signal to remove the host expression bias. H3 and H4 abbreviations were used to define the variants obtained from Sort 3 and Sort 4 V_H Libraries, respectively.

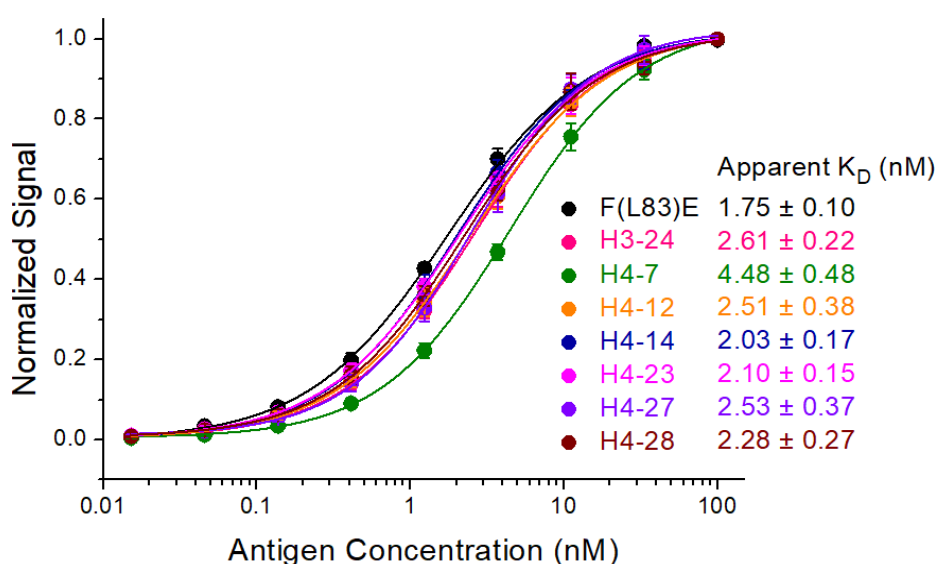


Figure 28. Antigen binding curves of F(L83)E and the seven stable variants obtained from V_H library. The antigen binding curves of the scFvs displayed on yeast were obtained by flow

cytometry. Mouse anti-Myc tag primary antibody and goat anti-mouse secondary antibody AlexaFluor 488 couple was used to monitor scFv expression. Biotinylated VEGF and streptavidin AlexaFluor 647 couple was used to monitor VEGF binding. The antigen-binding signal normalization was applied based on expression signal to remove the host expression bias. H3 and H4 abbreviations were used to define the variants obtained from Sort 3 and Sort 4 V_H Libraries, respectively.

5 DISCUSSION

One of the objectives of this thesis was to develop next-generation anti-angiogenic antibodies, given the critical role of anti-angiogenic therapy in the treatment of solid tumors, most frequently in combination with chemotherapy [13, 15-20]. Bevacizumab is the first humanized anti-VEGF monoclonal antibody in IgG format developed for anti-angiogenic therapy and is still being used in variety of indications of cancer [15, 16, 21]. On the other hand, it is known that the large size of the full-length antibodies (IgGs) precludes them from penetrating large masses of biological tissue and decreases their biological efficacy, especially in solid tumors [22, 23]. On the other hand, the single-chain variable fragments (scFvs) show better tissue penetration owing to their smaller size (~30 kDa) compared to IgGs (~150 kDa) [139]. In addition to that, as scFvs still have their parental paratope region, they can show the same binding activity as their parental antibodies in IgG format unless the orientation of the variable domains is changed due to the lack of the constant domains or the effect of the linker used to link the two variable domains [26]. On the other hand, due to the lack of constant domains, scFvs have some drawbacks in terms of their stability, which is essential in therapeutic applications [26-28]. That brought us to the second aim of this thesis, which was to develop a general antibody stability improvement method that could be further applied to different types of antibodies and antibody fragments.

First, we used two different flexible linkers to design anti-VEGF scFvs (scFv1 and scFv2) derived from bevacizumab. One of the linkers was a non-repetitive linker with the sequence of "SPNSASHSGSAPQTSSAPGSQ" (scFv1) and the other one was a repetitive linker with the sequence of "(G₃S)₄Q" (scFv2). Then, scFv1 and scFv2 were produced in the bacterial expression system. First, after verifying the purity of the scFvs by SDS-PAGE, the binding kinetics of scFv1, scFv2 and bevacizumab (obtained commercially) were analyzed against VEGF by SPR. ScFv1 and scFv2 showed comparable affinities for VEGF with K_D

values of 2.51 ± 0.01 and 0.83 ± 0.02 nM, respectively, to their parental antibody bevacizumab (0.38 ± 0.02 nM) (Figure 10B). Since bevacizumab is a divalent antibody, whereas scFvs are monovalent antibodies, the affinity values indicated that the generated anti-VEGF scFvs almost completely retain their parental binding activity to VEGF.

