

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**DETECTING BINDING ACTIVITY OF A THERAPEUTIC MONOCLONAL
ANTIBODY TARGETING VASCULAR ENDOTHELIAL GROWTH FACTOR
USING SURFACE PLASMON RESONANCE**



M.Sc. THESIS

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Department of Molecular Biology - Genetics and Biotechnology

Molecular Biology - Genetics and Biotechnology Programme

JULY 2024

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**VASKÜLER ENDOTELYAL BÜYÜME FAKTÖRÜNÜ
HEDELEYENTERAPÖTİK MONOKLONAL ANTİKORUN BAĞLANMA
AKTİVİTESİNİN YÜZEY PLAZMON REZONANS İLE BELİRLENMESİ**

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ABBREVIATIONS

SPR	: Surface Plasmon Resonance
Ig's	: Immunoglobulins
mAb	: Monoclonal Antibody
Fab	: Antigen-Binding Domain
CDR	: Complementarity-Determining Region
VEGFR	: Vascular Endothelial Growth Factor Receptor
USP	: Upstream Process
DSP	: Downstream Process
CQA	: Critical Quality Attributes
HPLC	: High Performance Liquid Chromatography
CE	: Capillary Electrophoresis
ELISA	: Enzyme-Linked Immunosorbent Assay
SCK	: Single Cycle Kinetic
CV %	: Coefficient Variation Percentage
RI	: Refractive Index



SYMBOLS

k_a	: Association Rate Constant
k_d	: Dissociation Rate Constant
K_D	: Equilibrium Constant
R_{max}	: Maximum Analyte Binding Capacity of The Surface
χ^2	: Chi Square





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DETECTING BINDING ACTIVITY OF A THERAPEUTIC MONOCLONAL ANTIBODY TARGETING VASCULAR ENDOTHELIAL GROWTH FACTOR USING SURFACE PLASMON RESONANCE

SUMMARY

The use of biopharmaceuticals, particularly monoclonal antibodies (mAbs), has become a major application following advancements in recombinant DNA technology. The treatment of a wide range of diseases, among them cancer, asthma, cardiovascular conditions, infections, inflammatory and autoimmune disorders, including allergies, has become possible with the development of mAbs. Surface plasmon resonance (SPR) is a well-known and reliable method that can evaluate biomolecular interactions in real-time in terms of binding affinity and kinetics without requiring labeling procedures. Using SPR technology to determine the kinetic parameters of a mAb molecule resulting from its binding with its target receptor is a common approach to characterize the binding activity of the final product. To characterize the binding kinetics between two molecules, one molecule is first stabilized on the sensor chip surface by immobilization or capturing steps. Then, the other molecule is flowed over the sensor chip surface at variety concentrations in a running buffer to observe the interaction between the two molecules and to determine the resulting kinetic parameters.

In this study, after an IgG monoclonal antibody was captured on a Protein A chip surface, the target VEGF121 growth factor was flowed over the surface at five different concentrations. By employing single-cycle kinetic analysis, the kinetic parameters were measured, providing information about the interaction between the two molecules. To develop a robust kinetic assay, it was necessary to optimize several parameters, including the capture molecule (mAb) concentration, the concentration range of the VEGF121 molecule, dissociation time, regeneration time, and the established method sequence. While evaluating the robustness and reliability of the method, important factors considered included quality control parameters from the evaluation software, plotted residuals, and the consistency of $R_{\max}$ and K_D values between replicates. As a result of the method development studies, a capture concentration of 13.4 nM, a dissociation time of 240 seconds and a regeneration time of 30 seconds were decided upon, ensuring that the deviation in K_D values between repeated studies was less than 20 %. After the K_D value was obtained with low deviation between replicate studies, qualification studies including specificity, system precision, repeatability and intermediate precision parameters were initiated. As a result of 6 replicate studies conducted by two different analysts, the percentage difference between the analysts was determined to be 0.14%. Additionally, kinetic binding studies were performed with mAb molecules from 12 different production batches to demonstrate the effectiveness of the developed method in determining the binding activity of mAb molecules in different lots. These studies showed quite similar kinetic parameters for different production batches.



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ÖZET

Biyofarmasötiklerin ve bu grubun en büyük üyesi olan monoklonal antikorların (mAb'ler) kullanımı, rekombinant DNA teknolojisindeki ilerlemelerden sonra oldukça kritik bir uygulama haline gelmiştir. Kanser, astım, kardiyovasküler rahatsızlıklar, enfeksiyonlar ve alerjiler dâhil olmak üzere inflamatuvar ve otoimmün bozukluklar gibi çeşitli hastalıkların tedavisi, monoklonal antikorların geliştirilmesiyle mümkün hale gelmeye başlamıştır.

Monoklonal antikorların biyolojik olarak aktif olabilmeleri için glikolizasyon gibi post translasyonel modifikasyonlara ihtiyaç duyarlar. Bu modifikasyonların meydana gelebilmesi için üretimin gerçekleştireceği konakçı hücrenin memeli hücresi olması gerekmektedir. Mikrobiyal konakçı hücrelerindeki protein katlama mekanizması birçok memeli proteinin ifadesi için yetersiz olduğundan monoklonal antikor üretimi karakterize edilmiş memeli hücre bankaları kullanılarak yapılır.

Üretimin ilk adımı olan üst akım işlemi 12 ila 18 gün boyunca kontrollü hücre kültürü işlem parametreleri altındahedef gen ile transfekte edilmiş memeli hücre hattı kullanılarak gerçekleştirilir. Üst akım işleminin amacı, hücre canlılığını ve konsantrasyonunu yüksek tutarak maksimum kapasitede hedef monoklonal antikor üretimini gerçekleştirmektir. Hücre kültürü koşullarında meydana gelen ufakdeğişiklikler glikozilasyon ve safsızlık profilleri de dahil olmak üzere çeşitli kalite özelliklerine etki edebilir. Bu nedenle kültür koşulları dikkatlice kontrol edilmelidir. Hücrelerin çoğaltılması ve büyütülmesi için üretim veriminidoğrudan etkileyen oksijen, pH, sıcaklık ve karıştırma hızı gibi çevresel parametrelerin kontrollü olarak takip edilmesinisaglayan, biyofarmasötiklerin üretilmesindebiyoreaktörler kullanılır.

Üst akım işleminden sonra, monoklonal antikorların önce hücre kültüründen hasat edilmesi ve ardından terapötik amaçlar için kullanılmadan önce saflaştırılması gerekir. Biyofarmasötik üretimde, ürünün hücre kültürü ortamından verimli bir şekilde eldesi ve saflaştırılması süreçteki kritik adımlardır. Saflaştırma sırasında uzaklaştırılması gereken safsızlıklar prosese bağlı safsızlıklar (örneğin, konak hücreden türetilen DNA ve proteinler) ve ürüne bağlı safsızlıklar (asidik-bazik varyantlar, parçalar, dimerler, agregatlar) olmak üzere ikiye ayrılır. Ayrıca, saflaştırma prosesi ürün güvenliğini sağlamak için virüslerden de etkin bir şekilde uzaklaştırma yapabilme kapasitesine sahip olmalıdır. Saflaştırma prosesi, kimyasal fonsiyonlar kazandırılmış rezinler veya filtreler ile proteinlerin biyokimyasal ve / veya fiziksel özellikleri arasındaki etkileşimi kullanarak, istenmeyensafsızlıkları gidermek için temel olarak kolon kromatografisine ve filtrasyona dayanır. Hücre kültürüprosesinin geliştirilmesinde olduğu gibi, her bir operasyonel parametrenin (ör. pH, iletkenlik,bağlama kapasitesi veya akış hızı) ürünün kalitesini nasıl etkileyebileceğinkarakterizeetmek önemlidir.

Krite kalite özelliklerinin belirlenmesi ile monoklonal antikorların karakterizasyonu ara veya bitmiş ürünün etkinliğini ve güvenliğini belirlemek için oldukça önemli bir adımdır. Rekombinant DNA teknolojisindeki ve antikor mühendisliğindeki gelişmeler ile farklı işlevsel özelliklere sahip çeşitli antikor üretimleri gerçekleştirilebilmeye başlanmıştır. Monoklonal antikorların yapılarının karmaşık olması ve canlı bir sistem tarafından ifade edildiği için ve üretimden üretime değişkenlik gösterebildiği için kapsamlı karakterizasyon çalışmalarının yapılması gerekmektedir.

Terapötik monoklonal antikorlarının karakterizasyonu konvansiyonel ve küçük molekülü ilaçlara kıyasla oldukça zordur. Ürünün yapısal ve işlevsel özelliklerinin belirlenmesi ve düzenleyici yönergelerle uyumlu olarak proses-ürünle ilgili safsızlıkların tespit edilmesi gerekir. Monoklonal antikorların karakterizasyonu temel olarak iki başlık altında incelenebilir. İlki, protein yapıda olan monoklonal antikorların birincil, ikincil ve yüksek yapılarını belirlemek için yapısal karakterizasyonlar; ikincisi ise üretilen monoklonal antikorun biyolojik aktivitesini göstermeyi hedefleyen fonksiyonel karakterizasyondur.

Monoklonal antikorların birincil yapı analizlerinde amino asit dizilerinin tanımlanması hedeflenir ve kütle spektroskopisi ve ters faz HPLC yöntemleri yaygın olarak kullanılır. Monoklonal antikorların ikincil, üçüncül ve dördüncül yapıları proteinin fonksiyonel aktivitesini belirleyen yüksek dereceli yapılardır ve molekülün üç boyutlu yapısından ve uygun katlanmasından sorumludur. İkincil yapı karakterizasyonunda α -heliks ve β -yaprak yüzdeleri belirlemek için dairesel dikroizm ve fourier dönüşümlü kızılötesi spektroskopisi (FT-IR) yaygın olarak kullanılır. Monoklonal antikorların üç-boyutlu yapısını belirlemek için çeşitli kütle spektroskopisi yaklaşımları uygulanır.

Biyolojik olarak aktif bir ürün eldesi monoklonal antikor üretim sürecinin en kritik noktalarından biridir. Bu nedenle fonksiyonel karakterizasyon yöntemleri uygulayarak üretilen ürünün biyolojik aktivitesinin belirlenmesi gerekir. Üretilen monoklonal antikorun hedef antijeni ile etkileşimi birincil kalite özelliğidir. Enzime bağlı immünosorbent analizleri (ELISA) ve yüzey plazmon rezonansı (SPR) teknolojisi antikor antijen etkileşimini belirlemek için kullanılan temel yöntemlerdir. ELISA ve SPR yöntemleri, bağlanma aktivitesini belirlemek için tamamlayıcı teknikler olarak kabul edilirken, SPR teknolojisi bağlanma kinetiğini kantitatif olarak ölçebilir. Monoklonal antikorların farmakokinetiği ve biyolojik aktiviteleri, hedef antijene bağlanmalarına dayanır ve monoklonal antikorların karakterizasyonu için oldukça önemlidir. Monoklonal antikorların etki mekanizmasını anlamak için hücre-tabanlı analizler çok önemlidir. Monoklonal antikorlar, canlı bir sistemde hedef antijene bağlandıktan sonra aşağı doğru sinyal iletimine sebep olan geç yanıt etki mekanizmasına sahip olabilir. Bu nedenle, biyolojik aktiviteyi yalnızca SPR ve ELISA gibi ligand bağlama analizleri ile belirlemek yeterli olmayabilir. Hücre-tabanlı analizler geç yanıt mekanizmasını belirlemek için en etkili yöntemdir.

Yüzey plazmon rezonans (SPR) teknolojisi, etiketleme işlemi yapılmasına gerek olmadan biyomoleküler etkileşimleri gerçek zamanlı olarak tespit edebilen, yaygın ve güvenilir bir yöntemdir. Bir mAb molekülünün hedef reseptörü ile olan etkileşimi sonucu ortaya çıkan kinetik değerlerin belirlenmesi ve bitmiş ürünün bağlanma aktivitesini karakterize etmek için SPR yönteminin kullanılması etkili bir yaklaşımdır. İki molekül arasındaki bağlanma kinetiğini karakterize etmek için öncelikle moleküllerden biri (ligand), immobilizasyon veya yakalama teknikleri uygulanarak sensör çipi yüzeyinde stabilize edilir. Ardından, diğer molekül (analit), hareketli bir mobil faz içerisinde farklı konsantrasyonlarda sensör çipi yüzeyinden geçirilir.

Analit molekülü, kompleks bir yapı oluşturmak için sensör yüzeyine bağlı ligand molekülüyle etkileşime girebilirse, sensör yüzeyindeki yerel kırılma indisi değişir ve bu da SPR açısında bir değişikliğe neden olur. Etkileşim gerçek zamanlı olarak izlenebildiğinden, ligand-analit kompleks oluşumunun ilişki hızı sabiti, yansıyan ışığın yoğunluğundaki değişikliklere göre hesaplanabilir. Etkileşen ve ayrışan analit miktarı birbirine eşit olduğunda, analitin konsantrasyonuna bağlı olan etkileşimden elde edilen yanıt seviyesi bir platoya ulaşır. Bağlanma aşamasını, kompleks formun ayrışma süresini/hızını belirlemek için analit molekül içermeyen bir yıkama tamponu ile sensör yüzeyinin yıkanması takip eder. Bu adım ayrışma adımı olarak nitelendirilir. Son adımda, sensör çipinin yüzeyi kalan tüm kompleksleri uzaklaştırmak için bir rejenerasyon solüsyonuyla yıkanır. Tüm yanıtlar rezonans birimlerinde ölçülür ve elde edilen yanıtlar zamana karşı çizilen bir sensörgramda gösterilir.

Bu çalışmada, Protein A çip yüzeyine ilgili IgG monoklonal antikor yakalandıktan sonra hedef VEGF121 büyüme faktörü, 5 farklı konsantrasyonda yüzey üzerinden geçirilmiştir. Tek döngülü kinetik analiz kullanılarak kinetik parametreler belirlenmiş ve iki molekül arasındaki etkileşim incelenmiştir. Sağlam bir kinetik analiz geliştirmek için, yakalama molekülü (mAb) konsantrasyonu, VEGF121 molekülünün konsantrasyon aralığı, ayrışma süresi, rejenerasyon süresi ve yöntem sekansı dahil olmak üzere çeşitli parametrelerin optimize edilmesi gerekmiştir. Yöntemin sağlamlığını ve güvenilirliğini değerlendirirken, değerlendirme yazılımından alınan kalite kontrol parametreleri, kalıntı grafikleri, tekrarlar arasındaki R_{max} ve K_D değerlerinin tutarlılığı gibi önemli faktörler dikkate alınmıştır. Yöntem geliştirme çalışmaları sonucunda tekrarlı çalışmalar arasındaki K_D değerlerindeki sapmanın % 20'den az olmasını sağlayan 13.4nM yakalama konsantrasyonuna, 240 saniye ayrışma süresine ve 30 saniye rejenerasyon süresine karar verilmiştir. Tekrarlı çalışmalar arasındaki K_D değeri düşük sapma ile elde edildikten sonra özgüllük, sistem kesinliği, tekrarlanabilirlik ve yöntem kesinlik parametrelerini içeren kalifikasyon çalışmalarına başlanmıştır. Sonuç olarak iki ayrı analist tarafından 6 tekrarlı yapılan çalışmalar sonucunda analistler arasındaki % fark 0.14 % olarak belirlenmiştir. Ayrıca, geliştirilen yöntemin farklı lotlardaki mAb molekülünün bağlanma aktivitesini belirlemede ne kadar etkili olacağını göstermek için 12 farklı üretim serisine sahip mAb molekülleri ile kinetik bağlanma çalışmaları yapılmıştır. Bu çalışmalar farklı üretim serilerine sahip mAb moleküllerindenbirbirine oldukça benzer kinetik parametreler elde edilmiştir.



1. INTRODUCTION

1.1 Monoclonal Antibodies and Their Crucial Role in Biotechnology

Antibodies belonging to the immunoglobulin family are large glycoprotein structures found in higher eukaryotes in dissolved form (blood or plasma) or membrane-bound form (B cell receptors). They are the most important structures in the human immune system, providing defense against pathogens such as invading bacteria and viruses by recognizing and neutralizing them (Parray H. et al., 2020).