Next, the melting temperatures (T_m s) of scFv1 and scFv2 were measured using differential scanning fluorimetry (DSF) as an indicator of their thermal stability. Both scFv1 and scFv2 had significantly lower thermal stabilities with T_m values of 53.3 ± 0.5 °C and 50.2 ± 0.6 °C, respectively (Figure 11), compared to previously developed thermally stable scFvs with T_m values of 60 °C or higher [135-137]. That was an expected result, as scFvs generally show poorer thermal stability compared to their parental Fab and IgG formats and need further engineering studies to improve their stability [26, 28]. Along with that, high thermal stability is essential for therapeutic applications to allow significant antibody enrichment at the target tissue under the physiological conditions. For instance, Willuda *et al.* previously developed an anti-epithelial glycoprotein-2 scFv with a high affinity (3.9 nM) [27]. However, this scFv fragment was unable to enrich effectively at lung tumor xenografts in mice, owing primarily to its insufficient thermal stability. They overcame this limitation by grafting the antigen-binding residues of the anti-epithelial glycoprotein-2 scFv to a more stable scFv framework (4D5) and applying an additional modification, namely transferring eight residues of the parent antibody's heavy chain variable domain core. As in this example, insufficient thermal stability is one of the critical parameters to be addressed in early antibody development studies.

However, before proceeding with further stability improvement studies, we aimed to test the anti-angiogenic effects of the generated anti-VEGF scFvs. To that end, scFv1 and scFv2 were tested in terms of their biological efficacy in comparison with bevacizumab using the zebrafish subintestinal vessel (SIV) assay [43]. Previously, it was demonstrated that VEGF induces SIV development in zebrafish, and that this process can be inhibited by bevacizumab [43]. Likewise, we tested the angiogenic effects of scFv1 and scFv2 in comparison with that of bevacizumab in the same experimental setup. PBS (negative control), scFv1 (55 µM), scFv2 (55 µM) and bevacizumab (27.5 µM) were injected into the yolk of zebrafish embryos at the initiation phase of SIV formation between 48- and 52-h post fertilisation (2dpf), and the anti-angiogenic effect of the antibodies was evaluated the following day by examining the SIV area of the zebrafish larvae. Bevacizumab was used at

half the concentration of scFvs in the experiments due to the difference in the stoichiometric ratio with respect to VEGF. Bevacizumab is divalent and can bind two VEGF while the scFvs are monovalent and binds one VEGF (Figure 1 and 2). In comparison to the PBS control, scFv1 and scFv2 reduced SIV area by 26% and 34.4%, respectively (Figure 12F). Surprisingly, bevacizumab, had no significant effect at 27.5 μ M. When we increased bevacizumab concentration 2-fold (55 μ M), a 35.6% reduction in SIV area was observed, similar to the effect of scFv2 at 55 μ M. It was not totally an unexpected result, since it is known that the biodistribution coefficient (a tissue distribution estimation depending on plasma concentration) of smaller-sized antibody fragments is higher compared to IgGs, which can increase drug efficacy in targeted tissue [140]. Likewise, we also observed a better efficacy for anti-VEGF scFvs compared to their parental IgG format. As a conclusion, in the first part of the thesis, it was revealed that scFv1 and scFv2 derived from bevacizumab retain their affinity for VEGF. Moreover, they had higher anti-angiogenic activity on the transgenic zebrafish model compared to their parental antibody, bevacizumab (IgG). On the other hand, they demonstrated inadequate thermal stability in terms of an antibody being developed for therapeutic purposes [26-28].