Immunoglobulins (Ig's) can be produced by isolating candidate B cells from spleen cells, evaluating their titers for several months after the immunization of animals. The immune system of the animals produces and processes these essential white blood cells to generate proteins called antibodies (Castelli M. S. et al., 2019). In 1975, Georges Köhler and César Milstein introduced a breakthrough technique, *hybridoma technology*, which fuses antibody producing B cells with cancerous cell lines such as myeloma cells (Fig. 1.1). This results in hybrid cells, making it possible to produce specific antibodies continuously (G. Kohler and C. Milstein, et al., 1976). Hybridoma cells are able to grow in cell culture and produce immunoglobulins with the same amino acid sequence, resulting in the same affinity for a selected single epitope of an antigen (Posner J. et al., 2019). After the introduction of hybridoma technology, the development of monoclonal antibodies for therapeutic purposes rapidly spread worldwide.

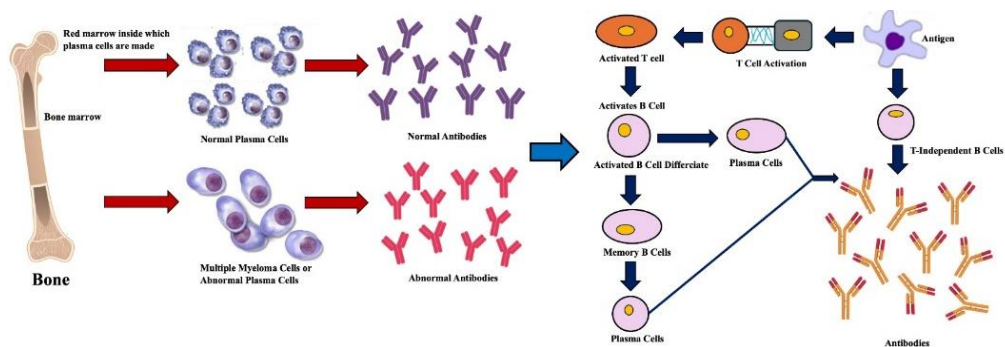


Figure 1.1 : Summary of producing antibodies from myeloma cells (modified from Mitra, S., Tomar, P.C et al., 2021).

The emergence of monoclonal antibodies (mAbs) has played a crucial role in the effective treatment of various diseases. The number of new mAbs under development continues to increase every year. The majority of mAbs are directed against two types of diseases: immune-inflammatory conditions and malignancies. Inflammatory conditions include diseases such as rheumatoid arthritis, systemic lupus, psoriasis, ulcerative colitis, and Crohn's disease. Malignancies include melanoma, breast cancer, some lymphomas, leukemias, and multiple myeloma. In addition, a wide variety of therapeutic mAbs have been developed for many indications (Posner et al., 2019). The first mAb molecule approved was Orthoclone OKT3 (muromonab-CD3), used to prevent kidney transplant rejection in 1986. Since Orthoclone OKT3 was a murine mAb, it was identified as a foreign substance and led to human anti-mouse antibodies (HAMAs) development in patient bodies, resulting in decreased half-life and neurotoxicity (Wang Z. et al., 2022). Subsequently, advancements in genetic engineering have facilitated the development of chimeric, humanized, and fully human monoclonal antibodies, addressing issues related to immunogenicity.

1.2 Structures and Functional Features of Monoclonal Antibodies

The basic structure of antibodies is Y shaped proteins consisting four polypeptide chains, two identical light chains and two identical heavy chains bound together by disulfide bonds, with a total molecular weight of ~150 kDa (Castelli M. S. et al, 2019). In terms of biological function, a single antibody chain is divided into variable and constant regions, located at the amino and carboxyl terminal halves, respectively. The variable region exhibits significant diversity in amino acid sequences among antibodies and serves as the antigen-binding domain, while the constant region ascertains the isotype of the antibody and effector functions.

Two types of light chains (λ and κ) and five types of heavy chains (α , δ , ϵ , γ , and μ) are present. The assembly of two heavy chains via disulfide bonds through covalent interactions constructs the antibody's core, while a heavy chain connected to a light chain through another disulfide bond forms one arm of the antibody. Each chain consists of constant (C_H and C_L) and variable domains (V_H and V_L). The variable domains of the heavy and light chains (V_H and V_L) form the antigen-binding region, which includes the complementarity-determining region (CDR), crucial for the high binding specificity of monoclonal antibodies (mAbs) to the target antigen.

The Fab, or antigen-binding fragment, constitutes the "V" portion of the Y-shaped mAb structure, comprising the VH and VL domains of the variable region, as well as the CH1 domains of the heavy chain. The stem part of the Y-shaped mAb structure is considered the Fc region and contains the CH2 and CH3 domains of the heavy chain (Posner et al., 2019).

1.3 Classification of Immunoglobulins

Immunoglobulins can be grouped into five different classes: IgG, IgM, IgE, IgA, and IgD, according to the variances in amino acid sequences found in the constant region of the heavy chains (Fig. 1.2).

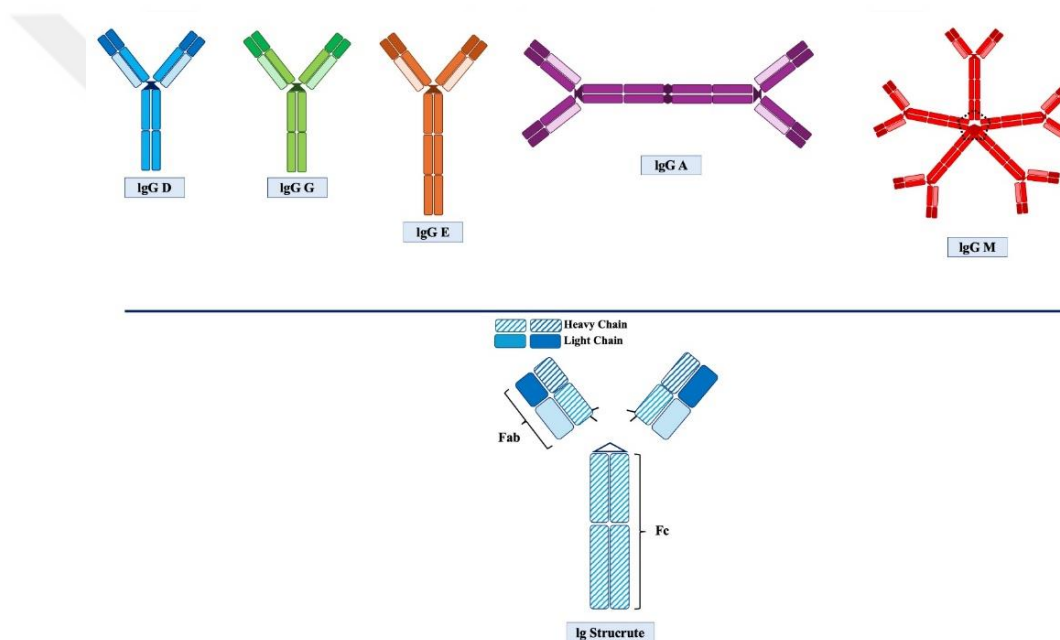


Figure 1.2 : Basic structures of five classes of immunoglobulins. IgD: Immunoglobulin D, IgG: Immunoglobulin G, IgE: Immunoglobulin E, IgA: Immunoglobulin A, IgM: Immunoglobulin M.

Immunoglobulin G is the most commonly used one for therapeutic purposes (Moorthy, B. et al, 2015) because of its longer half-life in circulation and ease of production (Ko, S., Jo, M. & Jung, S.T., 2021). The structures of the IgG antibodies differ from other isotypes and are subdivided into four subclasses: IgG1, IgG2, IgG3, and IgG4. Therapeutic mAbs are immunoglobulin G (IgG) molecules with a basic Y-shaped structure consisting of four polypeptide chains, having two identical heavy chains (~50 kDa each) and two identical light chains (~25 kDa each) (Buss et al., 2012).

1.4 VEGF-Mediated Angiogenesis

Angiogenesis is a highly regulated physiological process responsible for the formation of new blood vessels from pre-existing vascular networks. Angiogenesis is essential for development, wound healing, the menstrual cycle, and pregnancy, but it also participates in pathological conditions such as neurovascular disorders, rheumatoid arthritis, and cancer (tumorigenesis) (Sullivan L. A. & Brekken R. A., 2010). Due to the intricate nature of angiogenesis, it relies on extensive signaling networks between endothelial cells (ECs); human umbilical vein endothelial cells (HUVECs), endothelial colony-forming cells (ECFCs)), associated mural cells such as vascular smooth muscle cells (VSMCs), pericytes and various other cell types (such as immune cells) (Armulik A. et al, 2005). The biomolecular regulation of angiogenesis is mediated by a balance between the expression of different genes acting as pro-angiogenic and anti-angiogenic factors, which participate in various steps of the angiogenesis pathway (Carmeliet P., 2000).

The most essential and abundant molecules involved in the angiogenesis pathway are vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietins, epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) as angiogenic factors; and angiostatin, endostatin and thrombospondin as anti-angiogenic factors (Tahergorabi Z. & Khazaei M., 2012). Although various angiogenic factors involved in the pathway have been identified, VEGF is the most well-characterized factor and plays a pivotal role in the regulation and pathological angiogenesis (Ferrara N., 2002).

The VEGF family consists of seven secreted proteins, including VEGF-A, -B, -C, -D, -E, -F, and placenta-derived growth factor (PlGF). The VEGF family has specific binding abilities to three tyrosine kinase (TK) active receptors (vascular endothelial growth factor receptor (VEGFR) 1, 2 and 3: VEGFR-1, VEGFR-2 and VEGFR-3). In addition, VEGF-A may bind to Neuropilin 1 (NP-1), VEGF-C and VEGF-D may bind to Neuropilin 2 (NP-2) receptors (Vural, 2018). VEGF, also known as VEGF-A, is a dimeric polypeptide that binds to cell surface receptor tyrosine kinases termed VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2), as well as to non-tyrosine kinase receptors known as co-receptors such as neuropilin 1 (NRP-1) and neuropilin 2 (NRP-2) (Lange, C. et al, 2016).

The biological activity of VEGF is mainly mediated by the interaction of VEGF with its VEGF-2 receptor (Ferrara et al., 2003). VEGF-A is the most studied VEGF member and has been defined as a "vascular permeability factor" because it initiates vascular leakage. It has been revealed that VEGF and VEGFR expression is increased in most cancer tissues. VEGF-A is linked to tumor growth and spread. Due to its role in tumor angiogenesis, VEGF and its receptors are primary targets for the development of various drugs as angiogenesis inhibitors (Vural, 2018).

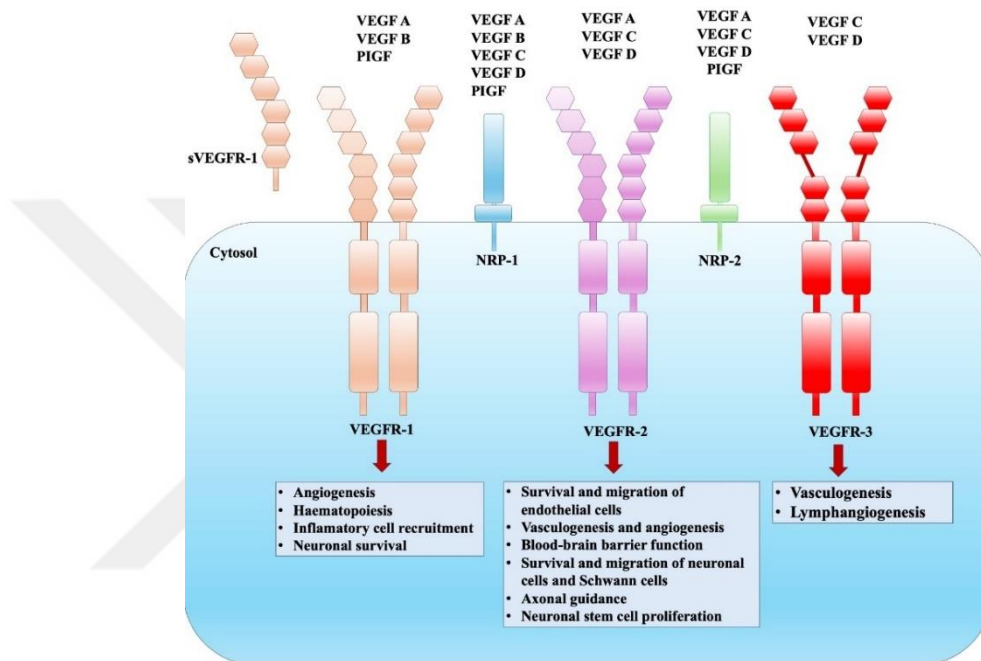


Figure 1.3 : Interactions of VEGF family members (VEGF-A, -B, -C, -D, and PlGF) with VEGFR-1,-2,-3 and NP-1,2. PlGF: Placenta-derived growth factor; VEGFR-1,-2,-3: Vascular endothelial growth factor receptors-1,-2,-3; neuropilins-1,2: (NP-1,2) (modified from Lange, C. et al, 2016).

There are four abundant isoforms of VEGF known as VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆ generated generated with the alternative exon splicing mechanism. VEGF₁₂₁ is an acidic polypeptide that cannot bind to heparin protein, resulting in a soluble VEGF isoform (Ferrara, N., et al., 2003). All isoforms, except for VEGF₁₂₁, have a heparin-binding domain that interacts with heparan sulfate proteoglycans (HSPGs) to mediate adherence to the extracellular matrix (ECM).

1.5 Therapeutic Molecules Targeting VEGF and VEGFR

Abnormal angiogenesis is one of the main factors that cause tumor progression and metastasis in different cancer types.

Since the significant effect of the interaction between VEGF and VEGFR in angiogenesis is known, monoclonal antibodies and small molecule inhibitors targeting VEGF and VEGFR, respectively, have been developed for therapeutic purposes (Liu, Y. et al., 2022). Monoclonal antibody-based therapies are an efficient way to treat patients with solid tumors, immunological disorders, eye diseases, etc. Over 100 monoclonal antibodies approved by the Food and Drug Administration (FDA) have been utilized to treat different groups of diseases so far (Mullard A., 2021).

Bevacizumab (Avastin®, Genentech, San Francisco, CA, USA) is a humanized monoclonal antibody (immunoglobulin G) that can bind and specifically target all VEGF-A isoforms, thus inhibiting interactions with VEGFR-1 and VEGFR-2. Bevacizumab is the most commonly used anti-VEGF molecule, combined with chemotherapy, to treat various cancer types such as glioblastoma, non-small-cell lung cancer, metastatic breast cancer by inhibiting the angiogenesis of the new blood vessels (Garcia, J., et al., 2020). Since bevacizumab has been clinically validated as a specific inhibitor of the interaction between VEGF-A and VEGFR-2, A growing number of monoclonal antibodies under development are focusing on VEGF as a promising molecular target for anti-angiogenesis. Ranibizumab is a humanized antibody derived from Bevacizumab but with higher VEGF-A binding activity and consisting of a single antigen-binding domain (Fab) (Schmucker et al., 2011).

Tyrosine kinase inhibitors are defined as anti-angiogenic drugs that interfere with VEGFR-1, VEGFR-2, platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptors (FGFR), and Tie2 signaling (Hartmann et al., 2009). Besides VEGFRs, two receptors known as Tie receptors are also found on endothelial cells. These receptors are in a different subfamily class. Tie2, and endothelium-specific receptor tyrosine kinases, plays a central role in the formation of blood vessels (Jones et al., 2001). Examples of therapeutics used as tyrosine kinase inhibitors include Sunitinib and Sorafenib as VEGFR-1 and VEGFR-2 tyrosine kinase inhibitors, Imatinib as PDGFR tyrosine kinase inhibitor, Nintedanib as FGFR tyrosine kinase inhibitor, and Regorafenib as Tie2 tyrosine kinase inhibitor (Gurkan-Alp et al., 2019). Aflibercept, the VEGF-trap protein obtained by fusion of the VEGF-binding domain of VEGFR-1 and VEGFR-2, which acts as a "VEGF ligand trap", is another therapeutic drug approved for the treatment of metastatic colorectal cancer (Gaya et al., 2012).

Ramucirumab is a fully human therapeutic monoclonal antibody targeting VEGFR-2 (Hsu et al., 2009). The mechanisms of action of some drugs having anti-VEGF activities are shown in Figure 1.4.