Nieba *et al.* previously showed that hydrophobic residues at the former variable/constant domain interface (V/C interface) may be significant contributors to the instability of scFvs [29]. In that study, they used an anti-fluorescein scFv fragment (the scFv fragment of 4-4-20) as a model scFv fragment that show a significantly poor *in vivo* folding characteristic (2% is native when expressed in the periplasm of *E.coli*). By applying a serial mutation to the three newly exposed hydrophobic residues at the former V/C interface, they aimed to improve the *in vivo* folding of the scFv fragment of 4-4-20. Surprisingly, by just a single mutation (V84D in the V_H domain), the functional periplasmic expression yield was increased 25-fold, and the thermally induced aggregation rate was decreased. Likewise, we also identified the three newly exposed hydrophobic residues at the former V/C interface of anti-VEGF scFvs; F(L83), L(H11), and L(H108). Subsequently, by selecting scFv2 as the template due to its higher binding activity compared to scFv1 (Figure 10), we generated three anti-VEGF scFv variants, each including a single mutation, by substituting those newly exposed hydrophobic residues, at the former V/C interface, with the charged amino acids; F(L83)E, L(H11)D, and L(H108)D. Following the bacterial production of these scFv variants with single mutations, their binding kinetics and melting temperatures were determined as

previously described (Figure 13B, 14). While the affinity values of each variant were comparable to scFv2, F(L83)E was the only variant that demonstrated an insignificant slight increase in thermal stability compared to wild type scFv2. The other variants demonstrated even lower thermal stability (Figure 14). As a result, this finding revealed the need to apply different approaches to improve the thermal stability of scFv2.

There are several different factors that would be taken into account to improve scFv stability, such as hydrophobic amino acids on the scFv surface, hydrophilic residues in the interior beta sheets, and hydrophilic residues in the normally hydrophobic V_L/V_H interface [26, 28]. In addition to that, it is also possible to apply CDR grafting to a known stable scFv framework to generate a new stable scFv fragment that contains the CDR regions of the unstable former scFv and the framework regions of the stable scFv [27]. However, there is not a general method that can be applied to all antibody fragments, including the scFvs. In addition to that, generating protein variants with single mutations and/or their combinations one by one and testing the effects of these mutations in terms of desired features is a laborious, time-consuming, and costly process. To address this, a number of computational tools for predicting the thermodynamic or thermal stability of proteins have been developed over the years using a variety of methods, most commonly based on the prediction of a difference in free energy of unfolding [31-33]. Due to the current limitations of these methods, their success is not at the desired level, and it is advisable to utilize multiple methods based on multiple different approaches to increase the feasibility of obtaining the desired increase in stability [33]. All these factors show that it is important to develop a general method for improving the stability of antibody fragments.

In accordance with this purpose, we generated a synthetic library design strategy by utilizing different types of stability prediction methods and some additional tools to improve the thermal stability of the anti-VEGF scFv fragments (for details, see Section 3.5.14 in the methods and Table 24 in this section). In this strategy, we utilized more than one prediction method to overcome the individual limitations of these methods. In addition to that, we selected mutation types depending on the antibody natural repertoires instead of directly applying the mutations determined by the prediction tools, which means we utilized these tools to only determine those residues that are more available to mutate to increase the scFv stability. We also considered rare framework residues in the wild type scFv sequence as previously described by Chowdhury and Vasmatzis [28] which is a strategy depending on the

importance of the framework residues in forming the Fv scaffold. Since framework residues largely define the folding and stability of the Fv fragment, rare framework residues, which are normally conserved over the other Fv sequences, could be more susceptible to the instability of the scFv structure [28]. With some other critical steps (see method section and Table 24) we designed two scFv libraries using *F(L83)E* as the template DNA sequence, namely the V_L and V_H libraries.

Table 24. The steps of the synthetic library design for improved scFv stability

Step 1	Selection of Mutable Sites	If one or more favorable mutations are predicted by at least two stability prediction tools (FoldX, CUPSAT, DeepDDG, PoPMuSIC, MAESTRO, HoTMuSIC)
		If the wild type framework residue is rare in the natural repertoire (<5%) (AbYsis)
Step 2	Exclusion	If the wild type residue is found as an interfacing and/or interacting residue with the antigen (PDBePISA)
		If the wild type residue is conserved (≥ 50) and/or has the highest frequency in the natural repertoire (AbYsis)
		If the wild type residue predicted as a stabilizing residue (SCide)
Step 3	Selection of Mutations for Mutable Sites	Amino acids that have $\geq 3\%$ or higher frequency in the natural repertoire (AbYsis)