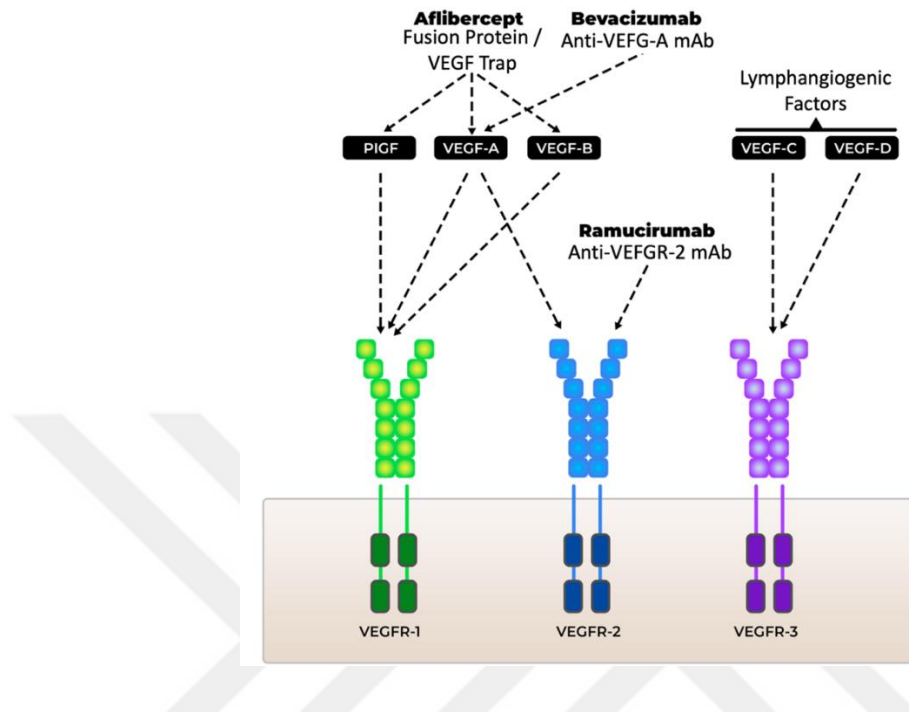


Figure 1.4 : Mechanism of action of anti-VEGF agents. mAb; monoclonal antibody, PlGF; Placental growth factor, VEGF; Vascular endothelial growth factor, VEGFR; Vascular endothelial growth factor receptor (Topaloğlu Y., 2023).

1.6 Production of Monoclonal Antibodies

1.6.1 Upstream process (USP)

The use of monoclonal antibodies as therapeutic agents has become increasingly common. mAbs require post-translational modifications (PTMs), such as glycosylation, to be biologically active and can only be produced in mammalian host cells, such as Chinese Hamster Ovary (CHO) cells, that can make these modifications. The protein folding mechanism in microbial host cells is inadequate for the expression of many mammalian proteins, making them unsuitable for use in humans (Carman et al., 2021). Upstream processing is performed using a mammalian cell line transfected with a gene of interest under controlled cell culture process parameters for 12 to 18 days (Wilson, L. J., 2020). The main goal of the upstream process is to ensure that the cell density grows with high cell viability to obtain a high concentration of the target monoclonal antibody (Bhoskar et al., 2013).

Therefore, in mAb production, certain production parameters (temperature, CO₂, % humidity, mixing speed) must be applied and followed throughout the production process to reach high viable cell numbers (Pan et al., 2017).

Cell culture process for mAb production can be carried out with batch, fed-batch and continuous processes. Figure 1.5 shows the three different production types.

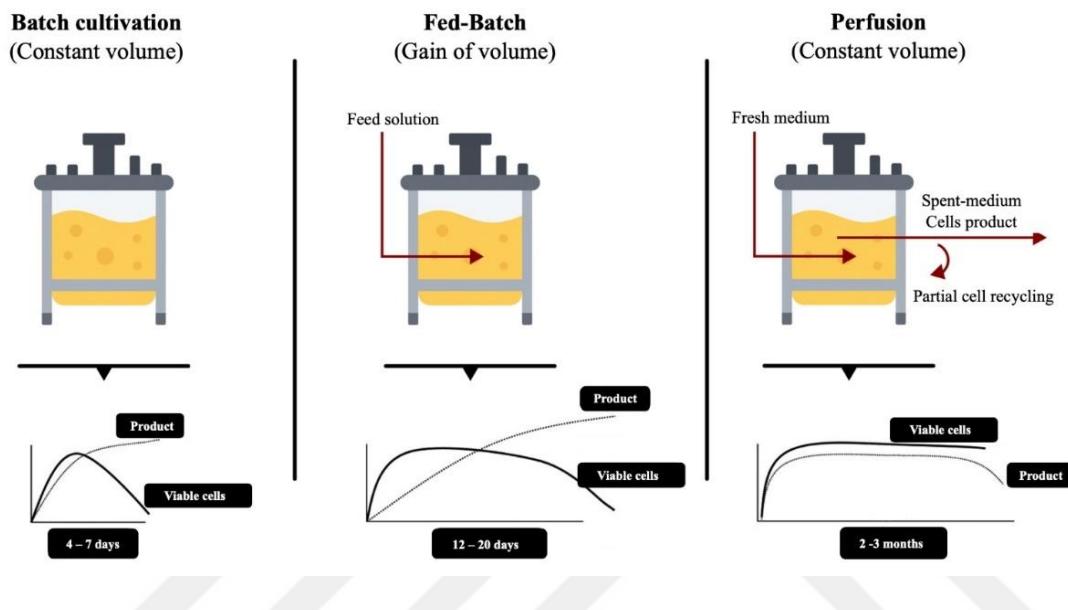


Figure 1.5 : mAb production processes applied in the upstream process (Topaloğlu Y., 2023).

Batch process which is the simplest production type, consists of a closed system in which a medium containing all the nutrients necessary for cellular development and mAb production is prepared and then cells are inoculated. It does not include the addition of fresh medium and feeding later in the production process. As production progresses, nutrient concentrations in the environment begin to decrease, and waste metabolites that reduce cell viability start to accumulate.

Fed-batch process is the second type of production of mAbs and is the most preferred production for industrial purposes. In this process, cell density reaches $8\text{--}12 \times 10^6$ viable cells/mL, and cell viability is increased by controlled nutrient addition at regular intervals. The production process can take between 12–20 days (Chartain et al., 2008). The medium used for feeding can be adjusted to enhance cell growth or to stimulate molecule production. Because different components can change the behavior of cells by altering their metabolism for different purposes (Carvalho et al., 2017).

A continuous process is the third type of production method. The continuous process can be performed with two different culturing methods which are chemostat and perfusion cultures. In chemostat cultures, fresh medium is continuously added to the bioreactor while fermented medium containing the cells is removed at a constant flow rate to keep the culture volume consistent. The dilution rate controls cellular growth, and when it equals the cell growth rate, the bioreactor reaches equilibrium. This keeps cell, nutrient, and product concentrations stable, and the cell culture can be maintained for several months at cell densities of $10\text{--}30 \times 10^6$ viable cells/mL (Chartain et al., 2008). To prevent the loss of viable cells, many manufacturers have developed the perfusion culture method which provides the recycling of the cells (Carvalho et al., 2017). The disadvantages of continuous fermentation are the use of large amounts of expensive culture medium and the difficulty of recovering the highly diluted product (Wurm, F. M., 2004).

1.6.2 Downstream process (DSP)

After cell culture, mAbs need to be harvested from the cell culture first and then purified before being used for therapeutic purposes. In biopharmaceutical production, the recovery and purification of mAbs from cell culture media are crucial steps in the process (Erkal et al., 2021). There are two separate impurities which need to be eliminated during the purification which are process-related impurities (e.g., DNA and proteins derived from host cell, cell culture ingredients such as animal serum) and product-related impurities (acidic-basic variants, fragments, dimers, aggregates). Moreover, the downstream process should have the capability to remove viruses, ensuring product safety in case of undetected contamination. (Shukla et al., 2007).

A general mAb purification process begins with the removal of cells and cell residues from the medium by applying clarification to the mAb obtained from the mammalian cell before the chromatographic purification steps (Erkal et al., 2021). Mammalian cells produce the target protein and release it into the medium. These proteins are collected from the medium by centrifugation or filtration, depending on the amount. After the production of mAbs obtained from mammalian cells, a clarification process is first applied to remove cells and cell residues from the medium for purification (Liu et al., 2010). The remaining product after this process is called cell culture liquid (supernatant).

Depth filtration or centrifugation combined with depth filtration is often used for clarification. Then, as the first chromatography step, the capture step is performed with Protein A chromatography, which takes advantage of specific interactions between the Fc region of mAbs and Protein A (Man et al., 2019).

According to FDA's Q5A guideline, there are established procedures for virus removal, along with chromatographic steps, to guarantee the safety of biotechnological products produced with mammalian cell culture. A single virus removal method cannot provide the level of virus removal expected by pharmaceutical authorities. Therefore, virus filtration and virus inactivation methods are used in a complementary manner. This combination helps achieve overall virus removal to meet the requirements presented by regulatory authorities (Kalyanpur, 2002). With virus inactivation, retroviruses can be successfully inactivated for various biotechnology products, usually at low pH values.

The subsequent chromatographic purification step or steps can be selected from different chromatographic methods. These can include ion exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, or multimode chromatography methods. After chromatographic purification techniques, the virus filtration step is applied to remove viruses. Finally, the final concentration of the target protein is adjusted, and buffer exchange is performed for the final formulation. These steps are performed using the tangential flow filtration (TFF) method, in which ultrafiltration (UF) and diafiltration (DF) pass through the filter tangentially to the filter surface (Erkal et al., 2021). An example of downstream process flow was summarized in Figure 1.6.

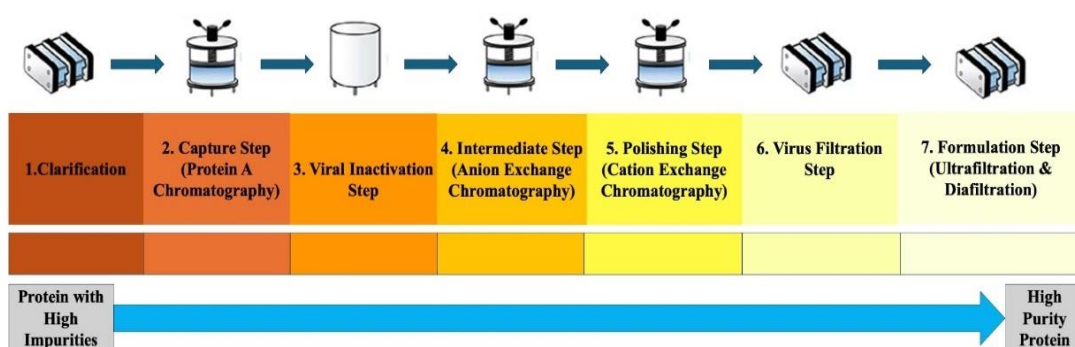


Figure 1.6 : An example of the general purification process for mAbs (Modified from Man et al., 2019.)

1.7 Characterization of Monoclonal Antibodies

Characterization of a monoclonal antibody is an important step to determine the efficacy and safety of the final or intermediate product by identifying critical quality attributes (CQAs). Recent improvements in antibody engineering technologies have made a multitude of antibody formats with different functional properties possible. Due to the variability and complexity in their structures, therapeutic mAbs require extensive characterization studies to ensure their safety and efficacy for human use (Kim et al., 2018).

Characterization of therapeutic mAbs could be a challenging process compared to small molecule drugs. This process is conducted for both upstream processing (USP) and downstream processing (DSP) of developing therapeutic monoclonal antibodies (mAbs). It involves evaluating the structural and functional features of the product and detecting the process-product related impurities all in alignment with regulatory guidelines. (Alhazmi et al., 2023). To identify CQAs, characterization studies are crucial for screening mAb molecules. CQAs encompass the chemical, biological, and physical properties of the mAb, which must fall within certain ranges to determine product quality. Moreover, characterization studies are essential for determining process-related impurities and for quality control of mAbs. For biosimilar approval, it is mandatory to conduct comparative characterization studies with the reference drugs (Kim et al., 2018). In Figure 1.7, analytical methods were categorized in order to characterize mAbs.

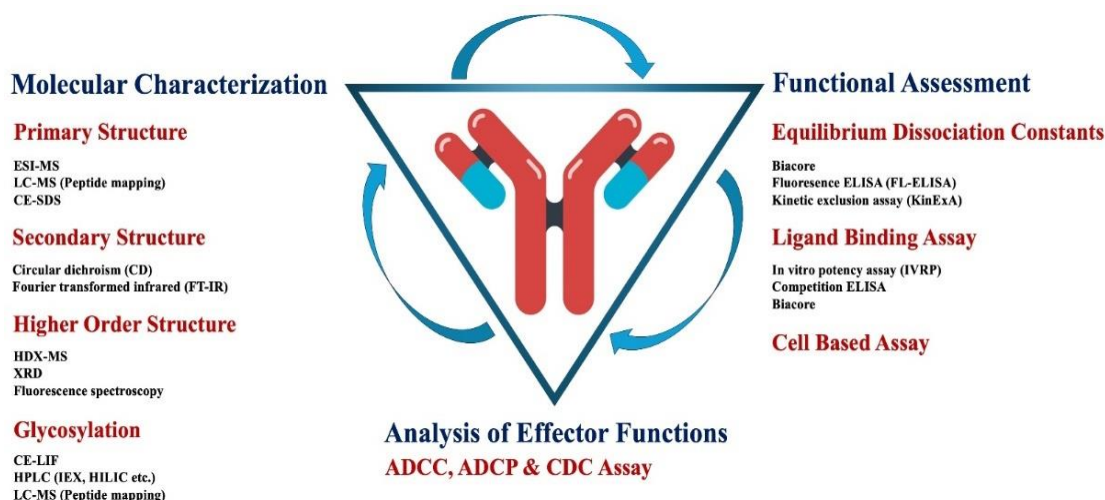


Figure 1.7 : Overview of the methods of molecular structural analysis and functional assessment (Wang et al., 2018).

1.7.1 Structural characterization of monoclonal antibodies

The majority of mAbs are derivatives of IgG1. However, their production by recombinant technology causes product heterogeneities. Moreover, culture media and storage conditions can also cause heterogeneity. For this reason, various analyses are applied to analyze mAb attributes.

1.7.1.1 Primary structure

The primary structure is revealed by identifying DNA and amino acid sequences. Mass spectrometry and reverse-phase HPLC methods are generally used in mAb characterization to determine the primary structure (Rosati et al., 2014; Jung et al., 2014). To understand lot-to-lot variation, these methods can also infer the amino acid sequence by peptide mapping of enzyme-digested protein (Fekete et al., 2013).

1.7.1.2 Secondary and higher-order structure

Secondary, tertiary, and quaternary structures are important parameters in the characterization of mAbs, known as higher-order structures (HOS), which express the functional activity of the mAb. They are responsible for the three-dimensional shape and appropriate folding of the mAb (Qiu et al., 2017). Circular dichroism (CD) and Fourier-transform infrared spectroscopy (FT-IR) are generally used to determine the secondary structure and percentages of α -helix, β -sheet, and random coils (Telikepalli et al., 2014). Disulfide bonds, which provide structural and functional stability to the mAb, are generally analyzed by collision-induced dissociation (CID) or electron-transfer dissociation (ETD), followed by Mass Spectrometer (MS) (Alhazmi et al., 2023). The tertiary structure (three-dimensional structure) of mAbs can be analyzed using fluorescence spectroscopy (Huang et al., 2017). Recently, highly sensitive spectroscopy methods have been developed to monitor HOS, such as hydrogen-deuterium exchange MS and ion-mobility MS (Terral et al., 2016; Wei et al., 2014).

1.7.1.3 Glycosylation assessment

Post-translational modifications such as N-linked glycosylation are very common in mAbs and have an effect on mAb effector functions. Thirty-two oligosaccharides may be added to Asn297, and random pairing of heavy chains can create many glycoforms. To separate the oligosaccharides from heavy chains (Jefferis, 2009), PNGase F reagent is usually used and then, released N-glycosylation is analyzed using MALDI-TOF.

The CE-SDS method is another application which can assess the glycosylation states of mAbs without the release of oligosaccharides (Esterman et al., 2016).

1.7.2 Product related heterogeneity

1.7.2.1 Charge variants

Monoclonal antibodies can show diversity in their biochemical and biophysical characteristics because of various post-translational modifications and degradation processes. The reason of this heterogeneity is due to the presence of miscellaneous of variants differing in molecular weight and charge. These variants are commonly referred to as acidic or basic variants compared to the main protein. The presence of abundant charge variants can significantly impact the in vitro and in vivo behavior of mAbs. Studies carried out with chemically modified antibodies have demonstrated that charge variants can affect the binding affinity of the antibody to its target proteins or cell membrane receptors thereby influencing the pharmacokinetic characteristic of the product (Khawli et al., 2010).