On the other hand, preparing a purpose-based synthetic library is not enough to be able to obtain antibody fragments with the desired characteristics. Besides that, it is essential to employ a screening step to be able to sort those variants with the desired characteristics from the generated libraries. Display techniques (e.g., phage display, yeast surface display) are the most powerful screening techniques for those kinds of purposes, as they enable very rapid screening of antibodies with the desired characteristics from synthetic/semi-synthetic or natural antibody libraries [35-42]. Previously, Traxlmayr *et al.* utilized the yeast surface display technique to further improve the thermal stability of IgG1-Fc antibodies that were already stable. In that study, they developed highly stable IgG1-Fc variants with T_m values of up to 85 °C using that approach. In this technique, they added a heat shock step to the yeast surface display technique at 79 °C for 10 min before sorting yeast cells by FACS. That heat shock step allows to only sort those antibody variants that still retain their proper folding characteristics after the heat shock. On the other hand, since yeast cells almost lose their

viability completely at 50 °C for 10 min [38], in this approach, they performed plasmid isolation after each round of stability sorting. Subsequently, after amplifying those plasmids, they were used to reconstruct the sorted yeast libraries by transforming yeast cells with those plasmids. This strategy was also successfully used to develop loop-inserted GFP scaffolds [37] which means this approach is also applicable to proteins other than antibodies. Likewise, we also utilized the same strategy to sort thermally stable scFv variants from the generated libraries. First, to evaluate the applicability of the yeast surface display technique for anti-VEGF scFvs, we verified the scFv expression level and antigen binding activity of the scFvs on the yeast. According to the results, the scFv display level on yeast cells was typically in the range of ~50-55% (Figure 15). In addition to that, the findings showed that the anti-VEGF scFvs maintain their binding affinities to VEGF (Figure 16).

Following the validation of the yeast surface display system for anti-VEGF scFvs, antigen binding affinity and thermal stability of wild type scFv2 and F(L83)E were assessed on yeast. The results showed that scFv2 and F(L83)E had similar properties, with F(L83)E having slightly better apparent K_D (1.71 ± 0.24 vs 1.08 ± 0.25) and melting temperature (54.3 ± 1.00 vs 55.7 ± 0.9) (Figure 17 and 18).

Next, we evaluated whether we could use Protein L as a conformational specific ligand for the stability sorting process (alone or in combination with VEGF). For that, we performed a flow cytometric analysis to assess the binding activity of F(L83)E against Protein L and VEGF, by using each ligand individually and simultaneously (Figure 19). The results showed that Protein L binds to F(L83)E displayed on yeast, and it is also possible to monitor both Protein L and VEGF interaction with anti-VEGF scFvs simultaneously (Figure 19). To further test if Protein L and VEGF can be used to monitor the folding stability of the scFvs simultaneously, we carried out a heat titration assay. After subjecting the scFv displaying yeast cells to heat shock step at different temperatures, Protein L and VEGF binding signals were evaluated by flow cytometry (Figure 20). We observed that the decrease in VEGF binding was well correlated with the increase in temperature. Although, Protein L binding was also well correlated with the temperature change, the VEGF binding capability of the scFvs was almost lost at the first significant decrease in Protein L binding. However, as we were aiming to sort those scFv antibodies from the libraries that maintain their VEGF binding affinity while showing better thermal stability, we concluded that using VEGF alone for

stability sorting is more efficient as it allows us to observe the effect of the temperature dramatically over a wider range of temperatures.

After the optimization and validation studies, a series of sorting steps were conducted to obtain thermally stable anti-VEGF scFvs (Figure 21). Due to the relatively high library diversity ($\times 10^7$) we aimed to decrease library sizes to a level that would be sorted more effectively by FACS, especially to make sure that each unique variant in the libraries is processed during the sorting. For that reason, we first applied two-step MACS sorting before FACS. First, to remove truncated, misfolded, and low affinity scFvs from the generated scFv libraries (V_L and V_H libraries), we applied affinity sorting by MACS (Sort 1). Then, to further decrease the library sizes and enrich the thermally stable scFv variants in the libraries, we performed one round of stability sorting by MACS (Sort 2) with a heat shock step at 58 °C which was selected as the heat shock temperature after conducting a heat titration assay at a range of 38-78 °C (Figure 22 and 23). It was observed that, at 58 °C both libraries lose more than half their binding to VEGF (Figure 23) and maintain the triangular shape of the antigen-binding positive cell population (Figure 22) which is critical for the stability sorting process by FACS. Additionally, as another outcome of this experiment, we selected 1 nM VEGF concentration as the antigen concentration for FACS applications. Because the primary objective was to sort scFv variants that retain their parental scFv binding characteristics but are thermally more stable, we kept the antigen concentration close to the apparent K_D values of wild type scFv2 and F(L83)E (Figure 18) in order to sort the scFv variants with higher affinities.