Charge variants can be detected via different analytical techniques including isoelectric focusing (IEF) gel electrophoresis, cation-exchange high performance liquid chromatography (CEX-HPLC) and anion-exchange high performance liquid chromatography (AEX-HPLC). Acidic variants have lower pI values while basic variants have higher pI values. Acidic variants and basic variants are identified by their retention times relative to the target protein peak (main peak). Acidic variants can be detected prior to the main peak in CEX-HPLC or later than the main peak in AEX-HPLC. Basic variants, on the other hand, determined after than the main peak of CEX-HPLC or earlier than the main peak of AEX-HPLC (Du et al., 2012).

1.7.2.2 Size variants

Monoclonal antibodies are increasingly recognized as a therapeutic class, primarily due to their extended serum half-life, favorable toxicology profile, and manufacturing process. Nevertheless, their intricate and diverse molecular compositions emphasize the need for characterization and comprehension of their structures and functions (Liu et al., 2008; Beck et al., 2013). High molecular weight species (HMWS) are one of the critical quality attributes (CQA) of mAbs as they can negatively affect product efficacy and cause immunogenicity in patients (Goyon et al., 2018; Yang et al., 2015).

Low molecular weight species (LMWS) are another type of structural product related impurities which can cause the decrease in the efficiency of mAb and reduced serum half-life due to missing Fab or Fc fragments (Yang et al., 2015). LMWS occurs as impurities generally because of cleavage at the IgG hinge region (Cordoba et al., 2005). Since the variety of impurities have different influence of the quality of the product, reliable and robust characterization methods should be developed to evaluate the size variants (Yang et al., 2015). Size variants are commonly evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion-high-performance liquid chromatography (SEC-HPLC) (Tous et al., 2005). Using SEC-HPLC, HMWS, monomer and LMWS levels are determined and their distribution in terms of total peak percentage is evaluated (Hong et al., 2012; Eurofins, 2018).

Aggregation

mAb aggregation and dimerization are challenges that can dramatically affect biological function. Moreover, aggregation can pose additional difficulties for DSP (Robets, 2014). Therefore, characterization and detection of aggregates are significant. SEC-HPLC is the most commonly used method for aggregate analysis due to its ease of application. Additionally, other preferred methods are analytical ultracentrifugation (AUC), dynamic light scattering (DLS), and high accuracy (HIAC) particle counting (Johnson, 2013; Krayukhina et al., 2013; Fekete et al., 2014; Marassi et al., 2014).

1.7.3 Functional characterization of monoclonal antibodies

Function is a critical feature for therapeutic mAbs. Analyzing the functional features is important to identify the toxicity, efficacy and safety of therapeutic mAbs. Before clinical studies, these properties should be determined through in vitro studies and animal studies. Functional characterization should be performed during the drug development phase, for batch release and to track commercially produced mAb products (Wang et al., 2018).

1.7.3.1 Ligand binding assays

Antibody-antigen interaction is the primary quality attribute. Enzyme-linked immunosorbent assays (ELISA) and surface plasmon resonance (SPR) based technologies are the two most popular methods for ligand binding analysis.

While both ELISA and SPR are considered complementary techniques to determine binding activity, the SPR technique can quantitatively detect binding kinetics by measuring the equilibrium dissociation constant (K_D value). Pharmacokinetics and biological activities of mAbs are based on their binding to the target antigen and this issue is significant for characterization of mAbs (Heinrich et al., 2010). SPR technology is a very successful method for measuring mAb binding to antigens, binding to receptors and the active concentration value required for this binding (Alhazmi et al., 2023).

1.7.3.2 Cell-based assay

In order to understand the mechanism of action of mAb, cell-based analyses are crucial. mAbs may have a late response mechanism of action that may involve downstream cell signaling after ligand-binding.

For this reason, only performing binding activity may not indicate mAb potency reliably. Downstream biomarkers (e.g., induction of apoptotic factors, inhibition of growth factors, cell adhesion, etc.) must be well-defined, and biological activity needs to be determined using quantitative or qualitative analysis. Cell-based assays could provide better results about the mechanism of action of a mAb than ligand-binding assays (Tada et al., 2014). Comparing to ligand binding assays, cell-based assays give an idea about chemical modification on biological activity. Cell-based assays should be applied for mAb characterization during the development phase, for batch release, and for monitoring of mAbs produced as commercial products (Wang et al., 2018).

1.7.3.3. Assessment of effector function

Therapeutic mAbs target soluble receptors or cytokines on the cell surface; by this way, they initiate downstream signaling. During original therapeutic mAb and biosimilars development phases, complement activation and other effector functions should be well-analyzed (Beck et al., 2013).

The mechanism of action and ligand binding are identified by the Fab region of mAb. In addition, the Fc region of mAb has a significant impact by affecting the serum half-life and effector functions such as antibody-dependent cell-mediated phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC).

1.8 Surface Plasmon Resonance Technology

1.8.1 Working principle of surface plasmon resonance

Surface Plasmon Resonance (SPR) is an optical method that enables the detection of biomolecular interactions in real time without applying any labeling procedures, such as attaching radioactive isotopes, fluorophores, or reporter tags covalently to the target biomolecules. Since labeling strategies may cause structural configuration changes or steric hindrance on the labeled molecule, the ability of the molecule to interact with its target molecule can be limited, consequently reducing the affinity characteristics of the complexes. However, SPR technology has overcome these challenges by eliminating the need for specialized labeling procedures and enabling sensitive kinetic affinity measurements (Moran, K. L. M. et al., 2018).

SPR allows the quantitative and qualitative characterization of a variety of interactions, such as antibody-antigen, ligand-receptor, and proteins with oligonucleotides or carbohydrates, protein-DNA, and DNA-DNA (P. Schuck et al., 1997). The utilization of SPR for qualitative applications ranges from various purposes such as small molecule and drug screening, epitope mapping and molecular assembly. The SPR biosensor can also be used as a quantitative tool to determine various parameters related to molecular interactions, including kinetics of reaction (association rate constant, k_a , and dissociation rate constant, k_d), affinity constants and, mechanisms underlying receptor-ligand interactions (Pattnaik P. et al., 2005).

A basic SPR instrument consists of a light source, an imaging optical system, an optical detector, which measures the changes in light, a sensor chip with a metal surface, which consists of a fluidics system allowing the flow through of the ingredients. The phenomenon of SPR relies on the Kretschmann configuration, in which surface plasmons emerge from free electrons at the interface of distinct materials. Since metals have high electron conductivity, they are particularly suitable to utilize the working principle of SPR. Therefore, metal-coated glass prisms are used as a surface where this phenomenon occurs (Nguyen H. H., et al., 2015). Under total internal reflection conditions (TIR), polarized light passing through a metal-coated glass prism at a well-defined angle excites the electrons on the metal surface and generates plasmons at a certain angle known as the "SPR angle."

The resonance causes a slight decrease in the intensity of the light, resulting in minimal reflected light as some of the energies are transferred to the electrons on the surface (P. Schuck et al., 1997). The depth of the evanescent field of plasmons can be influenced by the wavelength of the incident light and refractive indices (RI) of the materials used. If there are small changes in RI because of molecular interactions within this transient plasmon field, the SPR angle starts to shift, and electrons cannot be excited as in previous conditions, which results in the decreasing of plasmon formation and increasing of reflected light. By monitoring SPR angle shifts and measuring changes in reflected light, molecular interactions can be observed in real time by producing a plot referred to as a sensorgram. Since the intensity of the reflected light depends on the RI near the metal surface (solution in contact with the metal surface), the SPR angle starts to increase as the mass attached to the metal surface increases measurements (Moran, K. L. M. et al., 2018) (Pattnaik P. et al., 2005). The basic working principle of SPR is based on the steps shown in Figure 1.8.

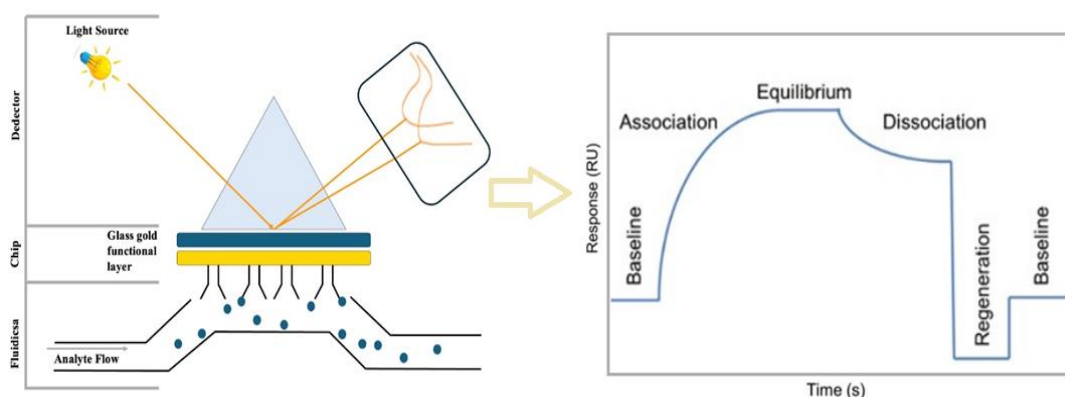


Figure 1.8 : Schematic illustration of SPR working principle. Left side of the figure, SPR system components are illustrated. On right side an example sensorgram is shown to explain the steps of molecular interaction.

As a first step, after attaching or immobilizing one molecule, referred to as the *ligand*, to a sensor surface, the other molecule, *the analyte*, is flowed over the sensor surface in a solution during the association phase of the experiment. If the analyte molecule can interact with the ligand molecule attached to the sensor surface to make a complex formation, then the local refractive index changes, resulting in a change in SPR angle. Since the interaction can be monitored in real time, the association rate constant of the ligand-analyte complex formation can be calculated according to changes in the intensity of the reflected light.

When the amount of analyte associating and dissociating are equal to each other, the response level obtained from the interaction, which depends on the concentration of the analyte, reaches a plateau. The association phase is followed by the dissociation phase, which includes the washing of the sensor surface with a running buffer absent from the analyte molecule in order to record the dissociation time of the complex form. In the last step, the surface of the sensor chip is washed with a regeneration solution to remove any remaining complex. All responses are measured in resonance units and illustrated in a sensorgram, which shows the obtained responses against time (Yu, H.-W. et al., 2015).

1.8.2 Sensor surfaces and immobilization strategies

The sensor surface is one of the most critical components of an SPR system, which provides necessary environmental conditions for molecular interactions. Various coating strategies are applied to the metal surface of the sensor chip to monitor specific molecular interactions. Gold is the most preferred material as a metal surface due to its biocompatibility, providing similar *in vivo* conditions during molecular interactions, and resistance to different conditions including corrosion and extreme pH values (Topor C. V. et al., 2023). The gold surface is coated with an organic layer to allow the attachment and stabilization of the ligand molecule. Various coating methods such as thiol anchoring, dextran-based hydrogel attachment, and silane attachment can be used on the chip surface according to the purpose of the experiment and the advantages and disadvantages of the coating method. For example, Carboxymethylated dextran (CMD) is commonly utilized for protein immobilization on the sensor chip surface. Dextran modified with carboxymethyl groups can easily interact with the free-amino functional groups of proteins (Mullett, W. M., et al., 2000). In the Biacore system, almost all sensor chip gold surfaces are first coated with alkanethiol molecules, preventing the molecules from directly interacting with the gold surface and providing an environment suitable for the attachment of the surface matrix. Then, the sensor chip surface is covered with a flexible hydrogel matrix consisting of carboxyl-methylated dextran, which increases the immobilization surface by providing a porous three-dimensional linking matrix (Biacore sensor surface handbook). The immobilization procedure aims to attach the recognition molecule (*ligand*) on a sensor chip surface with its active form to provide experimental conditions to observe the molecular interaction.

There are different types of techniques that can be applied to attach the ligand molecule to the sensor chip surface such as covalent immobilization, and oriented immobilization.

1.8.2.1 Covalent immobilization

Covalent immobilization technique attaches the ligand molecule to the sensor chip surface permanently by creating a heterogeneous subpopulation of reactive ligand molecules. The covalent immobilization technique can be utilized according to the functional groups present on the ligands. There are several strategies to perform covalent immobilization including amine coupling, maleimide coupling, and aldehyde coupling. Amine coupling, which can also be named as EDC / NHS coupling reaction, is the most common and generally the first option of the covalent immobilization technique as it utilizes the primary amine groups on the ligand following the activation of carboxyl groups on the sensor chip surface with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Zhao, H. et al., 2017).

1.8.2.2. Oriented immobilization techniques

The oriented immobilization technique, also named capture approaches, is applied when the ligand is not stable and amenable to chemical modifications. In this method, the ligand molecule is captured at a specific orientation on the chip surface, which is already immobilized by the capture agent. This technique is a reversible method that allows the removal of the captured ligand molecule from the surface by washing with a regeneration solution. Since the regeneration solution removes the captured agent from the surface, in every analysis, the ligand must first be captured freshly as an initial step of the analysis.

One of the most significant parameters when applying the oriented immobilization technique is the presence of a high-affinity interaction between the ligand and capturing agent immobilized on the sensor chip surface to inhibit the significant detachment of the ligand molecule while performing binding assays. Several applications are present to perform oriented immobilization techniques include but are not limited to these such as streptavidin-biotin capture, antibody-based capturing. Another method of specifically immobilizing antibody molecules on the sensor chip surface is based on the specific interaction of IgG antibodies with protein A and protein G from the Fc region.

This interaction ensures that the antibodies are attached to the chip surface from the Fc region while keeping the Fab region exposed, which allows them to interact with the target molecule (Topor C. V. et al., 2023). Different studies utilize the protein A/G-functionalized SPR biosensor for detecting the antibodies' binding activity such as in the study of Parkkila et al. 2022, extracellular vesicles (EVs) were captured on protein A/G immobilized sensor chip surface and obtained responses were evaluated.

The Aim of the Study

The purpose of this study is to characterize the binding kinetics of an anti-VEGF monoclonal antibody (mAb), known to inhibit angiogenesis by interacting with the VEGF121 protein, an isoform of the VEGF-A growth factor, using the surface plasmon resonance (SPR) method. To conduct this study, the mAb molecule was captured with high affinity at a certain concentration on a chip surface where Protein A capturing agents were already immobilized. The VEGF121 molecule was then flowed over the chip surface in the running buffer at five different concentrations. The binding kinetic parameters were examined to determine the affinity of the analyte VEGF121 molecule for the ligand mAb molecule on the chip surface. After optimizing various critical parameters during method development, a qualification study was conducted to determine the reproducibility of the method. Binding kinetic studies were carried out with the developed method using mAb molecules from different lots, and the differences between the kinetic parameters of mAb obtained from different production batches were examined.

2. MATERIALS AND METHODS

2.1 Laboratory Equipments, Consumables and Chemicals

The equipments and consumables used in this study are listed in Appendix A.

The chemicals used in this study are given in Appendix B.

2.2 Methods

2.2.1 Preparation of the required solutions

2.2.1.1 Preparation of 1M NaOH (0.1 L)

80 mL of ultrapure water is added to a 100 mL glass bottle. Then, 0.4 g of NaOH is weighed and added to the bottle. The mixture is stirred using a magnetic stirrer until NaOH dissolves homogeneously. Finally, ultrapure water is added to bring the total solution volume up to 100 milliliters.

2.2.1.2 Preparation of 5M HCl solution (0.01 L)

Prepared for used in pH adjustment. The solution is made by adding 4.17 mL of 37 % stock HCl solution to 5.83mL ultrapure water.

2.2.1.3 Preparation of running 1X HBS-EP (+) buffer

8.766 g of NaCl, 2.383 g of HEPES and 0.877 g of EDTA are dissolved in 90 mL of ultrapure water. After the solution becomes homogeneous, the final volume is completed to 99.5 mL. Then, 500 μ L of Tween 20 is added to prepare a 100 mL solution. After taking 20 mL of the prepared solution, diluting 180 mL of solution to 1X with ultrapure water and pH of the solution is prepared to 7.4 with 1M NaOH. Finally, the solution is passed through a 0.22 μ m filter to obtain 200 mL solution of running buffer for analysis. Also, 1X HBS-EP (+) buffer with a pH of 7.4 buffer is used for the dilution of samples and capture solutions.

It contains 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20, pH 7.4, supplied by Biacore, GE Healthcare.

2.2.1.4 Preparation of 10 mM glycine solution as regeneration solution, pH:2.0

150 mg of glycine is dissolved in 180 mL of ultrapure water. The pH of the solution is adjusted to 2.0 with 5M HCl and the final volume is completed with 200 mL of ultrapure water. The prepared regeneration solution is filtered (0.22 μ m) and properly degassed before use.