As previously discussed, since yeast cells are not viable after the heat shock at high temperatures, a library recovery process was performed as discussed in the methods after the stability sorting by MACS (Sort 2). Briefly, plasmid isolation, PCR amplification, and yeast re-transformation steps were applied. Then, the recovered Sort 2 libraries were further sorted by FACS with a heat shock step at 58 °C and 1 nM VEGF (Sort 3) (Figure 23). During the FACS, in a similar way described previously [37, 38] $\sim 1-3 \times 10^7$ cells were processed and the top 1% population ($0.9-1 \times 10^5$ cells) maintaining VEGF binding was collected (Figure 21).

After one round of stability sorting with FACS (Sort 3), all sorted V_L and V_H libraries (Sort1-3) and F(L83)E were compared to each other in terms of their thermal stabilities by a flow cytometric analysis to see if there is an enriched distinct population that possesses a higher thermal stability (Figure 24). As a result, we couldn't enrich a distinct stable

population in the V_L library at the end of Sort 3 (Figure 24). On the other hand, we could achieve enrichment of a distinct stable population in the V_H library (Figure 24). Moreover, that distinct population was not observed in the F(L83)E population. That result showed that a distinct and stable population enrichment was achieved in the Sort 3 V_H library. After that, the Sort 3 V_H library was sorted with one more round of stability sorting by FACS (Sort 4) at the same conditions, and 25 unique variants from Sort 3 and Sort 4 V_H libraries were taken into investigation in terms of their binding affinities to VEGF and thermal stabilities.

First, to determine the thermally more stable variants among the 25 variants obtained from the Sort 3 and Sort 4 V_H libraries, we conducted a stability screening assay. In this screening assay, we measured the antigen binding signals of the variants without heat shock and after a heat shock step at 58 °C by flow cytometry. As a result, we selected the seven most stable variants that maintained their 60% or higher antigen binding signal after the heat shock as the promising stable variants (Figure 25 and 26).

Finally, we analyzed the binding affinities and thermal stabilities of these seven promising variants (Figure 27 and 28). Encouragingly, all variants showed better thermal stabilities compared to the parental scFv variant, F(L83)E. Among them, H3-24 showed around a 10 °C higher transition mid-point ($T_{1/2}$) value compared to F(L83)E. Furthermore, we wanted to verify whether the high thermal stability of the variants was due to a bias caused by their high binding affinities, as stability and affinity sorting were performed simultaneously by FACS. To verify this, a flow cytometric analysis was used to obtain the antigen binding curves for all variants (Figure 28). However, the binding affinities of all variants (~2-4.5 nM) for VEGF were found to be very similar that of F(L83)E (~1.8 nM). As a conclusion, it was confirmed that all seven variants obtained from the V_H library have better thermal stability compared to F(L83)E.

6 CONCLUSION & FUTURE PERSPECTIVES

In this thesis, we developed next-generation anti-angiogenic antibody fragments in scFv format by utilizing the sequence information of bevacizumab. Further, we addressed the thermal stability issue of these generated scFvs, which is a common limitation of scFv fragments. While doing that, we developed a general, straight-forward antibody thermal stability improvement method by utilizing different computational methods and directed

evolution techniques, including yeast surface display technology. We believe that this strategy can be applied to any kind of antibody to improve its thermal stability.

For future studies, the biological efficacy of the thermally stable anti-VEGF scFvs generated in this study is yet to be investigated. As well, the developed antibody stability improvement method can be further tested in terms of its general applicability to other kinds of antibodies and antibody fragments, even different types of proteins other than antibodies, with some modifications to the method.

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8 APPENDIX

8.1 Ethics Approval from IBG Animal Experiments Local Ethics Committee (IBG-AELEC)



**İZMİR BİYOTİP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI**

Toplantı Tarihi : 11/03/2020 **Toplantı Günü** : Çarşamba
Toplantı Sayısı : 4 **Toplantı Saati** : 15:30

Sayın Doç. Dr. Sinan GÜVEN,

2020-013 Protokol No'lu; yürütücüsü olduğunuz "Kanser tedavisi için protein mühendisliği yaklaşımları ile geliştirilmiş anti-VEGF antikorlarının araştırılması" başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Doç. Dr. Güneş ÖZHAN BAYKAN Başkan	Vet. Hek. Kerem ESMEN Başkan Yardımcısı
Prof. Dr. H. Alper BAĞRIYANIK Üye (Katılmadı)	Prof. Dr. Ensari GÜNELİ Üye
Doç. Dr. Kasım DİRİL Üye (Katılmadı)	Dr. Öğr. Üyesi Ayşe Banu DEMİR Üye
(Projede araştırmacı) Dr. Gülçin ÇAKAN AKDOĞAN Üye	Vet. Hek. Umur KELEŞ Üye
H. Muammer KARAGÖZ Üye	

8.2 Curriculum Vitae in ARBIS format

MURAT KARADAĞ	
Kişisel Bilgiler	
İletişim Bilgileri	
İletişim Adresi	İzmir Uluslararası Biyotıp Ve Genom Enstitüsü/Merkezi (İBG-İZMİR) Dokuz Eylül
Telefon	
E-posta	
İnternet Sayfası	http://ibg.edu.tr/
Öğrenim Bilgileri	
01 Şubat 2018 - Şu Anda (4 yıl 3 ay) Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ, GENOM BİLİMLERİ VE MOLEKÜLER BİYOTEKNOLOJİ ANABİLİM DALI Ağırlıklı Genel Not Ortalaması: 3.86 / 4.0	
01 Eylül 2010 - 01 Ocak 2012 (1 yıl 5 ay) Yüksek Lisans, Tezli Program, EGE ÜNİVERSİTESİ, TÜRKİYE FEN BİLİMLERİ ENSTİTÜSÜ, BİYOKİMYA (YL) Tez Başlığı: Bazı Fonksiyonel ve Biyoaktif Yüzeylerin Elektrokimyasal ve Optik Biyosensör Uygulamalarında Kullanım Potansiyellerinin İncelenmesi Tez Konusu: Bu tez kapsamında iki kısımdan oluşan bir çalışma gerçekleştirilmiştir;İlk kısımda, poliamidoamin denimeri (PAMAM) ile modifiye edilmiş altın elektrot yüzeyinde altın nanopartiküllerin (AuNP) ve mediyatör olarak 6-(Ferrosenil) heksantiyol (FcSH)'ün kullanıldığı yeni bir amperometrik biyosensör geliştirilmiştir. İkinci kısımda ise, "click" kimyası ile hazırlanmış olan polisülfon (PSU-Py) yapılı polimerin mikrokuyucuk içeren plakaların tabanlarına bir membran olarak kaplanıp, oluşan bu platformun oksidaz ve hidrolaz/oksidaz temelli enzim analizlerindeki kullanım potansiyeli incelenmiştir. Her iki sensör platformunun kullanılabilirliği tez çalışması kapsamında test edilmiştir. Tarih: 2012 Tez Danışmanı: SUNA TİMUR Diploma Numarası: 5033 Ağırlıklı Genel Not Ortalaması: 94.8 / 100.0	
01 Eylül 2006 - 01 Haziran 2010 (3 yıl 10 ay) Lisans, Anadal/Normal Öğretim, EGE ÜNİVERSİTESİ, TÜRKİYE FEN FAKÜLTESİ, BİYOKİMYA BÖLÜMÜ Diploma Numarası: 16874 Ağırlıklı Genel Not Ortalaması: 3.26 / 4.0	
Deneyim / İşyeri Bilgileri	
01 Şubat 2016 - 01 Ekim 2017 (1 yıl 9 ay) (Tam Zamanlı) İDARI PERSONEL, AYBAK NATURA ANALİZ LAB. HİZ. TUR. SAN. VE TİC. LTD. ŞTİ. İdari Görev: YÖNETİCİ	
01 Eylül 2013 - 01 Şubat 2016 (2 yıl 6 ay) (Tam Zamanlı) ŞEF, EDGE GIDA YEM ÇEVRE SAĞ. ANALİZ LAB. HİZ. ARGE DAN. HİZ. S. TİC. LTD. ŞTİ.	