2.2.1.5 Aliquotting VEGF121 lyophilized protein

The lyophilized VEGF121 protein was dissolved in 80 μ L ultrapure water, resulting in a concentration of 0.25 mg/mL protein. To prepare a stock solution of 500 nM (5×10^{-7} M) VEGF121, the dissolved protein with a concentration of 0.25 mg/mL was diluted to 7 μ g/mL with 1x HBS-EP (+) buffer. The dilution was performed according to the equation 2.1 and equation 2.2.

$$5 \times 10^{-7} \text{ M} \times 14000 \text{ g/mol (molecular weight of VEGF121 protein)} = 7 \text{ } \mu\text{g/mL} \quad (2.1)$$

$$0.25 \text{ mg/mL} \times 80 \text{ } \mu\text{L} = 7 \text{ } \mu\text{g/mL} \times \alpha, \alpha = 2857 \text{ } \mu\text{L} \quad (2.2)$$

500 nM of stock VEGF121 solution was obtained first by adding 2777 μ L of 1X HBS-EP (+) buffer to 80 μ L of 0.25 mg/mL protein. Prepared VEGF121 stock solution was portioned to eppendorf tubes as 55 μ L and stored at 80°C.

2.2.2 Method development studies of detecting binding kinetics of VEGF specific mAb molecules with VEGF121

2.2.2.1 Capturing different concentrations of mAb on protein A chip surface

Within the scope of this study, we first determined the optimum concentration of the mAb molecule to be captured on flow cells of the Protein A sensor chip using the standard capture protocol proposed by Biacore X100 software. To prepare different concentrations of the mAb molecule for the chip surface, the 149 kDa mAb was diluted in 1X HBS-EP (+) buffer to obtain the highest capture concentration to be tested (13.4 nM). A 2-fold serial dilution was performed with 1X HBS-EP (+) buffer, starting from 13.4 nM, diluting the mAb to 1.675 nM. Each mAb concentration was captured via the Fc region onto the Protein A chip surface while the instrument was set to 25°C.

Additionally, 100 nM (1.40 µg/mL) of VEGF121 sample and running buffer were introduced in the same sequence to check for interference with the Protein A chip surface. The method conditions used in this study are given in Table 2.1 under the heading of experimental objective with analysis called capture concentration determining.

Table 2.1 : The summary of method development studies performed with Kinetic/Affinity template.

Experiment Purpose	Ligand Concentrations (nM)	Analyte Concentrations (nM)	Analysis Conditions
Capture Concentration	13.4	0 nM	Contact Time: 30 seconds
	6.7		Dissociation Time: 30 µL/min for 150 seconds
	3.35		Regeneration Solution Contact Time: 30 µL/min for 30 seconds
	1.675		
Optimization of Capture Concentration &Analyte Concentration	13.4	0.37	Capture Contact Time: 30 seconds
		1.11	Sample Contact Time: 30 µL/min for 180 seconds
		3.33	Dissociation Time: 30 µL/min for 150 seconds
		10.00	Regeneration Solution Contact Time: 30 µL/min for 30 seconds
	6.7	30.00	
Analyte Concentration Optimization	13.4	1.11	Capture Contact Time: 30 seconds
		3.33	Sample Contact Time: 30 µL/min for 180 seconds
		10.00	Dissociation Time: 30 µL/min for 150 seconds
		30.00	Regeneration Solution Contact Time: 30 µL/min for 30 seconds
		90.00	
Dissociation Solution Contact Time*	13.4	1.11	Capture Contact Time: 30 seconds
		3.33	Sample Contact Time: 30 µL/min for 180 seconds
		10.00	Dissociation Time: 30 µL/min for 150 / 240 /330 seconds
		30.00	Regeneration Solution Contact Time: 30 µL/min for 45 seconds
		90.00	

* Studies were carried out in triplicate.

2.2.2.2 Optimization of mAb concentration captured on protein A chip and first study to interpret VEGF121 molecule concentration range

After identifying two different mAb concentration candidates for capture on the Protein A chip surface, five different concentrations of the VEGF121 molecule (30.00 nM - 0.37 nM), which is expected to interact with the relevant mAb molecule, were injected onto the chip surface after dilution with 1X HBS-EP (+) buffer. Parameters applied in these studies are provided in Table 2.1 under the heading of analysis conditions. Additionally, blank buffer was sequentially injected to subtract baseline noise and evaluate binding kinetics. Each VEGF121 concentration was injected serially onto flow cell 2, while flow cell 1 was left empty to serve as a reference surface without any analyte on the chip surface. Binding kinetics parameters were calculated using a single cycle kinetic approach.

2.2.2.3 Optimization of VEGF121 concentration range

After determining the optimum capture concentration considering its interaction with VEGF121, a different study was conducted with a new VEGF121 concentration range, increasing the lowest analyte concentration by three times. Analysis conditions (Table 2.1) remained the same as the previous analysis conditions.

2.2.2.4 Application of three different dissociation times in three replicate studies

A total of four separate analyses were conducted for four different dissociation times (150s, 240s, 330s), with three repetitions for each dissociation time. The CV values obtained from these replicate studies were compared with each other. In the analysis conditions, unlike previous analyses, only the dissociation time was changed to observe how the obtained kinetic values were affected and to determine the deviation between repeated runs.

2.2.2.5 Changing the analysis template from kinetic/affinity to custom assay and regeneration solution contact time optimization

To control all cycles and their purposes during the analysis, the method was changed from the kinetic/affinity template to a custom assay, and the steps planned for the analysis were entered into the sequence one by one. The template used for the kinetic/affinity assay and the created method for the custom assay are shown for analyzing a single sample in Tables 2.2 and Table 2.3, respectively.

Table 2.2 : Kinetic/Affinity Template.

Cycle No	Steps	Cycle Type	Cycle Purpose
Cycle 1.	Buffer wash	Buffer wash	Start-Up
Cycle 2.	Buffer wash	Buffer wash	Start-Up
Cycle 3.	Sample (VEGF 0 nM)	Sample	Sample
Cycle 4.	Sample (VEGF 0 nM)	Sample	Sample
Cycle 5.	Sample (VEGF 1.11-90 nM)	Sample	Sample

Table 2.3 : Created Custom Assay Sequence.

Cycle No	Steps	Cycle Type	Cycle Purpose
Cycle 1.	Buffer wash	Buffer wash	Start-Up
Cycle 2.	Capture 1	Capture	Undefined
Cycle 3.	Sample (VEGF 0 nM)	Sample	Sample
Cycle 4.	Sample (VEGF 0 nM)	Sample	Sample
Cycle 5	Sample (VEGF 1.11- 90 nM)	Sample	Sample
Cycle 6.	Regeneration	Regeneration	Undefined

To determine the effect of changes in regeneration time on the CV % obtained from repeated studies, two different studies with regeneration times of 45 seconds and 30 seconds were conducted with three replicates each. The CV % values obtained from these two studies were compared. The analysis conditions used in the custom method are summarized in Table 2.4.

Table 2.4 : The summary of the studies carried out with Custom Assay.

Experiment Purpose	Ligand Concentrations (nM)	Analyte Concentrations (nM)	Analysis Conditions
Regeneration Solution Contact Time	13.4	0 nM	Capture Contact Time: 30 seconds Sample Contact Time: 30 μ L/min for 180 seconds Dissociation Time: 30 μ L/min for 150 seconds Regeneration Solution Contact Time: 30 μ L/min for 45/30seconds

Table 2.4 (continued) : The summary of the studies carried out with Custom Assay.

Experiment Purpose	Ligand Concentrations (nM)	Analyte Concentrations (nM)	Analysis Conditions
Conditioning Sensor Chip Surface	13.4		Capture Contact Time: 30 seconds
		0.37	Sample Contact Time: 30 μ L/min for 180 seconds
		1.11	
		3.33	Dissociation Time: 30 μ L/min for 150 seconds
		10.00	
		30.00	Regeneration Solution Contact Time: 30 μ L/min for 30 seconds

2.2.2.6 Conditioning Sensor Chip Surface with Regeneration Cycles

In previous studies, the chip surface was conditioned only with the running buffer before the first replicate of VEGF121 was flowed over the chip surface. Before flowing VEGF121 concentrations (samples) across the sensor chip surface, the chip was conditioned not only with the running buffer but also with the regeneration buffer in three separate cycles to ensure that the first sample among replicates was analyzed under conditions similar to the other replicates. The sequence created for conditioning the sensor surface is given in Table 2.5 for running only one sample.

Table 2.5 : Conditioning the sensor chip surface with regeneration cycle.

Cycle No	Steps	Cycle Type	Cycle Purpose
Cycle 1	Buffer wash	Buffer wash	Start-Up
Cycle 2	Regeneration	Regeneration	Undefined
Cycle 3	Buffer wash	Buffer wash	Start-Up
Cycle 4	Regeneration	Regeneration	Undefined
Cycle 5	Buffer wash	Buffer wash	Start-Up
Cycle 6	Regeneration	Regeneration	Undefined
Cycle 7	Buffer wash	Buffer wash	Start-Up
Cycle 8	1.Capture	Capture	Undefined
Cycle 9	Sample (VEGF 0nM)	Sample	Sample
Cycle 10	Sample (VEGF 1.11- 90nM)	Sample	Sample
Cycle 11	Regeneration	Regeneration	Undefined

2.2.3 Qualification Studies

During the qualification studies, the reliability and robustness of the developed method were determined by evaluating parameters such as system suitability, specificity, system precision, repeatability and intermediate precision as specified in the ICH Q2 guideline.

In the system suitability study, variables such as deviations in the baseline during the kinetic analysis and whether the quality control parameters determined by the evaluation software (e.g., reported kinetic values being within the detectable range by the device, bulk interference) met the necessary conditions were examined.

In the specificity study, it was shown that the mAb molecule could specifically bind only to its target protein. To demonstrate the specificity of the developed method, kinetic parameters were evaluated after the mAb interacted with a different protein that was theoretically known not to show binding activity. To determine this, a non-target analyte molecule was interacted with the mAb at five different concentrations using the developed kinetic binding assay.

The qualification parameter in which the similarity of the kinetic parameters obtained from six consecutive injections of five analyte concentrations within a single preparation set was demonstrated was system suitability.

In the repeatability study, analyst 1 prepared the mAb molecule and VEGF121 concentrations six separate times and performed six different kinetic cycles.

The quality control parameters obtained from these studies were examined, and the deviation between the kinetic parameters was evaluated. The aim of this study was to determine whether any variation arose from the sample preparation stage.

In the intermediate precision study, analyst 2 prepared the mAb molecule and VEGF121 concentrations six separate times and performed six different kinetic cycles. The results were evaluated to determine if they met the quality control parameters, examining the deviation between the kinetic parameters and the difference between the results obtained by analyst 1 and analyst 2.

2.2.4 Studies to Detect Binding Activity of Different Lot Numbers of mAb

Extensive biological characterization of the product should be performed on multiple lots to understand the variation between different production numbers of the product.

The coefficient of variation (CV %) between the obtained results was calculated to demonstrate the reliability of the developed method. Besides repeatability from lot to lot, the precision between different analysts is also an important parameter in analytical method development. A low percentage of differences between different analysts indicates the precision and robustness of the method.

2.2.5 Evaluation of the Data

Responses over time plotted in sensorgrams were obtained using Biacore X100 Evaluation Software, version 2.0.1 (Biacore, Uppsala, Sweden). The kinetic analyses were also conducted using the same software.

2.2.6 Statistical Approach

The reliability of the developed method has been demonstrated by comparing the CV % of the results obtained from the replicated experiments and studies of different analysts. Additionally, using CV % allows comparison of results obtained by exchanging different test materials, such as different lot numbers of the mAb product in the study. All analysts always performed analyses in triplicate. Furthermore, the variation % of average KD values between a total of six experiments with three replicates performed by two analysts was calculated.

3. RESULTS AND DISCUSSION

3.1 Method Development Studies

3.1.1 Capturing different concentrations of mAb on protein A chip surface

The surface of the Protein A sensor chip consists of a Protein A variant covalently attached to a carboxymethylated dextran matrix, enabling the binding of antibodies to the sensor surface in a specific orientation via the heavy chain within the Fc region. Unlike the Protein A chip, the CM3, CM4, and CM5 sensor chips, which are generally preferred for kinetic studies, contain only a carboxymethylated matrix covalently attached to the gold surface. Therefore, although these types of sensor chips are more preferred in kinetic studies, they do not contain molecules that can specifically attach the mAb to the chip surface as in this study. As a result, the antibody must be attached to the chip surface by covalent immobilization or high-affinity capture using additional kits instead of the Protein A capture technique.

In covalent immobilization, the ligand is irreversibly chemically bound to the carboxymethyl groups on the sensor chip. Since the ligand is chemically attached to the sensor surface, it remains on the surface for the lifetime of the chip and after each analysis, the chip surface is washed with regeneration buffers to remove non-covalently bound molecules. In this technique, unlike capture techniques, there is no agent that can attach the mAb to the chip surface at a specific site, leading to the possibility of random binding of the antibody to the chip surface. When determining the binding activity of the product, it is crucial that the antigen-binding site, the Fab region of the mAb molecule of interest, is exposed outwardly because this is where the interaction with the target molecule will take place. In the covalent immobilization technique, since the ligand is not attached to the sensor chip surface at a specific site, the Fab region where the analyte is targeted to bind may not be in the appropriate orientation or position. This may lead to a lower interaction than would normally be expected and consequently a lower detection of product activity.

Furthermore, in this technique, since the ligand remains attached to the chip surface throughout the life of the chip, it will be exposed to harsh solutions such as regeneration buffer that may damage its structure, causing the response value to decrease over time.

The high-affinity capture method, unlike the covalent immobilization method, involves the immobilization of a capture molecule that can bind to the Fc region of the antibody on sensor chip surfaces such as CM3, CM4, and CM5. This method has many advantages compared to covalent immobilization. Since the capture method does not require any chemical modification of the ligand molecule, it can work with molecules that are not suitable for covalent immobilization. In the capture method, unlike the covalent immobilization method, the ligand molecule attached to the chip surface is removed from the chip surface along with other molecules after the regeneration step. Although this method requires the ligand molecule to be re-prepared for each analysis, causing higher consumption, it also prevents the negative effects that the regeneration buffer may have on the activity of the ligand molecule by allowing the ligand to be prepared freshly for each new analysis.

In this study, the mAb molecule was captured as a ligand through its Fc region onto a Protein A chip surface, which was originally optimized for concentration studies but can also be valuable for affinity and kinetic analysis involving antibodies. Since Protein A chips already contain pre-immobilized Protein A molecules on their surface, the immobilization step for sensor surface preparation, which is necessary in the high-affinity capture method, was skipped.

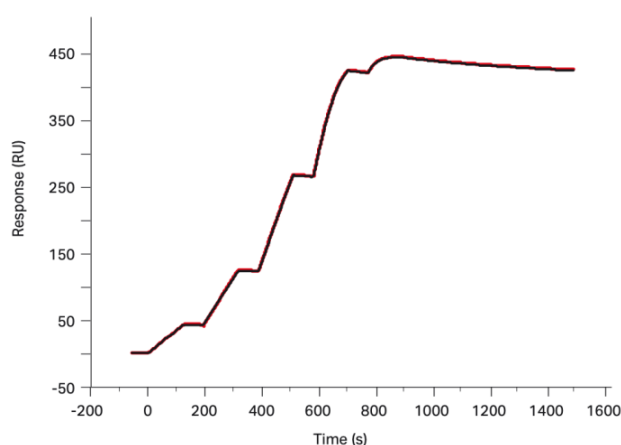


Figure 3.1 : Sensorgram of a reference study of single cycle kinetics using Protein A chip (Biacore Sensor Chip Protein A, 2020).

In an example study conducted to determine the interaction of the mAb molecule captured on the surface of the Protein A sensor chip with 1700 RU with its target protein, the sensorgram obtained as a result of single-cycle kinetic analysis is shown in Figure 3.1. Additionally, in a different study, the interaction of anti-VEGF molecules with VEGF165 was evaluated by calculating kinetic binding parameters using recombinant Protein A covalently immobilized on the CM5 chip surface (Sumner, G. et al., 2019).