01 Nisan 2011 - 01 Nisan 2013 (2 yıl 1 ay) (Yarı Zamanlı)
AR-GE PERSONEL, TÜRKLAB TIBBİ MALZEMELER SAN. VE TİC. A.Ş.

Yabancı Dil Bilgileri

İNGİLİZCE (Okuma: İyi, Yazma: İyi, Konuşma: İyi)

Bilimsel Teknolojik Faaliyet Alanları

Bilimsel Teknolojik Faaliyet Alanı Bilgileri

Temel Bilimler -- Yaşam Bilimleri -- Moleküler Biyoloji ve Genetik -- Protein Mühendisliği

Ar-Ge Yetkinlik

Kitaplar

H. ALOTAİBİ, M. KARADAĞ, S. KALYONCU UZUNLAR, M. C. YARIMÇAM, M. ARSLAN & N. E. KALELİ ÇOBANOĞLU, KODON OPTİMİZASYONU VE REKOMBİNANT PROTEİN ÜRETİMİNDEKİ YERİ, D. S. E. ACUNER [Editörler], PROTEİN: YAPISI, MÜHENDİSLİĞİ, ETKİLEŞİMLERİ, DİNAMİĞİ VE İLAÇ TASARIMINDAKİ YERİ(115 - 134), ISBN: 9786257564038, TÜRKİYE: Ankara Nobel Tıp Kitabevleri, 01 Ocak 2021, Kitapta Bölüm.

N. E. KALELİ ÇOBANOĞLU, M. KARADAĞ, M. ARSLAN & S. KALYONCU UZUNLAR, Physicochemical determinants of antibody-protein interactions, R. DONEV [Editörler], ADVANCES IN PROTEIN CHEMISTRY AND STRUCTURAL BIOLOGY(85 - 105), ABD: Academic Press, 20 Nisan 2020, Kitapta Bölüm.

Makaleler

M. ARSLAN, M. KARADAG, E. ÖNAL, E. GELİNCİ, G. ÇAKAN AKDOĞAN & S. KALYONCU UZUNLAR, Effect of non-repetitive linker on in vitro and in vivo properties of an anti-VEGF scFv, Scientific Reports, 2022, 2045-2322, 12, 1, 5449.

N. E. KALELİ, M. KARADAG & S. KALYONCU, Phage display derived therapeutic antibodies have enriched aliphatic content: Insights for developability issues, PROTEINS-STRUCTURE FUNCTION AND BIOINFORMATICS, 2019, 0887-3585, 87, 7, 607-618.

M. KARADAĞ, C. GEYİK, D. ODACI DEMİRKOL, F. N. ERTAŞ & S. TİMUR, Modified Gold Surfaces By 6-(ferrocenyl)hexanethiol/dendrimer/gold Nanoparticles As A Platform For The Mediated Biosensing Applications, MATERIALS SCIENCE & ENGINEERING C-MATERIALS FOR BIOLOGICAL APPLICATIONS, 2013, 0928-4931, 33, 2, 634-640.

M. KARADAG, G. YILMAZ, H. TOİSERKANİ, D. O. DEMİRKOL, S. SAKARYA, L. TORUN, S. TİMUR & Y. YAGCI, Polysulfone/Pyrene Membranes: A New Microwell Assay Platform for Bioapplications, MACROMOLECULAR BIOSCIENCE, 2011, 1616-5187, 11, 9, 1235-1243.

Bildiriler

M. KARADAĞ, D. KARADAĞ & S. KALYONCU UZUNLAR, Aggregasyona Dirençli Anti-VEGF Antikor Parçackların Geliştirilmesi , Sözlü Sunum, V. Türkiye in vitro Diyagnostik Sempozyumu, 19 Şubat 2020, 21 Şubat 2020.

M. KARADAĞ, M. ARSLAN, D. KARADAĞ, G. ÇAKAN AKDOĞAN & S. KALYONCU UZUNLAR, Tek zincirli antikor parçacğında kullanılan bağlayıcı peptitler afiniteyi ve anti-anjiyogenez etkinliğini değiştirmektedir, Sözlü Sunum, Onkolojide İz Bırakanlar 2019, 14 Kasım 2019, 17 Kasım 2019.

M. KARADAĞ, M. ARSLAN, D. KARADAĞ & S. KALYONCU UZUNLAR, Peptide Linker Characteristics of An Anti-VEGF scFv Antibody Affects Both Its Biophysical Properties and Efficacy In Cancer, Sözlü Sunum, 2nd International Cancer And Ion Channels Congress 2019, 22 Eylül 2019, 24 Eylül 2019.