An important factor when preparing a surface is the analyte binding capacity, identified as $R_{\max}$ for maximum response, as it will define the expected response range from the sample's concentration range. When the ligand molecule is attached to the sensor surface via covalent immobilization, the amount of ligand molecule to be immobilized on the sensor surface should be kept low. A basic calculation allows for an estimation of the amount of ligand to be attached to the surface, taking into account the size of both the ligand and the analyte equation 3.1

$$[\text{Response}_{\max} = (\text{Response}_{\text{Ligand}} \times \text{Mass}_{\text{Analyte}}) / \text{Mass}_{\text{Ligand}} \times \text{Stoichiometry}] \quad (3.1)$$

Before capturing the mAb on the Protein A Sensor Chip surface, the theoretical $R_{\max}$ value was calculated, as in the immobilization method, just as a reference. To calculate a theoretical $R_{\max}$ value of 20, the response level of the ligand should be targeted at 212.9 ($20 = \text{Response}_{\text{Ligand}} \times 14000 \text{ Da (mass of VEGF121)} / 149000 \text{ Da (mass of mAb)} \times 1$). As a first step in the method development studies, the mAb molecule was flowed over the chip surface (10 $\mu\text{l/min}$) in two replicates at four different concentrations (1.675 nM, 3.35 nM, 6.7 nM, and 13.4 nM) to find the optimum concentration of the ligand molecule to be captured on the Protein A chip surface. To detect possible interference from the running buffer and the VEGF121 molecule, they were also included in the same sequence as negative controls. According to the Figure 3.2, the responses obtained from the running buffer and the VEGF121 molecule were very close to the baseline at 5.9 and 23.8 RU, respectively, indicating no significant interaction between the chip surface and negative controls. It can be clearly seen in the sensorgram that the ligand responses increased as the concentration of the mAb molecule increased, showing that the mAb molecule can be captured on the Protein A Sensor Chip surface specifically.

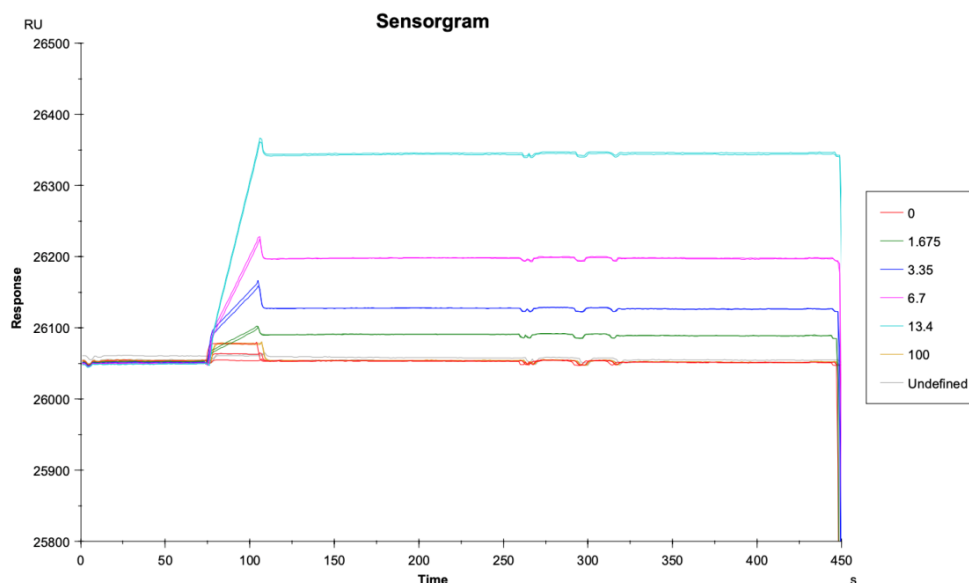


Figure 3.2 : Responses acquired from mAb captured with different concentrations. In the sensorgram x-axis shows time (seconds), y-axis shows responses. The yellow and red lines describe the 100 nM of the VEGF121 molecule and running buffer as blank, respectively. The green, dark blue, pink, and blue lines define the 1.675 nM, 3.35 nM, 6.7 nM, and 13.4 nM of capture molecule, respectively.

The average responses of two replicates of each ligand concentration are given in Table 3.1. As a result of the study, responses significantly different from the baseline were obtained with 6.7 nM and 13.4 nM ligand concentrations. Furthermore, since the maximum analyte binding capacity that can be obtained with the capture method is expected to be lower than the direct immobilization of the ligand, it was decided to conduct kinetic studies with ligand concentrations that gave high response.

Table 3.1 : Relative responses regarding to the different concentrations of mAb and 100 nm of VEGF121.

Sample Name	Concentration (nM)	Average of Relative Response (RU)
Running Buffer	-	5.9
VEGF121	100	23.8
Ligand	1.675	33.8
Ligand	3.35	82.1
Ligand	6.7	120.7
Ligand	13.4	210.3

3.1.2 Optimization of mAb concentration captured on protein A chip surface first study to interpret VEGF121 concentration range

The first kinetic binding analysis trials were conducted with two different optimum ligand concentrations determined by the capture study.

In the first kinetic study, after 6.7 nM of mAb was captured on the Protein A sensor chip surface, five different concentrations of analyte molecule (0.37 nM – 30.00 nM) were flowed over the chip surface with a flow rate of 30 $\mu\text{l}/\text{min}$ in running buffer to reduce mass transfer limitations. The study was performed using a singlecycle kinetic assay application. The ligand-analyte binding responses obtained against time are shown in the sensorgram using Biacore Evaluation Software. Kinetic constants k_a , k_d and K_D were calculated in the software using the 1:1 binding model. In the second study, 13.7 nM of mAb was captured on Protein A sensor chip surface and same range of analyte concentration with the previous experiment was applied. The responses are plotted in the sensorgram against time and kinetic constants were calculated by the software using the 1:1 binding model.

The sensorgram and residual plot obtained from the first kinetic binding analysis using 6.7 nM of the capture molecule are illustrated in Figures 3.3a-3.3b. Additionally, quality control parameters evaluated by the Evaluation Software are given in Figure 3.3c.

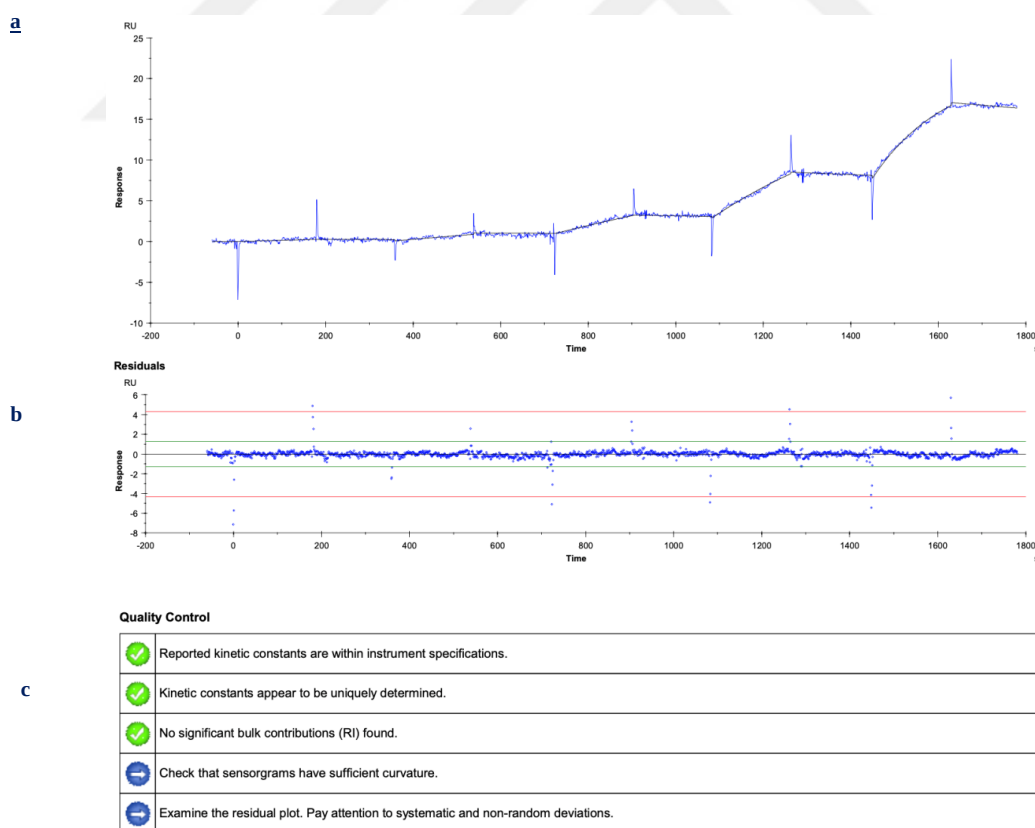


Figure 3.3 : Obtained responses against time by using 6.7 nM of ligand. Responses against time showed in sensorgram (a) and plotted residuals were shown (b). 3 separate quality parameters were evaluated in quality control section (c).

As a first evaluation, the appearance of the sensorgram and fitted curve were visually checked. For each concentration of the analyte molecule, the blue curve overlapped with the black curve, indicating a good fit (Figure 3.3a). Furthermore, the residual plot (Figure 3.3b) was checked for each concentration to evaluate how experimental data differentiated from the fitted curves. In the ideal fit, the residuals show only short-term noise during the ligand-analyte interaction, which means they should be scattered around zero response (typically $\pm 1-2$ RU). In the sensorgram, short-term noise was only seen during sample injection, but outside these time intervals, residuals were plotted between 1 ± 0.5 RU. Quality control parameters provided by software were used to evaluate the results. In Biacore systems, there is a detectable operating range for each kinetic parameter. While this value is between $10^3 \text{ M}^{-1}\text{s}^{-1}$ - $10^7 \text{ M}^{-1}\text{s}^{-1}$ for k_a , it is between 10^{-5} s^{-1} - 0.5 s^{-1} for k_d , and finally it is from picomolar to millimolar for K_D . Kinetic constants obtained outside the range that the device can measure do not represent reliable results. For this reason, it is important to work within the appropriate range that the device can detect for each kinetic parameter in method development studies. All reported kinetic constants fall within the range measurable by the instrument, as indicated in Figure 3.3c. In certain scenarios, it may not be possible to obtain an independent response for each kinetic parameter. The affinity constant (K_D), which is the most critical parameter in determining the interaction, is calculated as a result of k_d/k_a . However, the parameter uniqueness specified in the software evaluates the correlation between k_a , k_d and R_{max} values. If a significant correlation is found between these parameters, a warning is received that the relevant parameter cannot be detected independently. In this study, as it is shown in Figure 3.3c that all kinetic parameters are uniquely determined by the system. Following reference subtraction and blank subtraction, sensorgrams intended for kinetic assessment ideally should not include any bulk refractive index shifts (RI; parameter in quality control evaluation). Nevertheless, there might be instances where minor bulk refractive index shifts persist in reference- and blank-subtracted data, causing a warning in the quality control tab. As a result of this experiment, as shown in the quality control tab (Figure 3.3c), there were no bulk contributions. Kinetic constants obtained within the same study are given in Table 3.2. All kinetic constants (k_a , k_d and K_D), Chi^2 value, which allows for the calculation of the difference between experimental data and fitted curves, and U-value which expresses the uniqueness of the calculated kinetic parameters and R_{max} value, were examined.

Table 3.2 : Acquired kinetic constants and statistical parameters with 6.7 nM captured mAb.

ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi ² (RU ²)	U-Value
1.901E+5	2.669E-4	1.404E-9	22.71	0.269	9

One of the parameters that indicate a reliable fit curve is when the Chi-square value is less than 10% of the $R_{\max}$ value. In this study, the Chi square value was less than 10% (84.4%) of the $R_{\max}$ value, which shows that the fit curve was reliable. If the U-value is higher than 25, it indicates that more than one parameter is correlated, and they cannot be calculated individually. As shown in Table 3.2, the U-value (U=9) was lower than 25, indicating that the parameters were not significantly correlated.

Figures 3.4a-3.4b represent the sensorgram and residual plot resulting from the second kinetic binding analysis utilizing 13.4 nM of the capture molecule. Figure 3.4c displays the quality control parameters assessed by the Evaluation Software.

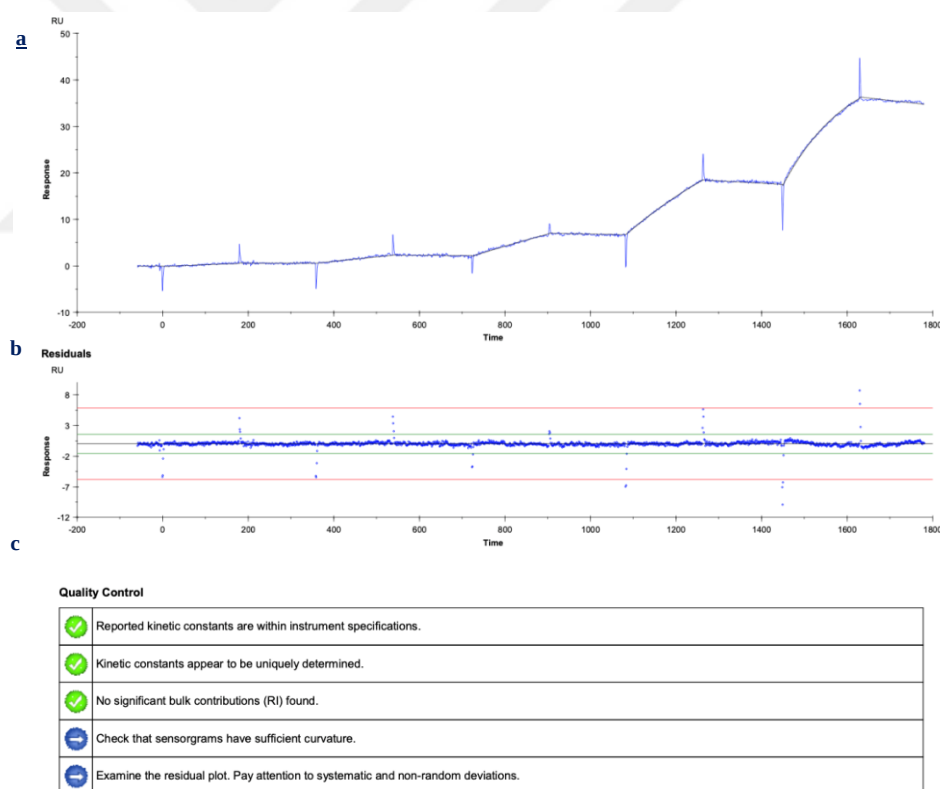


Figure 3.4 : The obtained responses over time utilizing 13.4 nM of the ligand.

Responses against time are illustrated in (a), while the corresponding plotted residuals are displayed in (b). Three distinct quality parameters were assessed in the quality control section (c).

As shown in Figure 3.4a, a well-fitted plot was achieved for every concentration of the analyte molecule.

As in the previous kinetic assay, the residuals responded at points close to 0 RU (Figure 3.4b). In the quality control tab (Figure 3.4c), it was determined that all kinetic parameters were independent of each other and within the range that the system could detect. No errors regarding bulk interference were detected. Kinetic constants obtained from this study are shown in Table 3.3.

Table 3.3 : Kinetic constants obtained from interaction between 13.4 nM of captured ligand and 0.37 nM – 30.00 nM VEGF121.

k_a (1/Ms)	k_d (1/s)	K_D (M)	R_{max} (RU)	χ^2 (RU ²)	U-Value
2.271E+5	3.023E-4	1.331E-9	46.10	0.447	7

Compared to the previous study, the obtained k_a and k_d values were higher, but the K_D value did not change significantly (3.8 %) since it was calculated by k_d/k_a . The most dramatic change was the nearly two-fold increase in the obtained R_{max} (increased from 22.72 RU to 46.10 RU), which was directly proportional to the increased ligand concentration. Since the obtained χ^2 value is related to the R_{max} value, the χ^2 value was higher than the previous study. However, while χ^2 value was equal to 84% of the R_{max} value in the previous study, it was equal to 103.1% of the R_{max} value in this study. This shows that the obtained fit curve can still be reliable. The U-value was equal to 7 which is even lower compared to the previous study. As the results showed similarities with the previous study in terms of kinetic constants, but considering that the recommended R_{max} value for kinetic studies falls within the range of 50-100 RU, the next study will be performed with a 13.4 nM capture concentration. However, upon inspecting the sensorgram obtained at 13.4 nM capture, uncertainty remains regarding whether the surface has been saturated with the highest achievable analyte concentration. Hence, to optimize the maximum analyte concentration that can bind to the surface, the next study set has been designed with an analyte concentration range between 1.11 nM and 90.00 nM.

3.1.3 Optimization of VEGF121 concentration range

In this study, to determine the maximum concentration of analyte that can saturate the chip surface, the highest analyte concentration was initiated at 90 nM, followed by a threefold serial dilution, reaching a minimum concentration of 1 nM. All other analysis conditions remained consistent with previous analyses.

Figures 3.5a-3.5b display the sensorgram and residual plot derived from the kinetic binding analysis conducted when the analyte was flowed over the sensor surface within a range of 1.11-90.00 nM. Figure 3.5c illustrates the quality control parameters assessed by the Evaluation Software.

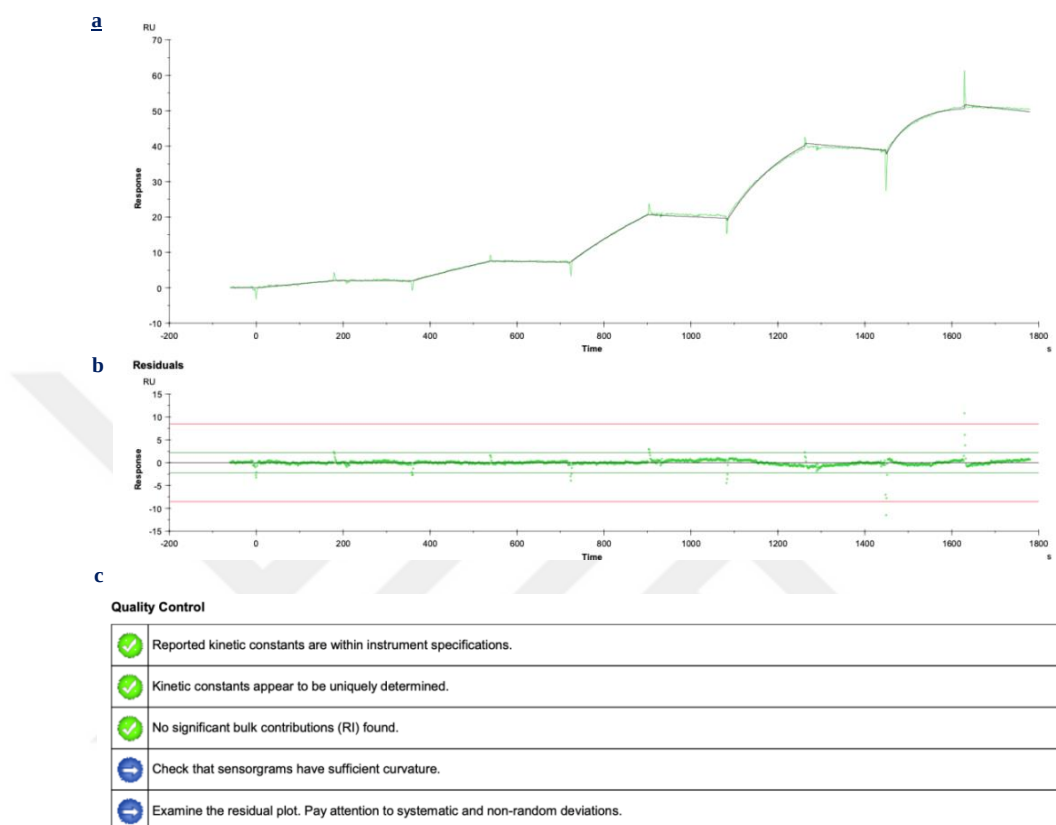


Figure 3.5 : The responses from molecular interaction between 13.4 nM of ligand and the range of 1.11 nM-90 nM analyte concentration was evaluated. Plotted sensorgram (a), with corresponding plotted residuals (b) were shown. Three distinct quality parameters were evaluated in the quality control section (c).

As in previous studies, a good curve fit was achieved in this study as well. Additionally, unlike previous analyses, it can be stated that the surface of the chip was saturated at the highest analyte concentration, and approximately the highest achievable $R_{\max}$ value could be reached when 13.4 nM of ligand was captured. Responses derived from residuals exhibited scattering only during needle sample draws, while deviations close to zero were observed in the remaining data. Additionally, all parameters specified in the quality control section have met the specifications. The results of this study regarding the kinetic constants can be found in Table 3.4.

Table 3.4 : Kinetic constants and statistical parameters obtained from molecular interaction between 13.4 nM captured mAb with 1.11nM - 90.00 nM VEGF121.

ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi ² (RU ²)	U-Value
2.027E+5	2.661E-4	1.312E-9	52.96	0.454	4

Despite changes in the k_a and k_d values, the K_D value remained almost the same as the previous study. Allowing for the binding of more molecules to the sensor surface under the same conditions resulted in an increased R_{max} value from 46.10 to 52.96 RU as it was expected. Chi² value was the 116 % of the R_{max} value, which was an even better result than the previous one. The decreased U-value from 7 to 4 shows that the kinetic parameters were even more uncorrelated from each other. Although the experimental conditions were different, the K_D values obtained from the three kinetic studies were very close to each other within a 5.19 % RSD. Therefore, two more replicates were included in the same sequence of this study, to examine the results with more than one sample sequence.

3.1.4 Application of three different dissociation times in three replicate studies

The averages of the kinetic constants (k_a , k_d , K_D) from the three replicates performed with 150 dissociation time and the R_{max} values from each replicate, are detailed in Table 3.5.

Table 3.5 : The interaction of 1.11 nM - 90.00 nM analyte concentration with 13.4 nM captured mAb was evaluated in three replicate studies.

ka (1/Ms) Average	kd (1/s) Average	KD (M) Average	Rmax (RU)	Chi ² (RU ²)	U-Value Average
2.138E+05	3.286E-04	1.532E-09	52.96	0.454	4
			54.20	0.91	
			57.86	2.14	

In this first study which was performed with 3 replicates, although the average K_D value was similar to the previous studies, a consistent increase in the R_{max} value was realized between the replicate samples. Furthermore, the highest Chi² level so far was acquired with an increase in the number of sample sets. The average capture level of the three replicates was 243 RU with a 1% RSD. Based on this, the reason for obtaining a higher R_{max} value could be related to the regeneration efficiency of the analyte molecule. Since a high sample number needs to be studied in the qualification parameters, it was very crucial to prevent the consistent increase of the R_{max} value.

The effect of the changing regeneration time was evaluated from that perspective. In the next three replicate study, while all analysis conditions were kept constant, only regeneration time was changed from 30 seconds to 45 seconds. By extending the regeneration time to 45 seconds, the increase in the $R_{\max}$ value (consistently around 50 RU) obtained from replicate studies was prevented. Upon examining the kinetic parameters, while the k_a values of the replicate samples were quite close to each other, there was significant deviation in the k_d values. Since the deviation in k_d value with a 60.97% CV affects the K_D value at a similar rate, the CV % of the K_D value between replicates was also quite high, 52.45 %. Additionally, the responses over time varied highly when 3 sensorgrams were overlaid and evaluated together, as seen in Figure 3.6a.

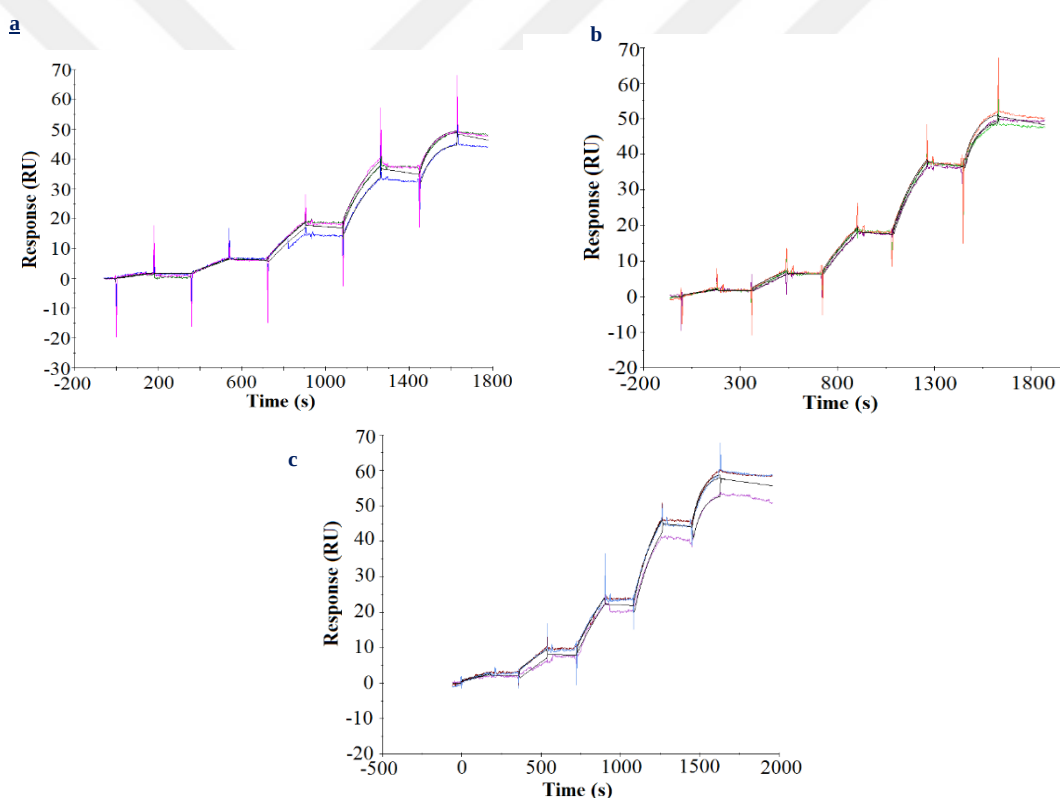


Figure 3.6 : The sensorgram of the study performed with 45 second regeneration times with three different dissociation times. 150 seconds dissociation time(a), 240 seconds dissociation times (b), 330 second dissociation times (c).

Considering the result, and given the uncertainty in determining the optimal dissociation time, the dissociation time was set at 240 seconds for the next three replicate studies. The sensorgrams obtained as a result of this study are shown in Figure 3.6b. Merely evaluating the sensorgram, it can be easily understood that the responses are closer than in the previous study.

While extending the dissociation time had no negative impact on the $R_{\max}$ value, consistent responses were obtained without an increase in the $R_{\max}$ value. When examining the deviation between k_a , k_d , and K_D parameters of the three replicate samples, a significant improvement is noticed compared to the previous study by decreasing CV% from 52.45 % to 25.46 %. However since CV % value was still higher than 20 % (ICH Q2 (R2)) and it could be decreased by extending dissociation time, 330 seconds dissociation time was studied for the next run. Furthermore, the time extension of dissociation was also applied to determine whether the highest analyte concentration could not be removed from chip surface resulting variety in K_D value. The sensorgram of the study when dissociation time was set to 330 seconds was illustrated in Figure 3.6c. With this study, K_D value was lower than previous studies by having 0.5E-09 nM response. The CV % of the K_D value between replicates was 35.25 %. Since a higher CV% value was obtained by applying 330 seconds dissociation time, the next run was performed with 240 seconds of dissociation time, which gave the lowest CV % value.

3.1.5 Changing the analysis template from kinetic/affinity to custom assay regeneration solution contact time optimization

Since it was not possible to control conditions such as deciding how many times capture steps would be applied in the analyses performed with the kinetic assay template included in Biacore Assay Software, a new analysis sequence was created with the custom assay method. One of the major points of changing the template to the custom assay method was that the consumption of the ligand molecule was high due to the kinetic assay template injecting the capture molecule in each cycle. Additionally, as the custom method gives the user full control over all parameters, it was believed that it could contribute to decreasing the CV % values obtained in previous studies by understanding each condition. The kinetic parameters acquired from the first analysis using the custom assay method are given in Table 3.6.

Table 3.6 : Kinetic parameters from first custom assay method trial.

k_a (1/Ms) Average	k_d (1/s) Average	K_D (M) Average	$R_{\max}$ (RU)	χ^2 (RU ²)	U-Value Average
1.691E+05	9.165E-05	5.406E-10	122.80 120.30 116.90	0.23 0.26 0.93	5.67

There has been an increase in the $R_{\max}$ values compared to the previous analyses by changing the kinetic assay template to the custom assay method. However, since the responses from kinetic analysis will decline over time by applying regeneration cycles, this increase was not prioritized for the optimization. The most important output from this trial was observing the average k_d value too low with 45 seconds regeneration and 240 seconds dissociation cycles. Furthermore, CV % from 3 replicates was 25.29 % which was quite similar to analysis performed with the kinetic assay template by applying 45 second regeneration and 240 seconds dissociation cycles mentioned in the section 3.1.4. Additionally, the $R_{\max}$ values in this analysis decreased progressively, and it was considered that due to the excessive implementation of regeneration cycle with 45 seconds, which may cause the regeneration of the capturing agent as well within this method. Therefore, instead of 45-second regeneration times, 30-second regeneration times were applied in the next study to prevent the decrease of the $R_{\max}$ value as well as to detect k_d values which are closer to the measurable range. The kinetic parameters measured after applying the 30-second regeneration cycle are given in Table 3.7.

Table 3.7 : Kinetic parameters obtained from custom assay method by applying 30 seconds regeneration cycles.

k_a (1/Ms)	k_d (1/s)	K_D (M)	$R_{\max}$ (RU)	χ^2 (RU ²)	U-Value
Average	Average	Average			Average
1.480E+05	1.095E-04	9.614E-10	94.85	0.17	7.05
		6.388E-10	85.18	0.58	
		7.310E-10	89.02	0.88	

It can be clearly seen that decreasing the regeneration time allowed obtaining a higher average k_d value which made it possible to detect more consistent and reliable results since the k_d values were in the measurable range. The CV % between the replicates was 21.92 % which was a slightly less than the previous deviations. When K_D value and $R_{\max}$ response of the first replicate were compared with the last 2 replicates, it was determined that the deviation was generally coming from the first replicate. It was anticipated that the reason for this deviation might be because, unlike in the other replicates, the chip was not conditioned with the regeneration solution before the first replicate. Therefore, in the next study, conditioning of the chip with regeneration solution as well as with running buffer as a washing reagent of the regeneration buffer was performed.

3.1.6 Conditioning sensor chip surface with regeneration cycles

In this study, the method flow was modified by adding only regeneration and extra buffer washing steps before the first repeat, while keeping other parameters exactly the same, and the new sequence shown in Table 6 was created. The aim was to condition the sensor chip surface for the first replicate the same as with the last 2 replicates. The results of the kinetic parameters after this modification are shown in Table 3.8.

Table 3.8 : Measured kinetic parameters after applying conditioning regeneration cycles.

ka (1/Ms) Average	kd (1/s) Average	KD (M) Average	Rmax (RU)	Chi ² (RU ²)	U-Value Average
9.807E+04	1.196E-04	1.118E-09	74.01	0.12	4.39
		1.248E-09	74.24	0.15	
		1.287E-09	71.62	0.24	

As shown in Table 3.8, the K_D values from 3 replicates were closer to each other compared to previous studies. The CV % value of K_D parameter between 3 replicates was 7.27% which was the lowest CV % value obtained so far in method development studies. Besides, consistency between R_{max} values was achieved by conditioning the sensor chip surface with regeneration and buffer wash cycles prior to running first replicate. As the CV % value was finally obtained below 20 %, the method development studies were completed with consistent results of 3 replicates, and the studies proceeded with method qualification.

3.2 Qualification Studies

3.2.1 Specificity parameter

The first parameter performed for method qualification studies was specificity. The specificity of the developed method was determined by evaluating the binding responses between the mAb molecule and a protein that has no binding activity with ligand mAb. As clearly seen in Figure 3.7, there was no increase in the response as the concentration of the non-specific analyte molecules increased, which shows that there is no interaction between the ligand mAb and non-specific analyte molecules and it shows the specificity of the developed method.

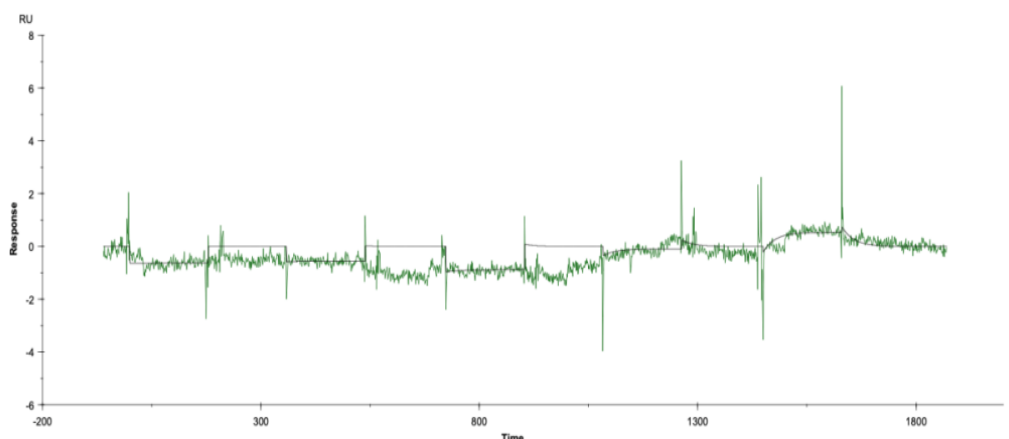


Figure 3.7 : Responses obtained from interaction between mAb and non-specific protein. Green line shows the responses from 5 different concentrations of non-target analyte molecule.