M. KARADAĞ, N. E. KALELİ & S. KALYONCU UZUNLAR, Faj Gösterim Tekniği Antikorların Antijen Bağlama Bölgelerini Alifatik Rezidüleriyle Zenginleştirir, Sözlü Sunum, TBD ULUSLARARASI BİYOKİMYA KONGRESİ 2018 / 29. ULUSAL BİYOKİMYA KONGRESİ, 26 Ekim 2018, 30 Ekim 2018.

N. E. KALELİ ÇOBANOĞLU, M. KARADAĞ & S. KALYONCU UZUNLAR, Physicochemical Properties Determine Poly-reactivity and Self-interaction of Monoclonal Antibodies, Poster Sunumu, 6th International Congress of the Molecular Biology Association of Turkey (molbiyokon2018), 05 Eylül 2018, 08 Eylül 2018.

M. KARADAĞ, M. YÜKSEL, D. ODACI DEMİRKOL & S. TİMUR, 6-(ferrosenil)hegzantiyol/PAMAM (Poliamidoamin)/Laktat Dehidrogenaz (LDH) Modifiye Altın Yüzeylerin Hazırlanması ve Laktik Asit Analizinde Kullanılması, Poster Sunumu, 24. Ulusal Kimya Kongresi, 29 Haziran 2010, 02 Temmuz 2010.

M. YÜKSEL, M. KARADAĞ, D. ODACI DEMİRKOL & S. TİMUR, Fermente Gıda Örneklerinde Laktatın Analizlenmesine Yönelik Laktat Oksidaz ve Dehidrogenaz Temelli Elektrokimyasal Biyosensörlerin Tasarımı ve Uygulamaları, Sözlü Sunum, 24. Ulusal Kimya Kongresi, 29 Haziran 2010, 02 Temmuz 2010.

M. KARADAĞ, S. SUNGUR, C. GEYİK, D. ODACI DEMİRKOL & S. TİMUR, Effects of gold nanoparticles on the response of 6-ferrocenyl-1-hexanethiol mediated and polyamidoamine modified glucose oxidase biosensor, Poster Sunumu, Conference on Nanomaterials and Nanosystems (Nanomats2009), 10 Ağustos 2009, 13 Ağustos 2009.

S. SUNGUR, M. KARADAĞ, C. GEYİK, Ç. ÖZDEMİR, D. ODACI DEMİRKOL & S. TİMUR, 6-(ferrosenil)hegzantiyol ve PAMAM (Poliamidoamin) dendrimerleriyle modifiye ikinci nesil glukoz oksidaz (GOX) biyosensörü, Poster Sunumu, 23. ULUSAL KİMYA KONGRESİ, 16 Haziran 2009, 20 Haziran 2009.

Ödüller

Diğer, Ulusal, En İyi Sözlü Sunum, Aggregasyona Dirençli Anti-VEGF Antikor Parçacıklarının Geliştirilmesi, Ödül Alınan Kurum: V. Türkiye in vitro Diyagnostik Sempozyumu (Sistemde kayıtlı olmayan kuruluş), TÜRKİYE, 21 Şubat 2020.

Diğer, Uluslararası, En İyi İkinci Sözlü Sunum, Peptide Linker Characteristics of An Anti-VEGF scFv Antibody Affects Both Its Biophysical Properties and Efficacy In Cancer, Ödül Alınan Kurum: 2nd International Cancer & Ion Channels Congress (Sistemde kayıtlı olmayan kuruluş), TÜRKİYE, 24 Eylül 2019.

TÜBİTAK Burs ve Destekleri

BİDEB Destekleri

MURAT KARADAĞ, Araştırma Burs ve Destekleri Müdürlüğü, 2214-A Yurt Dışı Araştırma Burs Programı (Doktora öğrencileri için), Destekleniyor, 2020 - 1,

17.01.2021 - 17.07.2021.

MURAT KARADAĞ, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2211-A Genel Yurt İçi Doktora Burs Programı, Burs/Destek Durduruldu, 2019 - 2, 01.06.2019 - 31.05.2023.

Panelistlik/İzleyicilik/Raportörlük Sayısı

Hakemlik/Panelistlik/Dış Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
İzleyicilik/Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
Raportörlük Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0

8.3 Publication list

1. Karadag, M., et al., *Physicochemical determinants of antibody-protein interactions*. Advances in Protein Chemistry and Structural Biology, 2020. **121**: p. 85-114.
2. Arslan, M., et al., *Effect of non-repetitive linker on in vitro and in vivo properties of an anti-VEGF scFv*. Scientific Reports, 2022. **12**(1): p. 1-8.