3.2.2 System precision parameter

To determine whether the system was properly working and obtaining consistent results, 6 consecutive runs were performed from a single preparation set (with 5 concentrations of VEGF121 molecule and 1 concentration of ligand molecule), and the obtained kinetic parameters were compared. The responses from this study are given in detail in Table 3.9.

Table 3.9 : The kinetic parameters revealed with system precision study with 6 consecutive runs.

ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi ² (RU ²)	U-Value
1.10E+05	9.38E-05	8.53E-10	76.19	0.39	6.89
1.05E+05	1.05E-04	9.97E-10	76.02	0.34	5.30
1.09E+05	1.22E-04	1.12E-09	75.55	0.47	5.30
1.02E+05	6.84E-05	6.67E-10	75.88	0.61	11.65
9.88E+04	7.34E-05	7.42E-10	75.45	0.25	6.89
1.10E+05	9.98E-05	9.11E-10	77.78	0.89	8.96

6 studies were evaluated one by one and the suitability of the system and quality control parameters were examined. While each study met its own quality control parameters, no deviation from baseline was observed in any study. In addition, the residuals distribution was determined between -2 RU and 2 RU for each sample set. When the kinetic parameters of the 6 studies are compared, similar results are garnered for each parameter (the deviation for the K_d value is below 20%), and the fact that the Chi^2 values are less than 10 % of the R_{max} values indicates that a reliable fit graph is obtained for each study.

Furthermore, responses from the 5 concentrations of VEGF121 molecule are given in a bar chart in the Figure 3.8 which shows the similarity of responses of 6 replicate studies.

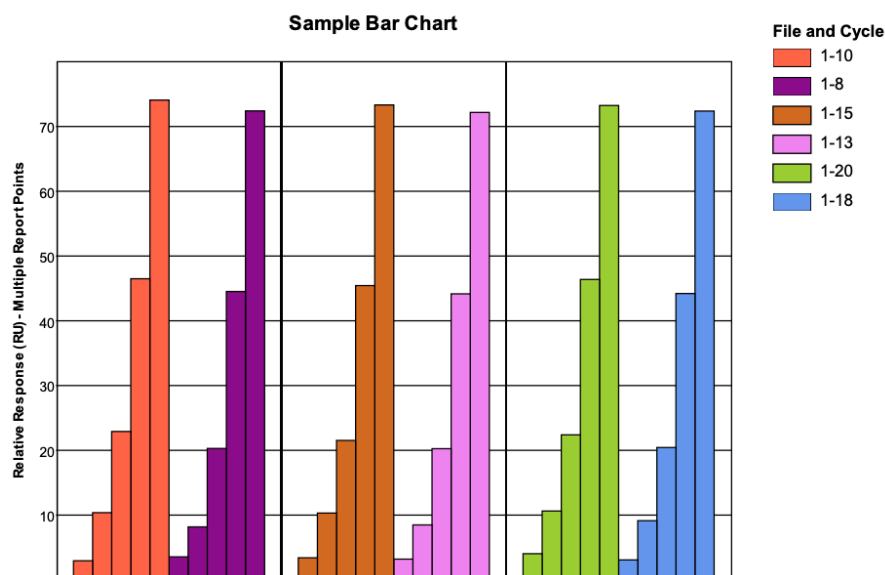


Figure 3.8 : System precision parameter results were illustrated in bar graph. 6 different colors of bar graphs show the responses obtained from each concentration of VEGF121 molecule within 6 consecutive sample sets.

3.2.3 Repeatability studies

In the parameter of repeatability, mAb and 5 concentrations of VEGF121 were prepared 6 separate times and 6 different kinetic analyses were performed by analyst 1 in order to find out if there was any reason for variation coming from the sample preparation procedure. As a result of this study, the kinetic parameters from 6 different preparations compared to each other and quality parameters were evaluated. Table 3.10 illustrates the measured kinetic parameters as a result of repeatability study.

Table 3.10 : The kinetic parameters obtained from repeatability studies.

ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi ² (RU ²)	U-Value
1.04E+05	6.42E-05	6.20E-10	78.78	0.65	15.14
1.13E+05	6.80E-05	6.04E-10	74.77	0.20	6.89
1.10E+05	7.49E-05	6.84E-10	74.73	0.23	6.89
1.12E+05	7.53E-05	6.71E-10	75.77	0.34	6.89
9.30E+04	6.98E-05	7.51E-10	75.15	0.55	11.65
8.80E+04	6.39E-05	7.26E-10	74.97	1.06	15.14

As a result of the repeatability studies, consistency in $R_{\max}$ and K_D values was observed among 6 replicate studies, and average K_D value was obtained as $6.760\text{E-}10$ with a deviation of 8.52 %.

3.2.4 Intermediate precision studies

In the intermediate precision study, analyst 2 followed exactly the same procedure which analyst 1 was performed in repeatability study by preparing mAb and 5 concentrations of VEGF121 for 6 independent times and performing 6 different kinetic cycles. The main idea of this parameter is to show that performing the developed method by a different analyst has no effect on the results and the robustness of the method is independent from analyst. To show the developed method is analyst-independent, the average K_D values obtained as a result of 6 replicate studies conducted by two analysts were compared with each other, and the deviation between of them was reported. The measured kinetic parameters of this study were detailed in Table 3.11.

Table 3.11 : The kinetic parameters as a result of intermediate precision studies which was performed by analyst 2.

k_a (1/Ms)	k_d (1/s)	K_D (M)	$R_{\max}$ (RU)	χ^2 (RU ²)	U-Value
1.060E+05	6.436E-05	6.071E-10	67.50	0.14	7.00
1.096E+05	7.457E-05	6.803E-10	66.67	0.33	9.00
1.095E+05	8.414E-05	7.688E-10	67.97	0.26	7.00
1.156E+05	8.846E-05	7.650E-10	66.94	0.23	7.00
1.060E+05	6.487E-05	6.118E-10	66.52	0.25	9.00
1.178E+05	7.268E-05	6.171E-10	66.34	0.24	9.00

Although the $R_{\max}$ values resulting from the analysis performed by a different analyst were lower than the values in the repeatability study, the average K_D value was $6.750\text{E-}10$ with a deviation of 11.26%. When the average K_D value as a result of this analysis was compared with the average K_D value from the repeatability study, the deviation between them was calculated as 0.14% which shows the developed method is robust and independent from the analyst. Besides, responses from 5 different concentrations of VEGF121 molecule in 6 replicates were illustrated in a bar chart in Figure 3.9. Even though there are slight differences between responses from 6 replicates, overall responses were quite similar to each other as shown in Table 3.11.

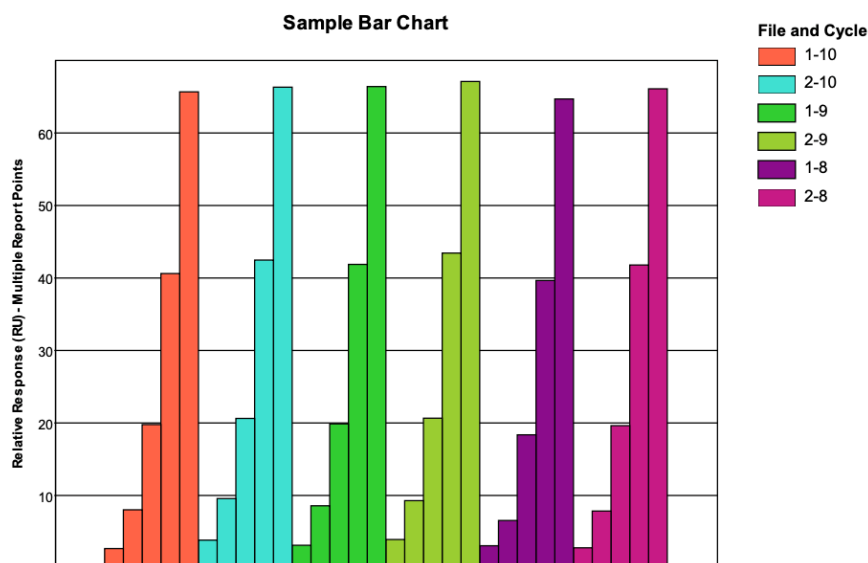


Figure 3.9 : Responses of 5 concentrations of VEGF121 from 6 different kinetic cycles performed from analyst 2.

3.3 Studies to Detect Binding Activity of Different Lot Numbers of mAb

Kinetic studies were conducted with 12 different production batches supplied from different markets of the monoclonal antibody to determine how effective and consistent the developed and qualified method was in detecting the binding activity of different production batches. While the k_a and k_d values measured as a result of kinetic studies are shown in Figures 3.10 and 3.11, respectively, the KD values calculated by proportioning the k_a and k_d values are illustrated in Figure 3.12.

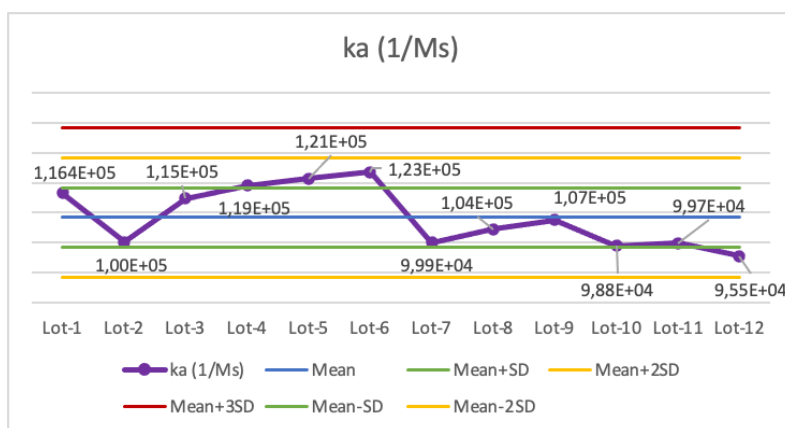


Figure 3.10 : Measured k_a values of 12 different production batches of mAb.

While making the comparison, the average of the kinetic parameters was calculated, standard deviation differences (Mean $\pm$ SD/ $\pm$ 2SD/ $\pm$ 3SD) were determined and distribution of the parameters was drawn in a chart.

It was evaluated whether the parameters obtained from each mAb product were within the specified statistical range. Figure 3.10 shows the k_a profiles obtained from each lot as a result of the interaction of mAbs in different lots with VEGF121, the average k_a value of 12 different series ($1.084\text{E}+05$) and the sample mean $\pm\text{SD}/\pm 2\text{SD}/+3\text{SD}$ of k_a values. The k_a values of all batches remained within the mean $\pm 2\text{SD}$ range, and the mAb molecule exhibited binding to the VEGF121 with similar rates.

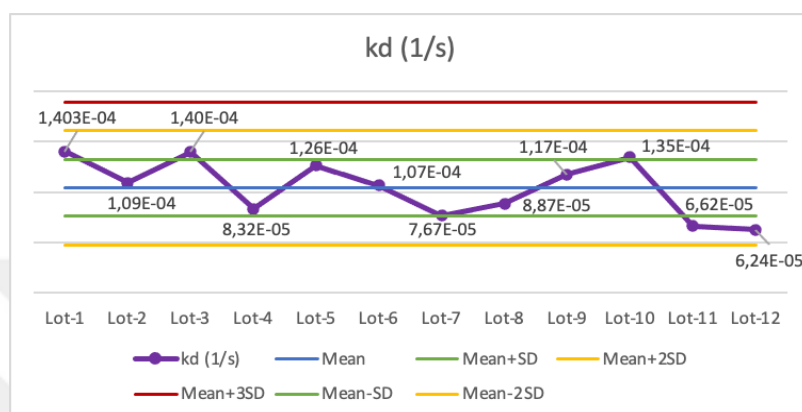


Figure 3.11: Measured k_d values of 12 different production batches of mAb.

Figure 3.11 shows the k_d profiles measured from 12 different lots, the average k_d value of the series ($1.043\text{E}-04$) and the sample mean $\pm\text{SD}/\pm 2\text{SD}/+3\text{SD}$ of the k_d values. The k_d values obtained from mAb molecules in different lots remained within the mean $\pm 2\text{SD}$ range.

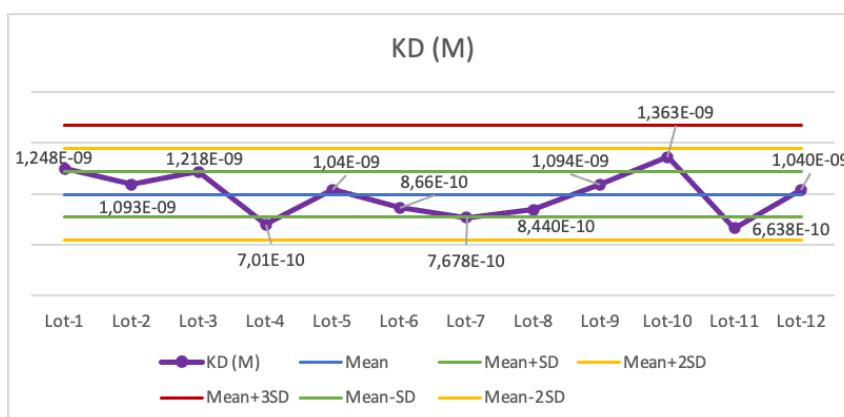


Figure 3.12 : Calculated K_D values of 12 different production batches of mAb.

Figure 3.12 shows the K_D profiles from different batches, the mean K_D value of the batches ($9.949\text{E}-10$) and the sample mean $\pm\text{SD}/\pm 2\text{SD}/+3\text{SD}$ of the K_D values. The obtained K_D values remained within the mean $\pm 2\text{SD}$ range and a similar binding profile was observed between lots.



4. CONCLUSIONS

The SPR method is a highly preferred and reliable method for examining the interaction of two molecules. This functional characterization method has become increasingly common for characterizing the binding activity of mAb molecules with their target receptors or proteins by measuring kinetic values. In this study, the interaction between a therapeutic mAb used to inhibit vascular angiogenesis and VEGF121, one of its target proteins, was examined using the SPR method.

During the method development studies, the mAb concentration captured on the Protein A chip surface was determined as 13.4 nM, while the VEGF121 concentrations flowed over chip surface were optimized in the range of 1.11nM to 90.00 nM. Analyte contact time and analyte dissociation time, which affect the k_a and k_d values used in the calculation of the equilibrium constant (K_D), were optimized to be 180 and 240 seconds, respectively.

The parameters described in ICH Q2, Revision 2 were studied to demonstrate the reliability of the developed method by evaluating the specificity and precision parameters, with each parameter meeting the acceptance criteria. Finally, a lot to lot variation analysis was performed with mAb molecules from 12 different production batches, and the difference between lots for each kinetic parameter remained within the ± 2 SD range.



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APPENDICES

APPENDIX A : Laboratory Equipments and Consumables

APPENDIX B : Chemicals and kits



APPENDIX A : Laboratory Equipments and Consumables

All the laboratory equipments and consumables used in this study were shown in Table A.1.

Table A.1 :Used equipments and consumables in this study.

Equipment	Supplier Company
Analytical Balances	Mettler Toledo
Refrigerators	Sanyo
pH meter	Horizon 11.14, Whatman,
Surface Plasmon Resonance	Biacore X100, Cytiva
Magnetic stirrer	Heidolph Standard
Otomatikpipetler	Eppendorf
Protein A Chip	Cytiva
Falcons (15 mL, 50 mL)	Isolab
Eppendorf Tubes (2 mL)	Interlab
1.5 mL tubes	Cytiva
Rubber caps	Cytiva
0.2 µm filters	Millipore

APPENDIX B: Chemicals and kits

Chemicals and kits are in below Table B.1.

Table B.1 : Used chemicals and kits in this study.

Chemical	Supplier Company
Anti-VEGF özelliği gösteren mAb standardı	Roche
NaCl	Merck, Cat #: 1.06400.500
EDTA	JTB, Cat #: 6381-92-6
HEPES	Sigma Aldrich, Cat #: H4034
Tween 20	JTB, Cat #: 4116-04
Glycine	Sigma-Aldrich, Cat #:68898
HCl	Merck, Cat #: 1.00314.25000
Regeneration Solution (Glycine, pH: 2.0)	Cytiva, BR100355
HBS-EP (+) Buffer	Cytiva, BR100826
BIAMaintenance Kit	Cytiva, BR10066
Purified water	Milli-Q Water

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